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The effects of dietary fibres on the behaviour and growth of colonic epithelial cells in vivo and in vitro

The effects of dietary fibres on the behaviour and growth of colonic epithelial cells *in vivo* and *in vitro*.

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Abstract

Dietary fibre is believed to protect against the development of a number of western diseases including colorectal cancer. The aim of this study was to investigate whether dietary fibres exert a chemopreventative effect in respect of colorectal neoplasia using the 1,2-dimethylhydrazine (DMH) mouse model, and to elucidate their biological mechanism of action focusing on the cadherin/catenin complex. Examination of DMH-induced adenomas revealed alterations in the cadherin/catenin complex. Within the dysplastic epithelium there was an increase in cytoplasmic localisation of β-catenin, α-catenin, p120 and E-cadherin. This was accompanied by nuclear localisation of β-catenin and the possible overexpression of all components of the cadherin/catenin complex. Within the feeding study which examined the effects of dietary fibre supplementation on DMH-induced mouse colonic tumours the dietary fibre pectin was found to significantly increase tumour incidence. This was accompanied by a possible increase in tumour development and an increase in colonic cells undergoing DNA replication as assessed by flash labelling experiments with the thymidine analogue bromodeoxyuridine. On examination of the cadherin/catenin complex there was no apparent difference in the pattern of expression, within the non-dysplastic and dysplastic epithelium, between the different fibre-supplemented diets. In *in-vitro* studies the dietary fibres did not affect total cell number or β-catenin expression, although the SCFAs butyrate and propionate, products associated with fibre fermentation, were shown to result in a decreased total cell number. Within the cell line HCT116, butyrate was also shown to result in the redistribution of β-catenin from the cell membrane to the cytoplasm. In summary, these studies have shown that dietary fibre may not account for the chemopreventive actions of fruit and vegetables, although the production of butyrate from the fermentation of fibre may elicit some protective effects. This is in keeping with the hypothesis that the chemopreventive biological effects for fibre are mediated via short chain fatty acids.

To my husband Tim

Without whom this would not have been completed,
But without whom this would have been completed 12 months ago!

"You are one in a million. I love you"

and
to all my family and friends.

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Publications

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Tucker, E.L, Pignatelli, M. (2000) Catenins and their associated proteins in colorectal cancer. *Histol Histopathol.* 15: 251-260.

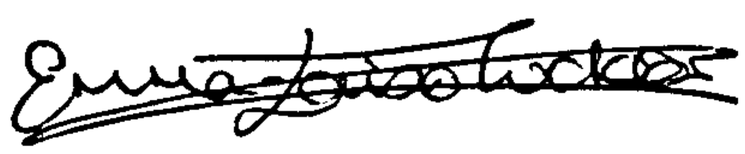
Tucker, E., Buda, A., Janghra, N., Baker, J., Code, J., Moorghan, M., Havler, M., Dettmar, P., Pignatelli, M. (2003) Abnormalities of the cadherin/catenin complex in chemically induced colorectal carcinogenesis. *Proc Nut Soc.* 62: 229-236

Declaration

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of this dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

SIGNED:  DATE: 9th April 2003

Contents

List of Figures.

List of Tables.

Abbreviations

A	Acetate
aa	Amino acid
ACF	Aberrant Crypt Foci
ANOVA	Analysis of Variance
AOM	Azoxymethane
APC	Adenomatous Polyposis Coli
B	Butyrate
BrdU	Bromodeoxyuridine
BrdU-LI	Bromodeoxyuridine-labeling index
BSA	Bovine serum albumin
Buty	Butyrate
COOH-terminal	Carboxy-termnial
COX-2	Cyclooxygenase
CMC	carboxymethylcellulose
CRC	Cancer Research Campaign
DAB	Diaminobenzidine
DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagles Medium
DMH	1,2-dimethylhydrazine
DMSO	Dimethylsulphoxide
DoH	Department of Health
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbant assay
FCS	Foetal calf serum
FH	Faecal homogenate
FITC	Fluorescein isothiocyanate
GSK-3β	Glycogen synthase kinase-3β
g	gram
HCl	Hydrochloric acid
HDL	High Density Lipoprotein
HDLG	Human discs large
HGF	Hepatocyte growth factor
HMG-CoA	3-hydroxy-3methylglutary coenzyme A
HRP	Horseradish Peroxidase
IGF	Insulin growth factor
IgG	Immunoglobulin G
IH	Ispaghula husk
IP	Intraperitoneal
kD	Kilodalton
LDL	Low Density Lipoprotein
mA	milliampere
mg	Milligram
min	Minute

MAM	Methylazoxymethanol
Ml	Millilitre
MMP	Matrix Metalloproteinase
mRNA	Messenger ribonucleic acid
MSM	Mineral salts media
MW	Molecular weight
Na/K	Sodium/potassium
NIDDM	Non-insulin dependent diabetes mellitus
NSAID	Non-steroidal anti-inflammatory drugs
NSP	Non-starch polysaccharide
N-terminal	Amino-terminal
OD	Optical density at wavelength
P	Probability
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA-LI	Proliferating cell nuclear antigen – Labeling index.
PKC	Protein kinase C
Prop	Propionate
PTP	Protein tyrosine phosphatase
RB1	Retinoblastoma
SCFA	Short chain fatty acids
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TBST	0.5% Tween 20/TBS buffer
TCF/LEF-1	T cell factor/lymphoid-enhancer-binding factor-1
V	Volt
WHC	Water holding capacity
w/v	Weight per unit volume
µg	Microgram
µl	Microlitre

Chapter 1

Introduction

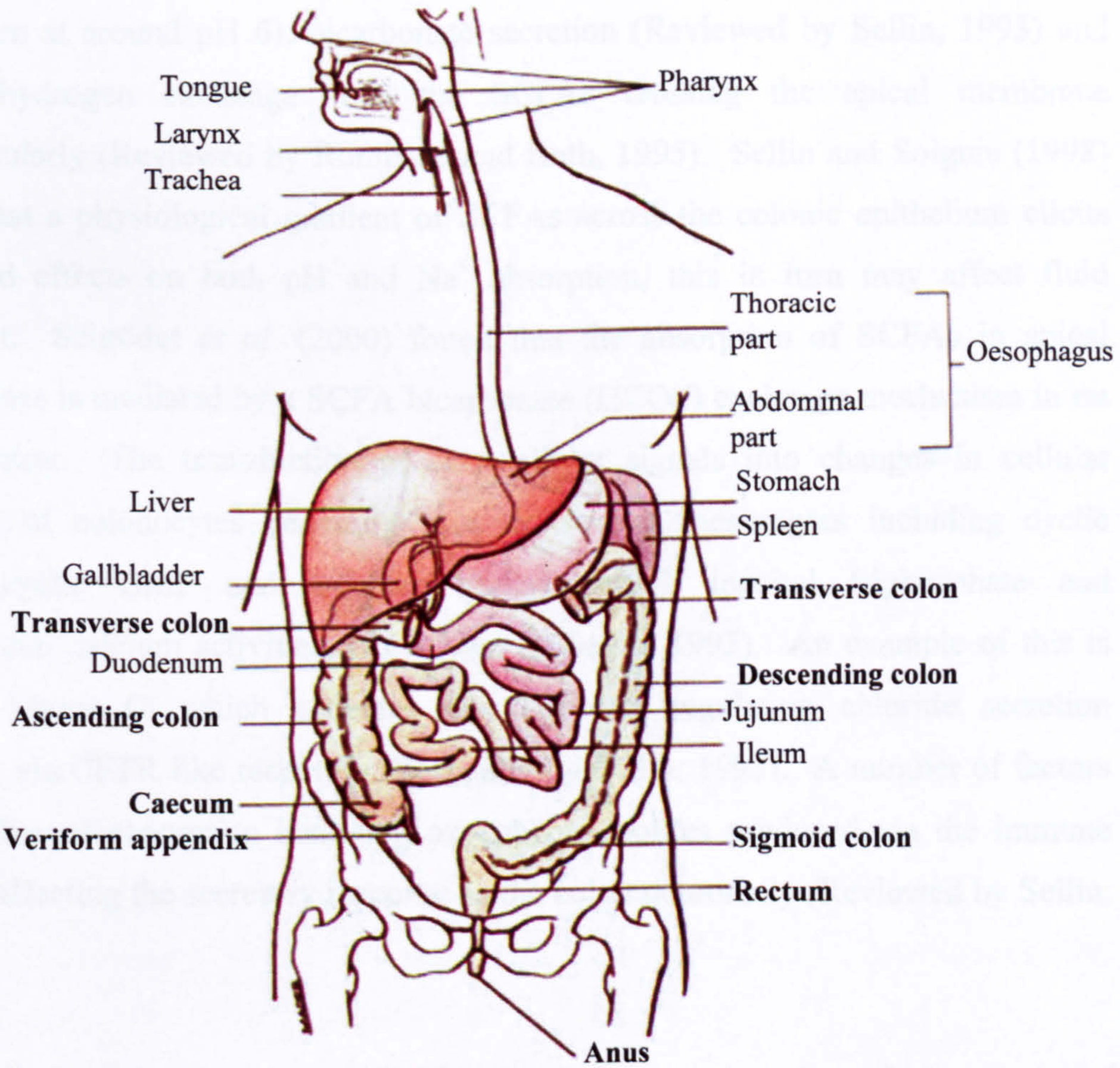
1.1 The Large Intestine.

1.1.1 The physiology of the Large Intestine.

The human large intestine (colon) is over 1.5 meters long and is made up of the caecum, vermiform appendix, ascending colon, transverse colon, descending colon, sigmoid colon, rectum and anal canal (Fig 1.1) (Reviewed Davies and Davies, 1962; Reviewed by Moore and Agur, 1995; Reviewed by Ross *et al.*, 1995). The large intestine can be regarded as two organs; the right side, which consists of the caecum, a fermenter where bacteria metabolise materials including bile passing from the ileum of the small intestine (Reviewed by Brydon, 1995), and the ascending (proximal) colon where most water absorption occurs (Reviewed by Moore and Agur, 1995), and the left side consists of the descending and sigmoid (distal) colon which are areas primarily involved in stool formation (Reviewed by Brydon, 1995).

The large intestine innervature is supplied by parasympathetic and sympathetic nerves of the mesenteric ganglia (Superior and Inferior) with blood supply via the mesenteric artery and removal via the mesenteric vein (Reviewed Davies and Davies, 1962; Reviewed by Moore and Agur, 1995; Reviewed by Ross *et al.*, 1995). Secretion and absorption are important processes in the colon with numerous proteins being involved (Reviewed by Sellin, 1993). Chloride secretion and absorption is facilitated by three main types of channel; calcium mediated, cyclic-AMP-mediated and volume-induced channels, the latter two bearing some resemblance to the cystic fibrosis transmembrane conductance regulator (CFTR) receptor, a target for both protein kinase A (PKA) and protein kinase C (PKC) (Reviewed by Sellin, 1993). CFTR is itself shown to form a small chloride channel exchanging for bicarbonate being linked to sodium-hydrogen exchange (Reviewed by Sellin, 1993). There is a family of sodium and hydrogen exchangers located on both the apical (vectorial transport) and basolateral (housekeeping, maintaining pH)

Figure 1.1 The Gastrointestinal tract



(Reviewed by Moore and Agur,1995)

membrane (Reviewed by Sellin, 1993). Short chain fatty acids (SCFAs) produced from the fermentation of dietary fibre and protein are absorbed in the colon but the precise mechanism is not known (Reviewed by Rombeau and Roth, 1995). SCFA absorption is shown to be associated with luminal alkalinization (helping to maintain the lumen at around pH 6), bicarbonate secretion (Reviewed by Sellin, 1993) and sodium-hydrogen exchange with the SCFAs crossing the apical membrane transcellularly (Reviewed by Rombeau and Roth, 1995). Sellin and Soignie (1998) found that a physiological gradient of SCFAs across the colonic epithelium elicits polarized effects on both pH and Na^+ absorption, this in turn may affect fluid transport. Schröder *et al.* (2000) found that the absorption of SCFAs in apical membranes is mediated by a SCFA bicarbonate (HCO_3^-) exchange mechanism in rat distal colon. The transduction of extracellular signals into changes in cellular function of colonocytes generally involves second messengers including cyclic AMP, cyclic GMP and calcium (diacylglycerol, inositol triphosphate and intracellular calcium activities) (Reviewed by Sellin, 1993). An example of this is protein kinase C, which activates diacylglycerol regulating chloride secretion possibly via CFTR like receptors (Reviewed by Sellin, 1993). A number of factors may influence absorption including oxygen metabolites produced via the immune system affecting the secretory response in the colon neuronally (Reviewed by Sellin, 1993).

Mammalian species can be classified into several groups based on characteristics of their respective digestive tracts. Colonic digesters, sacculated digesters such as man, do not have much caecal capacity whereas caecal fermenters such as the mouse exhibit large caeca and unsacculated colons (Reviewed by Van Soest, 1995). The large intestine is host to a large and diverse commensal microflora consisting of 50 different genera/families with over 400 different species, at a level of approximately 10^{11} (10^{10} Tannock) cells (Reviewed by Bingham, 1990; Reviewed by Tannock, 1994; Reviewed by Hill, 1995a). These bacteria tend to be anaerobes, such as *Bacteriodes* and *Bifidobacter*, with few facultative anaerobes such as *E.coli* and eubacteria (Reviewed by Tannock, 1994). There is site specificity for bacteria within the large intestine with anaerobes persisting in the proximal colon and numbers of facultative anaerobes increasing in the distal colon. This variation of bacteria affects the types of metabolic products found in an anaerobic environment. The metabolism

of non-starch polysaccharides (NSP) in an anaerobic environment leads to the production of short chain fatty acids (SCFAs), carbon dioxide (CO_2), methane (CH_4) and hydrogen (H_2) (Reviewed by Macfarlane and Gibson, 1994; Reviewed Rombeau and Roth, 1995).

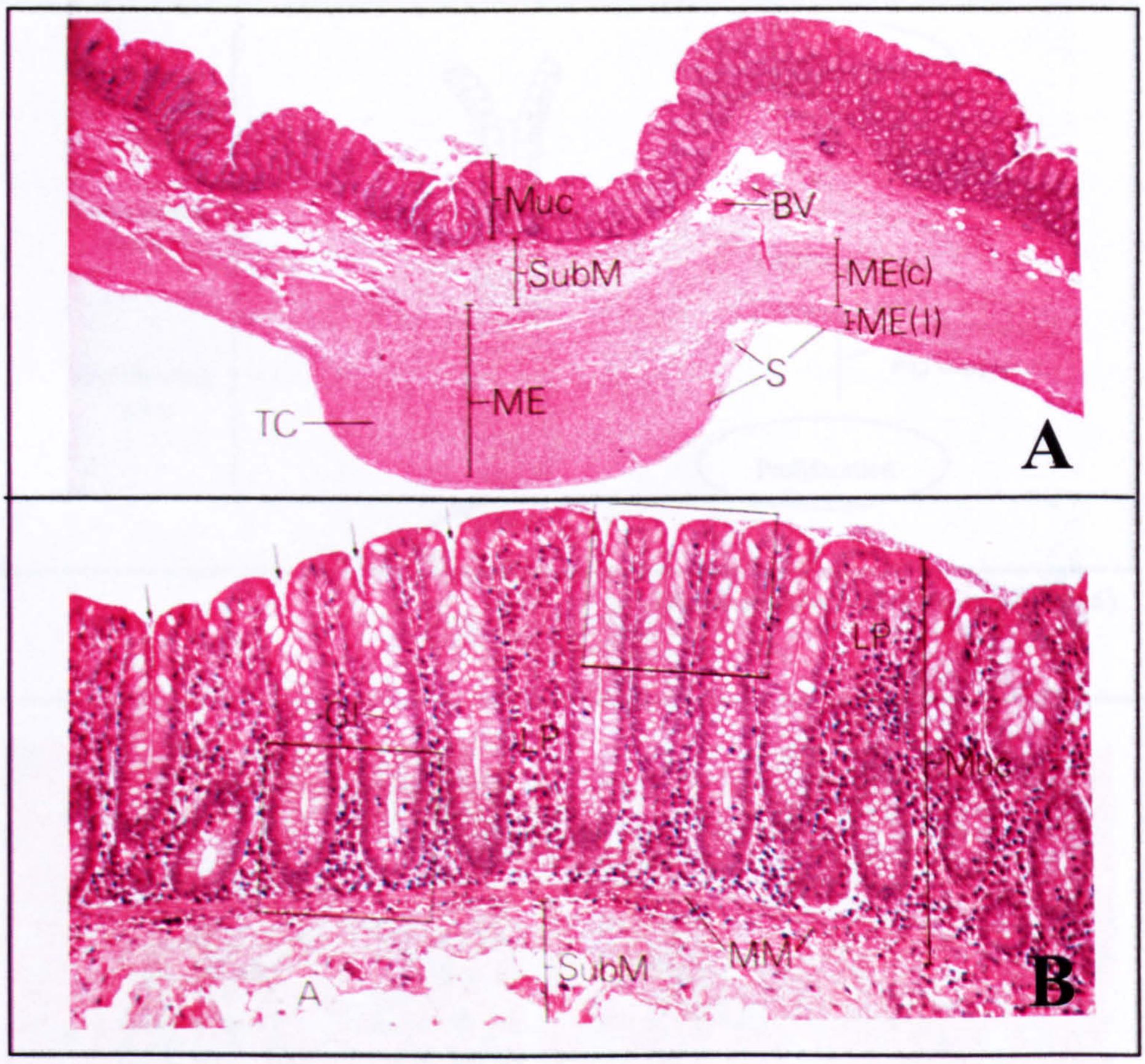
The acquisition of the gastrointestinal microflora has been studied in the mouse (Reviewed by Tannock, 1994). Neonatal mice have environmentally derived bacteria, ten days after birth the numbers of environmentally derived bacteria decrease dramatically, lactobacilli flourish in the proximal colon and enterococci and *E.coli* flourish in the distal colon (Reviewed by Tannock, 1994). Obligate anaerobic bacteria (*bacteriodes* and *fusiform*) become established in the gastrointestinal tract at infancy leading to marked reduction in facultative anaerobes (*enterococcal* and *E.coli*) (Reviewed by Tannock, 1994). Genetics and the environment play a role in determining predominant species within the large intestine. The effect of the environment and genetics on microflora colonisation of the gastrointestinal tract can be demonstrated by the colonisation of the colon by methogenic bacteria. Methogenic bacterial colonisation varies not only by geography but also race. An example of this being the higher incidence of methogenic bacterial colonisation in black South Africans when compared to white South Africans, and again a higher rate of colonisation in those black South Africans living within rural rather than urban areas (Reviewed by Walker, 1995).

1.1.2. The Colonic Epithelium.

The wall of the colon (large intestine) and rectum is made up of four distinct layers; the mucosa, an epithelial lining with underlying connective tissue (lamina propria) and muscularis mucosae (smooth muscle), the submucosa (connective tissue), muscularis externa, and the serosa (Figure 1.2) (Reviewed by Ross *et al.*, 1995).

The colonic epithelium is a rapidly proliferating tissue with a high turn over of cells (Reviewed by Goodlad and Wright, 1990; Reviewed by Cassidy and Fitzpatrick, 1995; Tappenden and McBurney, 1998). The epithelium is arranged in test-tube shaped invaginations known as colonic crypts (crypts of lieberkuhn), which have a defined spatial organisation (Fig 1.3) (Reviewed by Williams *et al.*, 1996; Reviewed

Figure 1.2 The colonic mucosa

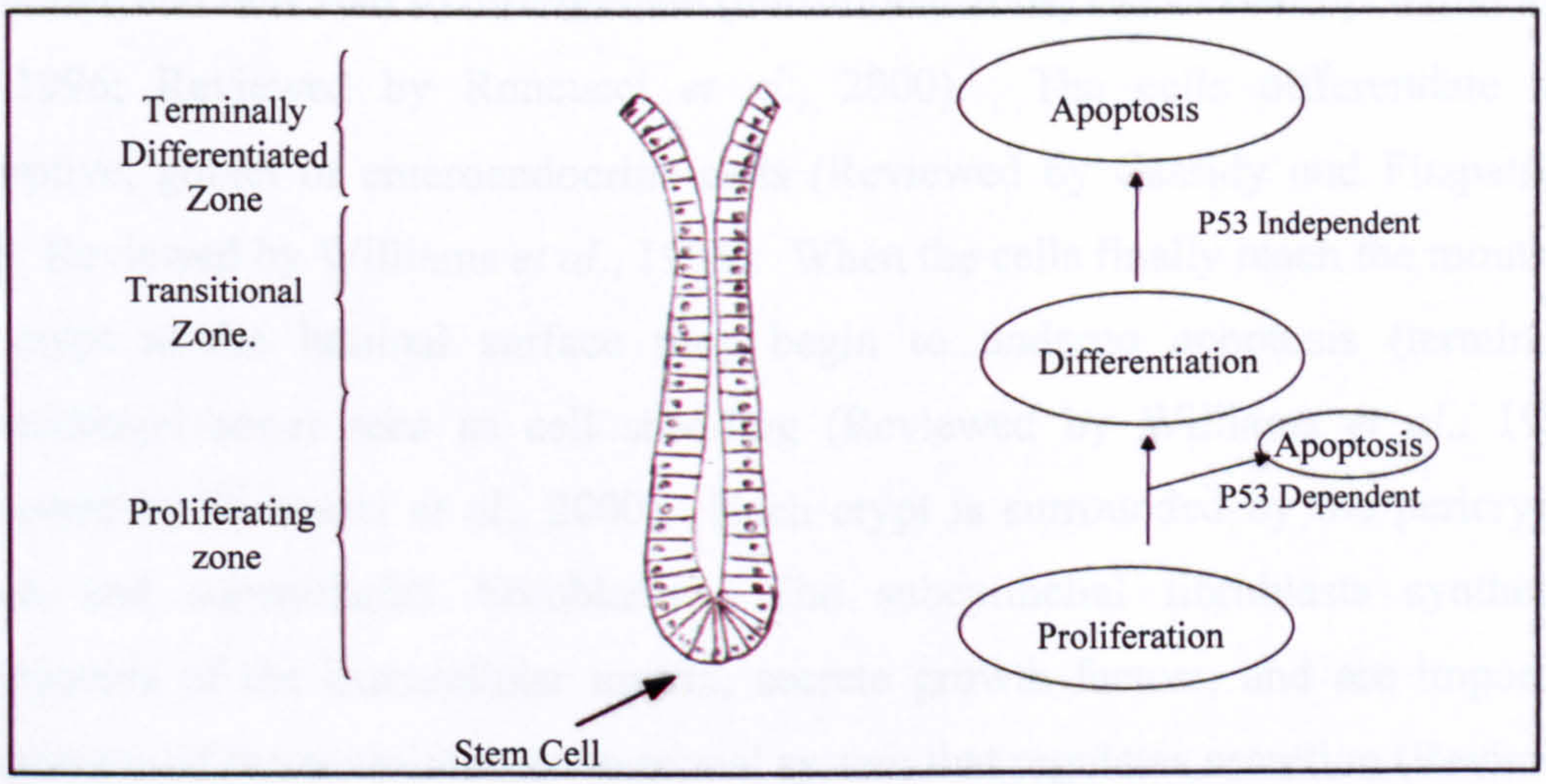


(Reviewed by Ross et al., 1995).

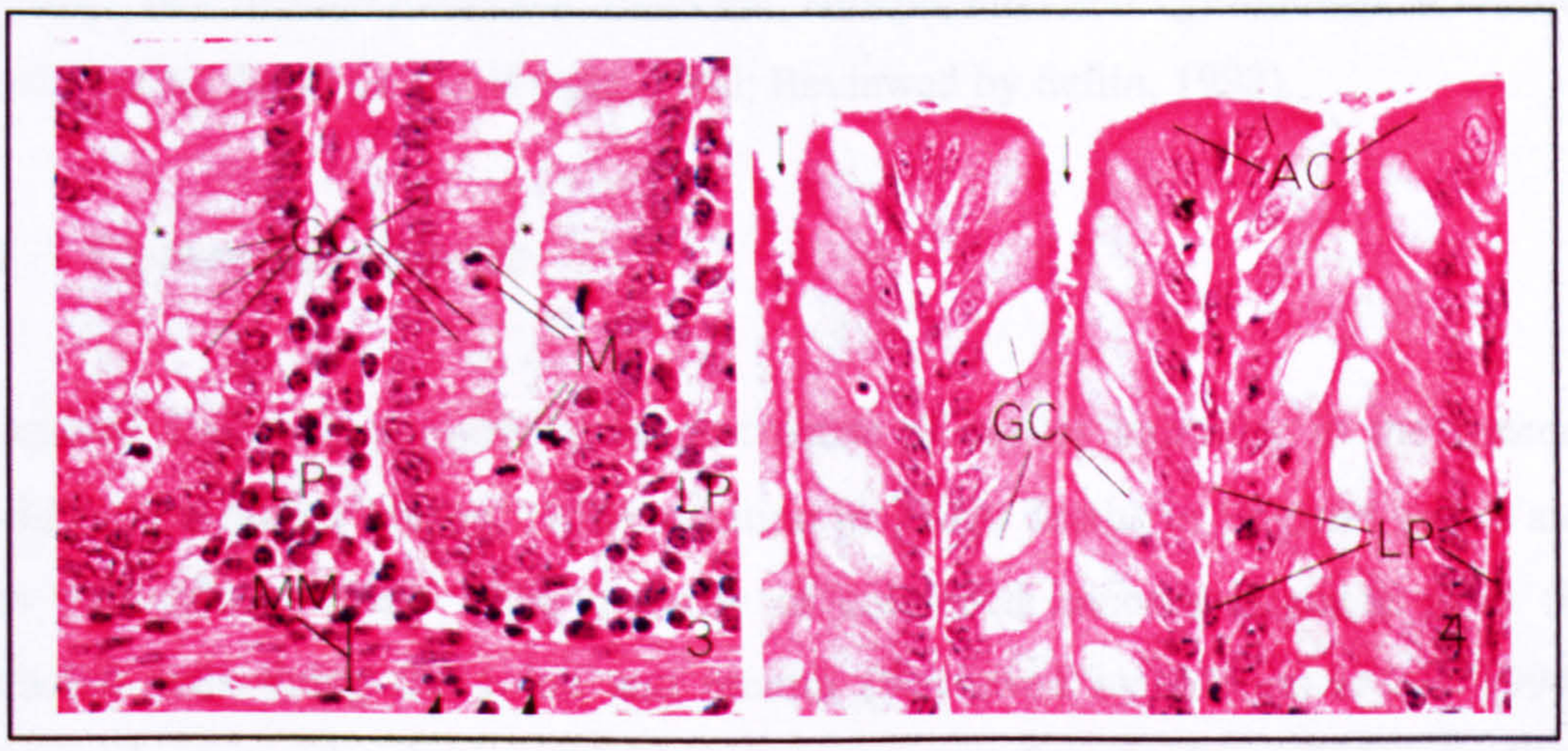
Key. M, Mucosa; LP, Lamina Propria; MM, Muscularis Mucosa; SubM, Sub Mucosa;.ME, Muscularis externa; ME(c), circular layer of muscularis externa; ME(l), longitudinal layer of muscularis externa; S, Serosa; BV, blood vessels.

Figure 1.2 **A**. A cross section of the large intestine from monkey (x30). The wall of the colon (large intestine) and rectum is made up of four distinct layers; the mucosa (**M**), an epithelial lining with underlying connective tissue (lamina propria) and muscularis mucosae (**MM**) (smooth muscle), the submucosa (**SubM**) (connective tissue), muscularis externa (**ME**), and the serosa (**S**) (Reviewed by Ross et al., 1995). **B**. The mucosa (x140) contains straight, unbranched, tubular glands (crypts of lieberkuhn) that extend to the musularis mucosae (**MM**). Between the glands (**GL**) is a lamina propria (**LP**) that contains cells of the immune system. The colonic crypt lies within the pericryptal sheath and undergoes cross talk with the surrounding fibroblasts (Reviewed by Ross et al., 1995) .

Figure 1.3 The colonic crypt



(Reviewed by Williams et al., 1996)



(Reviewed by Ross et al., 1995).

Key: MM, Muscularis Mucosa; LP, Lamina Propria; M, Mitosis; GC, Goblet Cells; AC, Absorbative cells.

Fig 1.3 The cells within the crypt originate from a single stem cell, these cells migrate up to the luminal surface of the crypt, as they migrate they start to differentiate forming absorptive (**AC**), goblet (**GC**) or enteroendocrine cells. At the mouth of the crypt the cells become terminally differentiated, undergoing apoptosis being sloughed off into the lumen (Reviewed by Williams *et al*., 1996; Reviewed by Ross et al., 1995).

by Roncucci *et al.*, 2000). The bottom third of the crypt consists of the proliferating cells (proliferative compartment), each progeny being derived from a precursor stem cell. The cells migrate up the crypt, as they migrate they lose their ability to proliferate and thus start to differentiate (transitional zone) (Reviewed by Williams *et al.*, 1996; Reviewed by Roncucci *et al.*, 2000). The cells differentiate into absorptive, goblet or enteroendocrine cells (Reviewed by Cassidy and Fitzpatrick, 1995; Reviewed by Williams *et al.*, 1996). When the cells finally reach the mouth of the crypt at the luminal surface they begin to undergo apoptosis (terminally differentiated zone) seen as cell shedding (Reviewed by Williams *et al.*, 1996; Reviewed by Roncucci *et al.*, 2000). Each crypt is surrounded by the pericryptal sheath and subepithelial fibroblasts. The subepithelial fibroblasts synthesise components of the extracellular matrix, secrete growth factors, and are important components of the neuroimmunohormonal system that regulates secretion (Reviewed by Sellin, 1993). Epithelial cell function is governed by a large number of signalling pathways that include hormonal, neuronal, subepithelial and cytoskeletal molecules (Reviewed by Goodlad and Wright, 1990; Reviewed by Sellin, 1993).

1.2 Colorectal Cancer

Colorectal Cancer is the second biggest cause of cancer mortality in the western world (Reviewed by Mason, 1998) resulting in 17000 deaths in England and Wales each year (DoH, 1998). The majority of colorectal cancers originate from the epithelial cells lining the colon and rectum (Reviewed by Alberts *et al.*, 1994). Colorectal cancer development is the consequence of a number of abnormalities resulting in a loss of colonic cell homeostasis, allowing tumour formation through excessive cell proliferation and/or a lack of differentiation (Reviewed by Bingham, 1999). Mutations in four or five genes are thought to be required for the development of a malignant tumour, although fewer may lead to the formation of benign tumours (Reviewed by Fearon, 1991). There are two main types of colon cancer; those inherited including familial adenomatous polyposis (FAP) and hereditary non polyposis colorectal cancer (HNPCC), accounting for approximately 15% of all colorectal cancer; and those which sporadically appear accounting for approximately 85% of all colorectal cancers (Reviewed by Kinzler and Vogelstein,

1996). Cancer develops along a three step process; firstly initiation, secondly promotion and thirdly progression (Reviewed by Bingham, 1999), genes and the environment are tightly linked to each stage (Fig 1.4) (Reviewed by Hill, 2001). Thus the development of colorectal cancer is thought to arise from the interaction of genes and their environment (Reviewed by Bingham, 1990; Reviewed by Hill, 1997d).

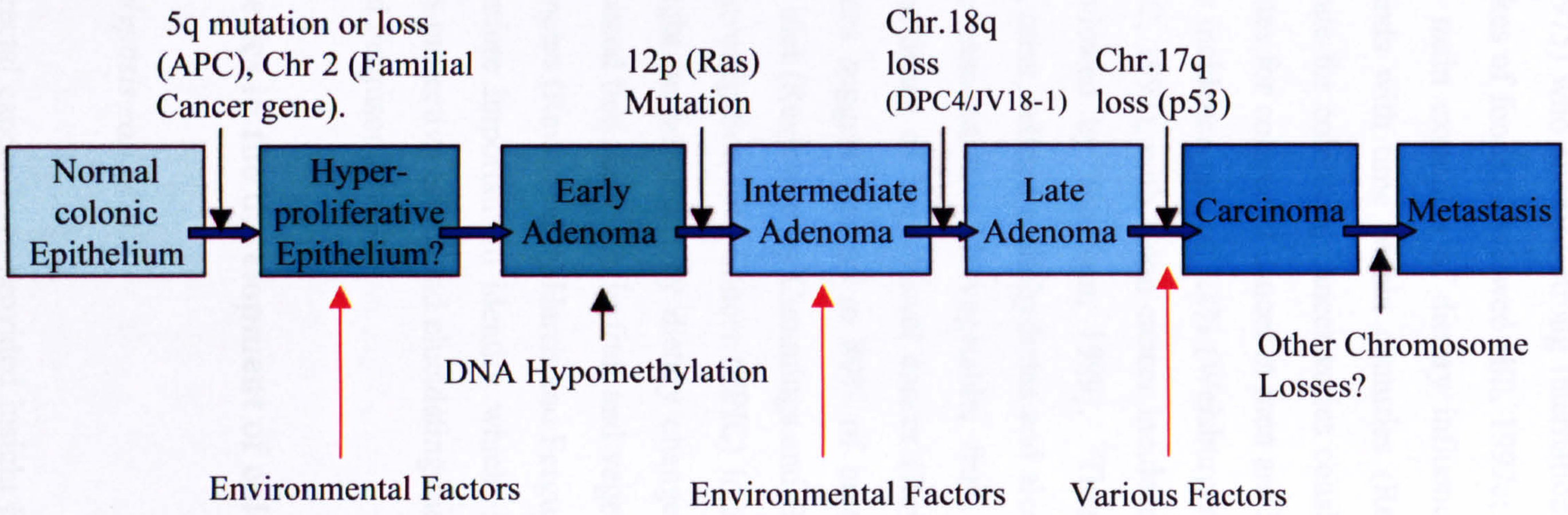
1.2.1 The role of genetics in colorectal cancer development.

The genetic steps involved in the development of colorectal cancer have been well studied and the adenoma to carcinoma sequence has been well elucidated (Figure 1.4) (Fearon and Vogelstein, 1990; Reviewed by Kinzler and Vogelstein, 1996; Reviewed Gryfe *et al.*, 1997; Reviewed by White, 1998). The first genetic alteration in the multistep process, in inherited and sporadic tumours, is the loss of the *APC* tumour suppressor gene (Reviewed by Kinzler and Vogelstein, 1996). Other genetic changes are also summarised in Fig 1.4, revealing the role of loss of a number of tumour suppressor genes, the induction of a number of oncogenes and the acetylation of DNA (Fearon and Vogelstein, 1990; Reviewed by Kinzler and Vogelstein, 1996; Reviewed Gryfe *et al.*, 1997; Reviewed by White, 1998). This helped to elucidate that colon cancer provides a clear example of tumour progression where adenomatous polyps are believed to be the precursors of colorectal cancers (Reviewed by Alberts *et al.*, 1994). Other genetic factors may also play a role in influencing an individuals' cancer risk, with individuals inheriting different enzymatic capacities to detoxify or activate carcinogens (Reviewed by Bingham, 1999).

1.2.2 *Environmental factors in the development of colorectal cancer*

The majority of major cancers; breast, colon, lung, prostate and oesophageal, occur in epithelial tissues (Reviewed by Alberts *et al.*, 1994; Reviewed by Bingham, 1999), and appear to be influenced by environmental factors (Reviewed by Bingham, 1999). The environmental factors for colon cancer include a number of socio-economic factors such as education (in terms of what a good diet consists of), age, physical

Figure 1.4 Environmental and Genetic factors associated with the adenoma to carcinoma sequence (Adapted from Fearon and Vogelstein, 1990; Reviewed by Kinzler and Vogelstein, 1996; Reviewd by Hill, 2001).



activity, diet, alcohol consumption, tobacco smoke and obesity along with psychological variables such as stress (Jarosz and Perczynska, 2001).

The work on the relationship between diet and cancer was prompted by the studies of Armstrong and Doll (1975) who showed strong international associations between rates of cancer and intakes of foods (Reviewed Hill, 1997c; Reviewed by Bingham, 1999). There are two main examples of dietary influences involving migration studies and marked trends with time within countries (Reviewed by Key *et al.*, 2002). The incidence rate for colorectal cancer varies considerably geographically, in the USA incidence rates for colorectal cancer in men are 34.1%, whereas in India men have a much lower incidence rate of 1.8% (Weisburger *et al.*, 1977; Reviewed by Bingham, 1990; CRC, 1993), with colon cancer incidence rates varying 20 fold between countries (Reviewed by Bingham, 1999). There is a strong positive association between fat, meat, refined carbohydrates and alcohol consumption along with a strong negative association with vegetable, fruit, vitamins and calcium consumption and the incidence of large bowel cancer (Reviewed by Kim, 2000). Epidemiological estimates suggest that up to 80% of breast, bowel and prostate cancer is attributable to diet (Reviewed by Cummings and Bingham, 1998) with the European Prospective Investigation into Cancer (EPIC) indicating that 9 out of 10 colon cancer deaths might be prevented by dietary change (Reviewed by Mason, 1998). It has been proposed that diets rich in fruit and vegetables may decrease the risk of many of these cancers (Reviewed by Harris and Ferguson, 1993; Reviewed by Kim, 2000). It is therefore important to identify which, if any, of these dietary factors contribute to this protective effect and elucidating their mechanism of action (Reviewed by Harris and Ferguson, 1993).

1.3 The role of Genetics in the development of colorectal cancer

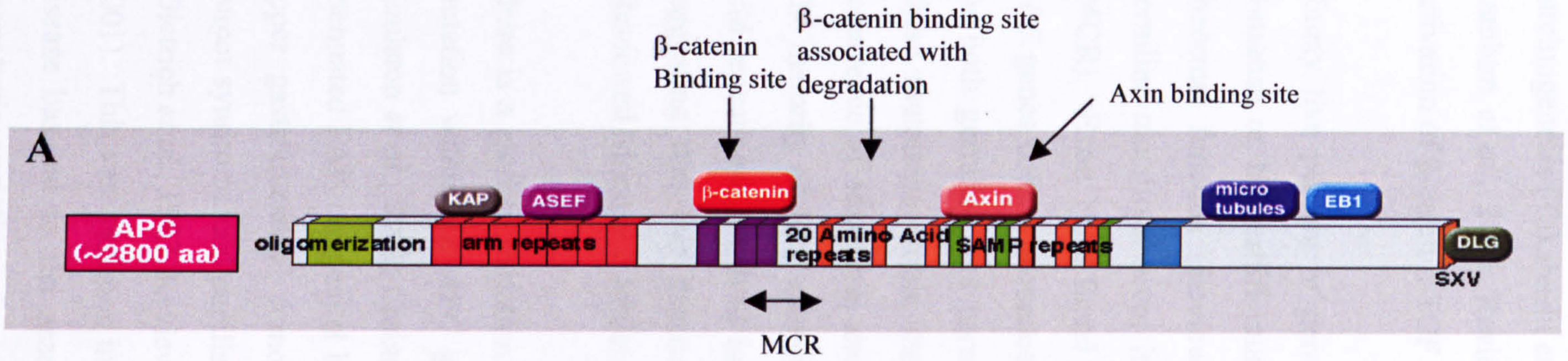
1.3.1 Adenomatous polyposis coli (APC)

Inherited forms of colorectal cancer have provided insight into the genetic changes associated with disease development. Familial Adenomatous Polyposis (FAP) is an inherited autosomal dominant disease resulting in the inheritance of a mutant *APC*

gene, followed by a second somatic mutation, leading to the early onset of colorectal cancer (Levy *et al.*, 1994; Reviewed by Kinzler and Vogelstein, 1996; Reviewed Gryfe *et al.*, 1997; Reviewed by Fearnhead *et al.*, 2001). Studies into FAP led to the identification of the *APC* gene and was shown to be the first genetic alteration in the development of colorectal cancer (Reviewed by Kinzler and Vogelstein, 1996; Reviewed Ilyas and Thomlinson, 1997; Reviewed by White, 1998; Reviewed by Goss and Groden, 2000) with up to 95% of patients with FAP carrying inactivating mutations in *APC* (Larken *et al.*, 1999). 80-85% of sporadic adenomas are thought to carry an *APC* mutation, resulting in loss of protein function (Reviewed by Nakamura, 1997). *APC* has been described as a gatekeeper or tumour suppressor gene regulating growth of tumours through the regulation of colonic homeostasis by inhibiting cell growth and/or promoting cell death (Reviewed by Kinzler and Vogelstein, 1996; Morin *et al.*, 1997a; Reviewed by Morin *et al.*, 1997b; Reviewed by Kinzler and Vogelstein, 1998; Mulkens *et al.*, 1998) with APC mutations being associated with increased proliferation within adenomas (Mulkens *et al.*, 1998). The APC protein also has a number of functional roles in cell signalling, intercellular adhesion, migration and apoptosis (Vleminckx *et al.*, 1997; Reviewed by Fearnhead *et al.*, 2001).

The *APC* gene has been mapped to chromosome 5q21 and shown to consist of 8535 base pairs spanning 21 exons encoding for a 2843 amino acid protein (Reviewed by Morin *et al.*, 1997; Reviewed by Fearnhead *et al.*, 2001). A homologue of APC, APC2, has also been described and is thought to have functions comparable to that of APC (van Es *et al.*, 1999). The APC protein consists of a large number of domains (Figure 1.5); including oligomerisation, armadillo region (*Arm*), 15 amino acid (aa)repeat region that binds β-catenin, 20 aa repeat motif TPXXFSXXXSL that also binds β-catenin, basic domain, EB1 domain and HDLG (human discs large) domain (Reviewed by Fearnhead *et al.*, 2001) that binds HDLG (Matsumine *et al.*, 1996). APC is a cytoplasmic protein found in association with γ- and β-catenin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993; Reviewed Jawhari *et al.*, 1997a; Reviewed by Moon and Miller, 1997) and is thought to function by controlling the cytoplasmic and nuclear levels of β-catenin (Reviewed by Moon and Miller, 1997; Reviewed by Morin *et al.*, 1997b; Reviewed by Ben-Ze'ev and Geiger, 1998; Reviewed by Gordon, 1998;

Figure 1.5 The structure and molecular interactions of (A) APC and (B) β-catenin



A) APC: this molecule consists of several functional domains and repeat motifs, the mutational cluster region (MCR) results in the loss of the C-terminal region, which results in a protein that is unable to degrade β-catenin. B) β-catenin contains multiple binding sites particularly involved in cell adhesion including α-catenin and E-cadherin, β-catenin cell signalling via binding to Tcf and β-catenin degradation by the binding and phosphorylation of the protein by GSK-3 β, APC and Axin. APC and E-cadherin binding sites within the *ARM* repeat region are thought to overlap (Hülsken et al., 1994) (Adapted from Ilyas et al., 1997; Nusse, 2002).

Reviewed by White, 1998). The stabilisation and accumulation of cytoplasmic β-catenin, resulting from 'loss of function' mutations in *APC*, or 'gain of function' mutations in *β-catenin* gene have been found to be associated with colon carcinogenesis (Takahashi *et al.*, 1998; Rubinfeld *et al.*, 1996; Müller *et al.*, 1998; Lamlum *et al.*, 2000; Reviewed by Fearnfead *et al.*, 2001) and is coupled to the activation of β-catenin-TCF signaling (Morin *et al.*, 1997a).

Ninety five percent of germline mutations in the *APC* gene of FAP patients are nonsense or frameshift mutations that result in a truncated APC protein with an abnormal function (Reviewed by Fearnhead *et al.*, 2001). The most common germline mutations occur in codons 1061 and 1309, the mutational cluster region (MCR). Exon 15 is found within the MCR, Exon 15 contains more than 75% of *APC* genes coding sequence, possibly explaining why it is the most common target for both germline and somatic mutations (Reviewed by Fearnhead *et al.*, 2001). These mutations within the MCR have been associated with an increase in the occurrence of adenomas and these tend to be larger in size (Mulkens *et al.*, 1998). The majority of APC mutants lack part, if not all, the 20-aa repeats. These amino acid repeats are involved in the binding and regulation of β-catenin protein levels suggesting that this function is targeted for elimination during tumourigenesis (Reviewed Moon and Miller, 1997; Reviewed Fearnhead *et al.*, 2001).

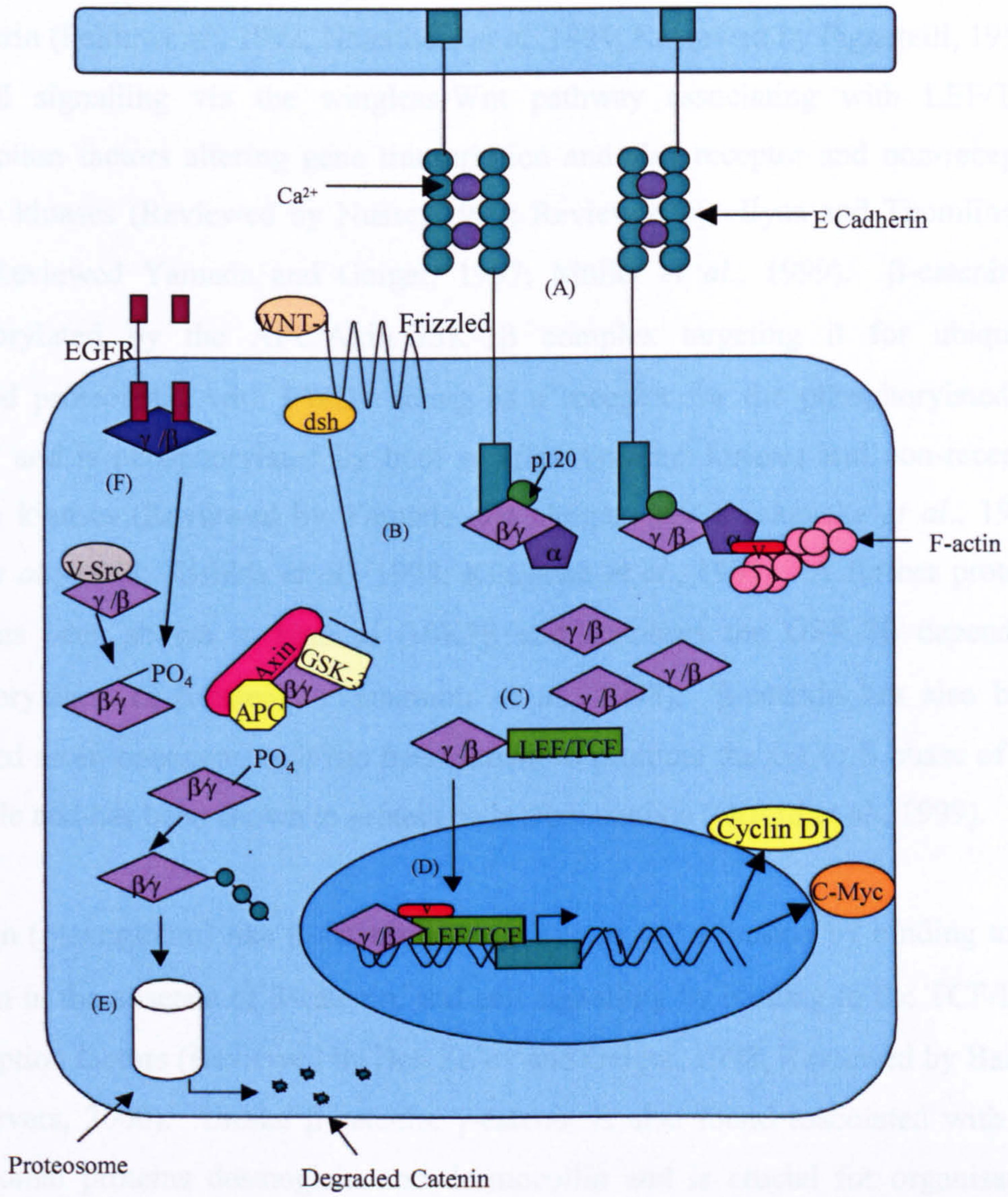
There is a marked variation in phenotype of FAP depending on the position of the mutation within the *APC* gene (Reviewed by Fearnhead *et al.*, 2001; Reviewed Houlston *et al.*, 2001; Crabtree *et al.*, 2002). Phenotypic variations of FAP include attenuated FAP, congenital hypertrophy of the retinal pigment epithelium (CHRPE), upper gastrointestinal tumours (periampullary carcinoma), Gardner's syndrome, Turcot syndrome and papillary carcinoma of the thyroid and adrenocortical tumours (Dietrich *et al.*, 1993; Reviewed by Fearnhead *et al.*, 2001; Reviewed Houlston *et al.*, 2001). This vast variation in germline *APC* gene mutations and the phenotype of the disease has led to the search for modifier genes that may be involved in this phenotypic variation, for example mom-1 (Dietrich *et al.*, 1993; Reviewed by Fearnhead *et al.*, 2001; Reviewed Houlston *et al.*, 2001; Crabtree *et al.*, 2002).

1.3.2 The Catenin Family.

The catenins are a family of cytoplasmic proteins involved in a number of cellular processes including cell adhesion and signal transduction (figure 1.6). The multigene family comprises of α- (102Kda), β- (92Kd), γ- (Plakoglobin) (83Kd) *catenin*, and *p120*$^{(CAS/ctn)}$, and are found on chromosomes 5q21-q22, 3p (3p22-p21.3), 17q21 and 11q11 respectively (Peifer *et al.*, 1992; McPherson *et al.*, 1994; van Hengel *et al.*, 1995; Reynolds *et al.*, 1996; Liu *et al.*, 1997; Reviewed by Smith and Pignatelli, 1997; Aono *et al.*, 1999; Liang and Goltesman, 2003). β-catenin is also homologous to the armadillo protein found in Drosophila, suggesting that the catenins have been evolutionarily conserved (Reviewed by Gumbiner, 1995; Reviewed by Ilyas and Thomlinson, 1997). The catenin proteins have three structurally distinct regions; an amino and carboxyl terminal domain together with a hydrophobic region comprising of arm repeats, each repeat consisting of 42 amino acids. The number of arm repeats vary between the catenins with both β- and γ-catenin having 13 arm repeats, whereas p120 has 11 (Reynolds *et al.*, 1994; Ruiz *et al.*, 1996; Troyanosky *et al.*, 1996; Reviewed by Ilyas and Thomlinson, 1997). Of the catenins, γ-catenin and β-catenin share the greatest homology, with the γ-catenin arm repeat region being 85% homologous to β-catenin (Troyanosky *et al.*, 1996). The catenins have been found to be associated with a large number of proteins namely APC, cadherins (in adhesive junctions), epidermal growth factor receptor (EGFR), C-erbB2, Tyrosine Kinases (v-src) and Phosphatases (Vandate and PTPμ), vinculin, α-actinin, fascin, paxillin, axin, GSK-3β and the transcription factors TCF (T cell Factor) and LEF (Leukocyte enhancing factor) (Brady-Kalnay *et al*, 1995; Reviewed by Nakamura, 1997; Hiscox and Jiang, 1998a; Ikeda *et al.*, 1998; Reviewed by Pignatelli, 1998; Reviewed by Hirohashi, 2000).

β-catenin (Fig 1.5) is the most widely studied, having diverse binding specificities and as a consequence functionality. Many of the activities of β-catenin are regulated through the formation of functional complexes with other molecules such as APC, GSK-3β, axin, E-cadherin, α-catenin and hLEF-hTCF, or the phosphorylation of β-catenin by tyrosine kinases such as EGFR, ErbB, c-MET and Src (Shibamoto *et al*, 1995; Reviewed by Ilyas and Thomlinson, 1997; Reviewed Yamada and Geiger,

Figure 1.6 Catenins and their associated proteins.



(A) E-Cadherin forms calcium dependent homotypic interactions with adjacent cells. The cytoplasmic domain of E-Cadherin forms a complex with either β-catenin/α-catenin or γ-catenin/α-catenin, with α-catenin linking it to the actin cytoskeleton via interactions with α-actinin, vinculin or fascin (V) (Näthke *et al*., 1994; Barth *et al*., 1997a; Hulsken *et al*, 1994; Imamura *et al*., 1999). (B) Wnt-1 pathway leading to the inhibition of GSK-3β which leads to the stabilisation and accumulation of β-catenin within the cytoplasm. (C) Cytoplasmic accumulation of β-catenin can result its association with the transcription factors hTCF-4/hLEF-1, resulting in translocation into the nucleus (Reviewed by Nusse, 1997; Müller *et al*., 1999; Reviewed by Ilyas and Thomlinson, 1997; Reviewed Yamada and Geiger, 1997). (D) In the nucleus β-catenin/Tcf complex binds to the DNA resulting in gene expression, targets include *c-Myc* (He *et al*., 1998) and *CyclinD1 (*Shtutman *et al*., 1999*)* . (E) β-catenin is phosphorylated by a complex consisting of APC, GSK-3β and Axin resulting in the targeting of β-catenin for ubiquitin dependent proteosomal degradation, and (F) tyrosine kinases and phosphatases including c-Src and EGFR (Sakanaka *et al*., 1998; Ikeda *et al*., 1998; Kishida *et al*., 1998; Kitagawa *et al*., 1999; Reviewed by Yamada and Geiger, 1997).

1997; Hart *et al.*, 1998; Hiscox and Jiang, 1999b). Both E-cadherin and APC compete for binding to β-catenin and this interaction is mediated by the internal *Arm* repeats (Hülsken *et al.*, 1994). β-catenin plays a role in cell adhesion via binding to E-cadherin (Peifer *et al.*, 1992; Nagafuchi *et al.*,1994; Reviewed by Pignatelli, 1998), and cell signalling via the wingless-Wnt pathway associating with LEF/TCF transcription factors altering gene transcription and also receptor and non-receptor tyrosine kinases (Reviewed by Nusse, 1997; Reviewed by Ilyas and Thomlinson, 1997; Reviewed Yamada and Geiger, 1997; Müller *et al.*, 1999). β-catenin is phosphorylated by the APC/Axin/GSK-3β complex targeting it for ubiquitin mediated proteolysis, with FWD1 acting as a receptor for the phosphorylated β-catenin, and is phosphorylated by both receptor tyrosine kinases and non-receptor tyrosine kinases (Reviewed by Yamada and Geiger, 1997; Sakanaka *et al.*, 1998; Ikeda *et al.*, 1998; Kishida *et al.*, 1998; Kitagawa *et al.*, 1999). A further protein, Axil, has been shown to bind to GSK3β and enhances the GSK-3β dependent phosphorylation of β-catenin (Yamamoto *et al.*, 1998). β-catenin has also been described as an oncogene as it has been shown to promote the G1 to S phase of the cell cycle and has been shown to protect cells from anoikis (Orford *et al.*, 1999).

γ-catenin (plakoglobin) like β-catenin plays a role in cell adhesion by binding to E-cadherin in the absence of β-catenin, and cell signalling by binding to the TCF/LEF transcription factors (Reviewed by Ben Ze'ev and Geiger, 1998, Reviewed by Barker and Clevers, 2000). Unlike β-catenin, γ-catenin is also found associated with the desmosomal proteins desmoglein and desmocollin and is crucial for organisation desmosomal junctions (Birchmeier et al., 1996; Troyanosky *et al.*, 1996; Ruiz *et al.*, 1996).

α-catenin has two isoforms of the protein, α-E which is found in epithelial tissue and α-N which is found in neuronal tissues (Hirano *et al.*, 1992; Hoschuetzky *et al.*, 1994). α-catenin binds directly to a number of proteins, including γ-catenin and β-catenin, APC, vinculin, α-actinin and v-src. It has also been shown to bind indirectly to E-cadherin via γ-catenin and β-catenin, having an essential role in cell adhesion (Oyama *et al.*, 1994; Reviewed by Barth *et al.*, 1997b; Weiss *et al.*, 1998; Reviewed by Hirohashi, 1998) playing a functional role at adherens junctions. α-catenin-

vinculin complexes have also been shown to play a fundamental role in the assembly of apical junctional complex (tight junctions) (Watabe-Uchida *et al.*, 1998). α-catenin and the actin cytoskeleton has been shown to be essential for the strong state of the E-cadherin adhesion system (Imamura *et al.*, 1999).

p120 (Cas, cadherin-associated src substrate, ctn, catenin) was originally identified as one of several substrates of the tyrosine kinase pp60src (Reynolds *et al.*, 1994; Liu *et al.*,1997). Subsequently $p120^{(ctn)}$ was shown to associate with β-catenin and E-cadherin in cell adhesive complexes (Shibamoto *et al.*, 1995; Liu *et al.*, 1997) but its role in cell adhesion still remains to be elucidated. Unlike β- and γ- catenins, p120 has been shown to have at least 4 different isoforms, which are expressed differentially in a variety of cell types (Reynolds *et al.*, 1994).

1.3.3 The Catenin family (α-, β-, γ-), p120 and E-Cadherin in Cell Adhesion.

Cell adhesion to neighbouring cells, via cadherin-catenin interactions plays an important role in cell motility, growth, differentiation and survival (Pignatelli *et al.*, 1992; Watabe *et al.*, 1994; Reviewed by Ben-Ze'ev and Geiger, 1998; Reviewed by Efstathiou and Pignatelli, 1998a; Reviewed by Pignatelli, 1998). Perturbation of any of these interactions results in changes in intercellular adhesion and cell transformation (Pignatelli *et al.*, 1992; Valizadeh *et al.*, 1997; Guilford *et al.*, 1998).

The cadherins are a family of transmembrane glycoproteins that mediate calcium dependent adhesion (Gagliardi *et al.*, 1995; Chitaev and Troyanovsky, 1998), sharing common amino acid sequences. They have been divided up into more than 10 subclasses dependent on their tissue distribution, including E- (Epithelial), N- (Neuronal) and P- (Placental) cadherin (Shiozaki *et al.*, 1994; Reviewed by Smith and Pignatelli, 1997), with *Fat* a protein homologous to cadherin, found in Drosophila, suggesting evolutionary conservation (Mahoney *et al.*, 1991).

The *E-cadherin* gene (*CDH 1*), found on chromosome 16q22.1, was first isolated by Berx *et al.* (1995), and was found to span 100Kb consisting of 16 exons (Berx *et al.*, 1995). E-cadherin (uvomorulin, L-Cam, Arc 1 and cell-CAM 120/80) (Gagliardi *et*

al., 1995) is located at zipper like adherens junctions forming calcium dependent homophilic interactions between cells (Shiozaki *et al.*, 1994; Gagliardi *et al.*, 1995; Reviewed by Smith and Pignatelli, 1997) tending to concentrate at the apical membrane (Näthke *et al.*, 1994). E-cadherin homotypic dimer formation at the cell membrane is promoted by calcium (Nagar *et al.*, 1996). E-cadherin functional adhesive complexes are linked to the actin cytoskeleton via interactions with β- or γ-catenin and p120 and indirectly by α-catenin, α- actinin, vinculin, IQGAP and calmodulin (figure 1.6) (Hülsken *et al*, 1994; Näthke *et al.*, 1994; Barth *et al.*, 1997a; Imamura *et al.*, 1999). Changes in the expression levels of cadherins and catenins have been shown to affect cell-cell adhesion (Reviewed by Adams and Nelson, 1998). Catenins are thought to mediate E-cadherin adhesive functions (Reviewed by Kemler, 1993; Reviewed by Smith and Pignatelli, 1997) and in the absence of β-catenin the E-cadherin cytoplasmic domain becomes unstructured (Huber *et al.*, 2001).

β-catenin has been shown to mediate epithelial cell adhesion by binding to the cytoplasmic domain of E-cadherin (Jou *et al.*, 1995), subsequently linking E-cadherin to the actin cytoskeleton by binding to α-catenin via its amino terminal (Reviewed by Yamada and Geiger, 1997), or directly associating with vinculin, α-actinin or fascin (an actin bundling protein) (Reviewed by Smith and Pignatelli, 1997; Reviewed by Polakis, 1998). γ-catenin has also been shown to bind to E-cadherin (Knudsen and Wheelock, 1992; Troyanosky *et al.*, 1996; Reviewed by Barth *et al.*, 1997b; Reviewed Ben Ze'ev and Geiger, 1998), with evidence pointing to β-catenin and γ-catenin forming mutually exclusive complexes (Reviewed by Barth *et al.*, 1997b). The domains of γ-catenin and β-catenin, which associate with cadherins and the cytoskeleton, have been mapped. It was found that while the central arm repeats of catenins interact with cadherins, the amino terminal domain, as well as the first arm repeat bind to α-catenin (Ruiz *et al.*, 1996). Unlike β-catenin, γ-catenin is also found associated with desmosomal cadherins, mainly desmoglein (Dsg) and desmocollin (Dsc) (Troyanosky *et al.*, 1996; Reviewed by Barth *et al.*, 1997b) in desmosomes. γ-catenin functions to sort and assemble desmosomal junctional components (Reviewed by Barth *et al.*, 1997b; Lewis *et al.*, 1997) with the suggestion that γ-catenin maybe a negative regulator of adheren junction formation

(Ruiz *et al.*; 1996). Troyanosky *et al.* (1996) suggested that it is the *Arm* repeat region of plakoglobin that mediates these functions. The *Arm* repeat region was shown to comprise of two functionally distinct regions, with the first 5 *Arm* repeats involved in specific binding of E-cadherin and desmoglein, with the remaining arm repeats being involved in targeting plakoglobin/cadherin complexes to junctional structures (Troyanosky *et al.*, 1996).

α-catenin, an actin bundling protein *in vitro,* is involved in adhesion by indirectly linking cadherin to the actin cytoskeleton (Reviewed by Adams and Nelson, 1998). α-catenin mediates the connection between the cadherin-catenin complex with the actin filaments (Hoschuetzky *et al.*, 1994; Reviewed by Efstashiou *et al*, 1998a; Reviewed by Hirohashi, 1998). Furthermore αE-catenin has been shown, in association with vinculin, to play a role in the assembly of apical junctional complex within the epithelium (Watabe-Uchida *et al*, 1998). Adams and Nelson (1998) have suggested that vinculin, a protein homologous to α-catenin, may bind to the cadherin/β-catenin or cadherin/γ-catenin complex in the absence α-catenin as a form of compensation (Shiozaki *et al.*, 1994; Nagafuchi *et al*, 1994; Reviewed by Adams and Nelson, 1998). p120, like β- and γ-catenin, has been shown to bind directly to E-cadherin, and is found associated with both the E-cadherin/β-catenin and the E-cadherin/γ-catenin complexes (Jou *et al.*, 1995; Shibamoto *et al*, 1995; Troyanosky *et al.*, 1996; Reviewed by Barth *et al.*, 1997b). Unlike β- and γ-catenin p120 does not bind α-catenin (Jou *et al.*, 1995). Skoudy *et al* (1996) results suggest that p120 plays a role in regulating E-cadherin, although the exact role remains a matter of debate (Skoudy *et al.*, 1996; Reviewed by Adams and Nelson, 1998). It has been suggested that p120 plays a role in blocking cellular adhesion (Aono *et al.*, 1999). Cadherin-p120 complex maybe formed initially to increase the rates of homotypic recognition and binding between cadherin molecules on two adjacent cells. Subsequently α-catenin, β-catenin and the actin cytoskeleton may associate with and stabilize the cadherin-p120 cluster and, thereby, strengthen the cell-cell adhesion. This interpretation of Skoudy *et al* (1996) results would explain why cells that overexpress cadherin with an intact β-catenin binding region, but lacking the p120 binding region, adhere very slowly (Reviewed Adams and Nelson, 1998). Alternatively as p120 is a major src substrate and is phosphorylated in response to

ligand stimulation of receptor tyrosine kinases (Reynolds *et al.*, 1994; Liu *et al.*, 1997; Valizadeh *et al.*, 1997) it may act to mediate the regulation of cadherin adhesion by these signalling pathways (Reynolds *et al.*, 1994; Hoschuetzky *et al*, 1994; Reviewed by Barth *et al.*, 1997b; Ozawa and Ohkubo, 2001). The proposed role for p120 in cellular adhesion is that it couples the formation and disruption of cadherin mediated cell to cell contacts during cell motility by triggering signalling pathways that involve the Rho family of GTPases (Grosheva *et al.*, 2001).

A number of other proteins have been shown to be associated with the E-cadherin/catenin complex. Catenins are involved in tyrosine kinases and phosphatase signalling pathways that regulate cell adhesion (Reviewed by Barth *et al.*, 1997b). A number of non-receptor and receptor tyrosine kinases, including small G proteins, Ras, cdc42 and Rac-1, involved in cell survival, death and the remodelling of the actin cytoskeleton (Reviewed by Braga, 2000), EGFR, and receptor tyrosine phosphatase, protein-tyrosine phosphatases (PTPμ and PTP 1B), alter the phosphorylation status of components of the cadherin-catenin complex (Hoschvetzky *et al*, 1994; Brady-Kalnoy *et al.*, 1995; Liu *et al.*, 1997; Balsamo *et al.*, 1998). Each of the different kinases/phosphatases has a different specificity for cadherin/catenin complex components. For example TFF3 (Trefoil factor 3) has been shown to cause rapid and specific tyrosine phosphorylation of β-catenin and epidermal growth factor receptor, but not E-cadherin or α-catenin (Liu *et al.*, 1997). Hepatocyte growth factor/scatter factor (HGF/SF) has been shown to induce the phosphorylation of p120, β- and γ- catenin (Shibamoto *et al*, 1995). The adaptor protein Shc has been shown to bind to and phosphorylate E-cadherin inhibiting the binding of β-catenin (Xu and Carpenter, 1999). Hoschuetzky *et al.* (1994) clearly demonstrated that EGF results in the tyrosine phosphorylation of β- and γ- catenin, whereas with v-src the primary targets are α-, β- catenin and $p120^{(Cas)}$ (Hoschuetzky *et al.*,1994; Liu *et al.*, 1997; Valizadeh *et al.*, 1997). Hoschuetzky *et al.* (1994) also demonstrated that this is also cell type specific. Tyrosine phosphorylation of β-catenin results in cytoplasmic accumulation and cellular migration, expression of PTP LAR (protein tyrosine phosphatase leukocyte common antigen) has been shown to inhibit this migration, colocalising with E-cadherin-catenin complex regulating cell adhesion (Müller *et al.*, 1999). The phosphorylation of tyrosine residues in the

β-catenin protein was observed in mammalian fibroblasts transduced with v-src, where src was shown to accumulate at adherens junctions and increase tyrosine phophorylation levels (Tsukita *et al* 1991). The precise biological consequence of tyrosine phosphorylation of the catenins is unknown. It has been suggested that it inactivates cadherin-mediated adhesion, with phosphorylated β-catenin being exclusively found in detergent soluble fractions, suggesting that tyrosine phosphorylation induces disassembly of the cadherin-catenin complex from the actin filament network, although other explanations are possible (Hoschuetzky *et al.*, 1994; Reviewed by Barth *et al*, 1997b; Liu *et al.*, 1997). This loss of cadherin-mediated adhesion, through tyrosine phosphorylation, resulting in destabilisation of the adherens junctions, was shown to induce cell dispersion/migration (Liu *et al.*, 1997) and is an important factor in determining the biological properties of human cancers (Reviewed by Hirohashi, 2000).

1.3.4 Cell Signalling and the regulation of the catenin family.

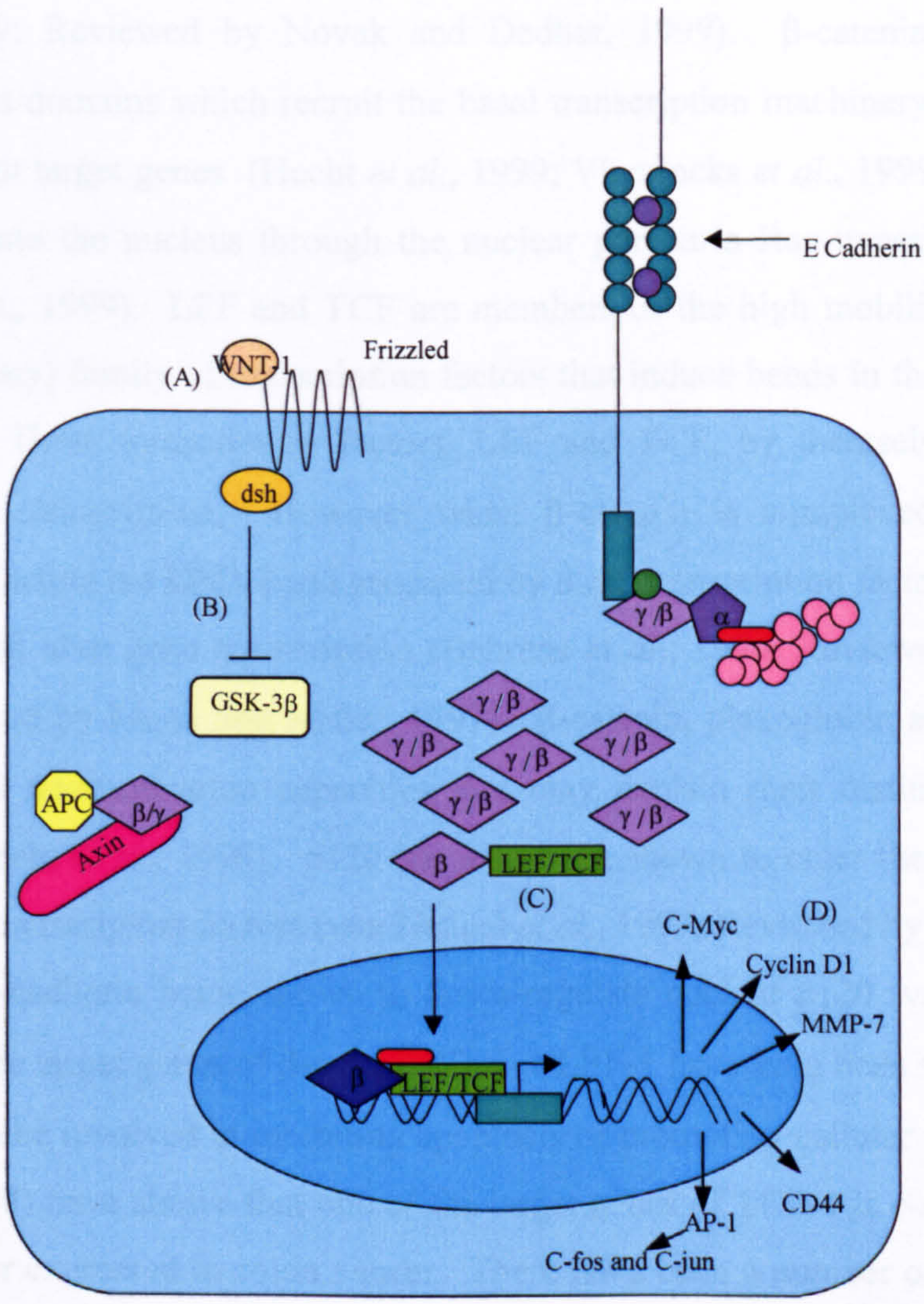
Studies in Drosophila revealed the role for armadillo in the wingless-wnt pathway that mediates cell fate determination (Reviewed by Barth *et al.*, 1997b). The WNT (wnt-1, wnt-2 and wnt-3) pathway has been shown to be involved in a number of cellular processes including cell polarity, growth, proliferation, differentiation and apoptosis (Reviewed by Gumbiner, 1995; Reviewed Gordon, 1998). As β-catenin is highly homologous to the armadillo protein it was proposed that β-catenin would play a part in the vertebrate wingless-wnt pathway (Reviewed by Gumbiner, 1995; Reviewed by Nusse, 1997; Reviewed by Ilyas and Thomlinson, 1997), subsequently revealing the importance of β-catenin in embryogenesis (Reviewed by Barth *et al.*, 1997b). β-catenin is a major player in the Wnt/wingless signalling pathway, Hinck *et al.* (1994a) showed that Wnt-1 expression resulted in the accumulation of β-catenin and γ-catenin in the cytoplasm. This cytoplasmic accumulation was coupled with an increase in cellular adhesion due to increased stability between the cadherins and catenins and increased E-cadherin expression (Bradley *et al.*, 1993; Hinck *et al.*, 1994a), and an increase in the stability between APC and the catenins (Papkoff *et al.*, 1996). E-cadherin has been shown to prevent β-catenin nuclear localisation coupled with the inhibition of β-catenin transcriptional activity (Orsulic *et al.*, 1999;

Stockinger *et al.*, 2001). Casein kinase II and GSK-3β have been shown to result in the phosphorylation of E-cadherin increasing the E-cadherin/β-catenin interaction in cell to cell adhesion, thus further regulating β-catenin (Lickert *et al.*, 2000).

The Wnt-1 pathway has been fairly well elucidated (figure 1.7), Cook *et al.* (1996) used 10T1/2 fibroblasts to show that Wnt acted through the wingless pathway to inhibit serine threonine glycogen kinase (GSK-3). Wnt-1 binds to its receptor frizzled, which in turn activates the vertebrate homologue of dishevelled (Holmen *et al.*, 2002). Activated dishevelled (dvl, dsh, Dvl, Dsh) inactivates GSK-3β through phosphorylation. GSK-3β is a serine/threonine kinase that negatively regulates β-catenin, when β-catenin levels increase to excess (Rubinfeld *et al.*, 1996; Reviewed by Jankowski *et al.*, 1997; Morin *et al.*, 1997a; Reviewed by Peifer, 1997). GSK-3β is involved in the phosphorylation of both APC (increasing APCs affinity for β-catenin) and β-catenin (on the N-terminal region) resulting in the degradation of β-catenin (Rubinfeld *et al.*, 1996; Reviewed by Moon and Miller, 1997) via ubiquitin dependent proteolysis (Aberle *et al.*, 1997). Inhibition of GSK-3β results in increased stability of β-catenin resulting in its cytoplasmic accumulation. GSK-3β and APC may regulate β-catenin independently of each other (Reviewed by Moon and Miller, 1997), or work in a complex comprising axin (conductin) (Reviewed by Gordon, 1998; Hart *et al.*, 1998). A further protein, casein kinase 1 is involved in targeting β-catenin for degredation, by phosphorylating β-catenin which then allows GSK-3β to phosphorylate β-catenin (Ding and Dale, 2002) and axin may mediate the interaction netween Dvl-2 and GSK-3β (Smalley *et al.*, 1999). Other studies have also suggested that other serine kinases may be involved in β-catenin degradation, when GSK-3β is inhibited (Easwaran *et al.*, 1999). Axin also aids in β-catenin degradation in cells lacking the axin binding sites within APC (Hart *et al.*, 1998).

This increase in cytoplasmic β-catenin leads to translocation of β-catenin into the nucleus where it associates with the transcription factors LEF and TCF (Reviewed by

Figure 1.7 The Wnt-1 signaling Pathway.



Wnt/Wg signaling pathway (A) Wnt binds to its receptor frizzled activating Dishevelled (dsh); (B) Activated dsh inhibits GSK-3β from phosphorylating β-catenin preventing β- or γ-catenin degradation; (C) This leads to an increase in cytoplasmic β-catenin leaving it free to associate with the transcription factors TCF/LEF, in the nucleus this complex binds to the DNA (Reviewed by Barth *et al.*, 1997b; Reviewed by Jankowski *et al.*, 1997; Korinek *et al.*, 1997; Reviewed by Nusse, 1997; Behrens *et al.*, 1996; Morin *et al.*, 1997); (D) This results in the expression of a number of target genes including *C-Myc* (He *et al.*, 1998), *Cyclin D1* (Shtutman *et al.*, 1999), *MMP-7* (Brabletz *et al.*, 1999), *CD44* (Wielenga *et al.*, 1999) and *AP-1* (Mann *et al.*, 1999) genes involved in colonic cell homeostasis and migration.

Barth *et al.*, 1997b; Behrens *et al.*, 1996; Reviewed by Jankowski *et al.*, 1997; Korinek *et al.*, 1997; Reviewed by Nusse, 1997; Morin *et al.*, 1997) and pontin52 subsequently displacing Groucho-related gene or CREB-binding protein corepressors from LEF or TCF consequently altering gene transcription (Reviewed by Roose and Clevers, 1999; Reviewed by Novak and Dedhar, 1999). β-catenin consists of transactivation domains which recruit the basal transcription machinery to promoter regions of Wnt target genes (Hecht *et al.*, 1999; Vlemincks *et al.*, 1999). β-catenin translocates into the nucleus through the nuclear pore in a Ran-unassisted manner (Yokoya *et al.*, 1999). LEF and TCF are members of the high mobility group sex determining (sry) family of transcription factors that induce bends in the DNA helix on binding. These transcription factors, LEF and TCF, by themselves are poor promoters of transcription. However when β-catenin is complexed with these transcription factors the DNA bend produced by these transcription factors is thought to enhance and alter gene transcription (Behrens *et al.*, 1996; Reviewed by Nusse, 1997; Reviewed by Moon and Miller, 1997). β-catenin, plakoglobin and armadillo have different transactivation capacities that may explain their distinct signalling properties (Hecht *et al.*, 1999). p120 has also been shown to enter the nucleus and interact with transcription factors (van Hengel *et al.*, 1999; Reviewed by Nollet *et al.*, 1999) with E-cadherin being shown to down-regulate nuclear p120 (van Hengel *et al.*, 1999). The target genes of β-catenin-TCF-4/LEF-1 have long been sort after, but are thought to be involved in inhibiting apoptosis or promoting cellular proliferation. He *et al.* (1998) have shown that one of the target genes of TCF-4 is *c-myc* a known oncogene over expressed in colon cancer. There have been a number of other target genes isolated including *Cyclin D1* (Shtutman *et al.*, 1999), *MMP-7* (Matrix metalloproteinase-7) (Brabletz *et al.*, 1999), *Matrilysin* (Crawford *et al.*, 1999), *CD44* (Wielenga *et al.*, 1999), *AP-1* (*c-jun* and *fra-1*) and indirectly *uPAR* along with a reduction in ZO-1 a protein involved in epithelial polarization (Mann *et al.*, 1999). *C-myc* has been shown to be more efficiently activated by plakoglobin than β-catenin, further supporting the theory that plakoglobin and β-catenin have different roles during Wnt signalling targeting different genes (Reviewed by Barker and Clevers, 2000). Wnt-1 activation has also been shown to result in elevated expression of E-cadherin and increased cell-cell adhesion (Bradley *et al.*, 1993). The β-catenin-LEF-1 complex has been shown to associate with the 5' end of the *E-*

cadherin gene suggesting that this signalling pathway can modulate E-cadherin expression and subsequently cell adhesion (Reviewed by Ben-Ze've, 1997). Cell-cell adhesion in turn is thought to play a major role in the transduction of transmembrane signals (Reviewed by Ben-Ze'ev, 1997). Simcha *et al.* (1998) showed that α-catenin, along with cadherins, sequester β-catenin to the cytoplasm inhibiting its transcriptional activity. Whether tyrosine phosphorylation of β-catenin is involved in the wingless-Wnt signalling pathway has not been clarified (Reviewed by Nakamura, 1997). LEF-1/β-catenin complex and activation of specific LEF-1 target genes is thought to be critical for tumourigenesis (Aoki *et al.*, 1999), although it has been suggested that a further component is required for gene activation (Prieve and Waterman, 1999). Increased expression of β-catenin and subsequent nuclear translocation has been shown to result in cellular hyperproliferation within a murine model of colonic hyperplasia (Sellin *et al.*, 2001).

γ-catenin has also been shown to enter the nucleus and interact with these transcription factors, thus suggesting some functional homology between the two proteins, β-catenin and γ-catenin. Simcha *et al.*, 1998 have shown that γ-catenin and β-catenin differ in their nuclear translocation and their association with the transcription factor LEF-1, with transcription being preferentially driven by β-catenin, with β-catenin binding to LEF-1 in association with vinculin.

1.3.5 The Catenins (α-, β-, γ-), p120 and E-Cadherin in colon cancer.

Stabilization and accumulation of cytoplasmic β-catenin as a result of mutations in either the *APC* or *β-catenin* genes are causatively associated with colon carcinogenesis (Rubenfeld *et al*, 1996; Iwao *et al.*, 1998; Takahashi *et al*, 1998; Müller *et al.*, 1998; Herter *et al.*, 1999). As discussed in section 1.3.1, *APC* mutations usually involve at least one of the β-catenin binding sites, thus resulting in increased stability of the β-catenin protein (Reviewed by Moon and Miller, 1997). This is shown in cells expressing mutant APC, as they possess an abnormally large pool of monomeric β-catenin (Reviewed by Moon and Miller, 1997). But where no *APC* mutations are observed there are a number of other possible candidate genes, and their proteins, that could be involved in colorectal carcinogenesis, including the

catenins. Somatic β-catenin activating mutations contribute to a small number of human colorectal cancer (Müller *et al.*, 1998).

Using immunohistochemical techniques Hao *et al.* (1997) showed that in normal tissues β-catenin was located at the cell membranes, but in the adenoma and carcinoma tissues there was a relocalisation of β-catenin resulting in reduced membranous staining which was accompanied with an increase in cytoplasmic and nuclear staining (Hao *et al.*, 1997; Hugh *et al.*, 1999). The redistribution of β-catenin appears to occur early in sporadic colorectal tumourigenesis (Herter *et al.*, 1999). This redistribution of β-catenin was also found to correlate with the progression from adenomas to carcinomas (Hao *et al.*, 1997), and serves as a criteria for estimating malignant potential of colonic tumours (Herter *et al.*, 1999). A further study revealed that β-catenin was not homogenously expressed within dysplastic tissues with strong nuclear expression being predominantly localised at the invasion front (Brabletz *et al.*, 1998). β-catenin mutations are also usually found within small colorectal adenomas rather than the larger adenomas and invasive carcinomas (Samowitz *et al.*, 1999). *β-catenin* mutations have been found within primary colorectal cancers involving exon 3, the absence of exon 3 has been shown to result in a stabilized protein which has a dominant oncogenic effect on colorectal tumourigenesis (Iwao *et al.*, 1998). Takahashi *et al* (1998) showed that in AMO (azoxymethane) treated rats there was an increase in both cytoplasmic and nuclear β-catenin. Further analysis of *β-catenin* revealed that in this model a mutation in the GSK-3β consensus motif resulted in the increased β-catenin stability due to decreased degradation. There are a number of other possible mutation sites in *β-catenin*, i.e. tyrosine phosphorylation due to a mutation in the coding sequences of the serine residues (Reviewed by Jankowski *et al.*, 1997), and an amino terminal deletion results in stabilisation of the protein along with hyperphosphorylation of APC (Munemitsu *et al*, 1996). Activating mutations in the *β-catenin* gene is thought to be responsible for the excessive β-catenin signalling involved in the majority of carcinogen induced colonic carcinomas (Yamada *et al.*, 2000). Yamada *et al.* (2000) found that β-catenin signalling may be involved in the initial stage, aberrant crypt foci, of colon carcinogenesis in azoxymethane treated male F344 rats. A further study revealed that crypts where β-catenin has accumulated are truly premalignant

lesions of colon cancer, and are independent of aberrant crypt foci (ACF) (Yamada *et al.*, 2001a). Further support for β-catenin mutations being involved in colon cancer was provided by Romagnolo *et al.* (1999) where transgenic mice containing a NH2-terminally truncated mutant *β-catenin* resulted in multifocal dysplastic lesions within the small intestine (Romagnolo *et al.*, 1999). β-catenin is expressed at epithelial cell membranes, except during cell signalling or disruptions in its regulation (i.e. mutations) where it is found in the cytoplasm and nucleus.

The increase of free β-catenin results in increased and inappropriate transcription of hLEF-1 and hTCF-4 (Reviewed by Moon and Miller, 1997) resulting in altered gene transcription such as *c-myc* expression (He *et al.*, 1998), this could be one cause of c-myc being overexpressed in colorectal tumours (Refer to section 1.3.4, page 24, for other target genes). The transcription factor TCF-4 has also been shown to maintain crypt stem cells of the small intestine, suggesting that when APC or β-catenin are mutated in human epithelial cells, these cells may regain stem cell characteristics thus resulting in malignant transformation (Korinek *et al.*, 1998). LEF-1/β-catenin complex formation resulting in the activation of LEF-1 target genes has also been implicated in tumourigenesis (Aoki *et al.*, 1999).

γ-catenin has also been shown to associate with APC and TCF/LEF entering the nucleus, suggesting there may be further functional homology with β-catenin. β-catenin mutations, with oncogenic activity, are found in many human tumours, whereas mutations in γ-catenin are rarely found, subsequently the suggestion that plakoglobin may act as a tumour suppressor gene was proposed (Williams *et al.*, 2000). However plakoglobin in the absence of stabilising mutations has been shown to induce neoplastic transformation of rat epithelial cells (Reviewed by Barker and Clevers, 2000). Plakoglobin has been shown to have a role in apoptosis by regulating the expression of the anti-apoptotic protein Bcl-2, with increases in plakoglobin expression correlating with increases in Bcl-2 protein (Hakimelahi *et al.*, 2000). Mutations in plakoglobin have failed to enhance its stability possibly explaining why mutations are rare (Williams *et al.*, 2000).

In normal colorectal epithelium E-cadherin is found associated with the cell membrane. In intestinal tumours, from transgenic mice and FAP patients, E-cadherin is located into the cytoplasm (Sloncova *et al.*, 2001). As early as 1989, Behrens *et al.* (1989) suggested that the loss of adhesive function of E-cadherin (uvomorulin) is a critical step in the promotion of the malignant phenotype of epithelial cells. E-cadherin is essential for the formation and maintenance of epithelia, and any perturbation of the adhesive complex leads to increased invasiveness and decreased differentiation (Vleminckx *et al.*, 1991; Frixen *et al.*, 1991; Pignatelli *et al.*, 1992; Berx *et al.*, 1995; Reviewed by Shiozaki *et al.*, 1996; Guilford *et al.*, 1998) a fundamental aspect of the neoplastic process (Reviewed by Jawhari *et al.*, 1999a). *E-cadherin* has been proposed as a tumour suppressor gene with the possibility that loss of cell-cell adhesion may convey signals that actively induce invasion and metastasis (Reviewed by Semb and Christofori, 1998; Reviewed by Christofori and Semb, 1999), along with an increase in genomic instability (Reviewed by Tisty, 1998). Mutations in E-cadherin itself, β-catenin, α-catenin and γ- catenin have all been shown to result in the perturbation of cellular adhesion (Gagliardi *et al.*, 1995). Gagliardi *et al.* (1995) used immunohistochemical techniques to look at the expression of E-cadherin in normal and cancerous tissues. They found that in normal colorectal epithelial cells there was typical membranous staining at the adherens junctions, but in the adenoma and carcinoma tissues there were changes in the immunoreactivity and cellular localisation. These changes correlating with tumour size, histopathology, growth patterns and degree of dysplasia (Dorudi *et al.*, 1993; Gagliardi *et al.*, 1995). Poorly differentiated adenocarcinomas and adenomas revealed a decrease in the expression of E-cadherin (Nigam *et al.*, 1993; Valizadeh *et al.*, 1997). Loss of E-cadherin mediated cell-cell adhesion could allow cells to escape normal growth control signals resulting in cell proliferation. Alternatively Guilford suggests that the cytoplasmic domain of E-cadherin may modulate the Wnt signalling pathway by inhibiting the availability of free β-catenin, similarly to APC (Guilford *et al.*, 1998). It has also been shown that there is an increase in the activation of several receptor tyrosine kinases, i.e. EGFR, v-src and c-MET in cancer cells, resulting in tyrosine phosphorylation of the catenin molecules, thus disrupting cellular adhesion (Behrens *et al*, 1993; Reviewed by Jankowski *et al.*, 1997). Other mechanisms of E-cadherin inactivation have also been described by

Yoshiura *et al* (1995) who showed that methylation on CpG islands near the promoter region also inactivated E-cadherin at least in ovarian, breast and prostate carcinomas. With hypermethylation of the promoter region of E-cadherin gene also found in sporadic colorectal cancer (Wheeler *et al.*, 2001).

Shiozaki *et al.* (1994) looked at whether the possible down regulation of α-catenin expression played a role in tumour invasion and metastasis through E-cadherin dysfunction. They found that normal epithelium expressed α-catenin strongly, without exception. However, α-catenin was reduced or absent in a number of primary tumours of the oesophagus, stomach and colon, thus suggesting that E-cadherin mediated adhesion may be abrogated by downregulation of α-catenin (Shiozaki *et al.*, 1994). An investigation into patients with colorectal cancer coupled with liver metastasis were examined for E-cadherin and α-catenin expression, α-catenin expression was found to be reduced and significantly correlated to invasion of the tumour whereas E-cadherin was downregulated in fewer cases and did not correlate to invasion (Gofuku *et al.*, 1999) suggesting that there may be further disruption of α-catenin at the cell membrane. Loss of α-catenin expression has been shown to significantly correlate with tumour de-differentiation (Hugh *et al.*, 1999). Down regulation of α-catenin has also been observed in cell lines, HCT-8, which have a more invasive phenotype (Vermeulen *et al.*, 1995).

Skoudy *et al.* (1996) also reported altered p120 expression in colorectal cancer. Skoudy *et al.* (1996) demonstrated that in the normal colon, p120-catenin was present in the crypt and surface epithelium, the cells showed reactivity in both the membrane and cytosol, with Valizadeh *et al* (1997) showing that the staining intensity was greatest in the proliferating crypt cells. Reduced expression of p120 was observed in 20% of adenomatous polyps, with loss of membranous p120 expression correlating with reduced E-cadherin expression. Decreased expression of p120 was also found to correlate with the larger size tumour (Skoudy *et al*, 1996). These findings are difficult to explain as the physiological role of p120 is unknown, but Skoudy *et al.* results suggest that changes in p120 catenin levels are a common event in colorectal tumours, and suggest that the distribution of this protein and E-cadherin is co-ordinately regulated.

1.4 Environment

1.4.1 Environmental Factors involved in the aetiology of colorectal cancer development.

A number of studies have revealed the importance of environmental factors in the aetiology of a vast majority of human cancers, further diet has been considered as important as tobacco smoking (Reviewed by Hill, 1997a). There are number of methods for determining the role of environmental factors in human cancer including epidemiological studies, animal models, *in vitro* studies and human intervention studies (Reviewed by Hill, 1997a). The strongest evidence for the role of diet in the aetiology of colon cancer has been provided by numerous epidemiological studies. These studies have identified potential promoters of colon cancer; including meat and fat intake, tobacco smoke, alcohol intake, lack of physical exercise, obesity (due to an energy imbalance), low intake of fruit, vegetables and whole grain cereals; with potential protectors including dietary fibre, antioxidants provided by fruits and vegetables, cereals and physical activity (Reviewed by Bingham, 1990; Reviewed Hill, 1997a; Reviewed Hill, 1997b, Reviewed Hill, 1997c; Reviewed by Cummings and Bingham, 1998). There are a number of human intervention trials that have been undertaken. These trials include the ECP trial involving 21 centres from 10 countries, which found that supplementation of a diet with ispaghula husk may have adverse effects on colorectal adenoma recurrence especially in patients with high calcium intake (Bonithon-Kopp *et al.*, 2000). Calcium on the other hand was shown to be associated with a small but not significant reduction in adenoma recurrence (Bonithon-Kopp *et al.*, 2000). Other trials include a study on wheat bran fibre and the polyp prevention trial (low fat, high fibre diet) both revealed that diet had no effect on the incidence of new colorectal adenomas (Reviewed by Byers, 2000). A prospective study of 88,757 women with no history of any bowel disease over a 16 year follow up period again revealed no association with fibre intake and colorectal cancer (Fuchs *et al.*, 1999). The Toronto polyp prevention trial found that high fibre low fat diets reduced the risk of neoplastic polyp recurrence in women with this being associated with a reduction in faecal bile acids. However this same diet resulted in an increased risk for men (McKeown-Eyssen *et al.*, 1994). This suggests

that there may be a gender-specific association with diet and colorectal cancer recurrence (McKeown-Eyssen *et al.*, 1994). Experiments using rodents also indicated the importance of diet in cancer development, including early work carried out by Tannenbaum and Silverstone (Reviewed by Hill, 1997a). *In vitro* methods reveal possible biological mechanisms behind dietary factors, including the potential role of short chain fatty acids (SCFAs, products of fibre fermentation), particularly butyrate, and a number of antioxidants and their role in colorectal cancer development or prevention (Reviewed by Hill, 1997a).

Denis Burkitt and Hugh Trowell hypothesised that dietary fibre could have a protective effect against a range of diseases that are prevalent in the western world (Reviewed Kritchevsky, 2001). Healthy image fibre-rich food products are now mainstream, with over 80% of American adults concerned about the effects of diet, especially dietary fibre, on their health (Reviewed by Dreher, 1995). One way to increase fibre intake is to eat fibre rich foods, and a second alternative way is to develop fibre rich processed foods (Reviewed by Dreher, 1995). Fibre has a number of health benefits and prophylactic uses including the lowering of cholesterol (Reviewed by Reiser, 1987), improvement of the glycaemic control of diabetes (Reviewed by Reiser, 1987; Reviewed by Spiller, 1994; Reviewed by Spiller, 1999), the relief of constipation (Reviewed Dwyer *et al.*, 1978; Reviewed by Spiller, 1994), diarrhoea (Reviewed by Spiller, 1994; Reviewed by Spiller, 1999) and a number of other large bowel disorders (Reviewed by Dwyer *et al.*, 1978; Reviewed by Kritchevsky, 2001). Dietary fibre had also been proposed to protect against colonic cancer with fibre subsequently becoming the health panacea of the 1980's. Subsequent reports have suggested that 'fibre supplemented foods may damage your health' (Reviewed by Wasan and Goodlad, 1996) with some fibres potentially increasing the risk of colon cancer development. A number of epidemiological studies examining the relationship between colon cancer and fibre intake have revealed that fibre has a protective role in colorectal cancer development (Reviewed by Bingham, 1990; Reviewed by Byers, 1995; Reviewed by Hill, 1997a; Reviewed by Hill, 1997b, Reviewed by Hill, 1997c; Reviewed by Cummings and Bingham, 1998). Fibre studies in animals have had varying results ranging from protection, no effect, to enhancement of colorectal cancer (Jacobs and Lupton, 1986; Reviewed by Jacobs, 1988; Reviewed by Harris and Ferguson, 1993; Reviewed by Harris and

Ferguson, 1999). On balance soluble fibre appears to be less protective than the insoluble fibres (Reviewed by Harris and Ferguson, 1993).

1.5 Dietary Fibre

1.5.1 Definition of Dietary Fibre

Dietary fibre is, as an entity, hard to define. There have been a number of attempts at a definition over the years. The initial definition being coined by Hipsley (1953), with further definitions by Cleave (1956), but the first definition accepted by scientists world-wide was that proposed by Huge Trowell in 1976 (Reviewed by Prosky, 2001; Reviewed by McCleary, 2001; Reviewed by Kritchevsky, 2001), 'dietary fibre is composed of the remnants of plant cells resistant to hydrolysis by human alimentary enzymes including all indigestible polysaccharides (cellulose, hemicellulose, oligosaccharides, pectins, gums, and algal polysaccharides) plus waxes and lignin' (Reviewed by Dreher, 1995; Reviewed by Lembo, 1998; Reviewed by Prosky, 2001; Reviewed by Kritchevsky, 2001). This definition was based not only his own observations but also those of Walker and Burkitt and reinforced by work of Cowgill and Anderson (1932), Williams and Olmstead (1936) and Dimock (1937) (Reviewed by Kritchevsky, 2001). Subsequent definitions referred to dietary fibre as 'non-starch polysaccharides' (Englyst *et al.*, 1994), 'ingested plant wall material and any non-starch polysaccharide not degraded by endogenous mammalian enzymes (Goodlad *et al.*, 1989; Ghatei *et al.*, 1997). Observations by Stephens (1983) revealed that this did not cover all the material available in the colon (Reviewed Hill, 1997b) as it did not include resistant starch polysaccharides which behaves physiologically as if NSP (Englyst *et al.*, 1996; Reviewed by Hill, 1997b; Reviewed by McCleary, 2001). Later studies revealed that resistant starch is a primary food source for colonic microflora (Reviewed by Blackwood *et al.*, 2000) with three times more starch possibly reaching the colon than NSP (Reviewed by Hill, 1997b). Thus if dietary fibre is not simply seen as residual plant cell wall material but all plant-derived carbohydrate entering the colon, then resistant starch is part of dietary fibre (Reviewed by Jenkins and Kendall, 2000). Most fibre definitions refer to fibre as crude fibre, total fibre or NSP, excluding

starch. It has been suggested that the definition of dietary fibre should be replaced with the term complex carbohydrate (CC) (Reviewed by Hill, 1997b). There are a number of other dietary and host components that resist digestion in the small bowel entering the colon thus acting as a microbial food source. These dietary and host components include other plant materials, including oligosaccharides (some being used a prebiotics such as fructooligosaccharides), proteins, sloughed cells and mucin (Reviewed by Rombeau and Roth, 1995). Consumption of dietary fibre has been shown to have a number of physiological effects on the gastrointestinal tract including increasing stool weight, altering gut transit time, altering the activity of the colonic microflora, modifying the absorption of fats, sugars, minerals and bile acids, influencing appetite, and absorbing toxins (Reviewed by Blackwood *et al.*, 2000). The Food and Agricultural Organisation (FAO) of the United Nation World Health Organisation (WHO) has also recognised that dietary fibre has not only constituents of plant origins but also animal origin, basically those resistant to enzyme attack through the alimentary canal (Reviewed by Prosky, 2001). The definition was subsequently extended, to not only include some of these new dietary fibres but also the health benefits associated with these dietary fibres;

'Dietary Fibre is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the small intestine with complete or partial fermentation in the large intestine. Dietary fibre includes polysaccharides, oligosaccharides, lignin and associated plant substances. Dietary fibre promotes beneficial physiological effects, such as laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation' (Reviewed by Prosky, 2001)

1.5.2 Quantification of Dietary Fibre.

A number of methods have been devised to not only determine the dietary fibre contents of food but as an aid in defining dietary fibre and its structure (Reviewed by Prosky, 2001). Methods can be classified as either chemical (Southgate, 1969; Theander and Aman, 1979; Englyst *et al.*, 1992), gravimetric (Asp *et al.*, 1983; Mongeau and Brassard, 1986), Neutral detergent fibre (Van Soest and Wine, 1967) or fall into the AOAC method (Prosky *et al.*, 1984) (Reviewed by Marlett, 1995). Methods for determining dietary fibre contents include Williams and Olmstead

(1935), Southgate (1969, 1982), Englyst and Cummings (1984, 1988), Prosky (AOAC) and methods for determining dietary fibre structure include Englyst (1983) (Reviewed by Prosky, 2001), methylation analysis (Edwards *et al.*, 2001) coupled with specific enzyme degradation (Blackwood and Chaplin, 2001). All methods have been extensively modified over the years, provide a crude measurement of dietary fibre content with the main aim to provide as much information about the fibre source to understand basis for the effect of fibre (Reviewed by Marlett, 1995). The procedures result in the removal of the digestible portion of the food using enzymes leaving an indigestible portion, mimicking the dietary fibre that enters the colon (Reviewed by Prosky, 2001). The determining factor for which procedure is preferentially used is not only time, modification but also geography (Reviewed by Prosky, 2001). The US Food and Drug Administration (1993) and the US Department of Agriculture (1993) and the WHO in America generally accepted the AOAC method for determining dietary fibre content of foodstuff (Reviewed by Prosky, 2001). In the UK, however, the Ministry of Agriculture, Fisheries and Food (MAFF) adopted the Englyst and Cummings method which determines the NSP content of foods (Reviewed by McCleary, 2001) thus adopting the term NSP to refer to dietary fibre (Reviewed by Prosky, 2001). The Englyst procedure has not received widespread adoption because, from a physiological point of view, NSP is less relevant than total dietary fibre (determined using the AOAC method) (Reviewed by McCleary, 2001).

1.5.3 The Physicochemical properties of Dietary Fibres.

Dietary fibre is heterogeneous in both structure and function having a wide variety of chemical and physiological (physical and metabolic) properties. The physiological effects of dietary fibres are partly predictable on the basis of their physicochemical properties (Reviewed by Blackwood *et al.*, 2000). These properties of dietary fibre are dependent on a number of factors not only intrinsic to the fibre and the host but also a number of extrinsic factors. Intrinsic factors include the dietary fibres conformation (branching, flexibility), monosaccharide content, monosaccharide availability, water holding capacity, solubility and subsequent viscosity (which in turn all affects molecular weight), level of exposed hydrophobic surfaces, particle size, interactions with other polysaccharides and components of the digesta such as

level of protein intake (Weisburger *et al*, 1977; Reviewed by Blackwood *et al.*, 2000; Reviewed by Morris, 2001; Reviewed by Kritchevsky, 2001). The conformation of dieary fibre affects the rate and extent of its fermentation with soluble fibres being more readily fermented and earlier in the colon, than insoluble fibres (Reviewed by Blackwood *et al.*, 2000). The monsaccharide composition also affects the rate of fermentation with polysaccharide chains being fermented at the terminal region first, and carbohydrates containing α-arabinose (pectin and ispaghula husk) and α-galacturonic acid (pectin) residues are more susceptible (Reviewed by Blackwood *et al.*, 2000). The site, rate and extent to which the fibres are fermented and absorbed in the colon and the type of products produced during this fermentation all affect the physiological properties of the fibre (Reviewed by Blackwood *et al.*, 2000). Host-derived factors affecting the physiological properties of the fibre include the colonic microflora, the pH of lumen, metabolic rate and genetic background. Extrinsic factors affecting dietary fibres physicochemical properties include pancreaticobiliary secretions, bacterial flora, and gastrointestinal hormones (Reviewed by Cassidy and Fitzpatrick, 1995), the extent of mastication, the extent of preparation (cooking, heating, processing) and the form of fibre (whole foods verses individual fibres) (Reviewed by Eastwood, 1995). Food preparation, in terms of processing and heating will also affect dietary fibres structure. Food processing will affect dietary fibre in terms of particle size and viscosity, for example sterilisation by gamma irradiation has been shown to affect ispaghula husks viscosity (M.Havler, personal communication).

Vast arrays of dietary fibres exist but the entire principal component are the non-starch polysaccharides and lignin (Reviewed by Blackwood *et al.*, 2000). Non-starch polysaccharides are composed of various structural polysaccharides including cellulose, hemicellulose (including arabinoxylans), and non-structural polysaccharides such as pectins, gums and mucilages (Reviewed by Dreher, 1995; Reviewed by Lembo, 1998; Reviewed by Blackwood *et al.*, 2000). Dietary fibre can also be sub-divided fairly crudely into soluble (non-structural polysaccharides such as pectin, ispaghula) verses insoluble (structural polysaccharides such as cellulose) fibres (Reviewed by Lembo, 1998) with a third group, the composite group a

combination of soluble (approximately 10%) and insoluble dietary fibre (Reviewed by Dreher, 1995).

Fibre structures can have very different geonometries ranging from flat ribbon assemblies (cellulose fibrils), double helices (starch) to buckled chains containing cavities that are occupied by bound ions (pectin) (Reviewed by Morris, 1995). Most polysaccharides of greatest importance are built up of six-membered, pyranose rings in a chair shaped conformation; for example, cellulose is made up of glucose chains of poly-β(1-4)-D-anhydroglucose (Reviewed by McCleary, 2001). The differences in the fermentation of cellulose, poly-β-D-Glucose, and resistant starch, poly-α-D-Glucose, is its conformation, axial versus equatorial, and due to its packing; for example, cellulose has a more ordered packing making it more resistant to enzymatic attack resulting in limited degradation during fermentation (Reviewed by Morris, 2001).

Water binding and viscosity are physical manifestations of fibre action and influence gastric emptying, faecal flow and absorption of nutrients (Reviewed by Eastwood, 1995; Reviewed by Blackwood *et al.*, 2000; Reviewed by Kritchevsky, 2001), enzyme activity, stool weight and facilitates fermentation (Reviewed by Eastwood, 1995). This also affects intestinal and colonic muscle activity, metabolic turnover rate of absorbed nutrients and endocrine hormone activity (Reviewed by Eastwood, 1995). The Blythe technique in humans, showed that there was an inverse relationship between water holding capacity (WHC) and stool weight providing evidence that fibre did not increase faecal weight by simply retaining water but that faeces also contained bacteria (Reviewed by Brydon, 1995). Thus fibre provides an energy source for bacteria through fermentation, resulting in their proliferation and subsequent increase in biomass (Reviewed by Brydon, 1995; Reviewed Lembo, 1998). An increase in stool mass is accompanied by a decrease in colonic transit time (Reviewed by Lembo, 1998).

Diet gives clues to the aetiology of many of the life style diseases, such as colon cancer and heart disease, which themselves have a large number of risk factors (Reviewed by Kritchevsky, 2001). Structural features of dietary fibre may influence

binding of bile acids and inorganic ions and potential carcinogens (Reviewed by Blackwood *et al.*, 2000; Reviewed by Kritchevsky, 2001). Bulking may increase the excretion of those substances trapped in the faecal mass (Reviewed by Kritchevsky, 2001). The mechanical action of dietary fibre, influencing stool weight, results in the relief of constipation and diverticular disease (Reviewed Dwyer *et al.*, 1978; Reviewed by Kritchevsky, 2001). There are a number of metabolic effects associated with fibres: these include reducing obesity; gallstones (through reducing cholesterol saturation and possibly affecting the bile acid pool); diabetes (found to help alleviate symptoms by normalising glucose tolerance and plasma insulin levels); coronary heart disease and colon cancer (Reviewed by Kritchevsky, 2001; Marcil *et al.*, 2002). It is now established that insoluble fibres (cellulose) do not influence serum lipids, whereas soluble fibres (pectin and psyllium due to their viscous nature) exert a hypocholesterolaemic effect (Reviewed by Reiser, 1987; Reviewed by Kritchevsky, 2001).

Fibrous carbohydrates are digested symbiotically by colonic microorganisms in all higher animals that have not evolved cellulases, hemi-cellulases or pectinases (Reviewed by Sakata and Yajima, 1984; Reviewed by Van Soest, 1995). Fibre is fermented by the microflora in the large intestine, to produce a number of metabolic by-products, particularly the short chain fatty acids (SCFAs) such as butyrate, acetate and propionate (Goodlad *et al.*, 1989; Reviewed by Mortensen and Nordgaard, 1995; Wang and Friedman, 1998). This can be shown by the administration of antibiotics, which was associated with a decrease in SCFA production highlighting the role of the microflora in their production (Reviewed by Sellin, 1993). Different fibres produce different levels of SCFAs, a wheat bran diet results in a colonic contents rich in butyrate with less propionate, whereas an oat bran diet yielded half as much butyrate but with a similar amount of propionate (Wang and Friedman, 1998).

An increase in colonic dietary fibre (NSP) also results in increased carbohydrate fermentation thus resulting in a decrease in protein metabolism and thus a reduction in ammonia formation (Reviewed by Macfarlane and Gibson, 1994; Reviewed by Eggum, 1995). Ammonia formation from colonic bacteria is of physiological significance to the host since concentrations as low as 10mM can alter the morphology and intermediary metabolism of intestinal cells, increase DNA synthesis

and increase their lifespan (Reviewed by Macfarlane and Gibson, 1994). Thus the colonic microflora influences colonic mucosal architecture, mucosal cell turnover and intestinal motility (Reviewed by Salyers, 1995). SCFA production, e.g. butyrate, has been shown to affect mucosal cell turnover thus an indirect way that the microflora affects the mucosa (section 1.5.5) (Goodlad *et al.*, 1989; Reviewed by Salyers, 1995; Goodlad *et al*, 1995). Diet has been shown to stimulate ornithine decarboxylase (ODC) intestinal activity, which is one of the earliest biochemical parameters associated with cellular turnover and development (Reviewed by Cassidy and Fitzpatrick, 1995). Diet has been shown to influence colonic morphology in rats, resulting in a physiological adaptation of the colonic epithelium and is essential for the maintenance of normal small and large intestinal mucosal structure and function (Reviewed by Cassidy and Fitzpatrick, 1995).

1.5.4 Fermentation of Dietary Fibre.

Fermentation is an important parameter of dietary fibre characterisation, in terms of the types of products formed, the rate at which they are formed and the site at which they are formed within the large intestine. The majority of fermentation occurs in the caecum (Reviewed by Lembo, 1998), which corresponds to the veriform appendix in humans (fig.1.1) (Reviewed Eastwood, 1995). The abundance of anaerobic, saccharolytic bacteria in the human colon provides an important milieu for the fermentation process (Reviewed by Rombeau and Roth, 1995). The majority of the carbohydrates entering the large bowel are polysaccharide chains (such as dietary fibres) and are fermented by the anaerobic bacteria using a series of energy-yielding reactions (Reviewed by Macfarlane and Gibson, 1994). With the exception of *bifidobacter*, the majority of saccharolytic anaerobes use the Embden-Meyerhoff-Parnas pathway (of anaerobic glycolysis) to ferment carbohydrates producing SCFA (Reviewed by Macfarlane and Gibson, 1994; Reviewed by Rombeau and Roth, 1995). Together with the other metabolites lactate, ethanol, hydrogen and succinate, they are produced to provide electron sinks to maintain a redox balance within the colonic lumen as fermentation results in the lowering of intraluminal pH (Reviewed by Macfarlane and Gibson, 1994; Reviewed by Lembo, 1998). The intermediates of fermentation such as lactic acid can also be utilised by other bacteria resulting in them being metabolised to SCFAs (Reviewed by Macfarlane and Gibson, 1994).

The major SCFAs produced from fermentation are butyrate, acetate and propionate (Walter, 1986; McBurney, 1991; Reviewed by Cummings, 1994; Reviewed by Livesey, 1995). Dietary fibre (NSP) is thought to be the largest contributor to SCFA production with the type of dietary fibre (NSP) influencing the pattern of SCFA production (McBurney, 1991; Edwards and Eastwood, 1992; Reviewed by Macfarlane and Gibson, 1994; Reviewed by Brydon, 1995; Reviewed by Rombeau and Roth, 1995; Wang and Friedman, 1998). The rate, site and extent of carbohydrate fermentation in the gut is also dependent on a number of factors associated with the fibre including solubility, chemical structure, availability of other more readily fermentable substrates (Reviewed by Blackwood *et al.*, 2000). SCFA production is dependent on a number of other factors including substrate precursors, composition of the microflora and a number of other host factors (Reviewed by Rombeau and Roth, 1995). Different dietary fibres are fermented to varying degrees, differing between whole foods and purified fibres (Reviewed by Wolever, 1995).

SCFAs are usually absorbed directly across the wall of the colon into the blood in the form of free acids (Reviewed by Van Soest, 1995) with absorption occurring to a much greater extent in the ascending (proximal) colon than the descending (distal) colon (Reviewed by Rombeau and Roth, 1995). The exact absorptive mechanism is unknown although SCFAs tend to move transcellularly across the apical membrane being dependent on sodium-hydrogen exchange and is associated with colonic alkalinisation (Reviewed by Rombeau and Roth, 1995). McNeil *et al.* (1978) suggested that SCFAs are absorbed by the large intestine with Sellin and De Soignie (1998) showing that SCFA affected pH and Na^+ absorption. The transport of butyrate across the luminal pole of the colon possibly involves a carrier protein, in the form of a pH activated anion exchange process/ anion exchanger (Ritzhaupt *et al.*, 1998). A broad range of anions are shown to exchange for butyrate, although its transport involves a single saturable transport protein (Ritzhaupt *et al.*, 1998). There is substantial evidence that transcellular flux of SCFA absorption is mediated by an anion exchange process with bicarbonate (Schröder *et al.*, 2000). SCFAs are rapidly absorbed as water and sodium are absorbed and bicarbonate is secreted in the colon (Review Cook and Sellin, 1998). The absorption of SCFAs maintains the pH of the colon above 6, which is required by the normal fibre-digesting bacteria (Reviewed by Van Soest, 1995). Butyrate, and to a much lesser extent propionate, are metabolised

by the colonic wall (colonocytes) as a maintenance source of energy (Reviewed by Van Soest, 1995; Reviewed by Behall, 1997; Reviewed by Lembo, 1998; Ritzhaupt *et al.*, 1998), and are used over and above other energy sources such as glucose and glutamine (Reviewed by Burrin and Reeds, 1997; Avivi-Green *et al.*, 2000b). Thus butyrate is essential for the maintenance of a healthy colon (Avivi-Green *et al.*, 2000b). This is reflected by the pattern of SCFA metabolism within different tissues of the body, acetate is largely metabolised by peripheral tissues (liver and muscles), propionate is mainly metabolised in the liver and butyrate by the colonic tissues (rumen epithelium) (Reviewed by Cummings, 1994; Reviewed by Van Soest, 1995, Reviewed by Cummings, 1997).

The colonic microflora has a symbiotic role in carbohydrate and protein metabolism, where the host provides dietary residues or endogenous substrates, for the bacteria which in turn supply metabolites such as short chain fatty acids for utilisation by the host (Reviewed Macfarlane and Gibson, 1994). Bacterial activities include breakdown of dietary and host polysaccharides, the breakdown of proteins, the modification of xenobiotic compounds, modification of bile salts and sterols, the production of faecal mutagens and the synthesis of many enzymes (Reviewed by Salyers, 1995). Changes in the microflora are seen due to influences of endogenous or exogenous origin. The substrate precursors include not only type and volume of unabsorbable carbohydrate (NSP and resistant starch), protein, nitrogen and endogenous/host polysaccharide sources including sloughed cells and mucus (Reviewed by Rombeau and Roth, 1995). Prebiotics are non-digestible food ingredients, such as oligosaccharides and dietary fibres, that selectively stimulates the growth and or activity of one or a number of limited bacteria in the colon that are beneficial to the hosts health (Reviewed by Gibson and Roberfroid, 1995).

Acquisition of the microflora influences host biochemistry such as enzymatic activity of the intestinal contents (e.g. β-D-Glucuronidase, azoreductase, bile salt hydrolyase), SCFA concentrations, oxidation-reduction potential, host physiology (e.g. rate of replacement of enterocytes), immunology (stimulation of reticuloendothelial tissues) and the modification of host-synthesized molecules (e.g. reduction of cholesterol to coprostanol, dehydroxylation of primary bile acids to

form secondary bile acids, mucin degradation) (Reviewed by Tannock, 1994). The colonic microflora influences colonic mucosal architecture, mucosal cell turnover and intestinal motility (Reviewed by Salyers, 1995). SCFA production, for example butyrate, affects mucosal cell turnover providing an indirect way that the microflora affects the mucosa (Reviewed by Salyers, 1995). SCFA production from fibres results in an increase in colonic cell proliferation (Goodlad *et al.*, 1989; Goodlad *et al.*, 1995). Stability in species composition of the normal microflora may be common, stability at the level of bacterial strains may be less common (Reviewed by Tannock, 1994). Colonic bacteria are very versatile and adaptable, changing to a constantly changing environment including changes in substrate availability (Reviewed by Salyers, 1995). The volume of substrate present is also important, increased substrate results in more bacteria having access, which will subsequently increase and alter the type of fermentation product formed (Reviewed by Macfarlane and Gibson, 1994). Increasing carbohydrate availability also affects bacterial growth rates resulting in an increase in bacterial biomass which can in turn influence the actions of the microflora such as depolymerising enzymes (Reviewed by Macfarlane and Gibson, 1994). The rate at which any fermentable substrate becomes available for assimilation by the bacteria along with substrate preferences will affect the type of fermentation products produced (Reviewed by Macfarlane and Gibson, 1994). There are a number of possible ways fibres interact with the colonic microflora changing total numbers, changing species and changes in bacterial products and activities (Reviewed by Salyers, 1995). For example *C.perfingens* has been shown to increase lactate production and decreases acetate production, thereby enabling the bacteria to maximise their energy (Reviewed by Macfarlane and Gibson, 1994; Reviewed by Rombeau and Roth, 1995). The production of these substances in fermentation therefore contributes to the diversity of bacteria found in the colon (Reviewed by Macfarlane and Gibson, 1994). There are marked differences of levels of bacterial metabolites, i.e. SCFAs and methane in different regions of the colon, along with a variation in species, and obligate versus facultative anaerobes. Edwards and Eastwood (1992) demonstrated that fibres were fermented at different sites within the colon they showed that wheat bran was mainly fermented in the caecum whereas ispaghula husk was fermented throughout the colon (Edwards and Eastwood, 1992; Reviewed by Brydon, 1995).

There are variations in the metabolic capacity of bacteria from colon to colon this may explain the differential response to non-absorbable carbohydrates from individual to individual (Reviewed by Sellin, 1993). The availability of inorganic electron acceptors, such as NO_3^- will affect the ratio or type of SCFA produced (Reviewed by Macfarlane and Gibson, 1994). Interestingly butyrate highlights another factor that must influence fermentation in that butyrate makes up 20% of the total SCFA pool but the level of butyrate producing bacteria in the faeces is very low (<1%). This suggests that they are very metabolically active, so numbers may not be the only important criterion (Reviewed by Macfarlane and Gibson, 1994).

The metabolism of proteins produces a number of by-products including ammonia, urea and free amino acids providing nitrogen for growth of saccharolytic bacteria and amino acids for fermentation by assaccharilytic species. Fermentation of amino acids also results in a wide spectrum of by products including ammonia, amines, phenols, indoles, organic acids (e.g. SCFAs mentioned earlier plus the branched SCFAs isobutyrate and isovalerate), alcohols and the gases H_2 and CO_2 (Reviewed by Macfarlane and Gibson, 1994; Reviewed by Rombeau and Roth, 1995). Some of these by-products are toxic to the host and are thus excreted in the faeces or transported to the liver where they are converted into urea and excreted in the urine (Reviewed by Macfarlane and Gibson, 1994). The more dietary fibre (NSP or starch) available in the colon the smaller the volume of protein metabolised by the colonic microflora (Reviewed by Eggum, 1995). Fibre affects protein/amino acid metabolism by decreasing transit, with an increase in transit time increasing phenol production by faecal bacteria, suggesting the breakdown of protein rather than carbohydrate (Reviewed by Macfarlane and Gibson, 1994). Some bacterial by-products are toxic and potentially carcinogenic, for example faecapentaenes produced by *Bacteriodes* from plasminogen precursor have been shown to cause mutations in DNA (Reviewed by Salyers, 1995). There are also potentially beneficial bacteria such as *Bifidobacteria* in the gut (Reviewed by Blackwood *et al.*, 2000). Rycroft *et al.* (2001) looked at the fermentation of some prebiotic oligosaccharides in 24 hour batch cultures. All prebiotics increased the numbers of *Bifidobacteria* and most decreased *Clostridia*, but different oligosaccharides affects these numbers to different extents and resulting in different fermentation profiles (Rycroft *et al.*, 2001).

1.5.5 Physiological significance of SCFA production

SCFAs are the predominant luminal anion (Reviewed by Sellin, 1993) with acetate, propionate and butyrate predominating, contributing to about 50%, 20% and 20% of the total SCFA (Reviewed by Macfarlane and Gibson, 1994). In humans approximately 200mMol of SCFAs are produced daily but is dependent on polysaccharide consumption (Reviewed by Rombeau and Roth, 1995). SCFA are rapidly absorbed through the colonic epithelium (Reviewed by Macfarlane and Gibson, 1994; Reviewed by Van Soest, 1995; Reviewed by Behall, 1997; Reviewed by Cook and Sellin, 1998; Ritzhaupt *et al.*, 1998; Schröder *et al.*, 2000). Acetate is metabolised in the brain, heart and peripheral tissues, whereas propionate appears to be mainly cleared by the liver (Reviewed by Macfarlane and Gibson, 1994; Reviewed by Rombeau and Roth, 1995). Butyrate formation by intestinal bacteria is of particular interest, because this SCFA is an essential fuel for the colonic epithelium particularly in the distal gut (Reviewed by Macfarlane and Gibson, 1994). Clinically, SCFAs have been studied as possible therapeutic agents in diversion colitis, ulcerative colitis, radiation proctitis, pouchitis and antibiotic associated diarrhoea (Review Cook and Sellin, 1998). SCFAs may be the effector of the beneficial role of fibre in prevention of colon cancer (Review Cook and Sellin, 1998).

The SCFAs profoundly alter the milieu of the colon and have a wide range of biological effects. The physiological effects of SCFA production include the enhancement of sodium absorption, stimulation of blood flow improving peripheral artery dilation (mainly by acetate), and helps regulate carbohydrate and lipid metabolism (Reviewed by Sakata and Yajima, 1984; Reviewed by Rombeau and Roth, 1995). SCFAs have been implicated in water retention in faecal matter (Reviewed by Brydon, 1995) and electrolyte absorption (Reviewed by Lembo, 1998). The fermentation of fibre also results an increase in bacterial biomass (Reviewed by Topping, 1995) by stimulating bacterial growth and this stimulation of biomass leads to increased faecal bulk and bacterial protein synthesis. *In vitro* these SCFAs, particularly butyrate, have been shown to be powerful promoters of differentiation, apoptosis and cell migration (Hague *et al.*, 1995; Wilson and Gibson, 1997; Basson *et al.*, 1998; Wang and Friedman, 1998; Bordonaro *et al*, 1999;

Siavoshian *et al*, 2000), inhibition of growth (Archer *et al*., 1998a; Archer *et al*., 1998b; Basson *et al*., 1998), membrane synthesis and sodium absorption of colonocytes (Schwartz *et al*. 1998) and changes in protein and gene expression (Archer *et al*., 1998; Tappenden and McBurney, 1998; Coradini *et al*., 2000; Buecher *et al*., 2001). *In vivo* Behall *et al* (1997) reviewed that SCFAs change intestinal morphology of rats. Colonic infusion of SCFAs resulted in greater mucosal height and DNA synthesis, but no change in absorption of water, electrolytes, or butyrate (Reviewed by Sellin, 1993). There is now evidence that these SCFAs can stimulate intestinal cell proliferation *in vivo* (Goodlad *et al*., 1989; Goodlad *et al*., 1995; Ichikawa and Sakata, 1998). The ratio of SCFA production is very important (Reviewed Eastwood, 1995), with different fibres producing different levels of these SCFAs (Edwards and Eastwood, 1992; Reviewed Rombeau and Roth, 1995; Wang and Friedman, 1998).

1.5.5.1 Acetate.

Acetate enters the portal system, is mainly metabolised in the liver into long-chain fatty acids and ketone bodies providing energy for the periphery (Reviewed by Rombeau and Roth, 1995). Acetate may be an important precursor for lipogenesis in the absence of adequate carbohydrate intake (Reviewed by Rombeau and Roth, 1995). The minimal metabolism of acetate by colonocytes suggests it will have little biological influence directly on them. Acetate has been shown to have some immunomodulatory effects, increasing IL-18 intracellular levels, an interleukin which has been shown to have potential anti-neoplastic activity (Kalina *et al*., 2002).

1.5.5.2 Propionate.

Propionate is partly metabolised by colonocytes but is primarily metabolised by the liver, thus propionate could elicit a biological effect on colonic epithelial cells (Reviewed by Rombeau and Roth, 1995; Reviewed by Macfarlane and Gibson, 1994). The fact that the majority of fibres that produce high levels of propionate lowers cholesterol lead to the suggestion that propionate may be a mediator of NSP in reducing cholesterol levels (Reviewed by Ink and Hurt, 1987; Reviewed by Topping, 1995; Reviewed by Rombeau and Roth, 1995). SCFAs are thought to

inhibit hepatic cholesterol synthesis (Reviewed by Behall, 1997), propionate has specifically been shown to inhibit cholesterol synthesis *in vitro* in isolated hepatocytes, an effect produced by certain drugs used to lower plasma cholesterol in humans with hypercholesterolemia (Reviewed by Topping, 1995; Reviewed by Rombeau and Roth, 1995) possibly by the redistribution of cholesterol from the plasma to liver (Reviewed by Rombeau and Roth, 1995). Propionate may also inhibit 3-hydroxy-3-methyl-glutary CoA reductase (HMG-CoA), the rate limiting enzyme in hepatic cholesterol synthesis (Reviewed by Ink and Hurt, 1987). Other fibres (gum arabic for example) which also produce high propionate levels have failed to reduce cholesterol *in vivo* (Reviewed by Topping, 1995) suggesting other factors are involved, possible factors include viscosity and butyrate production (Reviewed by Topping, 1995). Propionate has been shown to be a weak deactylase inhibitor thus possibly affecting gene expression although it is less effective than butyrate in altering NF-κB activity *in vitro* (Yin *et al.*, 2001). Propionate has also been shown to be a potent colonocyte mitogen *in vitro* (Friedman *et al.*, 1988), others have shown it to reduce invasion *in vitro* (Emenaker and Basson, 1998) and to induce apoptosis *in vitro* (Marchetti *et al.*, 1997)

1.5.5.3 Butyrate.

Colonocytes undergo apoptosis in the absence of SCFAs, interestingly, SCFAs, particularly butyrate, have the opposite effect on transformed cells in culture, inducing rather than suppressing apoptosis (Yin *et al.*, 2001). Singh *et al.* (1997) showed that butyrate is both growth stimulatory and growth inhibitory depending on the availability of other energy sources such as glucose. Tumour and normal cells may have different responses to butyrate and may rely on other energy sources such as glucose, this is also suggested by increased GLUT-2 expression in tumour cells (Reviewed Hague *et al.*, 1997). Others have suggested that the paradoxical effects of butyrate (*in vivo* versus *in vitro*) do not appear to represent inherent differences between normal and transformed cells (Gibson *et al.*, 1999). Rather, the response may be determined by the state of activation of the cells (Gibson *et al.*, 1999).

Butyrate is the most important fuel for colonocytes contributing to 70% of oxygen consumption during cell oxidation, even though it is produced at the lowest amount (Reviewed by Rombeau and Roth, 1995). Butyrate has a short half-life and is metabolised quickly (Reviewed by Rephaeli *et al.*, 2000), butyrate prodrugs are being developed for use in anti-cancer, haemoglobinopathies and protection against chemotherapy induced alopecia (Reviewed by Rephaeli *et al.*, 2000) revealing the diversity of butyrate activity. Butyrate has been shown to have a number of biological effects, *in vivo*, including the proliferation of epithelial cells (Reviewed by Rombeau and Roth, 1995). The mechanism of this increase in proliferation is unknown but could be due to energy source, increase blood flow, increase in enterohepatic gastrointestinal hormones and increases in pancreatic and biliary secretion (Reviewed by Rombeau and Roth, 1995). Butyrate causes differentiation, growth inhibition and apoptosis *in vitro*, and these may attribute to butyrates underlying mechanisms of protecting against colorectal cancer development (Hague *et al.*, 1995; Reviewed by Rombeau and Roth, 1995; Lührs *et al.*, 2002). Transformed Caco-2 cells treated with sodium butyrate where shown to lose their malignant phenotype undergoing reduced proliferation, enhanced differentiation, stimulation of apoptosis leading to decreased viability of cells, and stimulation of interleukin-8 secretion (Dzierzewicz *et al.*, 2002). SCFAs induce cell cycle inhibitors in colonocytes, with butyrate blocking cells mainly in the G1 phase of the cell cycle (Siavoshian *et al.*, 2000). Butyrate mediates growth inhibition of colonic cancer cells by inducing p21 mRNA and protein expression (Archer *et al*, 1998a; Archer *et al.*, 1998b; Siavoshian *et al.*, 2000) and also results in the induction of p27kip1 (Wang and Friedman, 1998) and Cyclin D3, with p21 being responsible for the G1 blockage (Buecher *et al.*, 2001) thus effecting proliferation and differentiation (Siavoshian *et al.*, 2000). Butyrate also induces histone H4 hyperacetylation (Siavoshian *et al.*, 2000) and reduces cyclin D1 and p53 levels (Coradini *et al.*, 2000).

Avivi-Green *et al.* (2000b) has shown that apoptosis cascade proteins are differentially localised along the lumen-crypt axis, and their expression and activity may be controlled by dietary components and may partially account for the documented protective effect of butyrogenic fibres on colorectal cancer (Avivi-Green *et al.*, 2000b). Butyrate induces apoptosis *in vitro* (Hague *et al.*, 1995; Marchetti *et*

al., 1997; Chen *et al.*, 2002; Menzel *et al.*, 2002) via Fas receptor dependent signal transduction (Fan *et al.*, 1999, Reviewed by Chapkin *et al.*, 2000). Avivi-Green *et al.* (2000b) found that high levels of butyrate inhibit early and late events in colon tumourigenesis by controlling the transcription expression and activity of key proteins involved in the apoptotic cascade including caspase 1 and Bcl-2. Butyrate-induced apoptosis correlated with an increase in Bak expression and a decrease in the expression of Bcl-xl (Menzel *et al.*, 2002). The induction of p21 and p27 and the down-regulation of Bcl-2 and Bcl-xl by butyrate suggests a link between the cell-cycle mechanisms regulating enterocyte differentiation and apoptosis (Litvak *et al.*, 1998). Litvak *et al.* (1998) showed that differentiation and apoptosis occurred simultaneously in Caco-2 cells, which suggested that apoptosis maybe linked to enterocyte differentiation (Litvak *et al.*, 1998). Butyrate has been shown to result in the inhibition of DNA synthesis in the G1 phase of the cell cycle and leading to differentiation (Reviewed by Rombeau and Roth, 1995). SCFAs affects mucosal colonocyte brush-border enzymes indicating enterocyte differentiation (Basson and Sgambati, 1998; Siavoshian *et al.*, 2000). Differentiation induced by butyrate in HT29 cells found parallel increases in the enzyme glutathione S-transferases and other detoxification systems activities (Kirlin *et al.*, 1999). TGF-β1 is required for butyrate induced Caco-2 cell differentiation (Schroder *et al.*, 1999) and has been shown to reduce protein kinase C (PKC) activity (Rickard *et al.*, 2000). Butyrate differentiation is thought to model the migration to the surface epithelium (Mariadason *et al.*, 2000). Dietary fibre may protect against invasive colon cancer through stimulation of tissue inhibitor matrix metalloproteinases (TIMPs) and the inhibition of urokinase plasminogen activator (uPA) activities, with butyrate inhibiting SW1116 invasion (Emenaker and Basson, 1998).

Other biological effects include the modifying cell morphology and ultrastructure (by organising actin and vimentin) (Reviewed by Rombeau and Roth, 1995). Butyrate is able to induce a variety of changes in the nucleus, including histone hyperacteylation, inhibition of histone phosphorylation and DNA methylation (Lührs *et al.*, 2002). Butyrate causes histone hyperacetylation through non-competitive and reversible inhibition of histone deacetylases (HDACs) (Reviewed by Cress and Seto, 2000; Lührs *et al.*, 2002). Histone hyperacetylation has been associated with both a

decrease in cell growth and activation of specific genes (Lührs *et al.*, 2002). Several studies have pointed to the possible involvement of histone deacetylases in human cancer, thus butyrate may have potent anti-tumour effects (Reviewed by Cress and Seto, 2000). Butyrate has also been shown to result in hyperacetylation of the nonhistone nuclear protein HMG-N2 (high mobility group) protein *in vitro*, which changes its binding properties to chromatin potentially changing gene expression (Lührs *et al.*, 2002). Modulation of this non-histone chromatin protein may be partly responsible for the wide range of butyrate effects (Lührs *et al.*, 2002).

The influence of butyrate on cellular gene expression has received attention due to its ability to inhibit histone deactylases (Yin *et al.*, 2001). A number of transcription factors are directly regulated by acetylation, and butyrate can enhance their activity (including GATA-1 and p53) (Yin *et al*, 2001). There are conflicting reports as to whether butyrate affects proteosome activity (Buecher *et al.*, 2001; Yin *et al.*, 2001) thus it maybe dependent on the protein being investigated and the cell system. If Butyrate suppresses proteosome activity in the cell this could have an impact on a number of colonocyte and colon cancer cell activities as many proteins that regulate the cell cycle (eg cyclins and β-catenin) and cell death (p53) are regulated by the ubiquitin-proteosome pathway (Yin *et al.*, 2001).

Retinoblastoma protein (RB) is involved in the regulation of the cell cycle, preventing entry into S phase, by binding to E2F, and has also been shown to bind to HDACs. There is a connection between butyrate exposure (a HDAC inhibitor) and retinoblastoma (RB) tumour suppressor gene function. p21 mRNA increases following treatment with butyrate, with p21 being essential for butyrate to induce growth arrest (Reviewed by Cress and Seto, 2000). Other studies have revealed that RB functions downstream from p21 resulting in growth arrest (Mal *et al.*, 2000). APC also works upstream from RB to prevent entry into S phase resulting in cell cycle arrest (Reviewed by Sellin, 2001). Basson *et al.* (1998) showed that the SCFA butyrate downregulated c-myc expression and altered the tyrosine phosphorylation status of some intracellular proteins in CaCo2 cells. Sodium butyrate has also been shown to result in a reduction of nuclear c-myc levels (Taylor *et al.*, 1992). Butyrate results in the reduction of protein kinase C isoforms, PKCα and PKCε, in colonic

cell lines (Rickard *et al.*, 2000). A number of luminal factors activate PKC such as diacylglycerol (DAG), secondary bile acids and faecapentenes (Rickard *et al.*, 2000). Secondary bile acids and faecapentenes are potentially positively associated with colorectal cancer thus if butyrate reduces PKC activity this could be another potential protective mechanism of butyrate against colorectal cancer (Rickard *et al.*, 2000). The Cdx genes CDX1 and CDX2 are expressed in the colorectal epithelium and are frequently downregulated during tumourigenesis, butyrate showed no regulation of these proteins when induced to differentiate (Qualtrough *et al.*, 2002).

Butyrate also appears to have anti-inflammatory activities (Yin *et al.*, 2001). IL-18 has been associated with anti-tumour activity and butyrate has been shown to activate the IL-18 promoter upregulating IL-18 protein expression in intestinal epithelial cells (Kalina *et al.*, 2002). The anti-inflammatory effects of butyrate has also been shown by its ability to modulate IL-12 and IL-10 receptor expression, down regulating IL-12R expression and upregulating IL-10R expression (Raingeard de la Blétière *et al.*, 2001a). Butyrate pre-treatment of a human colon cell line (HT29 cells) inhibited the tumour necrosis factor-α induced nuclear translocation of the proinflammatory transcription factor NF-κB (Yin *et al.*, 2001).

Butyrate has also been proposed to have an effect on cholesterol metabolism, again the mechanism is unknown, but as for propionate could be by shifting cholesterol from the plasma to the liver (Reviewed by Rombeau and Roth, 1995; Reviewed by Topping, 1995). Butyrate may regulate apolipoprotein A-1V (apoA-1V) secretion, a ligand for high density lipoproteins, thus modulating reverse cholesterol transport resulting in a 20-30% increase in cholesterol efflux, the moving of cholesterol out of the body (Nazih *et al.*, 2001). Butyrate has also been shown to have a small effect on glucose and glutamine metabolism (Reviewed by Rombeau and Roth, 1995). In Caco-2 cells butyrate was shown to modulate lipid synthesis, apolipoprotein biogenesis, and lipoprotein assembly thus suggesting a potential regulation of intestinal fat absorption and circulating lipoprotein concentrations (Marcil *et al.*, 2002).

1.5.6 Health Benefits of Dietary Fibres and their use in disease states.

Dietary Fibre is thought to have a number of properties that influence a number of prevailing western diseases. Diet gives clues to the aetiology of many of the life style diseases, such as colon cancer and heart disease (Reviewed by Kritchevsky, 2001). Metabolic effects of fibres include reducing obesity (by reducing calorie intake), gallstones (through reducing cholesterol saturation and possibly affecting the bile acid pool), diabetes (normalising glucose tolerance and plasma insulin levels), coronary heart disease and colon cancer (Reviewed by Kritchevsky, 2001).

1.5.6.1 Large Bowel Disorders.

The majority of diseases that occur in the human large intestine are of unknown aetiology, but diet and bacteria have been implicated in having a role (Reviewed by Gibson and Macfarlane, 1994). The mechanical action of dietary fibre includes influencing stool weight resulting in the relief of constipation, diahorrea, irritable bowel syndrome and diverticular disease (Reviewed by Lembo, 1998; Reviewed by Kritchevsky, 2001) with fibre therapy improving symptoms (Reviewed by Lembo, 1998). SCFA (Butyrate) intracolonic infusions have been shown to have beneficial effects on diversion colitis and distal ulcerative colitis (Reviewed by Gibson and Macfarlane, 1994; Reviewed by Rombeau and Roth, 1995). Antibiotic associated colitis, inflammatory bowel disease (IBD) and colon cancer are all thought to have an aetiology connected with the activities of the gut flora (Reviewed by Gibson and Macfarlane, 1994), which are in turn affected by fibre. In Crohn's disease fish oils including (n-3) PUFAs inhibit the inflammatory reaction (Reviewed by Rogers, 1997), showing a possible role of dietary modification of immune function.

1.5.6.2 Colorectal cancer.

Burkitt was the first to propose the link between fibre intake and susceptibility to cancer of the colon and rectum (Reviewed by Kritchevsky, 2001). A diet rich in plant foods contains a number of possible anti-carcinogenic phytochemicals which may augment fibre action, exert a specific action of their own, or even antagonize the action of other food components (Reviewed by Kritchevsky, 2001). Colon cancer is

a disease of excess, over nutrition, which is a symptom of westernisation, resulting in an increase intake of refined carbohydrates and fast foods (Reviewed by Hill, 1999a). This accompanied by a decline in the level of physical exercise has attributed to an increase in obesity (loss of energy balance) (Reviewed by Hill, 1999a). Physical activity is protective (Reviewed by Oliveria and Christos, 1997), consistently being associated with a low risk of colorectal cancer, breast and prostate cancers (Reviewed by Hill, 1999a; Reviewed by Gerber and Corpet, 1999). Obesity (energy imbalance) is positively associated with colorectal adenomas and cancer risk, with several determinants of obesity being related to colorectal cancer risk (Reviewed by Stephen, 1995; Reviewed by Gerber and Corpet, 1999; Reviewed by Hill, 1999a).

Whether fibre has a protective role is unclear (Reviewed by Kritchevsky, 2001). Overwhelming epidemiological evidence suggests that diet is related to colorectal cancer development, including dietary fibre (Reviewed by Cummings and Bingham, 1987; Reviewed by Bingham, 1990; Reviewed by Cummings and Bingham, 1998). Epidemiological studies on black Africans revealed a low incidence of colorectal cancer and this was associated with a high fibre, low calcium and low fat diets (Reviewed by Walker, 1995). The protective effects of fibre in laboratory animals against colorectal cancer have also been studied; these results have given confusing information suggesting a protective, neutral or even promotional effect on colon cancer development. These mixed results could be the result of the use of different fibres and differences in experimental models (Reviewed by Jacobs, 1990; Reviewed by Kurfeld, 1990; Reviewed by Harris and Ferguson, 1993; Reviewed Kritchevsky, 2001). There is a suggested association between insulin resistance and colon cancer with a plausible suggestion that low glycaemia index foods are protective for colon cancer through the systemic effects in reducing risk factors associated with the development of type 2 diabetes (Reviewed by Jenkins and Kendall, 2000). High calorie intake, particularly refined carbohydrate, may result in the synthesis of IGF-1 through the insulin-resistance syndrome (Reviewed by Gerber and Corpet, 1999). If carbohydrate has a protective role in colon cancer prevention, it may lie in the systemic effects of low glycaemic index foods (Reviewed by Jenkins and Kendall, 2000). Interestingly, calorie restriction is also shown to reduce the number of spontaneous and carcinogen induced colorectal cancers in animal models, even in the presence of high fat, again suggesting the importance of an energy balance

(Reviewed by Hill, 1997a; Reviewed by Gerber and Corpet, 1999; Reviewed by Hill, 1999). The role of energy restriction is thought to be due to reducing colonic epithelial cell proliferation and enhancing apoptosis (Reviewed by Gerber and Corpet, 1999).

Fat and red meat was found as a risk factor in colorectal cancer, dietary fat modifies many colonic parameters, including bile acid and fatty acid concentrations, gut flora metabolism of bile acids (Reviewed by Gerber and Corpet, 1999). Although not all fat is bad for you, there is a positive association with animal fat but there is a negative association with vegetable fat and with fish oils and colon cancer (Rodgers, 1997).

1.5.6.3 Possible protective mechanisms of dietary fibres.

A number of other mechanisms have been proposed for the protective effect of dietary fibre (Reviewed by Harris and Ferguson, 1993) including the effect on immune function, transit time (decreasing transit time thus reducing the length of time carcinogens are in contact with the colonic mucosa and changes in nutrient absorption), hormones (gastrin, motilin or glucagon, insulin, oestrogen, IGF-1), body fat and blood concentration of energy nutrients (glucose, triglycerides, fatty acids) (Reviewed by Stephen, 1995; Reviewed by Oliveria and Christos, 1997; Reviewed by Gerber and Corpet, 1999). These blood factors could in turn lead to insulin resistance, and enhance the proliferation of normal and neoplastic cells (Reviewed by Gerber and Corpet, 1999).

Possible protective effects of dietary fibre may be due to their bulking ability resulting in a reduced calorie density and digestibility as well as their own low calorie content (Reviewed by Gerber and Corpet, 1999). Fibre increases the stool bulk and speeds up intestinal transit thus results in the dilution of any possible carcinogen along with their contact with the colonic mucosa. Fibre is thought to increase bulk (McNeil, 1978) by increasing bacterial biomass, water and bulk content thus diluting down the carcinogen and decreasing transit time (Reviewed by Mason, 1998; Reviewed by Lembo, 1998). This decrease in transit time subsequently decreases the duration that the carcinogens are in contact with the colonic mucosa

(Reviewed by Greenwald and Clifford, 1995). The increasing fibre intake would reduce the amount of protein digested and the level of toxic by products produced (Reviewed by Eggum, 1995). The colonic microflora have been shown to play an important role in human health and disease (Reviewed by Rao, 1995). Interestingly in Africa there is a correlation between bacterial species and colorectal cancer incidence, those that possess methogenic bacteria appear to have the lowest colorectal cancer incidence (Reviewed by Walker, 1995). Tumours are also more common in the large bowel compared to the small bowel suggesting that the microflora may contribute towards the carcinogenesis (Reviewed by Gibson and Macfarlane, 1994). Diets high in fat and protein and low in fibre can result in an unfavourable microbial environment in the human colon. Different fibres and bacteria also differ in SCFA profiles when fermented in the colon, which could significantly effect the development of colon cancer. Different changes in microflora associated with different fibres in turn affect the ability of the microflora to ferment different fibres subsequently affecting SCFA profiles (section 1.5.4). The fermentation of fibre also results in butyrate production within the large intestine, which might protect against colorectal cancer as butyrate has been shown to induce differentiation, apoptosis, along with SCFAs lowering the pH of the lumen decreasing the levels of secondary bile acids being produced (Reviewed by Key *et al.*, 2002). Faecal butyrate levels have been shown to negatively correlate with tumour mass (Reviewed by Kritchevsky, 2001) and has been shown to protect against DMH induced tumourigenesis *in vivo* (McIntyre *et al.*, 1993).

The major mechanism by which the gut flora may be involved is by the production of carcinogens from non-toxic precursor molecules (Reviewed by Gibson and Macfarlane, 1994). Low risk populations contained lower populations of *Bacteroides* and *Bifidobacteria*, with increased counts of faecal streptococci and a low anaerobic: aerobic ratio (Reviewed by Gibson and Macfarlane, 1994). Faeces from individuals from the west frequently contain mutagenic substances (Reviewed by Gibson and Macfarlane, 1994). Faecapentaenes are the most prevalent genotoxin in the western diet being conjugated to lipids, which exert potent mutagenic activity (Reviewed by Gibson and Macfarlane, 1994). Microflora enzymes are readily induced by changes in the metabolic environment, which can be attributable to changes in diet, which as a consequence alters the spectrum of substrates hydrolysed,

reduced, degraded or synthesised by these flora (Reviewed by Bingham, 1990). This could be especially important in the activation or degradation of carcinogens (Reviewed by Bingham, 1990). Other enzymes that bacteria produce that may be carcinogenic are nitroreductases and azoreductases as they convert procarinogens into carcinogens (Reviewed by Gibson and Macfarlane, 1994). These compounds have adverse effects towards colonic epithelial cells and are potentially harmful (Reviewed by Gibson and Macfarlane, 1994).

Interestingly human populations with lower faecal pH show lower rates of colorectal cancers (Reviewed by Bruce, 1987). The fermentation of fibre lowers faecal pH (Ranhotra 2001), which in turn affects enzyme activity such as 7-α-hydroxylation and β-glucuronidase. β-glucuronidase is an inducible enzyme involved in the activation of procarcinogens and carcinogens, and may thus be a risk factor for the development of colon cancer (Rodgers, 1997). When pH decreases, due to the SCFA production, the bile acid solubility also changes. A decrease in pH results in a reduction in free primary bile acids in the faecal water, which subsequently decreases the number of primary bile acids free to be converted into secondary bile acids (Reviewed by Harris and Ferguson, 1993). Bile acids are also though to be a causal factor in colorectal cancer (Reviewed by Chaplin, 1998). Faecal bile acid concentrations correlates with the risk of large bowel cancer, it is thought that colonic bacteria convert bile acids into steroids, which have carcinogenic properties (Reviewed by Gibson and Macfarlane, 1994; Reviewed by Chaplin, 1998). Secondary bile acids having been shown to promote colorectal cancer development in animal models (Reviewed by Jacobs 1988; Reviewed by Chaplin, 1998; Reviewed by Blackwood *et al.*, 2000; Reviewed by Kritchevsky, 2001). Thus a decrease in pH should theoretically decrease the potential tumour promoter activity of secondary bile acids (Reviewed by Harris and Ferguson, 1993). The concentration of bile acids in faeces in populations at high risk have been shown to be elevated, probably because of less dilution of colonic content (Reviewed by Kritchevsky, 2001). With colorectal cancer the ratio of lithocholic acid and deoxycholic acids has been shown to be an important aetiological factor (Reviewed by Story, 1995; Reviewed by Kritchevsky, 1995). All together dietary fibre (NSP) itself could be protective by driving fermentation towards their own digestion rather than that of protein, which

produces toxic by products such as amines. Although a decrease in pH may also be seen as a risk factor due to the increase in cell proliferation this causes, potentially enhancing chemically induced/initiated carcinogenesis (Newmark and lupton, 1990). Thus changes in colonic homeostasis is also important (Reviewed by Roncucci, 2000) with a number of groups showing dietary fibre to effect proliferation (Robblee, 1989; Pell, 1995), changes in crypt number (McCullough *et al.*, 1998) and apoptosis (Avivi-Green *et al.*, 2000a; Aviv-Green *et al.*, 2000b).

1.6 Models of Colorectal Cancer

1.6.1 Animal Studies - In vivo models of colorectal cancer.

Animal studies are selectively designed to demonstrate how specific dietary components influence a given outcome (Rodgers, 1997). Rodent studies are a useful tool in studying the role of nutritional factors in cancer development determining how non-absorbable dietary components influence the development of colonic neoplasia (Rodgers, 1997). Animal models provide a relatively quick analysis, colon cancer can take up to 50 years to develop in humans, whereas a rodent life cycle can be completed within 3 years. Advantages of animal models include the fact that each mouse is genetically identical suggesting that they share the same microflora thus it would only be the luminal influences (Fibre) that would affect microflora and cancer development. Mice and rats are usually the model of choice because they are easy to house, easy to maintain and well characterised physiologically (Reviewed by Salyers, 1995). Using animal models individual stages of carcinogenesis can be studied helping eliminate some of the disadvantages of epidemiological studies (Reviewed by Hill, 1997a). They also have a number of disadvantages, rodents have a very low rate of spontaneous cancers, probably because of their short life span, therefore cancer needs to be induced with specific carcinogens which may not itself be an environmental carcinogen. There is also strain selectivity/sensitivity to the carcinogen thus giving a variety of results, but this is the same for the human population, and are probably due to differences in metabolism, genetics, being dependent on the initiator used, route of administration and sex of rodent (Reviewed by Hill, 1997a). The main problems with rodent

models in dietary studies come from differences in microflora to humans and also most rodents indulge in coprophagy (Reviewed by Salyers, 1995). Structural difference between mouse and man include the structure of the caecum, it is proportionally much larger in the mouse being the main fermentation site, whereas the caecum in man plays a less significant function. There are also differences in bacteria between man and mouse (Reviewed by Salyers, 1995), murine microflora have similar bacterial genres but has differences in species. In man there are also large differences between individuals. In human cancer the initiator is rarely known thus difficult to apply to animal studies (Reviewed by Hill, 1999). Diet manipulation is then studied to see whether the proportion with cancer can be decreased (protection) or increased (promotion) (Reviewed by Hill, 1999). In the case of colorectal cancer the main etiological agents, fat and fibre, act during the promotion stages, therefore if a carcinogen is used to initiate then can see if fat/fibre changes the rate of promotion/progression (Reviewed by Hill, 1997a).

There are a number of rodent models available including knockout mouse models where cancer related genes are over expressed (oncogenes) or inactivated (tumour suppressor genes) (Reviewed by Hursting *et al*, 1997). Those of particular interest are those involving the *APC* gene including APC (delta716) a model constructed by Oshima *et al* (1997) where *APC* gene contains a truncated mutation at codon 716 and were shown to develop numourous intestinal polyps, this model has been modified with other genes being knocked out including Smad4 and Smad2 (Takaku *et al*, 2002), APC(delta474) which also result in small intestinal adenomas (Sunayama *et al*., 2002) and APC(1638N) (Smits *et al*, 2000). The two most commonly used murine models are the multiple intestinal neoplasia (MIN) (Moser *et al*., 1990; Su *et al*., 1992; Dietrich *et al*., 1993; Moser *et al*.,1995; Barnes and Lee, 1998; Philipp-Staheli 2002; Anderassen, 2001; Roberts *et al*, 2002) and the 1,2-dimethylhydrazine mouse (Reviewed by Rogers and Nauss, 1985; Reviewed by Klurfeld, 1995; Moorghen *et al*., 1998; Jackson *et al*. 1999; Philipp-staheli *et al*., 2002).

1.6.3.2 Multiple Intestinal Neoplastic mouse.

FAP has provided a useful model for studying colorectal cancer and is characterised by the development of 1000's of polyps at a young age. An animal model of familial colon cancer has been developed known as the multiple intestinal neoplasia (MIN) mouse (Moser *et al.*,1990; Dietrich,1993; Moser *et al.*,1995; Barnes and Lee, 1998). The MIN mouse model is reflective of inherited (FAP) colorectal cancer (5% of all colorectal cancers), having an APC mutation (found in 80-85% of all colorectal cancers), which results in the production of a large number of small bowel tumours. The MIN mouse provides an excellent model to study genetic interactions and environmental influences such as diet on the colon (Dietrich, 1993; Moser, 1995).

1.6.3.3 1,2-dimethylhydrazine mouse (DMH) model.

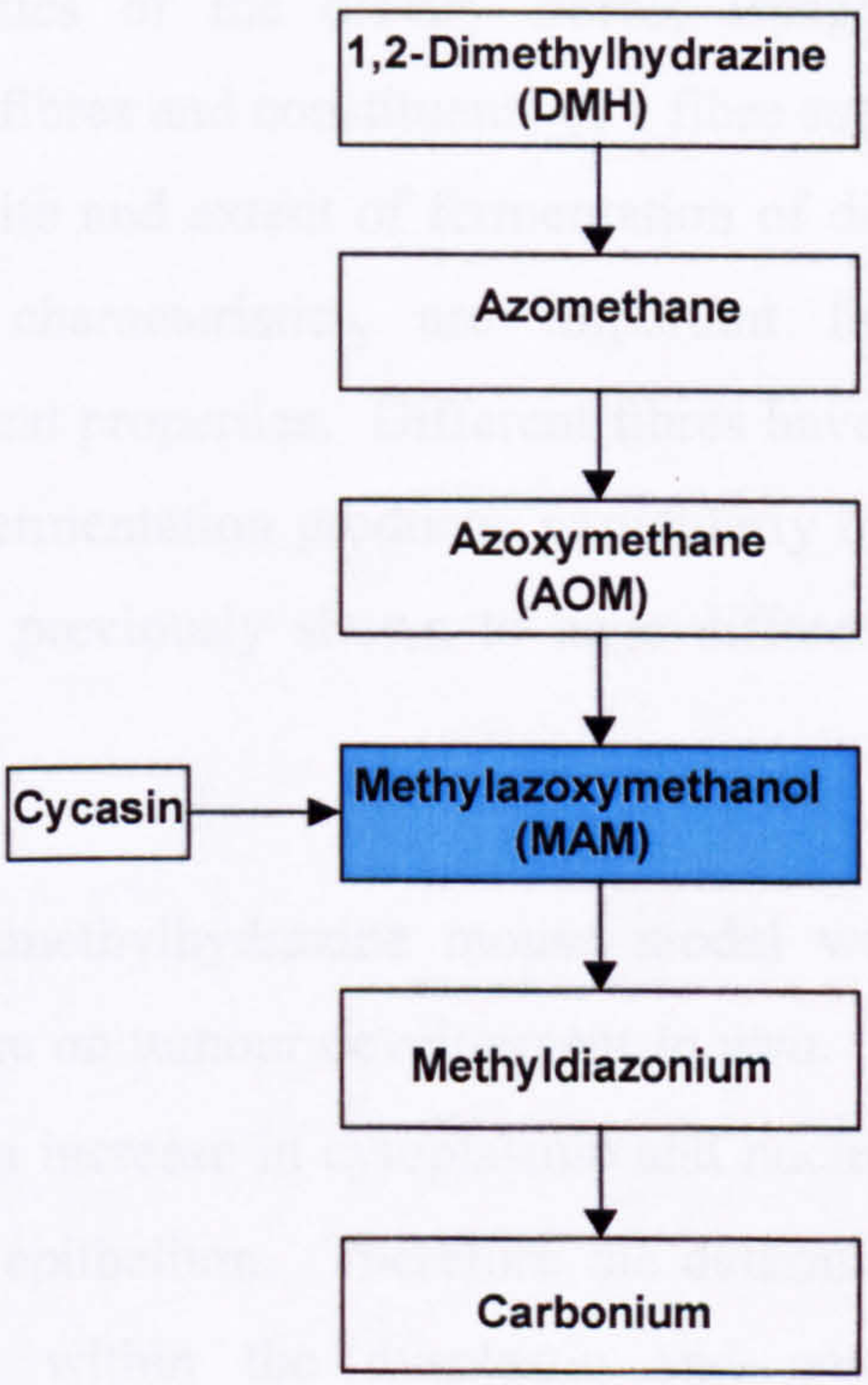
DMH is widely used to study chemically induced colon cancer (Reviewed by Rogers and Nauss, 1985; Reviewed by Klurfeld, 1995; Jackson *et al.* 1999) and provides a useful model for studying early carcinogenesis. In 1967 Druckrey discovered that 1,2-dimethylhydrazine (DMH) was a specific colon carcinogen (Reviewed by Pozharisski *et al.*, 1979; Reviewed by Rogers and Nauss, 1985; Reviewed by Klurfeld, 1995; Zhurkov *et al.*, 1996) being highly carcinogenic in rodents (Reviewed by Klurfeld, 1995). DMH mouse model is initiated using a chemical carcinogen, reflective of sporadic cancers (95% of all colorectal cancers). The chemical carcinogen is site specific to the large bowel. DMH is activated (metabolised) in the liver, and these active metabolites reach the dividing cells at the bottom of the crypt via the bloodstream (Weisburger *et al.*, 1977; Reviewed by Pozharisski *et al.*, 1979; Reviewed by Klurfeld, 1995). Pozharisski *et al.* (1979) also suggests DMH metabolites to also be excreted from the liver via biliary secretion but other groups have found no evidence for this biliary secretion (Reviewed by Sunter, 1980). The metabolism of DMH, suggested by Druckrey (Fig 1.8), results in the production of an intermediate compound methylazoxymethanol (MAM), an unstable compound in aqueous solution with alkylating properties being observed both *in vitro* and *in vivo* (Reviewed by Sunter, 1980). The evidence points to methyldiazonium producing the alkylating properties providing the carcinogenic effects of these compounds (Reviewed by Sunter, 1980).

The metabolite of DMH, methylazoxymethanol (MAM), is known to be a DNA akylating agent (Reviewed by Sunter 1980; Reviewed by Klurfeld 1995) and the pro-mutagenic lesion O^6-Methylguanine has been detected in DNA from various rat and mouse tissues following exposure to DMH (Jackson *et al*, 2000). Many groups have looked at the various genes found mutated in human colorectal cancer to see if they are affected. For example Rudzki *et al.* (1997) found that there was marked global overexpression of standard and variant CD44. Jackson *et al.* (1999) found that there was no mutations found in either codon of *K-ras* (a gene commonly mutated in human tumours). Another study revealed that *K-ras* mutations were an early event in colorectal tumourigenesis when examining tumours induced by DMH in rats (Jacoby *et al.*, 1991). It has been shown that AOM, a derivative of DMH, causes a mutation in the GSK-3β consensus motif of *β-catenin* in rats (Takahashi *et al.*, 1998), and mice (Takahashi *et al.*, 2000a; Takahashi *et al.*, 2000b). The role of p53 in DMH tumour development has been studied and revealed that IgG antibodies against tumour associated antigens including p53 resulted in the decrease in incidence and progression of the tumours, suggesting a role for p53 in DMH tumour development (Zusman *et al.*, 1996). Further studies found that germline mutations in *p53* enhance the incidence of DMH induced colon tumours (Lubet *et al.*, 2000). Subsequent studies have revealed *p53* mutations, with majority of mutations occurring in intron 6 (non-coding) and exon 4 region (Jenkins *et al.*, 1997). Refer to chapter 4 for more information on genetic changes within the DMH model.

Observations in the DMH mouse have revealed that the changes seen in the mouse bear many similarities with the morphological changes observed in human adenomatous polyposis (Reviewed by Rogers and Nauss, 1985; Carter *et al.*, 1994; Moorghen *et al.*, 1998). Subcutaneous administration of DMH initially results in necrosis of the colonic crypt epithelial cells with crypts appearing markedly reduced in size with the appearance of morphologically abnormal crypt cells (Reviewed by Sunter, 1980). Chronic administration of DMH results in hyperplasia (characterised by increased crypt length and proliferative compartment, dilatation, tortuosity and crypt branching) (Chang, 1978; Chang, 1980; Moorghen *et al.*, 1998) followed by the appearance of dysplastic foci (glandular fan shaped microadenomatas) near the

mucosal surface(Reviewed by Sunter, 1980; Chang, 1980). These microadenomas grow into macroscopic sessile polyps (adenomas), which can become invasive (invasive adenocarcinoma) (Carter *et al*., 1994; Moorghen *et al*., 1998).

Figure 1.8 Metabolism of DMH (cycasin-related Compounds) (Sunter, 1980)



1.7 Aims

The main aim of this study was to evaluate whether dietary fibre supplements elicit a protective effect against colorectal cancer development and if this is mediated via the cadherin/catenin complex since the regulation of β-catenin expression levels appears to be a fundamental component of colorectal cancer development.

Initially a study was conducted to determine the physical and chemical characteristics of the dietary fibres; ispaghula husk, pectin and cellulose, as individual fibres and constituents of a fibre supplemented diet used within this study. The rate, site and extent of fermentation of dietary fibres, which are based on their structural characteristics, are important factors in determining dietary fibre physiological properties. Different fibres have also been shown to produce different levels of fermentation products, particularly butyrate, propionate and acetate, which have been previously shown to have differential biological effects on the colonic epithelium.

The 1,2-dimethylhydrazine mouse model was chosen to examine the effects of dietary fibre on tumour development *in vivo*. Preliminary studies on archival tissues revealed an increase in cytoplasmic and nuclear localisation of β-catenin within the dysplastic epithelium. Therefore the determination of cadherin/catenin component expression within the dysplastic and non-dysplastic epithelium of the 1,2-dimethylhydrazine mouse model was examined.

The effects of dietary fibre supplements (ispaghula husk, pectin and cellulose) on 1,2-dimethylhydrazine induced colonic adenomas and cadherin/catenin expression was then investigated. This involved a 17 week feeding study, where mice were fed on a basal diet supplemented with the various dietary fibres accompanied by weekly injections of the carcinogen. A number of parameters were to be examined including tumour incidence, number, burden and cell proliferation. The parameters chosen were useful for examining tumour initiation and development.

The effects of dietary fibre were examined further to try and elucidate any biological mechanism of action from the effects observed *in vivo*. This involved the examination of the effects of luminal factors (dietary fibres and short chain fatty acids butyrate, acetate and propionate) on a parameters of colonic homeostasis and cadherin/catenin protein expression in human and murine colonic epithelial cell lines.

Chapter 2

Materials and Methods

2.1 Fibre Characterisation.

2.1.1 Fermentation Study using faecal slurries under anaerobic conditions.

Fresh human faecal stool samples from a male Caucasian, 51 years of age, and of good health with no recent intake of antibiotics, were collected into a polythene bag, immediately sealed to occlude oxygen, and stored at 37 °C for 1 hour. 0.1g of fibre (ispaghula husk, pectin, cellulose, resistant starch, Table 2.1) or 0.2g of diet (basal, basal supplemented with ispaghula husk, pectin or cellulose) were hydrated in 5 ml (2% w/v and 4% w/v respectively) of anaerobic mineral salts medium (MSM, Table), within the anaerobic cabinet (MACS-VA500 – Microaerophilic workstation, Don Whitley Scientific, Shipley, West Yorks) under anaerobic conditions (10% Carbon dioxide, 10% hydrogen, 80% nitrogen) for 2 hours.

Table 2.1

Dietary Fibre	Brand Name	Supplier
Ispaghula Husk	Fybogel	ReckittBenckiser Healthcare limited
Pectin	Genu Pectin Type FIT-1	Copenhagen Pectin A/S, Division of Hercules Incorporated, Denmark
Cellulose	Solkafloc	James River Corporation, NJ
Rice Starch		Special Dietary Services, Witham, Essex
Resistant Starch	Novelose (Zea Mais, Corn Maize)	National Starch and Chemical, Manchester, UK

The mineral salts medium (MSM, Table 2.2a and b) was previously placed in an anerobic environment for 24 hours. A faecal homogenate was achieved by adding 40g fresh stool sample to 200 ml of anaerobic MSM (16.67% w/v) in a class 2 cabinet (Endair). The faecal homogenate was stomached (Seward stomacher 400) in a stomacher bag for 30 seconds. The resulting slurry was centrifuged (3000 rpm for 10 minutes; MSE, Mistral 2000) separating out the solids from the supernatant (which

contained the relevant bacteria), and returned to the anaerobic cabinet to minimise the oxygen content.

Three different reaction mixtures were set up within the anaerobic cabinet; firstly the test sample (in triplicate) containing 5 ml of hydrated fibre in MSM diluent and 5 ml faecal homogenate supernatant (1% w/v fibre), a fibre control containing 5 ml of hydrated fibre in MSM diluent and 5 ml MSM (1% w/v fibre) and three faecal controls containing 5 ml of faecal homogenate supernatant and 5 ml MSM diluent. All were then agitated. Anaerobic conditions were monitored using internal and external oxygen meters and remained around 0.1% throughout.

At designated times (0, 3, 6 and 24 hours) 0.1 ml of reaction mixture was removed in duplicate and diluted (x10) with 0.9 ml acidified propan-2-ol containing 5.55mM internal standard (ethyl butyric acid) followed by centrifugation (13000rpm, 5 minutes). Aliquots of the clear supernatant were pipetted into crimp sealed gas chromatography injection vials and stored at 4°C until analysis by gas chromatography.

An external standard solution was prepared for gas chromatography analysis containing 5mM concentration of acetic acid, propionoic acid and butyric acid (the three principle short chain fatty acids produced by bacterial fermentation, Sigma). GC analysis (Supelco Nukol 24107 capillary column (30m x 0.25mm x 0.25μm) oven maintained at 160°C, injector and detector maintained at 250°C, with injection volume of 1μl. Carrier gas (helium at 16.6psi) flow rate of 8.2ml/min. Hewlett Packard instrumentation) coupled with external standard solutions (5mM concentration of acetate, propionate and butyrate) was performed in duplicate.

The concentration (Conc) of short chain fatty acids (SCFAs) in the sample was calculated using the following formula for each dietary fibre at each time point:

For each SCFA

Conc. SCFA in sample = (Conc of std/response of std) **x** response of sample

Therefore the standard (std) for butyrate would be used to calculate the level of butyric acid present within the sample.

The mean mM concentration minus the mean concentration for the faecal homogenate control was then determined for each dietary fibre or diet at each time point.

mM concentration of SCFA Within the sample	–	mM concentration of SCFA with faecal control

Table 2.2a Mineral Salts Media (MSM) pH 7

Compound	Weight (g)
Disodium hydrogen phosphate	2.00
Potassium dihydrogen orthophosphate	4.00
sodium chloride	6.00
Cysteine	0.8
sodium hydrogen carbonate	1.5
calcium chloride	0.1
Magnesium chloride	0.15
Cobalt chloride	0.1
manganese chloride	0.15
ferrous sulphate	0.005
haemin (1mg/ml in 001M KOH)	5 ml
purified water	made up to 1000 ml
Vitamin mixture (See Table 1b)	1ml

Table2.2b Vitamin mix

Compound	weight (mg)
Vitamin B12	0.5
Vitamin K	1.00
Biotin	1.00
Thiamin	2.00

2.1.2 Non-Starch Polysaccharide analysis using the Englyst technique.

Englyst (Modified from Englyst *et al.*, 1992) was used to determine total and insoluble non-starch polysaccharides (NSP) levels of ispaghula husk, pectin, resistant starch and cellulose (Table 2.1). It was further used to measure NSP levels of fibre based diets: basal, basal supplemented with ispaghula, basal supplemented with pectin and basal supplemented with cellulose. Isphagula husk also acted as a control sample as it has been characterised on numerous occasions by ReckittBenckiser Healthcare limited using the same technique.

100mg (fibre) and 200mg (diet) aliquots were weighed out into 50ml plastic screw top centrifuge tubes (TCR, Fischer), where 0.5ml acid rinsed sand (to prevent aggregation of sample) and a PTFE magnetic flea were then added to each tube. Dispersion of starch, to allow more effective starch hydrolysis, was achieved by boiling the samples in 2 ml of dimethylsulphoxide (DMSO, Sigma) for 30 minutes with continuous mixing. Enzymatic hydrolysis of starch was achieved by immediately adding 8 ml of 0.1M sodium acetate buffer (pH 5.2) and mixed (Table 2.3). Once samples had cooled to below 35°C 0.5 ml α-amylase (2150 units/ml, Sigma) and 0.1 pullulanase (1 unit/ml, Sigma) (Table 2.3) were added to each sample and incubated at 42°C (± 2) for 16 to 18 hours mixing after the first hour.

Depending on whether total or insoluble NSP was being determined the tubes were treated differently at this point. With total fibre analysis, the tubes were cooled to room temperature, 40 ml of absolute ethanol (BDH) added to each tube, mixed by inversion and placed on ice water for 30 minutes. The tubes were then centrifuged (2610rpm, 10 minutes), the supernatant aspirated and 10 ml 85% ethanol added. The samples were vortexed, filled to 50 ml with 85% ethanol, stirred for at least 5 minutes (at room temperature) and centrifuged (2610rpm, 10 minutes), supernatant aspirated.

With insoluble fibre analysis, samples were boiled for 30 minutes in 40ml of 0.2 M sodium phosphate buffer (pH7) (Table 2.3) with continuous stirring. The samples were then cooled in water for ten minutes, centrifuged (2610rpm, 10 minutes) and supernatant removed. 10ml of water added, vortex mixed and topped up to 50ml with

water. Samples were then stirred for 5 minutes, centrifuged (2610rpm, 10 minutes) and supernatant removed by aspiration.

All samples were treated the same from this point on: 10ml absolute ethanol (BDH) was added to all samples, mixed by inversion and filled to 50ml with absolute ethanol. After 5 minutes of mixing the tubes were centrifuged (2610rpm, 10 minutes) and the supernatant removed. 10ml acetone (BDH) was then added, vortex mixed and filled to 50ml with acetone. The tubes were stirred for 5 minutes, centrifuged (2610rpm, 10 minutes) and supernatant aspirated. The remaining residue was dried in a 80 °C water bath, with continual mixing.

Acid hydrolysis of the polysaccharides was achieved by adding 5 ml 12 M sulphuric acid (BDH) to the dry residue, vortex mixed and incubated at 35°C (±1) for 1 hour. The samples were periodically mixed to help disperse any cellulose. Twenty five ml of water was then rapidly added to each sample, vortex mixed, and boiled for 1 hour with continuous stirring. The samples were cooled, filtered (GF/C filter paper 110mm, Whatmann) into 8 dram glass vials and stored overnight at 5°C.

Alditol acid derivatisation of the sugars was achieved by aliquoting out 3ml of the hydrolysate or external sugars (0.5mg/ml rhamanose, xylose, arabinose, mannose, galactose and glucose in 2 M sulphuric acid) into 4 dram glass vials and 1 ml of allose internal standard (1.002mg/ml) added (Table 2.3). On ice, 2.4ml of 12M ammonium hydroxide (BDH) was added, followed by 0.4ml of sodium borohydride/ammonium hydroxide solution (200mg sodium borohydride (BDH) in 1ml 12M ammonium hydroxide (BDH) and 3ml of water) and 5µl of octan-2-ol (anti-foaming agent; BDH). After shaking, the vials were incubated at 40°C for 1 hour after which 0.8ml glacial acetic acid (BDH) was added.

Acetylation of the sugars was achieved by transferring 0.5ml of the acidified solution to an 8 dram vial where 0.5ml 1-methylimidazole (Sigma) and 5ml acetic anhydride (BDH) was added. The samples were shaken and left capped for 10 minutes. 1.25ml absolute ethanol was added, vials shaken, and left capped for 5 minutes. 10ml of water was added, shaken and left capped for a further 5 minutes. 0.5ml of 0.004%

bromophenol blue solution was then added to the vials, mixed and placed on ice water. 5ml 7.5M potassium hydroxide (BDH) was added to each vial followed by a further 5ml and then inverted to mix. The vials were left for 15 minutes while phase separation occurred. The clear phase was transferred to an autosampler vial containing a 250µl micro-insert for analysis by gas liquid chromatography (Hewlett Packard HP 5890 GC with HP7673 autosampler). Using the chemical/enzymatic Englyst method coupled with gas-liquid chromatography (GC, Supelco SP2380 capillary column (30m x 0.25mm) with flame ionisation detector, column maintained at 250°C, with the injector kept at 260°C and the detector at 280°C, carrier gas (helium) flow rate of 3ml min^{-1}) and colourimetery (UV spectroscopy), the total, soluble and insoluble NSP levels of the dietary fibres ispaghula husk, pectin, cellulose and resistant starch and their associated diets were determined.

For each run the response factors (RF) were calculated using the following formula:

$$RF = \frac{\text{Conc. of sugar in external standard (mg/ml)*3*allose peak area}}{\text{Conc. of allose in internal standard (mg/ml) * sugar peak area}}$$

This was then used to determine the concentration (%w/w) of the neutral sugars using the following formula:

$$\frac{\text{RFx 890 x conc. of allose soln. (mg/ml) x sugar peak area in sample}}{\text{Allose peak area x sample weight}}$$

Uronic acid derivatisation was achieved by transferring 0.3ml of the original hydrolysate to a plastic centrifuge tube. Samples were diluted with 1.2ml 2M sulphuric acid to ensure uronic acid concentration was less than 100 ppm. 0.3ml sodium acetate/boric acid solution and 5ml concentrated sulphuric acid was added to each tube and vortex mixed immediately. The samples were then heated in a 70°C oven for 40 minutes. The tubes were cooled in water at room temperature. 0.2ml 3,5-dimethylphenol solution (Table 2.3) was added followed by vortexing. After 15

minutes absorbance at 400nm and 450nm was measured against a de-ionised water reference.

The concentration (%w/w) of uronic acid in each sample was determined using the following calculation:-

$$\frac{(A_{450}-A_{400}-C)x2.9x0.89}{M \text{ x sample weight (mg)}}$$

where A_{450} is the absorbance at 450nm, A_{400} is the absorbance at 400nm, c is the Y intercept of the calibration graph and M is the gradient of calibration graph. A dilution factor was also included where necessary.

Table 2.3

Solution	Chemical	Weight	Supplier
Sodium Acetate Buffer	Sodium acetate anhydrous	8.2g	BDH
	Benzoic acid	50% Saturated	BDH
	Glacial acetic acid	To adjust to pH 5.2	BDH
	1M calcium chloride	4ml/L	BDH
	ddH_20	1L	
α-Amylase solution	α-amylase suspension	90µl	Sigma A-4268
	Sodium acetate buffer	1ml	
Pullulanase solution	Pullulanase suspension	10µl	Sigma P-5420
	Sodium acetate buffer	1ml	
Sodium Phosphate buffer	Anhydrous disodium hydrogen orthophosphate	28.4g	BDH
	ddH_20	1L	
	0.2M sodium dihydrogen orthophosphate solution	To adjust to pH7	BDH
0.2M sodium dihydrogen orthophosphate solution	Anhydrous Or Monohydrated salt	24g 27.6g	BDH
	ddH_20	1L	
Allose internal standard	allose	1mg/ml	Sigma
	Benzoic acid	50% saturated	BDH
3,5-dimethylphenol solution	3,5-dimethylphenol	0.1g	BDH
	Glacial acetic acid	100ml	BDH

2.1.3 Solubility of the fibres as determined by Image analysis

Individual fibres were placed onto microscope slides (BDH) and hydrated in ddH_2O for 1 hour, images of hydration were then observed (Zeiss microscope) and recorded using image analysis (Zeiss KS300 3.0).

2.2 1,2-dimethylhydrazine mouse model.

2.2.1 Housekeeping

Two week old Female Balb/C Mice (BNK) were caged in groups of 10 and housed under standard conditions (12 hour light and dark cycle at 25°C). The mice were randomly divided into 5 groups, group 1 consisting of 10 mice, the other 4 groups containing 30 mice. Group 1 were wild type female Balb/c mice fed on a powdered basal diet (fibre deficient diet, Special Diets Services, Table 2.4). The second group of mice were fed on the same basal diet but were injected with the carcinogen 1,2-dimethylhydrazine (DMH) (section 2.2.2). The third, fourth and fifth groups were fed on a basal diet supplemented with 14.5 % of Ispaghula husk (Reckitt Benckiser Healthcare plc), pectin (Hercules, Genu pectin type FIT-1, Denmark) and cellulose (Solkafloc, Special Diet Services, Witham, Essex) (Table 2.1) respectively and were also subjected to DMH administration. The mice were allowed free access (*ad libitum*) to the specified diet and water with their weight and water intake continually monitored throughout the duration of the experiment.

The diets were formulated and manufactured by Special Diets Services (SDS, Witham Essex), eradicating the irradiation of the feed to minimise structural damage of the fibres. Thus due to the nature of the diets they were received in three batches to maintain food quality at the highest nature.

Table 2.4 Composition of the Diets used in the Maintenance of Balb/C mice.

Diet /Composition.	Basal (%)	Basal plus (%) Ispaghula Husk	Basal plus Pectin (%)	Basal plus Cellulose (%)
Rice Starch	37.5	30	30	30
Ispaghula Husk	0	14.5	0	0
Pectin	0	0	14.5	0
Cellulose	0	0	0	14.5
Ca Caseinate	20	20	20	20
Sucrose	32.5	25.5	25.5	25.5
SoyaOil (0.5%)	5	5	5	5
VitaminMineral 2.5%	5	5	5	5

2.2.2 Treatment with 1,2-dimethylhydrazine

Female Balb/C mice were injected subcutaneously with 1,2-dimethylhydrazine hydrochloride (Aldrich Chemical Co. Ltd.) dissolved in EDTA (0.4%, sigma) and normal saline (0.9%) pH6.5 (25mg DMH base/Kg body weight) once weekly for 17 weeks. After 17 weeks, the mice were sacrificed by cervical dislocation and colons removed. The colon was opened longitudinally, pinned onto corkboard serosal side down thus exposing the colonic mucosa. The tissue was fixed in carnoys reagent for 3 hours followed by fixation in 10% formalin. Nodules were then counted, measured and position noted (fig.2.1 and fig. 2.2) using a dissecting microscope. Representative histological samples were taken from the macroscopic nodules, adjacent normal mucosa and midpoint regions (fig 2.1) in the distal portion of the colon for further analysis.

2.2.3 Bromodeoxyuridine (BrdU) as a measurement of proliferation

Female Balb/c mice were injected IP with BrdU (Sigma) and left for 1 hour (Flash labelling). The mice were sacrificed by cervical dislocation and colons removed. The colon was opened longitudinally, pinned onto corkboard serosal side down thus exposing the colonic mucosa. The tissue was then fixed as in section 2.2.2. Tissues were sectioned (4μm) using a microtome and mounted onto poly-L-lysene slides (BDH) so that every fifth section was assessed (to avoid crypt replication). The tissues were dewaxed and dehydrated using histoclear and graded alcohol washes. Endogenous peroxidase activity was blocked by incubating the slides in 0.6% H_2O_2 (Sigma). The paraffin sections were hydrolysed using 1 M hydrochloric acid (HCl) (BDH) at 60 °C for 8 minutes. The tissues were cooled using water and non-specific binding was blocked using Goat serum (1:20, DAKO). The tissues were incubated with the primary antibody, rat anti-BrdU (Harlan-seralab, 1:2000) for 2 hours at room temperature. Primary antibody was localised by incubating the tissues in secondary antibody, biotin conjugated goat anti-rat (MAS 250c Harlan-seralab, 1:2000) for 30 minutes. Tissues were then

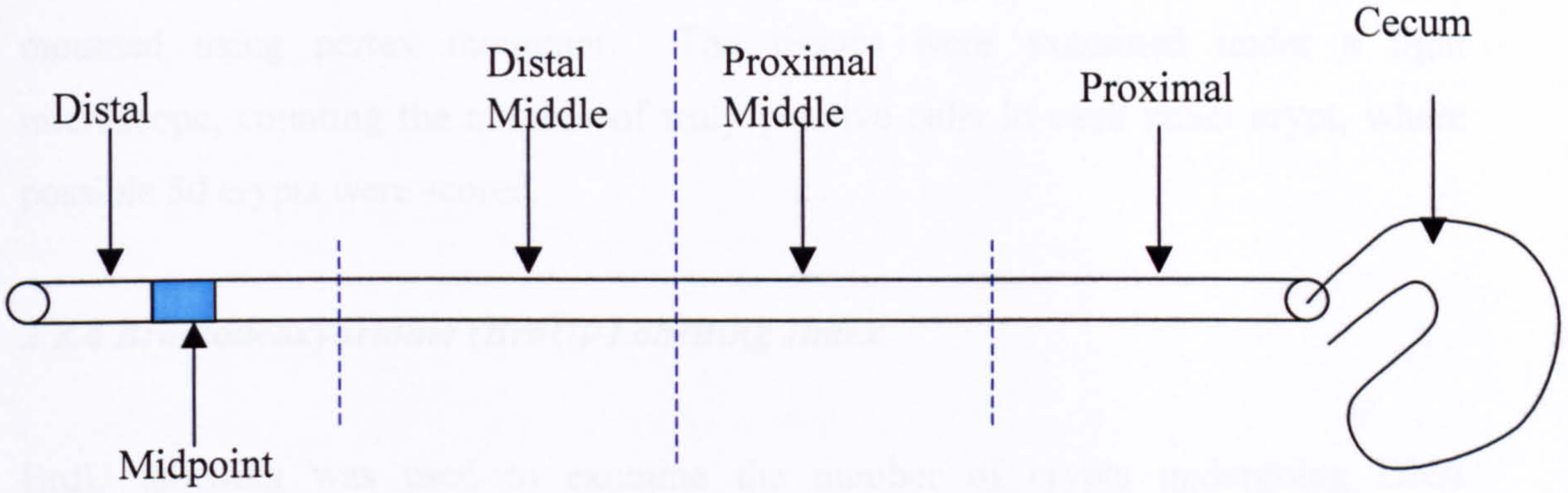


Fig 2.1 Representation of the colon revealing the colonic divisions made when locating the distribution of nodules and tumours.

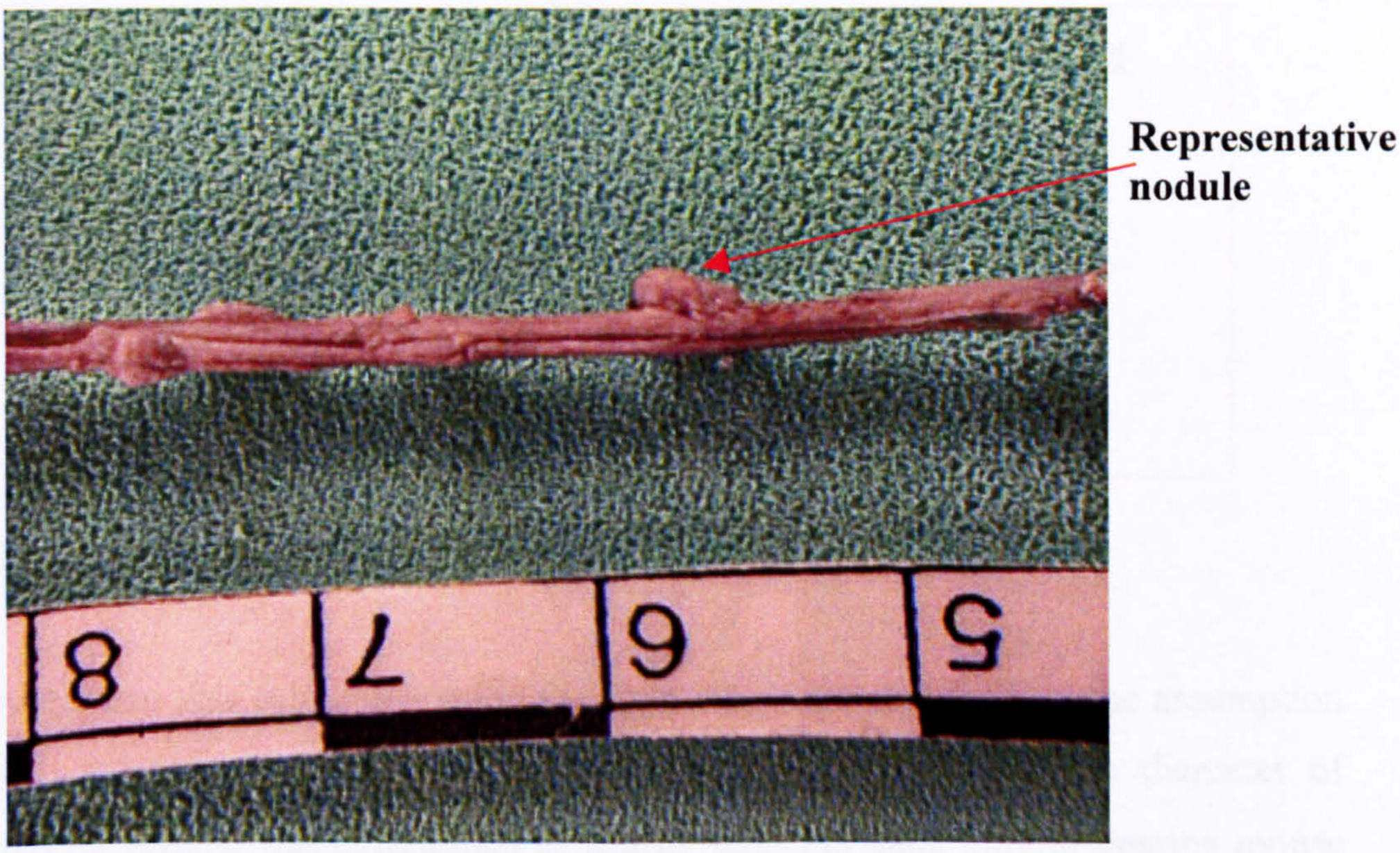


Figure 2.2 Representative nodules. Example of nodules excised from the colon, here tumours are found in the distal and distal middle parts of the colon (Mouse 5J, from the Pectin fed group).

incubated in streptavidine (1:250, DAKO) for 30 minutes, followed by DAB (Dako kit) and counterstained with Haematoxylin (Gills) and lithium carbonate (Scotts), and mounted using pertex mountant. The tissues were examined under a light microscope, counting the number of truly positive cells in each intact crypt, where possible 50 crypts were scored.

2.2.4 Bromodeoxyuridine (BrdU)-Labelling Index

BrdU labelling was used to examine the number of crypts undergoing DNA replication (in S phase of the cell cycle) as a measure of cell proliferation within the crypt. The BrdU – labelling index for each mouse was calculated as follows;

$$\text{Labelling index} = \frac{\text{Total number of positive cells for a single crypt}}{\text{Number of cells within that crypt}}$$

All 50 crypts from that mouse were added together and divided by 50 to give the percentage of positive cells representing the labelling index.

2.2.5 Tumour Burden

Tumour burden was calculated using the following formula based on the assumption that each tumour can be represented as a hemisphere with a mean diameter of $\frac{1}{2}(d_1+d_2)$ its volume was determined as $\pi_{/96}(d_1+d_2)$. For each tumour-bearing mouse the tumour volumes were added and the diameter 'd' of the hemisphere corresponding to the total volume was calculated:

$$\mathbf{d = \tfrac{1}{2}\sqrt[3]{\sum(d_1+d_2)^3}}$$

where **d** is the diameter of the sum total, thus this value was used as a measure of total tumour burden for each individual mouse. $\mathbf{d_1}$ and $\mathbf{d_2}$ are the length and width of each individual tumour for that mouse (Moorghen,1992).

2.3 1,2-dimethylhydrazine mouse model characterisation.

2.3.1 Histopathology

Identification of microadenomas and tumours were determined using haematoxylin and eosin (H&E) staining. 4μm sections of paraffin embedded tissues were mounted onto superfrost slides (BDH), dewaxed and rehydrated using histoclear and graded alcohol washes to water. The slides were stained with haematoxylin (Gills) for 3 minutes, Lithium carbonate (Scotts), eosin for 4 minutes, with ddH_2O washes between each step, then dehydrated in graded alcohol washes, histoclear and mounted. Tissues were examined by two independent observers (E. Tucker and Prof. M. Pignatelli, except microadenomas E. Tucker and Dr. J.Gupta), using a light microscope.

2.3.2 Immunohistochemistry

Expression and cellular localisation of catenins was determined using standard avidin-biotin complex (ABC) immunohistochemical technique on carnoys-fixed paraffin embedded tissue sections. 4μm sections of paraffin embedded tissues were mounted onto poly-L-lysene (BDH) slides. The tissue was dewaxed and rehydrated using histoclear and graded alcohol washes. Endogenous peroxidase activity was blocked by incubating the slides in 0.6% H_2O_2. Antigen retrieval was achieved by heating (microwave) the slides in 0.1M citrate buffer (pH 6) for 20 minutes. Non-specific binding was blocked using goat serum (Dako, 1:20) for 10 minutes. The tissues were incubated with the primary antibody (Table 2.5) overnight at 4°C. Localisation of the primary antibody was achieved using biotin labelled goat anti-mouse (DAKO, 1:250) for 30 minutes followed by peroxidase labelled streptavidin (DAKO, 1:100) for 30 minutes. Visualisation was achieved using diaminobenzadine (DAB kit, DAKO), counter stained using haematoxylin (Mayers) and lithium carbonate (Scotts). Slides were then dehydrated and mounted using pertex mountant. Tissues were examined under a light microscope by two independent observers (E. Tucker and Prof. M. Pignatelli) without knowledge of origin. The cellular localisation and immunoreactivity was always assessed relative to adjacent non-

malignant epithelium within the same tissue. Staining was assessed in terms of the percentage epithelial cells with membranous, cytoplasmic or nuclear staining. Tumours were scored as follows:- a score of 3 for >90% positive epithelial cells, 2 for 90-50% positive cells and 1 for <50%. A negative control was carried out for each round of staining (Figure 2.4)

Table 2.5 Antibodies.

Antibody	Supplier	Concentration
β-catenin	BD Transduction Labs	1:500
α-catenin	BD Transduction Labs	1:100
γ-catenin	BD Transduction Labs	1:100/1:250
p120	BD Transduction Labs	1:250
E-cadherin	BD Transduction Labs	1:500
BRDU	Harlan-seralab	1:1000

2.3.3 Tissue Lysates for varifying specificity of antibodies in mouse tissues

Frozen tissues were sectioned (30x25μm) on a cryostat at -30°C and stored at -80°C. H&E sections were taken at three stages of the cutting process to determine histology of the tissues. 300μl of soluble fraction lysis buffer (0.5% Triton-X, 50mM $NaCl_2$, 10mM Pipes (pH6.8), 3mM $MgCl_2$, 300mM Sucrose) were added to each tube, kept on ice for 20 minutes with periodic vortexing. The lysates were then centrifuged at 13000rpm for 10 mins at 4°C, supernatent removed and stored at -20°C (soluble fraction). The pellet was then lysed in 200μl of insoluble fraction (SDS) lysis buffer (15mM Tris-HCl (pH7.5), 5mM EDTA, 2.5mM EGTA, 1% SDS) at 100°C for 15 minutes, centrifuged (13000rpm) for 10 minutes at 4°C. The supernatant was collected, insoluble fraction, and stored at -20°C. Protein concentration was determined for each lysate using a BioRad protein assay (BioRad) and cricket graph. Western blot samples were then made using equal concentrations of protein extract , boiled in sample buffer for 5 minutes, cooled on ice, pulse spun and loaded. See section 2.6.1.2 for SDS-PAGE protocol.

2.3.4 Protein extract anaylsis to determine equal loading

Two different methods were implemented depending on whether a high level of detergent was found in the lysates. The BioRad colourimetric protein assay kit (Bio-Rad) was used per manufacturers instructions, bovine serium albumin (BSA) was used as a control and spectrophotometry was used to determine protein concentrations, with protein concentrations being calculated using the Gricket Graph or Microsoft Excel.

2.4 Mutational analysis

2.4.1 Primer design

β-catenin was amplified using primers designed by Takahashi *et al.* (2000a) obtained from MWG-Biotech.

2.4.2 Polymerase Chain Reaction (PCR) for β-catenin gene amplification

DNA was extracted from paraffin and frozen tissues using a Qiagen® kit (Qiagen Ltd, West Sussex, UK). PCR primers were used to amplify exon 3 of β-catenin containing the consensus sequence for GSK-3β phosphorylation. 5'-primer, GCTGACCTGATGGAGTTGGA; 3'-primer, GCTACTTGCTCTTGCGTGAA (Takahashi *et al.*, 2000a), with a PCR product length of 227bp obtained from MWG-Biotech. For frozen tissues 50 µl PCR reaction mixture consisted of 0.5µl (50pMol) of each primer, 5µl dNTP (2mM, Gibco), 5µl PCR buffer (x10, Gibco), 3µl $MgCl_2$ (50mM, Gibco), 0.3µl platinum Taq (5U/µl, Gibco), 6µl DNA (tissue lysate) and made up to 50µl with sterile distilled water. Forty cycles were then performed, each cycle consisting of a denaturing step (94°C for 1 minute) annealing step (58°C for 1 minute) and elongation step (72°C for 1 minute, except the last cycle which lasts 7 minutes) using a DNA engine, MJ block, Peltier Thermal cycler, Dyad™. A negative, and where possible, a positive control were run in parallel. Fifteen microlitre aliquots of DNA (PCR product) in 5µl ficol buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% ficoll (type 400; pharmacia) in water; Molecular Cloning, Maniatis) were then fractionated by gel electrophoresis on a 1% (w/v TBE) agarose gel containing 0.14 µg mL^{-1} ethidium bromide running in 1x Tris-Borate EDTA (TBE) buffer (0.045 M Tris-Borate, 0.001 M EDTA, pH 7.5) at 100V for 1 hour. The separated DNA was visualised on an ultra violet transilluminator, bands of relevent size were identified by comparison with a 100bp DNA marker, and recorded using a digital camera.

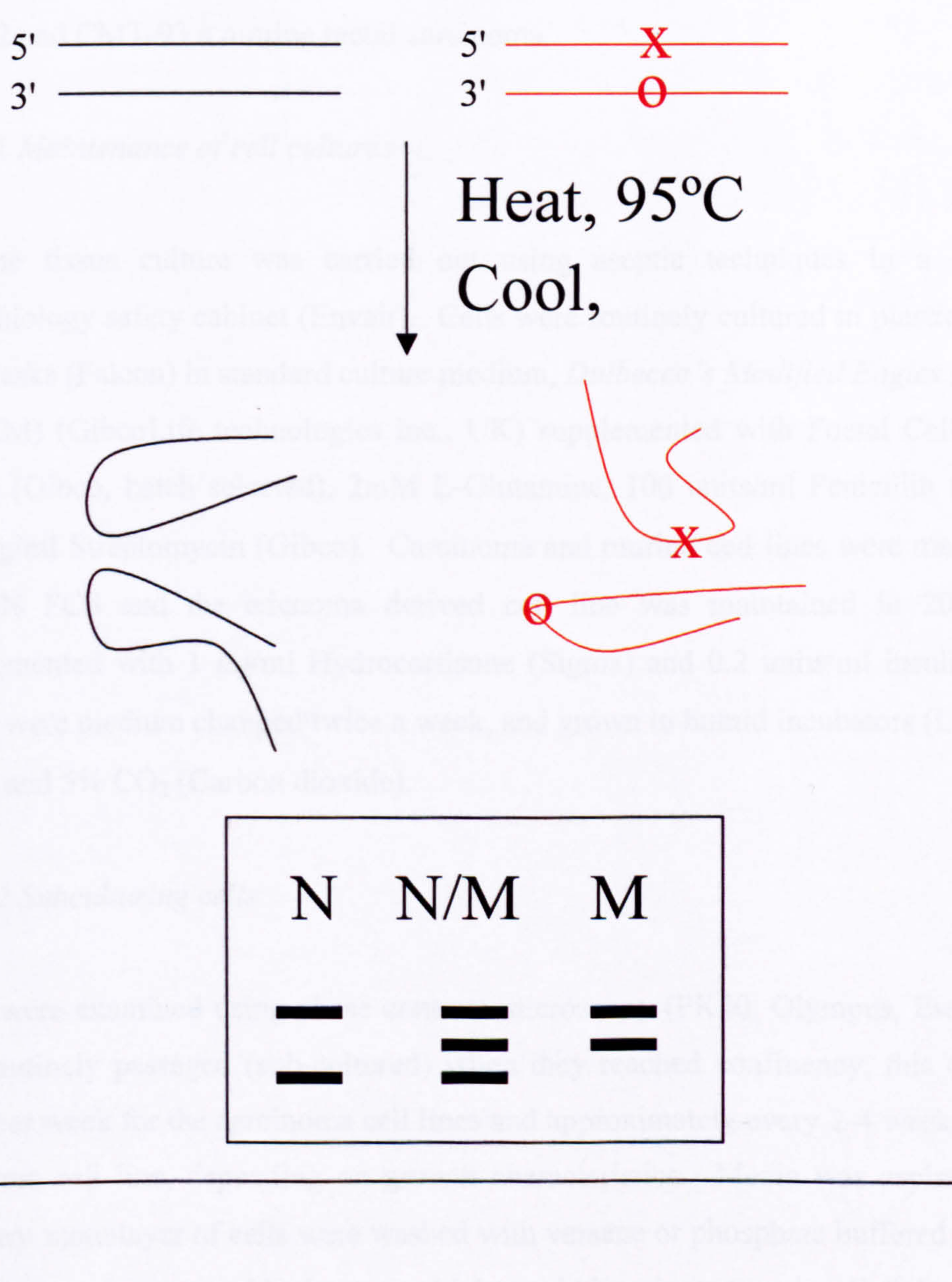
2.4.3 Single Strand Conformational Polymorphism (SSCP)

2-8µl DNA (PCR product) together with 2µl SSCP loading buffer (5M NaOH, 0.05M EDTA, 50% Glycerol, 5% BPB/Xylene cyanol and water, Orita) were heated to 37°C for 2 minutes, placed on ice for 5 minutes and then loaded. SSCP gel (6.3ml MDE, 3ml 5XTBE, 16.1ml ddH_2O, 60µl (TEMED), 400µl 10% APS) was run in 0.6xTBE at 4°C. For β-catenin the samples were run for 7 hours at 200V. Band visualisation was achieved by washing the gel in 10% ethanol and acetic acid (BDH), incubated in 0.1% silver nitrate ($AgNO_3$) for 15 minutes, washed with ddH_2O, developed using 1.5% NaOH and 0.1% formaldehyde (37%). Once developed the gel was fixed in 0.75% sodium carbonate (Na_2CO_3), visualised using a light box and photographed using a digital camera (Figure 2.3).

2.4.4 Sequencing

PCR products for sequencing were first cleaned up using Micron® kit, per manufacturers' instructions to remove excess primer and primer dimer. PCR primers were then added to the cleaned PCR product, made up to 14µl and sequenced by Department of Biochemistry, University of Bristol, BS8 1TD. The sequencing data was analysed using websites (Blast search Genebank database at ncbi, www.ncbi.nlm.gov/genebank).

Figure 2.3 A diagrammatic representation for single strand conformational polymorphisms.



2.5 *In vitro* Cell Culture.

2.5.1 Cell Lines (See Table 2.6)

The human colorectal adenocarcinoma cell lines used in this model were HT29, SW1222, HCT116 and LS174T along with a human colorectal adenoma cell line RG/C2 and CMT-93 a murine rectal carcinoma.

2.5.1.1 Maintenance of cell cultures

Routine tissue culture was carried out using aseptic techniques in a class II microbiology safety cabinet (Envair). Cells were routinely cultured in plastic T25 or T75 flasks (Falcon) in standard culture medium, *Dulbecco's Modified Eagles Medium* (DMEM) (GibcoLife technologies inc., UK) supplemented with Foetal Calf Serum (FCS) (Gibco, batch selected), 2mM L-Glutamine, 100 units/ml Penicillin (Gibco), 100 μg/ml Streptomycin (Gibco). Carcinoma and murine cell lines were maintained in 10% FCS and the adenoma derived cell line was maintained in 20% FCS supplemented with 1 μg/ml Hydrocortisone (Sigma) and 0.2 units/ml insulin. The flasks were medium changed twice a week, and grown in humid incubators (LEEC) at 36 °C and 5% CO_2 (Carbon dioxide).

2.5.1.2 Subculturing cells

Cells were examined using phase contrast microscopy (PK40, Olympus, Essex, Uk) and routinely passaged (sub-cultured) when they reached confluency, this occurred once per week for the carcinoma cell lines and approximately every 2-4 weeks for the adenoma cell line, depending on growth characteristics. Media was aspirated, the adherent monolayer of cells were washed with versene or phosphate buffered solution (PBS), removing any residual serum which would inactivate trypsin. Cell detachment was achieved by adding Difco Trypsin 0.25% (w/v 1mM EDTA in hanks buffered salts solution without $CaCl_2$, $MgCl_2$, $MgSO_4$) (Gibco) to the cells and incubating them for approximately 5 minutes at 37°C. Media containing FCS was then added, to stop the reaction, centrifuged for 5 minutes at 1000 rpm, the supernatant removed and

the cells were resuspended in the appropriate medium, at the required split ratio (see Table 2.5).

2.5.1.3 Storage and Retrieval.

Cells were resuspended in a mixture of 90% Foetal calf serum and 10% dimethylsulphoxide (DMSO; Sigma), alliquots were placed into cryotube vials and frozen at -70°C for 24 hours before being placed in liquid nitrogen for long term storage. Retrieval of cells was achieved by the cells being rapidly defrosted at 37°C and transferred to a T25 vented tissue culture flask containg fresh media. The cells were then incubated overnight as in 2.5.1.1 and media changed after 24 hours.

Table 2.6 Cell Lines	General Details	Growth	Morphology	Genetic status			References	Passage numbers
Colonic				APC	E-cadherin	β-catenin		Range
HT29 (Human)	Sporadic, Adenocarcinoma, Primary tumour, Clonogenic, Tumourigenic, Anchorage Independent.	Fast split 1:10 10% FCS	Poorly diff-erentiated Polyponal	Mutant Truncated 110KD	Wild type	Wild type	Fogh and Tremp, 1975	177-189
SW1222 (Human)	Adenocarcinoma Primary tumour Tumourigenic	Fast Split 1:10 10% FCS	Clumps	Mutant	Wild type	Wild type	Leibovitz *et al*., 1976	103-119
HCT116 (Human)	MMR deficient, (RER+) Adenocarcinoma, tumourigenic Metastatic potential	Fast Split 1:10 10% FCS	Poorly diff-erentiated	Wild type full length	Wild type ? (Heterozyg-ous Exon 3?)	Mutant	Brattain *et al*., 1981 Efstathiou *et al*, 1996	23-35
LS174T (Human)	RER+ Adenocarcinoma	Fast Split 1:10 10% FCS	Grape -like Branches, single cells, poorly cohesive clumps	Wild type full length	Mutant (Mutation in Exons 3 and 8)	Wild type? (mutation in Exon 3)	Tom *et al*., 1977 Efstathiou *et al*., 1996	17-51
RG/C2 (Human)	Sporadic Adenoma, Anchorage dependent, Non-tumourigenic, Clonogenic.	Slow Split 1:5 20% FCS + Insulin +Hydro	Well Differ-entiated	Wild type full length	Unknown	Unknown	Paraskeva *et al*., 1989. Hague *et al*, 1992.	42-54
CMT-93 (Murine)	Rectal polyploid Carcinoma Tumourigenic	Fast Split 1:4 – 1:10	Poorly Differ-entiated				Franks and Hemming, 1978	3-13

Key:- **RER+**, Replication error positive; **Hydro**, Hydrocortisone; **MMR**, mismatch repair.

2.6 Cell line characterization and protein expression

2.6.1 Primers/PCR/SSCP/Sequencing

RG/C2 and LS174T cell line was characterised in terms of β-catenin gene exon 3 using carcinoma cell lines as positive controls. Primers received from B.J.Wijnhoven, Rotterdam, Holland (Wijnhoven *et al.*, 2000). The primer pairs 1F (5'-primer CATTTCCAATCTACTAATGCT) and 1R (3'-primer CTGCATTCTGACTTTCAGTA

A) were used to amplify the β-catenin gene (Fukuchi *et al.*, 1998). The 50 μl PCR reaction mixture contained buffer (x10; Gibco) 5μl, dNTP (2mM, Gibco) 5μl, Magnesium chloride (50mM, Gibco) 3μl, 1μl of each primer at a concentration of 0.1μg per reaction, with 3μl DNA per reaction and 0.25μl Platinum Taq and made up to 50μl using injection water. PCR was performed for 40 cycles at an annealing temperature of 55°C. Mutational analysis was carried out using SSCP analysis (Section 2.4.3) for β-catenin it was run at 200V for 18-20 hours.

2.6.2 SDS-PAGE Western Blotting

2.6.2.1 Cell lysates.

A.

Cells were detached from the plates using trypsinisation or cell scraping (rubber policeman) and washed 3 times in PBS. Cells were resuspended in 1ml lysis buffer (50mM Tris HCL pH6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 1mM EDTA, complete protease inhibitor cocktail tablets (Boehringer Mannheim) (pH 8), boiled for 10 minutes, put on ice for 10 minutes and spun (10000g) at 4°C for 10 minutes. Protein concentration was determined using a BioRad colourimetric protein assay (Biorad protein assay kit) according to the manufacturers' instructions (BSA was used as the standard) and read at 595nm on a spectrophotometer. Protein concentrations were then determined using cricket graph and samples were stored at -20°C. Relevant protein lysate volumes were then diluted in sample buffer (to give equal protein concentrations) boiled for 5 minutes, cooled, spun for 2 minutes and were then ready to load.

B.

Cells were detached from the plates using trypsinisation or cell scraped using a rubber policeman, washed 3 times in phosphate buffered solution (PBS). Cells were then resuspended in a known volume of PBS (University stores), passed through an 18G syringe needle to obtain a single cell suspension. Cell number was determined using a haemocytometer (counting chamber), four fields of view were used and a mean number of cells obtained. 1x 10^6 were resuspended in 300µl lysis buffer (50mM Tris HCL pH6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 1mM EDTA, complete protease inhibitor cocktail tablets (Boehringer Mannheim) (pH 8), boiled for 10 minutes, ice for 10 minutes and centrifuged (10000g) at 4°C for 10 minutes. The supernatant was collected and placed into clean microfuge tubes and added to fresh lysis buffer containing bromophenol blue (Sigma) boiled for 5 minutes, cooled on ice, pulse spun and were then ready to load.

2.6.2.2 SDS-PAGE

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis was used to separate proteins in respect of their molecular weight. BioRad mini-PROTEAN 3 and mini-PROTEAN 2 electrophoresis kits were used. Resolving gel was prepared and allowed to set before being overlaid with the stacking gel which was also allowed to solidify (Table 2.7), for β-catenin 10% polyacrylamide gels were used. The gels were loaded with 15 µl of the relevant samples into each well under SDS-PAGE running buffer (Table 2.8) along with standard molecular weight markers. The gels were run vertically at 90V for 5 minutes and then 180V for approximately 45-60 minutes, transferred for 2 hours at 250mA onto PDVF membrane (Immobilon-P, Millipore) (Table 2.8). The PDVF membrane was blotted in milk (5% Marvel in 1xTBS, table) and incubated with primary antibody (Table 2.9) together with an α-tubulin loading control (Sigma), heatsealed into a plastic bag, overnight at 4°C on a rotating platform. The blots were washed with TBST (Tris-buffered saline and tween, Table 2.7) and incubated with the secondary antibody (rabbit anti mouse-HRP labelled, DAKO used 1:2000-1:3000) for 1 hour at room temperature, heat sealed in a plastic bag and placed on a rotating platform. The blots were then washed for 1 hour with frequent changes of TBST. Visualisation was achieved by using enhanced chemiluminescence

(ECL, amersham pharmacia Biotech UK Ltd) followed by exposure of the membranes to light sensitive film (Kodak). Blots were stripped by incubating them in stripping buffer (100mM 2-Mercaptoethanol, 2% SDS, 62.5mM Tris-HCL pH 6.7) for 30minutes at 50°C. The blots were washed twice with TBST, blocked in milk for 1 hour and re-probed by incubating in primary antibody as before.

Table 2.7 Acrylamide gels.

Chemical	5%	8%	10%	Stacking
29:1 Acrylamide	3.3ml	5.3ml	6.7ml	800µl
1.5M Tris pH 8.8	5.0ml	5.0ml	5.0ml	410µl (pH6.8)
50% Glycerol	1ml	1ml	1ml	----
DH20	10.5ml	8.5ml	7.1ml	3.74ml
10% SDS	200µl	200µl	200µl	50µl
Temed	32µl	32µl	32µl	5µl
10% APS	72µl	72µl	72µl	50µl

Table 2.8 Western blotting solutions.

Solutions	Chemical	Per 1000ml (g)
Running Buffer	Tris base	30g
5 x SDS Page	Glycine	144g
	SDS	10g
	ddH_2O	1000
Transfer Buffer	Tris base	3g
	Glycine	14.4g
	MeOH	200
	10% SDS	1ml
	ddH_2O	799ml
10 x TBS	Tris base	30.25g
	NaCl	84g
	HCl (conc)	20ml
	ddH_2O	980ml
TBST	10 x TBS	100ml
	ddH_2O	895ml
	Tween.	5ml

Table 2.9 Western blotting (WB) and immunocytochemistry (ICC) antibodies.

Antigen specificity	Antibody Type	Clone	Class	Supplier	Working Conc.	
					WB	ICC
β-catenin	Monoclonal		IgG1	Transduction labs	1:2000	1:1000
γ-catenin	Monoclonal		IgG1	Transduction labs	1:2000	1:500
α-catenin	Monoclonal		IgG1	Transduction labs	1:250	1:250
P120	Monoclonal		IgG1	Transduction labs	1:2000	1:1000
E-Cadherin	Monoclonal	HECD1	IgG	ICRF	1:500	1:500
E-Cadherin	Monoclonal			Transduction labs	1:500	1:500
α-Tubulin				Sigma	1:2000	-
Ki-67	Monoclonal	MIB-1		Coulter Electronics	-	1:50

2.6.2.3 SCFA and Fibres.

Each cell line was subcultured into T25 Flasks, grown to 50-60% confluency and then treated (in duplicate or triplicate) with the relevant SCFA or fibres at the relevant molar concentrations; The molar concentrations of Sodium Butyrate (Sigma) used were 0mM (control), 1mM, 2mM and 4mM, for Sodium Acetate (Sigma) 0mM, 6mM, 12mM and 15mM, and for Propionic acid (Sigma) 0mM, 3mM, 5mM and 10mM)). The fibres were used at a concentration of 0.1% diluted in ddH_20. The SCFAs and fibres were made up in sterile ddH_2O, and syringe filtered. The cells were incubated for 48hrs and then cell lysates made (Refer to section 2.6.1A and B) or used in the following experiments.

2.6.3 Immunocytochemistry (ICC)

Cells were grown to 50-60% confluency and treated with SCFAs as for section 2.6.2.3 for 48 hours. There were two different techniques employed cells were either embedded in agar or cytospun onto slides. If the cells were to be embedded in agar, the cells were then trypsinised or cell scraped using a rubber policeman fixed in buffered neutral formaldehyde, pelleted and embedded in 1% agar or cytospun directly onto slides and fixed in ethanol. If agar embedded 4μm sections were cut and mounted onto poly-l-lysine slides. These sections were then treated as in section

2.2.8, for primary antibody concentrations refer to Table 2.9. If the cells were cytospun, the cells were trypsinised or cell scraped (depending on whether E-cadherin was being examined), washed three times in PBS and then spun onto superfrost slides (BDH) using a cytospin (Shandon) at 100g for 5 minutes, cells were then fixed onto the slides in 100% ethanol, left to dry and frozen in a heat sealed bag containing silica gel (BDH) until used, the slides were then treated as in section 2..2.8 except no antigen retrieval (heating in citric acid buffer) was required. A negative control was used for each round of staining (Figure 2.4).

2.6.4 Cell Proliferation

Cells were cultured to 80-90% confluent in 10% FCS medium. The cells are then trypsinised and a single cell suspension was obtained via syringing (18G) and cell number obtained using a haemocytometer. 1x 10^6 (for carcinomas) or 2x10^6 (for adenoma) cells were plated out into T25 flasks. After 24 hours the flasks were treated (section 2.6.2.3), either in duplicate or triplicate (some single studies were also performed) and two T25 flasks were trypsinised and cell number determined using a heamocytometer to give the first time point (zero). At each time point the total number of cells in each flask for each treatment was determined.

2.6.5 3D-Collagen Gel Protocol.

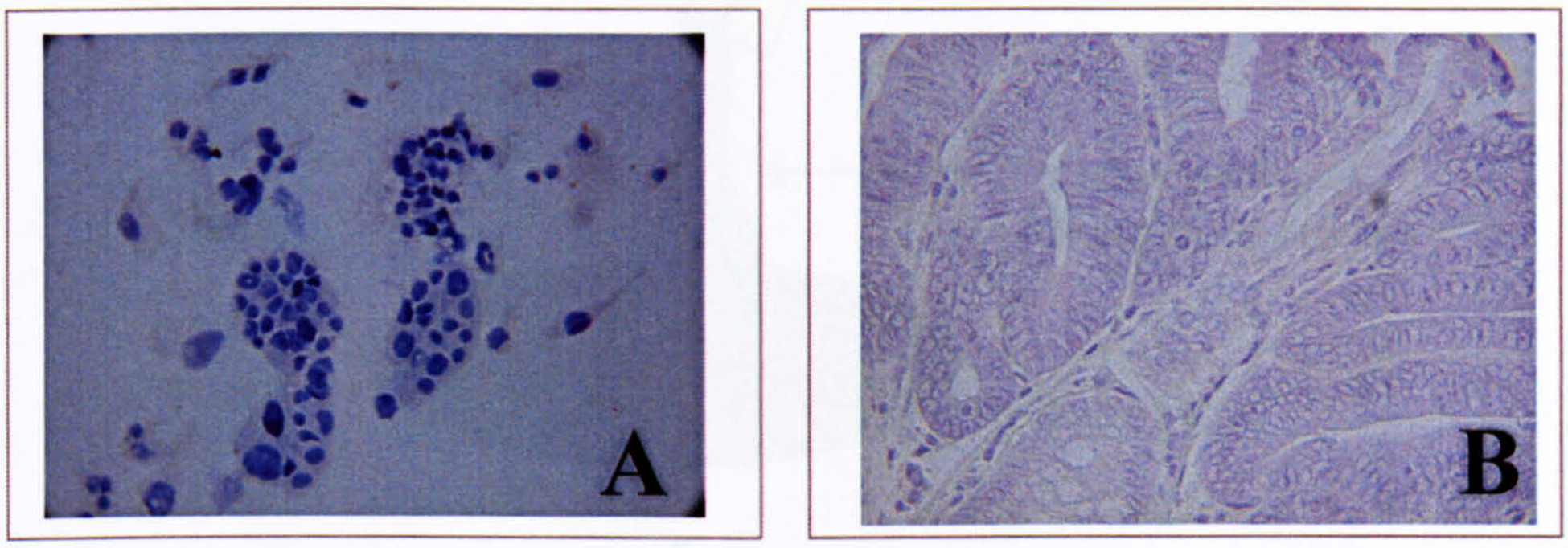
Cells were subcultured, cell number determined using a haemocytometer. The cells were resuspended to give a concentration of 1 - 2x 10^6 per ml (depending on growth rate), aliquoted out and pelleted. Vitrogen (Invitrogen) was neutralised (pH 7.4±0.2) using a number of drops of a solution consisting of 1ml 1M NaOH and 2ml (10 x) BME (Gibco) on ice. Cells were resuspended in neutralised vitrogen, plated out on 35mm petri dishes (Falcon), and set in the incubator (10-15 minutes). After the gels had set they were overlaid with 1.5ml of Media, incubated for 1 hour, fresh media containing the relevant SCFA or fibre (section 2.6.2.3) for up to 4-14 days, monitoring differentiation and media changing every 48 hours. To terminate the experiment the media was removed and 2ml BNF (fixative) was added to each well and left for around 24 hours. The gel was removed placed into a cassette and put into

70% alcohol. The blocks were processed using serial (increased) concentrations of alcohol, followed by histoclear. The gels were then embedded in paraffin wax, cut into 4μm sections and mount on poly-L-lysene slides (BDH). These sections were then treated as in section 2.2.8. Primary antibody concentrations Table 2.9, as for ICC

2.6.6 Immunofluorescence using Confocal Microscopy

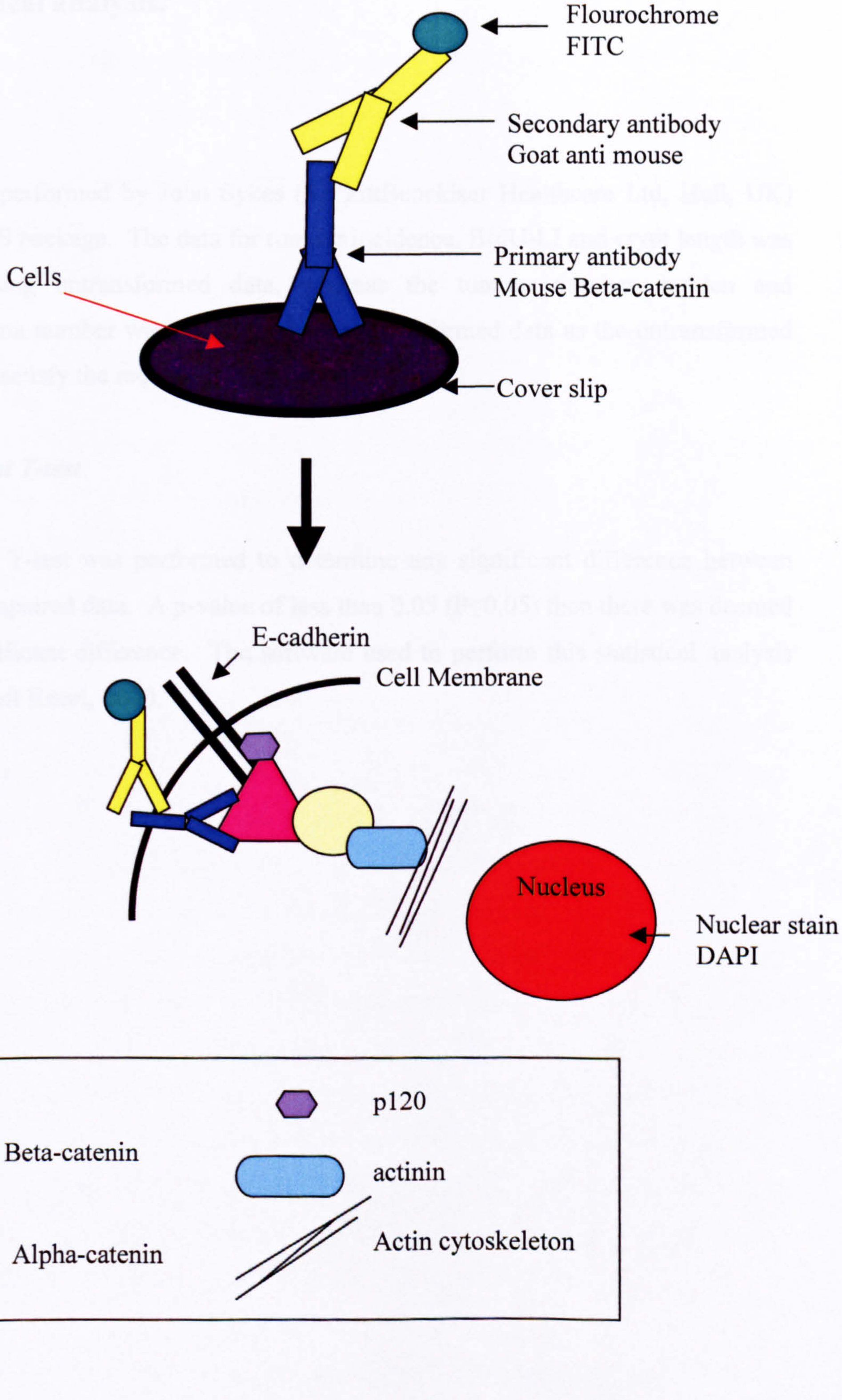
Cells were seeded, 1-2x10^4 or 10^5, onto a glass cover slip and then treated with varying concentration, 0, 2mM and 4 mM, of sodium butyrate (Sigma) for 48 hours. Cover slips were washed using PBS prior, cells were fixed for 5 minutes in 4% (w/v) paraformaldehyde and permeabilised using Triton-X 100 (0.05%) (Sigma, MO). β-catenin within the cells were immunofluorescently labelled (Figure 2.5) by incubating in mouse monoclonal antibodies for β-catenin (Pharminogen) for 45 minutes followed by incubation in FITC-conjugated goat anti-mouse F(ab')2 fragments (Dako, UK) for 30 minutes. Cover slips were then mounted onto glass slides using Vectashield containing 4',6-diamino-2-phenylindole dihydrochloride (DAPI) (Vector, Burlington, CA) with slides being stored at 4°C in darkness until visualisation. Slides were then viewed on a Leica TCS-NT confocal laser scanning microscope attached to Leica DM IRBE inverted epifluorescence microscope (Leica Microsystems, Mannheim, Germany). An oil-immersion objective lens x 63, NA 1.32 was used and imaging parameters were selected to optimize resolution.

Figure 2.4 Representative negative controls for Immunocytochemistry (A), Immunohistochemistry (B) and Immunoflouresence (C).



(**A**) Representative negative control for non-specific binding (no primary antibody) for immunocytochemistry (Magnification x40 plus telescopic zoom). (**B**) Representative negative control for non-specific binding (no primary antibody) for immunohistochemistry (Magnification x20). (**C**) Representative negative control for non specific binding (no primary antibody) for immunoflouresence; (1) field of view for non-specific binding, (2) Field of view for DAPI nuclear stain.

Figure 2.5 Immunoflouresence using confocal microscopy.



2.7 Statistical analysis.

2.7.1 Anova

Anova was performed by John Sykes (ReckittBenckiser Healthcare Ltd, Hull, UK) using an SAS package. The data for tumour incidence, BrdU-LI and crypt length was analysed using untransformed data, whereas the tumour number, burden and microadenoma number was determined using transformed data as the untransformed data did not satisfy the requirements of ANOVA.

2.7.2 Student T-test

The student T-test was performed to determine any significant difference between two-tailed unpaired data. A p-value of less than 0.05 ($P<0.05$) then there was deemed to be a significant difference. The software used to perform this statistical analysis was Microsoft Excel, 2000.

Chapter 3

The characterisation of the fibres Ispaghula husk, Pectin, Cellulose, Rice starch and Resistant starch and their associated diets.

3.1 Introduction.

Dietary fibre (NSP) has a number of physiological roles within the colon and these vary depending on the physical and chemical properties of the dietary fibre (Reviewed by Spiller, 1994; Reviewed by Cummings, 1997). The various physiochemical properties include viscosity, water-holding capacity, bile acid binding, faecal bulking capacity and fermentability (Reviewed by Davidson and McDonald, 1998). Thus it is important to explore a number of these parameters to determine their physiological roles.

A number of methods exist to analyse the dietary fibre content of food including the Prosky method (Reviewed by Cummings, 1997), modified AOAC method (Ohkuma *et al.*, 2000) and the Englyst methods (Englyst et al., 1992). A number of other techniques such as NMR (Black *et al.*, 1999), methylation analysis (Edwards et al., 2001), high performance anion-exchange chromatography (HPAEC) and glycosidic hydrolysis by carbohydrases coupled with molecular remodelling (Blackwood and Chaplin, 2001) can be used to determine precise structural information. The Englyst procedure defines fibre as non-starch polysaccharides (NSP, non-α-glucans) and is a measurement of total, soluble and insoluble NSP, providing an index of plant material (Englyst et al., 1992). Englyst also elucidates the constituent sugars within the varying fractions of NSP, thus helping to give an indication of its structure. The Englyst procedure is used to determine the NSP values of over 400 foods that are included in the UK food tables (Englyst et al., 1992) (section 1.5).

Fermentation is a very important parameter of dietary fibre characterisation, not only the rate, site and extent to which they are absorbed or fermented but also the type of products produced during this fermentation, being critical to their physiological

properties within the colon (Reviewed by Edwards, 1995; Reviewed by Chaplin, 1999; Reviewed by Blackwood *et al.*, 2000). Fermentation of dietary fibre (NSP) by colonic bacteria results in the production of a number of products, the short chain fatty acids (SCFAs, primarily butyrate, propionate and acetate), lactate, methane and hydrogen. Fermentation primarily occurs in the proximal colon of humans (caecum in mice) making it difficult to measure fermentation *in vivo* (Reviewed by Edwards, 1995). There are a number of techniques to determine the level of fermentation *in vivo*, such as breath hydrogen, methane production and faecal levels of SCFAs (Reviewed by Cummings, 1997), to animal models such as human flora associated (HFA) rats and human trials (Rycroft et al., 2001), but all have their limitations. A number of methods have been devised to determine the fermentative properties of dietary fibres (polysaccharide) and oligosaccharides (some used as prebiotics) *in vitro*; these include pure culture studies using a range of intestinal bacteria and mixed faecal cultures (Rycroft et al., 2001). The faecal slurries provide rapid (completed within 24hours), and comparative (several can be set up simultaneously) evaluation of individual polysaccharides representing a relatively diverse gut microflora (Rycroft et al., 2001) with analytical measurements of SCFA production using gas-liquid chromatography (Reviewed by Rombeau and Roth, 1995). SCFA production from ileal effluent correlated well with that from fibre isolates but not with the production from whole foods, and they concluded that fibre isolates, rather than whole foods provide the nearest estimate of colonic SCFA production (Reviewed by Brydon, 1995). A number of *in vitro* fermentation studies have already been carried out showing that the more soluble the NSP the more rapidly it is fermented (Reviewed by Blackwood et al., 2000). Different sugars have also been shown to give rise to different SCFAs (Reviewed by Toole and Toole, 1991), giving individual fibres unique SCFA profiles. A number of structural properties of the fibre; degree of stiffness and regularity of the polysaccharide chains, esterification, side chains and monosaccharide composition all affect the rate and extent of their fermentation (Reviewed by Blackwood et al., 2000), along with a number of intrinsic factors of the host including microflora composition. The ratios of different SCFA production is thus very important (Reviewed by Eastwood, 1995), with different fibres producing different levels of these SCFAs due to the differences in carbohydrate make up and accessibility (Wang and Friedman, 1998; M.Havler, ReckittBenckiser, personal communication).

3.2 Aim

To evaluate the physical and chemical characteristics of the dietary fibres; ispaghula husk, pectin and cellulose, as individual fibres and constituents of a fibre supplemented diet.

3.3 Objectives

1. To determine the solubility and NSP content of the dietary fibres of interest; ispaghula husk, pectin, cellulose, rice starch and resistant starch.
2. To determine the NSP content and solubility of the feeding study diets (Chapter 5); Basal, ispaghula husk supplemented, pectin supplemented and cellulose supplemented.
3. To determine the rate of fermentation of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch; and their individual SCFA profiles.
4. To determine the rate of fermentation of the diets; Basal, ispaghula husk supplemented, pectin supplemented and cellulose supplemented; and their individual SCFA profiles.
5. To relate the structural characteristics of the individual fibres and diets to their fermentation properties and physiological roles within the colon.

3.4 Methodology

These studies, dietary fibre measurements and fermentation properties, utilized methods proposed by M. Havler (ReckittBenckiser, Hull), the Englyst experiment was adapted from the Englyst et al. (1992). The Englyst procedure was undertaken with support and guidance from L. Lewis (ReckittBenckiser, Hull) and the fermentation study was undertaken with support and guidance from M. Havler (ReckittBenckiser, Hull).

3.5 Results.

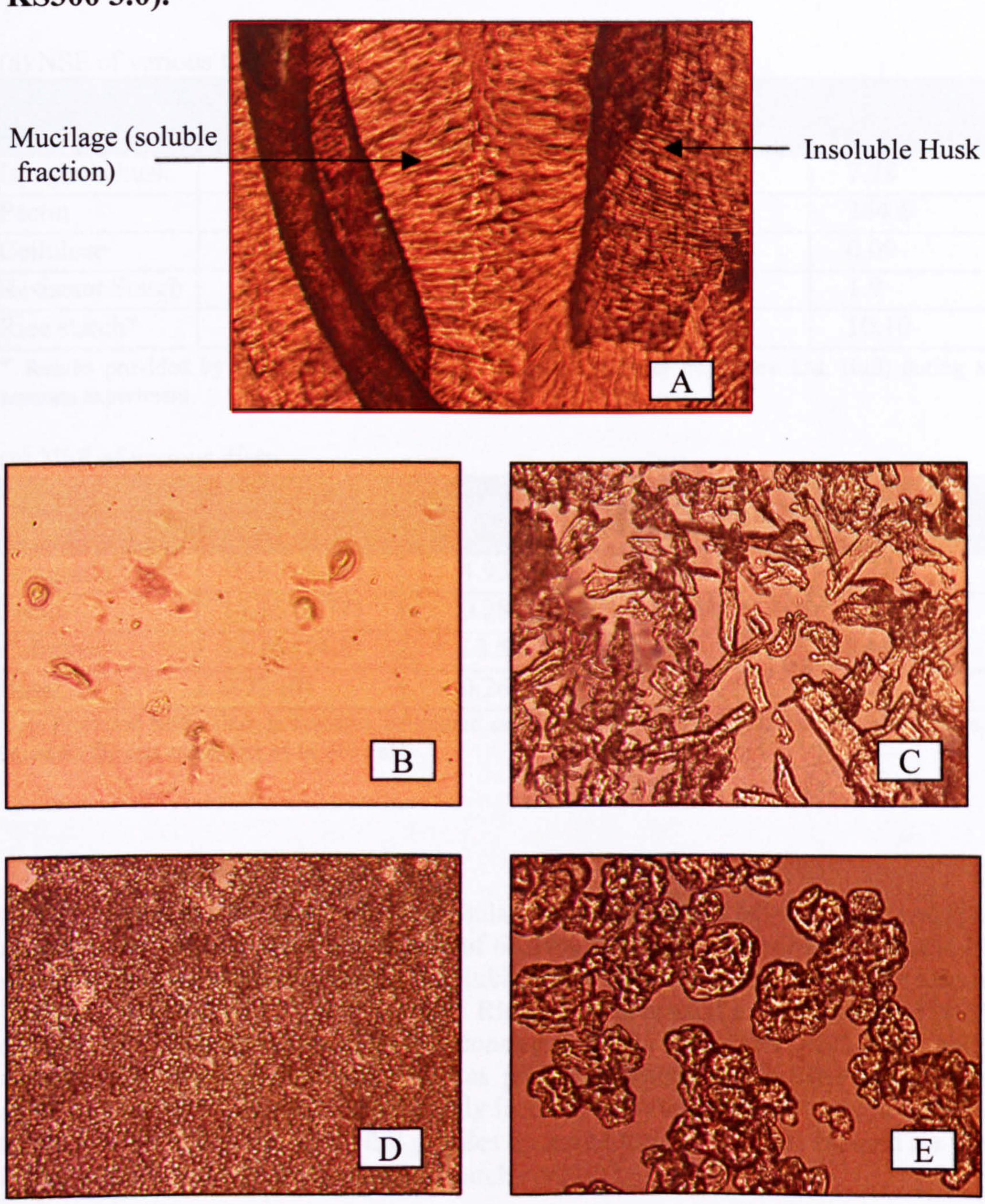
3.5.1 Solubility and NSP content of the fibres Ispaghula husk, Pectin, Cellulose, Rice and Resistant starch and their associated diets.

Using the chemical/enzymatic Englyst method coupled with gas-liquid chromatography (GC, Supelco SP2380 capillary column (30m x 0.25mm) with flame ionisation detector, column maintained at 250°C, with the injector kept at 260°C and the detector at 280°C, carrier gas (helium) flow rate of 3ml min^{-1}) and colourimetery (UV spectroscopy), the total, soluble and insoluble NSP levels of the dietary fibres ispaghula husk, pectin, cellulose and resistant starch (Table 3.1a) and their associated diets (Table 3.1b) were determined. Englyst, defining dietary fibre as NSP, determines the concentration of NSP in samples as a percentage of their weight (Englyst *et al.*, 1992).

3.5.1.1 *Ispaghula husk*

Ispaghula husk has been fairly well studied (Reviewed by Youngken, 1932; Kennedy *et al.*, 1979; Sandhu *et al.*, 1981; Reviewed by Dettmar, 1996). Reviewed by Dettmar (1996) published a mean NSP value for ispaghula of 80-85% (using the same technique as in this study) although others have found values as high as 99% using the Englyst procedure (Edwards and Eastwood, 1992). Table 3.1a gives a representative value (n=2) for ispaghula husk (batch 196/56. Reckitt Benckiser) of 81.43% total NSP, which lies within this range quoted by Dettmar (1996). The majority of this NSP was found to be soluble, 72.18% (88.6% of total), with the remaining fraction, 9.26% (11.4% of total), being insoluble, thus ispaghula husk has a solubility ratio of 7.78:1. The solubility of ispaghula husk is well known, forming a viscous mucilage in water (Reviewed by Youngken, 1932; Kennedy *et al.*, 1979; Sandhu *et al.*, 1981; Reviewed by Spiller, 1994). This was observed, figure 3.1, using Image analysis (Zeiss microscope, Zeiss KS300 3.0) of ispaghula after hydration in water for one hour. Observations during hydration have shown that the mucilaginous (soluble) fraction is released from, but remains attached to, the insoluble fraction. These observations have previously been described by Youngken

Fig 3.1 Solubility of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch, after hydration in water for approximately 1 hour as observed using an image analyser (Zeiss microscope, Zeiss KS300 3.0).



A. Ispaghula Husk (X5) is partially soluble forming a gel like structure (mucilage) which is eluted from the insoluble husk, B. Pectin (X10) shows almost complete solubilisation after 1 hour; C. Cellulose (X20) D. Rice Starch (X20) and E. Resistant Starch (X20) are all insoluble.

Table 3.1 Total, soluble and insoluble NSP content of (a) ispaghula husk, pectin, cellulose, rice and resistant starch (b) basal diet and basal diets supplemented with the following fibres ispaghula husk, pectin and cellulose, determined using the Englyst procedure.

(a) NSP of various fibres

	Concentration (%W/W)			Total
Fibres	Total	Insoluble	Soluble	Sol/Insol ratio
Ispaghula husk	81.43	9.26	72.18	7.78
Pectin	60.48	0.18	60.31	344.6
Cellulose	108.35	99.85	8.51	0.09
Resistant Starch	0.72	0.26	0.46	1.9
Rice starch*	1.86	0.17	1.69	10.10

* Results provided by M.Havler and K. Doyme (ReckittBenckiser healthcare Ltd, Hull) during a separate experiment.

(b) NSP of various diets

	Concentration (%W/W)			Total
Diets	Total	Insoluble	Soluble	Sol/Insol ratio
Ispaghula husk	10.97 (11.81)	4.93	6.04	1.22
Pectin	15.15 (8.77)	0.28	14.88	54.09
Cellulose	13.76 (14.5)	13.57	0.19	0.01
Basal	0.72 (0)	0.26	0.46	1.75

Key: Predicted NSP values in brackets, calculated as a % of NSP retrieved from the individual fibres, based on the diet consisting of 14.5% fibre.

(a) The results demonstrated that ispaghula husk consisted of 81.43% NSP and the majority was soluble. Pectin consisted of 60.48% NSP and was primarily soluble in nature. Cellulose was composed of insoluble NSP (108.35% Total). Resistant starch had trace levels of NSP, 0.72% in total. Rice starch consisted of a low level of NSP (1.86%). (b) The ispaghula husk diet consisted mainly of soluble NSP. The NSP within the pectin supplemented diet was primarily soluble. The NSP within the cellulose supplemented diet was primarily insoluble and the basal diet had only trace levels of NSP. Thus the diets NSP profiles appeared to reflect those obtained for the individual fibres (N=2, except for rice starch N=1).

(1932) and Havler and Spicer (1999), where Havler suggested the insoluble fraction acts as a support network, possible forming a cytoskeleton. Table 3.1b reveals that the ispaghula husk supplemented diet reflects the pattern obtained for the fibre. The diet consists of 10.97% NSP, this is slightly lower than the predicted value of 11.81%. The predicted value was calculated by dividing the fibre content of the diet (14.5%) by 100 and multiplying the % NSP obtained for the fibre used to supplement the diet (as in Table 3.1a). The majority of the NSP is soluble 6.04% (55% of total) with a slightly smaller insoluble fraction 4.93% (45% of total) thus giving a solubility ratio of 1.22:1. Thus there are some differences between the two with the diet consisting of a slightly lower NSP value than predicted and a larger insoluble fraction. This could be due to a non-homogenous mix of all constituents within the diet.

3.5.1.2 *Pectin*

Pectin is a known soluble dietary fibre, forming gels in water having considerable water-holding capacity (Reviewed by Spiller, 1994). The Pectin used in this study was fruit derived (orange) for human consumption (Hercules, GENU pectin type FIT-1). This pectin was shown to consists of 60.48% NSP (n=2), with the NSP being soluble in nature, 60.31% (99.7% of total), having a soluble to insoluble ratio of 2200.045:1. This is consistent with the image analysis, Fig 3.1b, where pectin was shown to be almost completely soluble forming a gel in water after hydration for one hour in water. The pectin supplemented diet (Table 3.1b) basically reflects the results found for the individual fibre (Table 3.1a) consisting of 15.15% NSP, which is higher than the predicted value of 8.77%, but is soluble, 14.88% (98% of total), having a soluble to insoluble ratio of 54.09:1.

3.5.1.3 *Cellulose*

Cellulose is a well defined dietary fibre, being insoluble in water although capable of binding some water (Reviewed Spiller, 1994). Cellulose (Solkafloc) was shown (Table 3.1a) to be solely NSP, 108.35%, with the majority of it being insoluble, 99.85% (92.2% of total), a small fraction being soluble, 8.51% (7.8% of total), giving it a soluble to insoluble ratio of 0.085:1. This is also shown in Fig 3.1, where

the cellulose is still intact after hydration in water for one hour suggesting that it is insoluble. The cellulose supplemented diet, Table 3.1b, reflects the results found for the individual fibre. Although the NSP content, 13.76% was slightly lower than predicted 14.5%, it did show that the majority of this NSP was insoluble, 13.57% (98.6% of total NSP), with a soluble to insoluble ratio of 0.014:1.

3.5.1.4 *Rice and Resistant starch*

Resistant starch, from corn (*Zea mais,* National starch and chemical/starch division), also enters the colon but is not included in the definition of dietary fibres as non-starch polysaccharides (Englyst *et al.*, 1992). Rice starch (provided by SDS) would not be expected to reach the colon but may provide it with some resistant starch. During the Englyst procedure all starch is removed by α-amylase and pullanase digestion, thus resistant starch and rice starch would be expected to contain no NSP. The results in Table 3.1a show that the resistant starch and rice starch have only trace levels of NSP, 0.72% and 0.19% respectfully. This small level of NSP could be the result of incomplete enzymatic removal of starch, possibly leaving traces of glucose (Table 3.5a) and in the case of rice starch possible contamination as also has trace sugars not seen in the basal diet that contains rice starch. Using image analysis (Zeiss microscope, Zeiss KS300 3.0), Fig 3.1, it was also shown that both the rice starch (used to supplement the basal diet) and the resistant starch are insoluble in water, as demonstrated after one hour hydration in water. The basal diet is supplemented with sucrose and rice starch (which may contain traces of resistant starch). The basal diet is also referred to as a fibre deficient diet (SDS, Witham, Essex) as it is said to contain only very low levels of crude fibre and no other specific dietary fibres (Table 3.6a and 3.7), thus it is expected to be NSP free. As Table 3.1b shows the basal diet is primarily NSP free (0.72%), only having trace levels, as for the resistant starch and rice starch. The diet mirrors the findings for resistant starch to a greater extent than the rice starch, which is intriguing as the basal diet consists of a large percentage of rice starch (37.5%). This could be accounted for as the rice starch results were obtained during a separate experiment or the rice starch could have had trace levels of contaminants from the other fibres being examined, such as ispaghula husk.

3.5.2 Constituent NSP sugars within the total, insoluble and soluble fractions of the fibres Ispaghula husk, Pectin, Cellulose and Rice and Resistant starch and their associated diets.

The Englyst procedure was used to determine the monosaccharide profiles of the NSP within the different fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch. Englyst results in the hydrolysis of the NSP into individual sugars which are then reduced and acetylated before analysis by Gas-liquid chromatography (Supelco SP2380 capillary column (30m x 0.25mm) with flame ionisation detector, column maintained at 250°C, with the injector kept at 260°C and the detector at 280°C, carrier gas (helium) flow rate of 3ml min^{-1}). The uronic acid content of the fibres was measured separately using colourimetric reagents and UV spectroscopy (ATI/UNICAM UV4 spectrometer, at 400nm and 450nm).

3.5.2.1 *Ispaghula Husk*

Ispaghula husk has been fairly well analysed (Kennedy *et al.*, 1979; Sandhu *et al.*, 1981). Ispaghula is known to have an arabinoxylan backbone (Kennedy *et al.*, 1979; Sandhu *et al.*, 1981; Reviewed by Spiller, 1994), thus we would expect to see arabinose and xylose within the sugar profile. A number of other sugars are also found associated with this backbone, such as uronic acids, rhamnose and glucose (Kennedy, *et al.*, 1979; Sandhu *et al.*, 1981; Edwards and Eastwood, 1992; Reviewed by Spiller 1994). Garcia (1976) showed that the mucilage (soluble fraction) consisted mainly of xylose, arabinose and galacturonic acids, with lower levels of rhamanose and galactose. Table 3.2a represented the constituent NSP sugars within total, insoluble and soluble fractions of ispaghula husk (n=2). The results demonstrate that the NSP within ispaghula husk is mainly composed of arabinose and xylose with lower levels of uronic acids (probably galacturonic acids). There are

Table 3.2 Constituent NSP sugars within total, insoluble and soluble fractions of (a) ispaghula husk and (b) ispaghula husk supplemented basal diet, as determined using the Englyst procedure.

(a) Ispaghula husk

	(%W/W)		
Monosaccharide	Total	Insoluble	Soluble
Rhamanose	1.17	0	1.17
Arabinose	17.31	4.03	13.28
Xylose	47.77	0.37	47.4
Mannose	0.74	0.6	0.14
Galactose	2.76	1.48	1.28
Glucose	2.84	2.65	0.19
Uronic Acid	8.87	0.15	8.73

(b) Ispaghula husk supplemented basal diet

	(%W/W)		
Monosaccharide	Total	Insoluble	Soluble
Rhamanose	0.03	0	0.03
Arabinose	1.35	0.4	0.95
Xylose	4.51	0	4.51
Mannose	0.12	0.09	0.03
Galactose	0.28	0.15	0.13
Glucose	4.1	1.73	2.37
Uronic Acid	0.59	0	0.59

The above tables represent the constituent NSP sugars within total, insoluble and soluble fractions of (a) Ispaghula husk (100mg) (b) Basal diet supplemented with ispaghula husk (200mg), using an adapted Englyst method and gas-liquid chromatography (n=2). (a) The NSP within ispaghula husk was mainly composed of arabinose and xylose with lower levels of uronic acids with trace levels of glucose, galactose, rhamanose and mannose. The soluble fraction primarily comprised of xylose, arabinose and uronic acids, whereas the insoluble fraction consisted of arabinose and glucose. (b) The majority of the ispaghula supplemented diet consisted of xylose, arabinose and glucose, with lower levels of uronic acids, galactose, mannose and rhamanose. The xylose and uronic acids were within the soluble fraction, whereas the glucose and arabinose were found within both the soluble (primarily) and insoluble fractions.

also low levels of rhamanose, mannose, galactose and glucose. The soluble fraction is mainly composed of the sugars; xylose, arabinose and uronic acids, while the insoluble fraction consists of arabinose and glucose. The insoluble fraction consisting of glucose is consistent with the theory that the insoluble fraction of ispaghula husk may be like cellulose, acting as a cell wall (personal communication with M.Havler, ReckittBenckiser). Table 3.2b represented the constituent NSP sugars within total, insoluble and soluble fractions of the ispaghula husk supplemented basal diet (n=2). The table reveals that the resulting profiles are consistent with those obtained for the individual fibre, ispaghula husk, used to supplement the diet. The majority of the ispaghula supplemented diet is made up of xylose, arabinose and glucose with lower levels of uronic acids. The xylose, arabinose and uronic acids are found within the soluble fraction, which is consistent with the findings for the dietary fibre. The insoluble fraction also consists of arabinose and glucose, as for the fibre, but the major difference is that the glucose is not only found within both the soluble and insoluble fractions, it is proportionally at a much higher level than expected. Reasons for this are unclear but could be related to the non-homogenous mixing of the diet resulting in a non-proportionate amount of sucrose being analysed.

3.5.2.2 *Pectin*

Pectin is a known galactouronic acid (Reviewed by Reiser, 1987; Reviewed by Spiller, 1994), found with areas of rhamanose and side chains composed of galactose (galacturonic acids), glucose (glucuronic acid), xylose and arabinose (Reviewed by Reiser, 1987; Reviewed by Spiller, 1994) thus high level of uronic acids would be expected from an Englyst profile. Table 3.3a represented the constituent NSP sugars within total, insoluble and soluble fractions of pectin (n=2). The results demonstrate that the NSP within pectin is primarily made up of uronic acids (54.26%, 89.7% of total NSP) and these are found within the soluble fraction. Within the soluble NSP fraction there is also a low level of galactose (4.52%, 7.47% of total NSP) which could also be derived from the galactouronic acids although other sugars have been found associated with pectin, for example galactose and arabinose (Reviewed by Spiller, 1994). There are also low levels of arabinose, rhamanose, glucose and mannose. Table 3.3b represented the constituent NSP sugars within total, insoluble

Table 3.3 Constituent NSP sugars within total, insoluble and soluble fractions of (a) pectin and (b) pectin supplemented basal diet as determined by the Englyst procedure.

(a) Pectin

	(%W/W)		
Monosaccharide	Total	Insoluble	Soluble
Rhamanose	0.53	0	0.53
Arabinose	0.97	0.1	0.87
Xylose	0	0	0
Mannose	0.08	0.08	0.01
Galactose	4.52	0	4.52
Glucose	0.13	0	0.13
Uronic Acid	54.26	0.01	54.25

(b) Pectin supplemented basal diet

	(%W/W)		
Monosaccharide	Total	Insoluble	Soluble
Rhamanose	0	0	0
Arabinose	0.19	0.07	0.12
Xylose	0	0	0
Mannose	0.04	0	0.04
Galactose	0.6	0	0.61
Glucose	1.05	0.21	0.84
Uronic Acid	13.27	0	13.27

The above tables represent the constituent NSP sugars within total, insoluble and soluble fractions of (a) Pectin (100mg) and the (b) Pectin supplemented basal diet (200mg), using an adapted Englyst method and gas-liquid chromatography (n=2). (a) The NSP within pectin primarily consisted of uronic acids and are within the soluble fraction. Within the soluble NSP fraction there was also a low level of galactose along with traces of arabinose, rhamanose, glucose and mannose. (b) The pectin supplemented basal diet primarily consisted of uronic acids found within the soluble fraction, along with glucose and traces of galactose, arabinose and mannose.

and soluble fractions of the pectin supplemented basal diet (n=2). The results show that the pectin supplemented basal diet is also primarily made up of uronic acids (13.27%, 87.6% of total NSP) found within the soluble fraction. Glucose is also present, mainly found within the soluble fraction, but probably from the diets' sucrose content as for the ispaghula husk supplemented diet. As for the fibre there are also low levels of galactose, arabinose and mannose.

3.5.2.3 *Cellulose*

Cellulose is a long unbranched glucose polymer made up of β(1-4) linked glucose residues (Reviewed by Spiller, 1994; Reviewed by Toole and Toole, 1991). Using the Englyst procedure we would expect to find high levels of glucose within the insoluble fraction of the NSP. Table 3.4a represents the constituent NSP sugars within total, insoluble and soluble fractions of cellulose (n=2). The results show that the NSP within cellulose is primarily made up of glucose, 97.87% (90.3% of total NSP), with a small fraction of uronic acids, 6.95% (6.4% of total NSP). These sugars are found within the insoluble fraction of cellulose, with a small fraction of glucose being soluble (7.78% of total glucose). Xylose and mannose are also found at low levels within the insoluble fraction. Table 3.4b represents the constituent NSP sugars within total, insoluble and soluble fractions of the cellulose supplemented basal diet (n=2). The cellulose supplemented basal diet reflects the profile found for the fibre, comprising mainly of insoluble NSP made up of glucose (91.13% of total NSP). It also contains low levels of uronic acids, mannose and xylose, this could be due to contamination.

3.5.2.4 *Rice and Resistant starch*

Resistant starch has a well elucidated structure consisting of α(1-4) and α(1-6) linked glucose residues forming amylose and amylopectin (branched) respectively (Reviewed by Spiller, 1994). The resistant starch in this study is of high amylose maize consisting primarily of straight chains of α(1-4) linked glucose residues. Table 3.5a represents the constituent NSP sugars within total, insoluble and soluble

Table 3.4 Constituent NSP sugars within total, insoluble and soluble fractions of (a) cellulose and (b) cellulose supplemented basal diet as determined using the Englyst procedure.

(a) Cellulose

	(%W/W)		
Monosaccharide	Total	Insoluble	Soluble
Rhamanose	0	0	0
Arabinose	0	0	0
Xylose	1.94	1.63	0.31
Mannose	1.62	1.46	0.16
Galactose	0	0	0
Glucose	97.87	90.26	7.61
Uronic Acid	6.95	6.51	0.44

(b) Cellulose supplemented basal diet

	(%W/W)		
Monosaccharide	Total	Insoluble	Soluble
Rhamanose	0	0	0
Arabinose	0	0.06	0
Xylose	0.23	0.17	0.06
Mannose	0.18	0.18	0
Galactose	0	0	0
Glucose	12.54	11.99	0.56
Uronic Acid	0.81	1.18	0

The above tables represent the constituent NSP sugars within total, insoluble and soluble fractions of (a) cellulose (100mg) and the (b) cellulose supplemented basal diet (200mg), using an adapted Englyst method and gas-liquid chromatography (n=2). (a) The NSP within cellulose primarily comprised of glucose with a small fraction of uronic acids. These sugars were found within the insoluble fraction of cellulose, with a small fraction of glucose being soluble. Xylose and mannose were also found at low levels within the insoluble fraction. (b) The cellulose supplemented basal diet consisted mainly of glucose within the insoluble fraction of the NSP. It also had low levels of uronic acids, mannose and xylose.

Table 3.5 Constituent NSP sugars within total, insoluble and soluble fractions of (a) resistant starch, (b) rice starch and (c) basal diet as determined using the Englyst procedure.

(a) Resistant Starch

		(%W/W)	
Monosaccharide	Total	Insoluble	Soluble
Rhamanose	0	0	0
Arabinose	0	0.06	0
Xylose	0	0	0
Mannose	0	0	0
Galactose	0	0	0
Glucose	0.72	0.21	0.51
Uronic Acid	0	0	0

(b) Rice Starch

	(%W/W)		
Monosaccharide	Total	Insoluble	Soluble
Rhamanose	0	0	0
Arabinose	0.22	0	0.22
Xylose	0.47	0	0.47
Mannose	0	0	0
Galactose	0.02	0	0.02
Glucose	0.59	0	0.59
Uronic Acid	0.56	0.17	0.39

(c) Basal diet

	(%W/W)		
Monosaccharide	Total	Insoluble	Soluble
Rhamanose	0	0	0
Arabinose	0.06	0.07	0
Xylose	0	0	0
Mannose	0	0	0
Galactose	0	0	0
Glucose	0.36	0	0.36
Uronic Acid	0	0	0

The above tables represent the constituent NSP sugars within total, insoluble and soluble fractions of (a) Resistant starch (100mg), (b) Rice starch (100mg) and the (b) Basal diet, using an adapted Englyst method and gas-liquid chromatography (n=2, except for rice starch n=1). (a) The low level of NSP found within the resistant starch consisted of soluble glucose. (b) The rice starch was found to primarily consist of soluble glucose along with soluble xylose and soluble uronic acids and trace levels of arabinose and galactose. (c) The low level of NSP found within basal diet was made up of soluble glucose and insoluble arabinose.

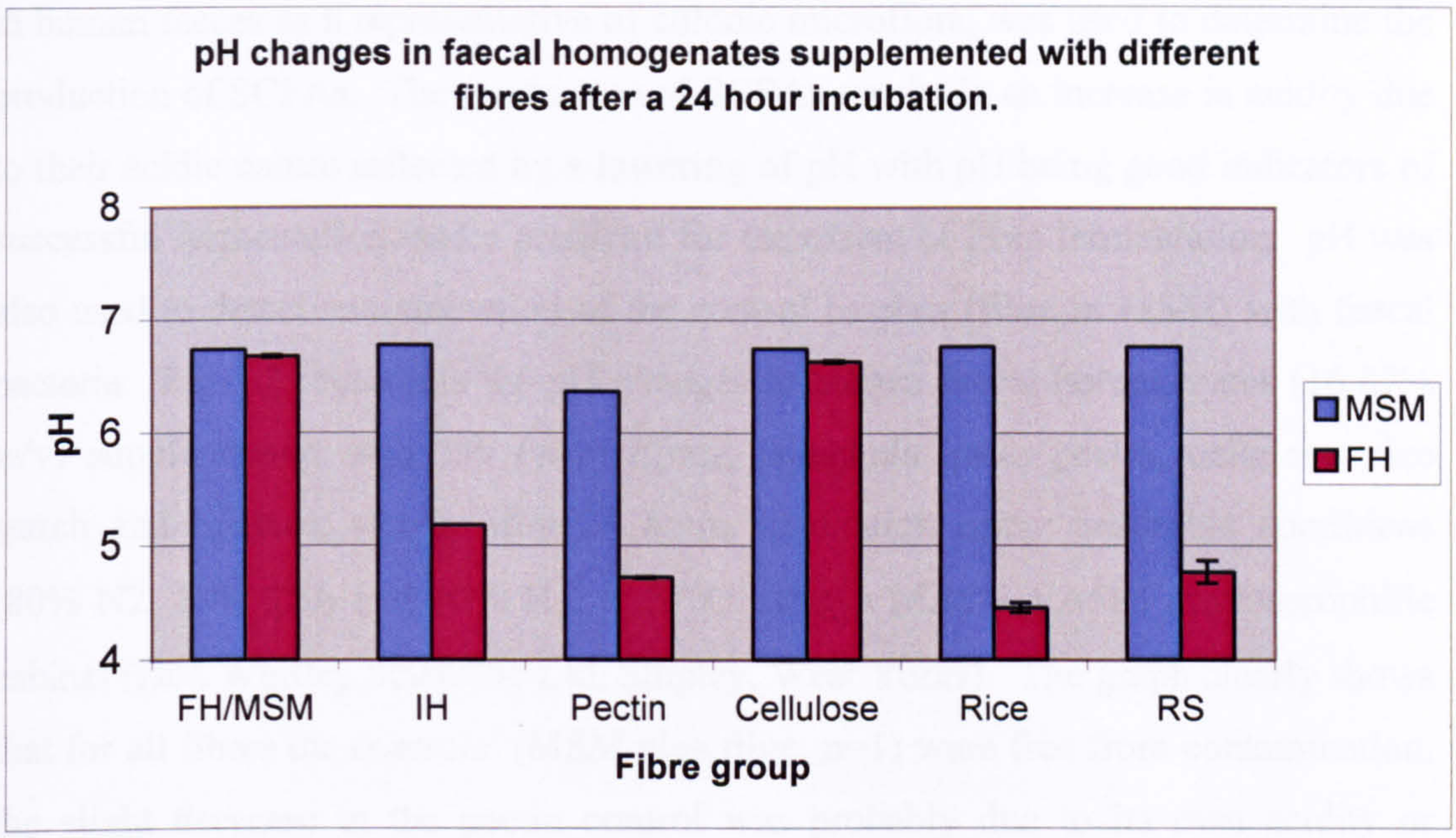
fractions of resistant starch. The results show that the low level of NSP found within the resistant starch is made up of glucose (100%). This sugar is found within both the soluble and insoluble fraction of the resistant starch but is primarily soluble (71.33% of total glucose). The origin of this glucose is unclear but could be derived from the incomplete enzymatic digestion of the starch. There are traces of the sugar arabinose in the insoluble fraction, which could be due to contamination. The rice starch was found to primarily consist of soluble glucose, which could be residual from the incomplete hydrolysis of starch, soluble xylose, and soluble uronic acids with trace levels of arabinose and galactose. The elucidation of the basal diet sugar profile would be predicted to follow the pattern for rice starch as it is one of its main constituents and expected to only contain low levels of resistant starch. The basal diet was shown to have very little constituent NSP reflecting the pattern observed for resistant starch. Table 3.5b represents the constituent NSP sugars within total, insoluble and soluble fractions of the basal diet (n=2). The results show that the low level of NSP observed within the basal diet consists of glucose and arabinose. The glucose is found within the soluble fraction whereas the arabinose is found within the insoluble fraction, thus principally reflecting the profile found for resistant starch. Variation between the basal diets' results and that of rice starch could be due to contamination of the sample or due to differences in experimental conditions as they were carried out separately.

3.5.3 Fermentation characteristics of the fibres; Ispaghula husk, Pectin, Cellulose, Rice and Resistant starch.

3.5.3.1 *pH changes*

When dietary fibre is fermented (anaerobically) by colonic bacteria it produces a number of products, most importantly the short chain fatty acids (SCFAs) butyrate, propionate and acetate. The SCFAs are thought to carry out many of the biological functions attributed to different dietary fibres, thus it is important to determine the SCFA profiles for the individual fibres along with their rate of production. Fermentation may be characterised by the rate, extent, site and products of

Fig 3.2 pH changes within human faecal homogenates supplemented with the dietary fibres; ispaghula husk, pectin, cellulose, rice and resistant starch.



Key: MSM = Mineral salts media supplemented with fibre, FH = Faecal homogenate supplemented with fibre.

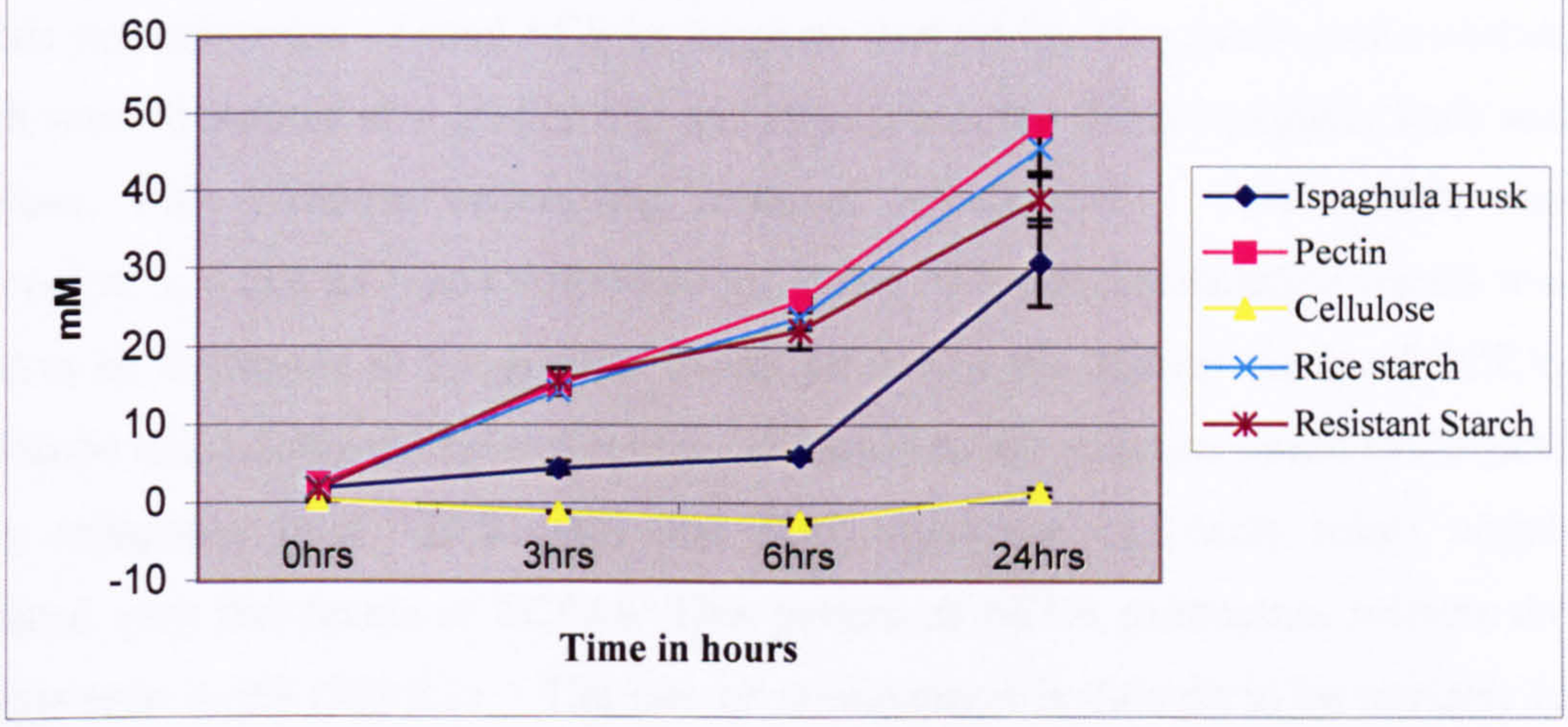
pH changes in human faecal homogenates supplemented with 1% (w/v) fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch, after 24 hours incubation under anaerobic conditions (80% N2, 10% CO2 and 10% H2) at 37°C using a MACS VA500 microaerophilic cabinet (Don Whitley Scientific Ltd, Shipley, West Yorks). This graphic representation showed that within all the fibre controls (MSM plus fibre, n=1) the pH was consistent the slight decrease in the pectin control was probably due to its own acidity. The fermentation of the fibres with faecal homogenate (n=3), revealed that pectin, rice starch (rice) and resistant starch (RS) resulted in the largest decrease in pH. Ispaghula husk resulted in a smaller reduction in pH. With cellulose producing no significant reduction in pH, as observed for the control (n=3).

fermentation. Each of these parameters determines the action of dietary fibre on stool output, colonic motility and cellular proliferation of the colonic mucosa (Reviewed by Edwards, 1995). An *in vitro* system, which utilises bacteria excreted in human faeces as a representative of colonic microflora, was used to determine the production of SCFAs. The production of SCFAs results in an increase in acidity due to their acidic nature reflected by a lowering of pH with pH being good indicators of successful fermentation, and a predictor for the extent of fibre fermentation. pH was also used to detect contamination of the control batches (fibre in MSM) with faecal bacteria. Fig 3.2 represents the pH changes in human faecal homogenates (16.67% w/v) supplemented with 1% (w/v) fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch, after 24 hours incubation under anaerobic conditions (80% N2, 10% CO_2 and 10% H_2) at 37°C using a MACS VA500 microaerophilic cabinet (Don Whitley Scientific Ltd, Shipley, West Yorks). The graph clearly shows that for all fibres the controls (MSM plus fibre, n=1) were free from contamination, the slight decrease in the pectin control was probably due to its own acidity or immediate utilization. The fermentation of the fibres with faecal homogenate (n=3), revealed that rice starch, resistant starch and pectin resulted in the lowest pHs, 4.46 (decrease of 2.31), 4.77 (decrease of 2), 4.72 (decrease of 1.65) respectively, suggesting that they have been readily fermented. Ispaghula husk resulted in a smaller reduction in pH, 5.18 (decrease of 1.6), although a similar decrease to pectin, suggesting it was partially fermented. With cellulose there was no significant change in pH, 6.64 (decrease of 0.11) suggesting little, or no, fermentation has occurred indicated by comparison with the mineral salts medium (MSM)/faecal homogenate control (pH 6.68, n=3). The MSM/faecal homogenate control was included to determine the intrinsic fermentation of the faeces.

3.5.3.2 *Total Short chain fatty acid production*

With the majority of the fibres resulting in a decrease in pH it suggested that fermentation had been successful, thus the level of SCFA production was determined. Figure 3.3 represents the total short chain fatty acid production, above control, from the fermentation of 1% (w/v) of fibre; ispaghula husk, pectin, cellulose,

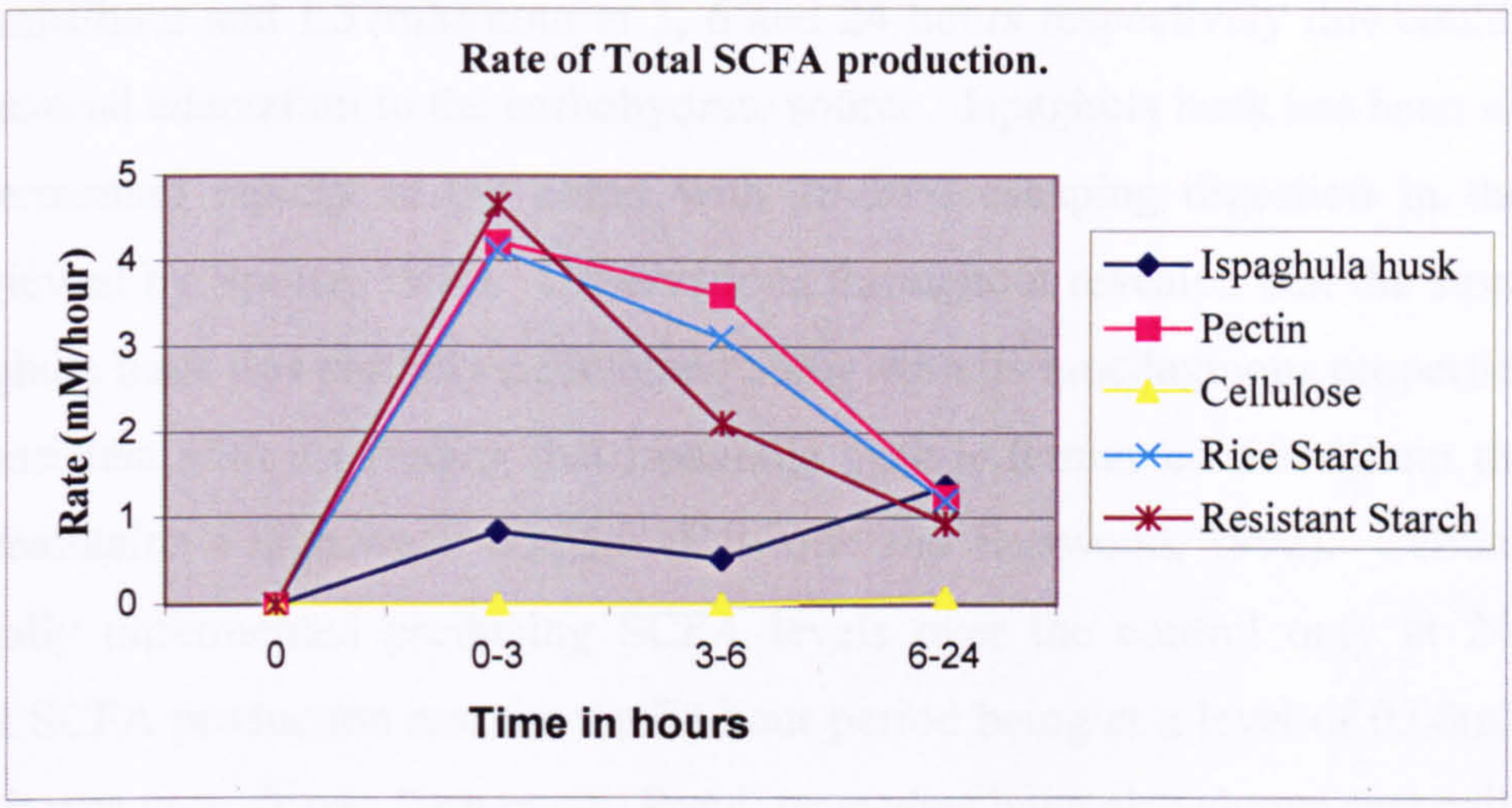
Figure 3.3 Total short chain fatty acid production from the fermentation of the dietary fibres ispaghula husk, pectin, cellulose, rice and resistant starch.



Total short chain fatty acid production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the levels of SCFAs were determined using GC analysis (Hewlett Packard). The graph revealed that pectin, rice starch and resistant starch were readily fermented ispaghula husk was fermented but at a slower rate initially when compared to the other fibres. Cellulose was not fermented well by the faecal homogenates.

rice starch or resistant starch; by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the levels of SCFAs were determined using GC analysis (Supelco Nukol 24107 capillary column (30m x 0.25mm x 0.25μm) oven maintained at 160°C, injector and detector maintained at 250°C, with injection volume of 1μl. Carrier gas (helium at 16.6psi) flow rate of 8.2ml/min. Hewlett Packard instrumentation) coupled with external standard solutions (5mM concentration of acetate, propionate and butyrate). The graphic representation of total SCFAs suggests that pectin, rice starch and resistant starch were fermented at a greater rate and extent than the fibres ispaghula husk and cellulose, with cellulose undergoing minimal fermentation. This pattern was observed at 3, 6 and 24 hours fermentation. After 24 hours fermentation pectin was found to be fermented to the greatest extent producing the highest levels of SCFAs (48.68mM total), followed by rice starch (45.71mM total), resistant starch (49.07mM total), ispaghula husk (30.81mM) and lastly cellulose (1.37mM total) which produced very low levels of SCFAs. This pattern of SCFA production reflects the changes seen in pH (Fig 3.2). The rate of fermentation is thought to be intrinsic to the physiological roles of dietary fibres (Reviewed by Edwards, 1995). Figure 3.4 provides a graphic representation of the rate of fermentation as determined by total SCFA production for the dietary fibres ispaghula husk, pectin, cellulose, rice starch and resistant starch. Pectin, rice starch and resistant starch fermentation started rapidly with a SCFA production rate of 4.23mM/hour, 4.13mM/hour and 4.66mM/hour respectively at 3 hours and 3.61mM/hour, 3.12mM/hour and 2.12mM/hour respectively at 6 hours. The rate of fermentation then tails off with rates for pectin, rice starch and resistant starch decreasing to 1.25mM/hour, 1.21mM/hour and 0.94mM/hour respectively. This decrease could be due to a lack of substrate due to its rapid fermentation within the *in vitro* fermentation system. In previous studies, pectin was metabolised rapidly within the colon of humans and the caecum of mice (Edwards and Eastwood, 1992; Reviewed by Spiller, 1994). Resistant starch was previously shown to be metabolised by colonic bacteria but at a slower rate and to a lesser extent than normal starch (Reviewed by Spiller, 1994). Observation for rice starch and resistant starch supplemented faecal homogenates

Figure 3.4 Rate of total SCFA production by the fibres ispaghula husk, pectin, cellulose, rice starch and resistant starch during fermentation by faecal homogenates.



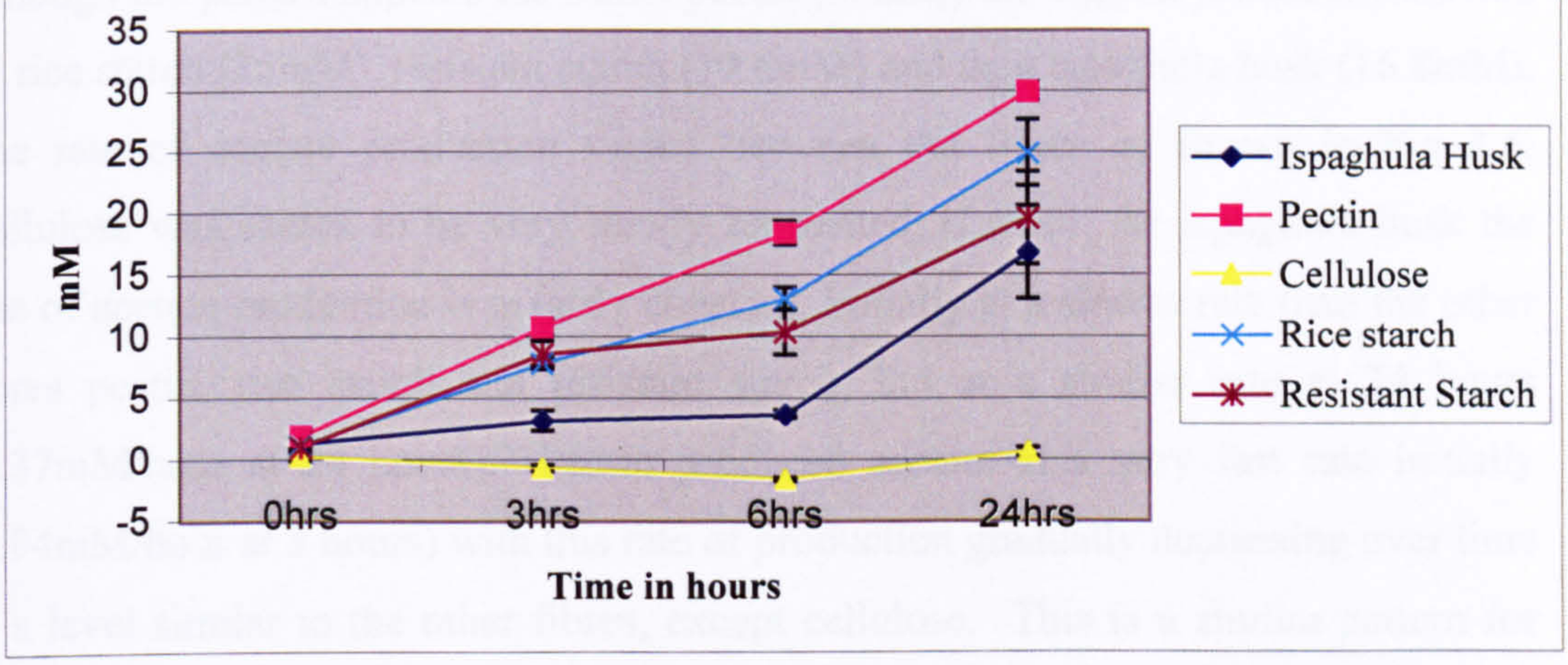
The rate of total short chain fatty acid production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the levels of SCFAs were determined using GC analysis (Hewlett Packard). The rate of SCFA production for the individual fibres revealed that initially resistant starch, pectin and rice starch were rapidly fermented, with their rate of SCFA production decreasing over time. The rate of ispaghula husks fermentation started slowly, in comparison to the other fibres, but gradually increased over time, whereas cellulose rate of fermentation was very slow throughout the duration of the experiment.

suggested that these fibres were not completely fermented, as residual fibre was still visible at the experiments termination. Ispaghula husk was shown to be fermented at a much slower and consistent rate when compared to the other fibres. Ispaghula husk was associated with a gradual increase in fermentation rate over time, 0.85mM/hour, 0.53mM/hour and 1.37mM/hour at 3, 6 and 24 hours respectively this could be due to bacterial adaptation to the carbohydrate source. Ispaghula husk has been shown to be fermented rapidly in the colon with 20-30% escaping digestion in the colon (Reviewed by Spiller, 1994). Observations throughout revealed that the structure of ispaghula husk was partially maintained along with its mucilaginous properties. This is consistent with the finding that ispaghula husk is fermented throughout the colon but maintains a high water content (Edwards and Eastwood, 1992). Cellulose was virtually unfermented producing SCFA levels over the control only at 24 hours. Total SCFA production rate for the 24 hour period being at a level of 0.08mM/hour, thus it was very slowly fermented. Previous studies have also shown that cellulose is largely unaltered through the gut, being variably fermented by colonic cellulases but up to 80% can be excreted in the faeces (Reviewed by Spiller, 1994). This lack of fermentation may be due to a lack of cellulases within the faeces or due to the fact that this experiment used purified cellulose, which has been shown to be less fermentable than cellulose found in plant cell walls (Reviewed by Wolever, 1995).

3.5.3.3 *Acetate production*

Acetate was consistently shown to be the main SCFA produced during the fermentation of these dietary fibres. Fig 3.5 shows the total acetate production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch by faecal homogenates (n=3). These fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals with the level of acetate being determined using GC analysis. Fig 3.5a shows that all fibres, except cellulose, produce measurable levels of acetate with pectin appearing to be the largest producer, followed by rice starch, resistant starch and then ispaghula husk. At 3 hours there was no comparative difference between

Figure 3.5 Total acetate production from the fermentation of the dietary fibres ispaghula husk, pectin, cellulose, rice and resistant starch.



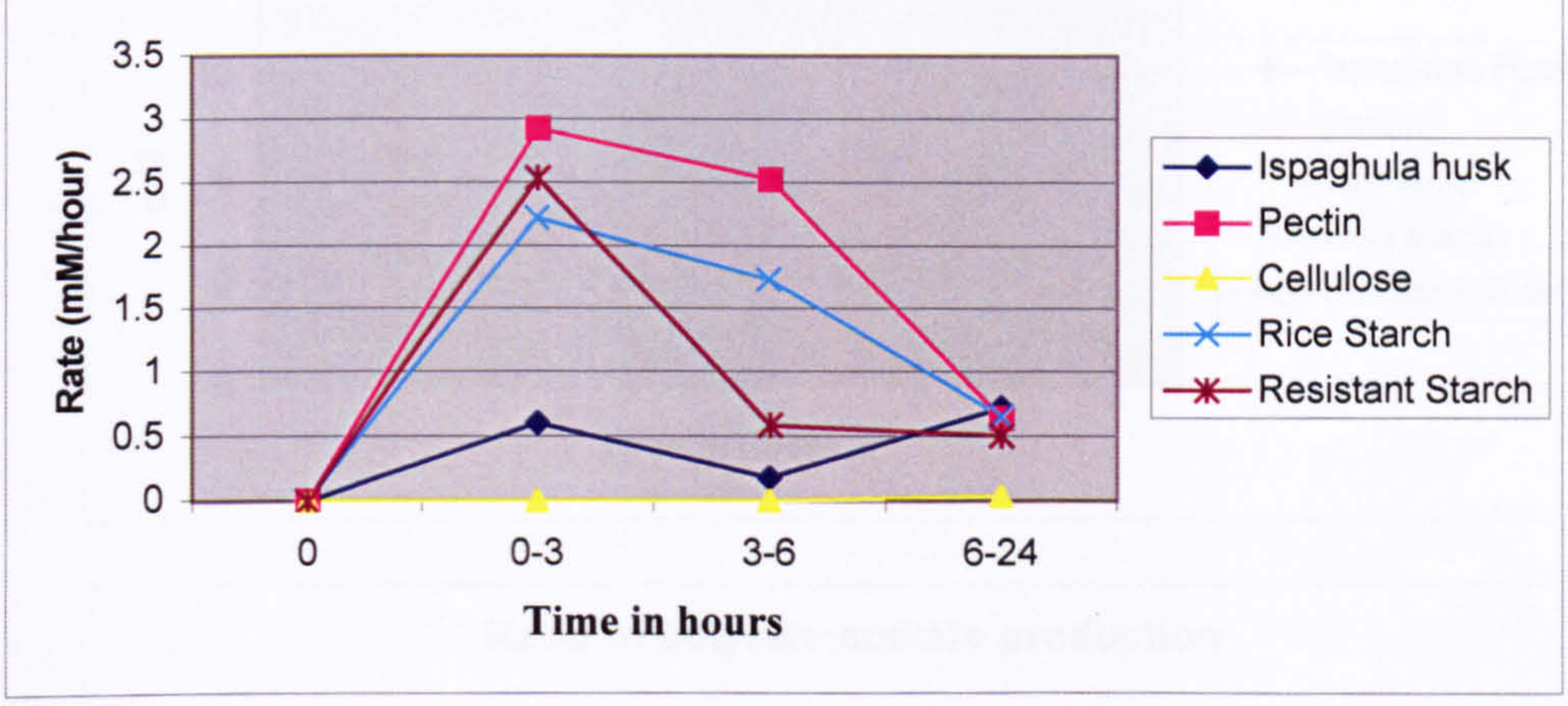
Total acetate production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the level of acetate determined using GC analysis. The graph revealed that all fibres except cellulose produced acetate. Pectin appeared to be the largest producer of acetate, followed by rice starch, resistant starch and then ispaghula husk. At 3 hours there was no obvious difference between pectin, rice starch and resistant starch, ispaghula husk produced less acetate. At 6 hours there was a larger distinction between the fibres, pectin produced the largest amount of acetate, followed by rice and resistant starch, with lower levels produced by ispaghula husk. At 24 hours there was no major distinction between the fibres although pectin appeared to be the greatest producer followed by rice starch, resistant starch and then ispaghula husk.

pectin, rice starch and resistant starch (10.79mM, 7.89mM and 8.66mM respectively), although pectin is the largest producer. Ispaghula husk produced less acetate (3.22mM) probably due to its slower fermentation rate. At 6 hours there was a greater distinction in acetate production between the fibres with pectin producing the highest levels (18.4mM), followed by rice (13.10mM) and then resistant starch (10.43mM) with lower levels being produced by ispaghula husk (3.74mM). At 24 hours there was no major distinction, as seen by the large standard deviations, although the pattern appears the same, pectin (30mM) the biggest producer followed by rice starch (25mM), resistant starch (19.6mM) and then ispaghula husk (16.8mM). The rate of acetate production varied between the fibres as shown in Fig 3.6. Cellulose was shown to be very slowly fermented, if at all, for ispaghula husk the rate of acetate production was fairly constant, initially at a slower rate than the other fibres pectin, rice starch and resistant starch, but at a similar rate at 24 hours (1.37mM/hour at 24 hours). Pectin produced acetate at a very fast rate initially (2.94mM/hour at 3 hours) with this rate of production gradually decreasing over time to a level similar to the other fibres, except cellulose. This is a similar pattern for rice starch, although not at such a rapid rate, reaching its height at 3 hours (2.23mM/hour). Resistant starch was rapidly fermented initially (2.54mM/hour at 3 hours), but this rate then dramatically reduced at 6 hours but then remained constant. Thus all fibres produced high levels of acetate, but at different rates and quantities, with pectin being the biggest producer. This pattern appears to reflect the pattern for total SCFA production, thus acetate is possibly a good indicator of rate and extent of fermentation.

3.5.3.4 *Butyrate production*

As all fibres appeared to produce high levels of acetate, it is thought that the determining factors for fibre profiles are their butyrate and propionate production. Fig 3.7(a) showed the total butyrate production with Fig 3.7(b) the ratio of butyrate to acetate production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch; by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the level of butyrate determined using GC analysis.

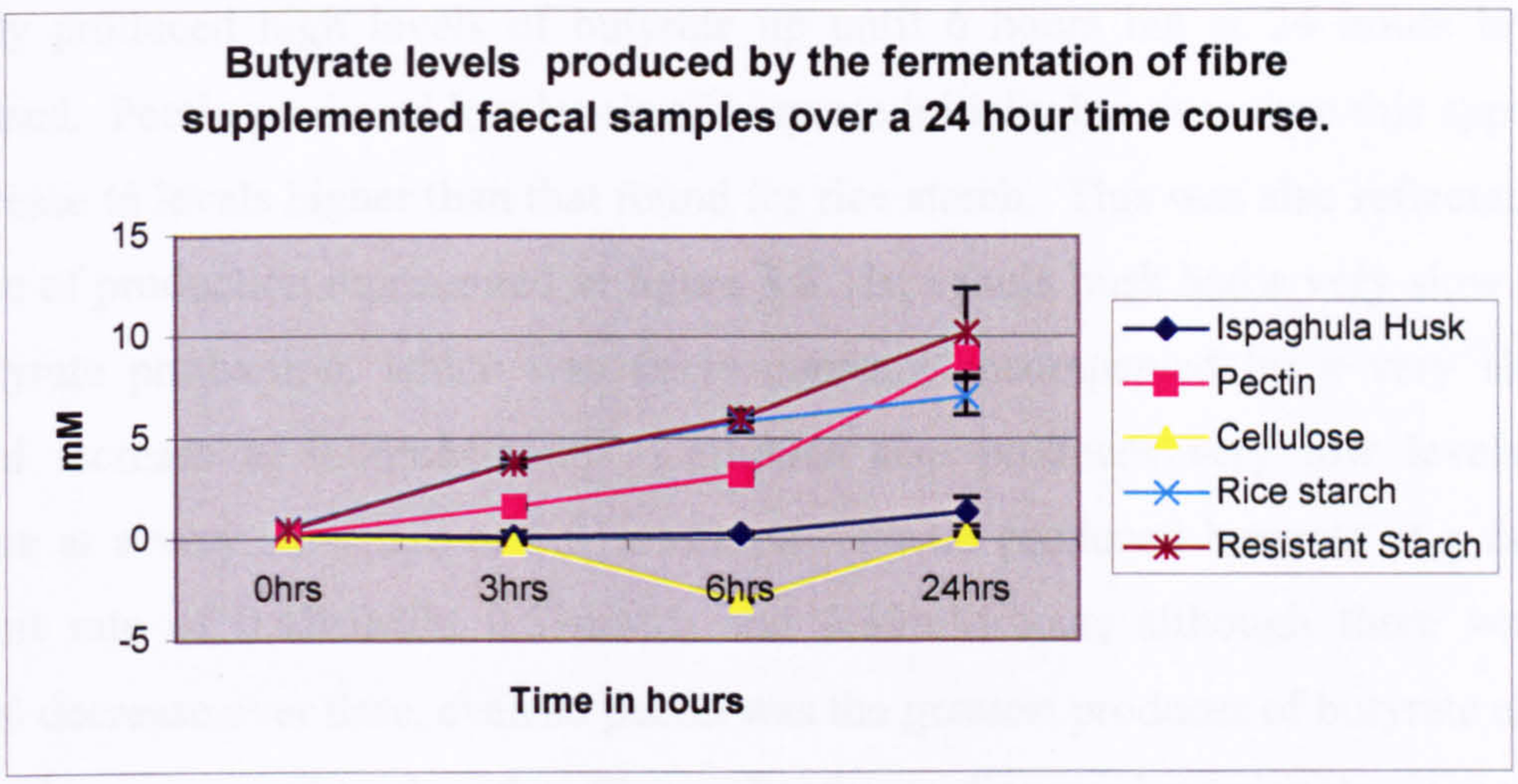
Figure 3.6 Rate of acetate production by the fibres ispaghula husk, pectin, cellulose, rice starch and resistant starch during fermentation in faecal homogenates.



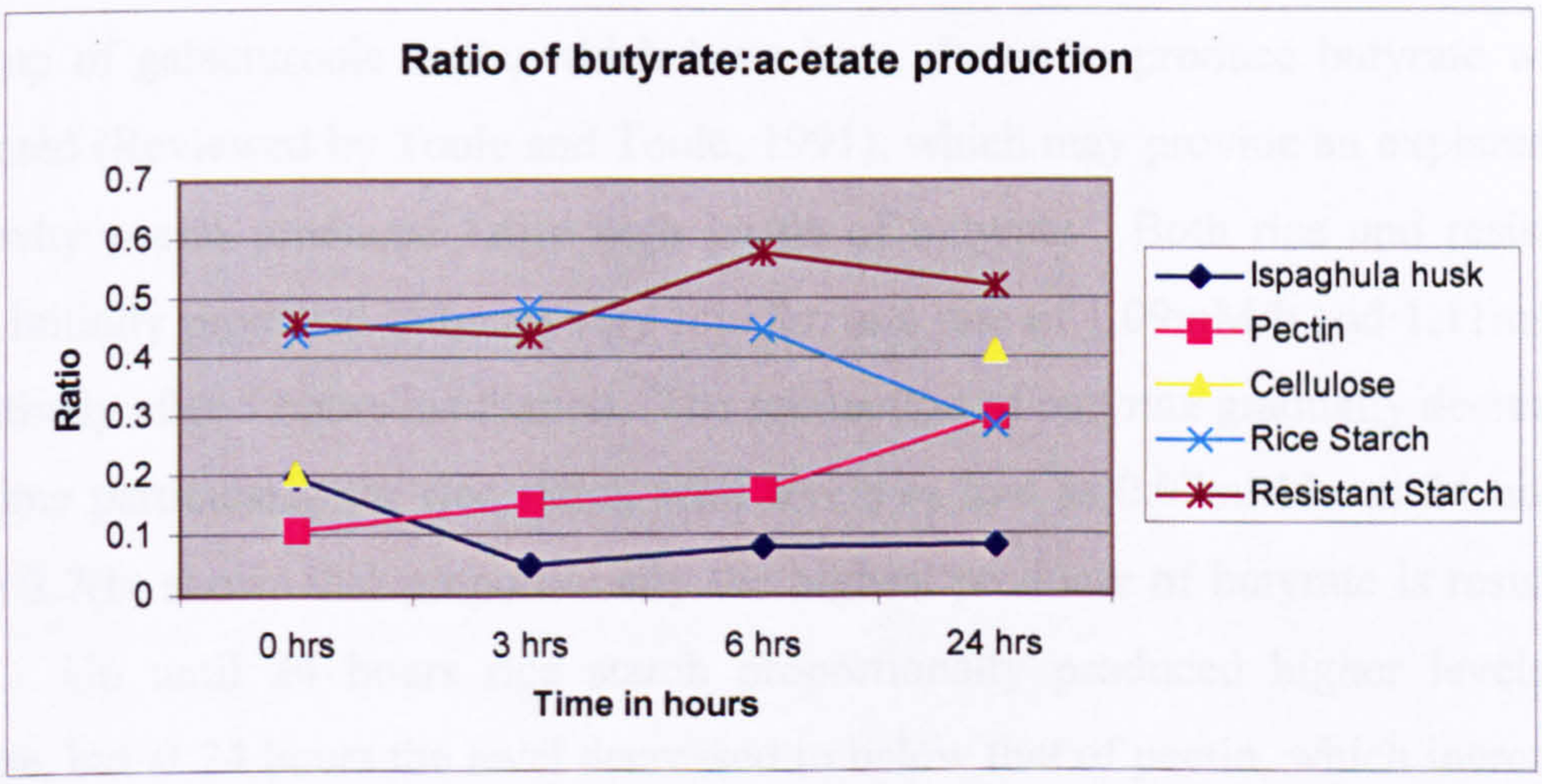
The rate of acetate production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the levels of SCFAs were determined using GC analysis (Hewlett Packard). The rate of acetate production appeared to fundamentally mirror that of total SCFAs revealing that pectin, resistant starch and rice starch initially produced acetate at the greatest rate, this was shown to steadily reduce over time, interestingly resistant starch rate of acetate production dramatically decreased between the 3rd and 6th hour suggesting a possible switch in SCFA production to propionate or butyrate. Ispaghula husk and cellulose rate of acetate production mirrored that of total SCFA production. At 24 hours the rate of acetate production was similar in all the fibres except cellulose.

Figure 3.7a) Total butyrate production (b) Ratio of butyrate:acetate production from the fermentation of the dietary fibres; ispaghula husk, pectin, cellulose, rice and resistant starch.

a)



b)



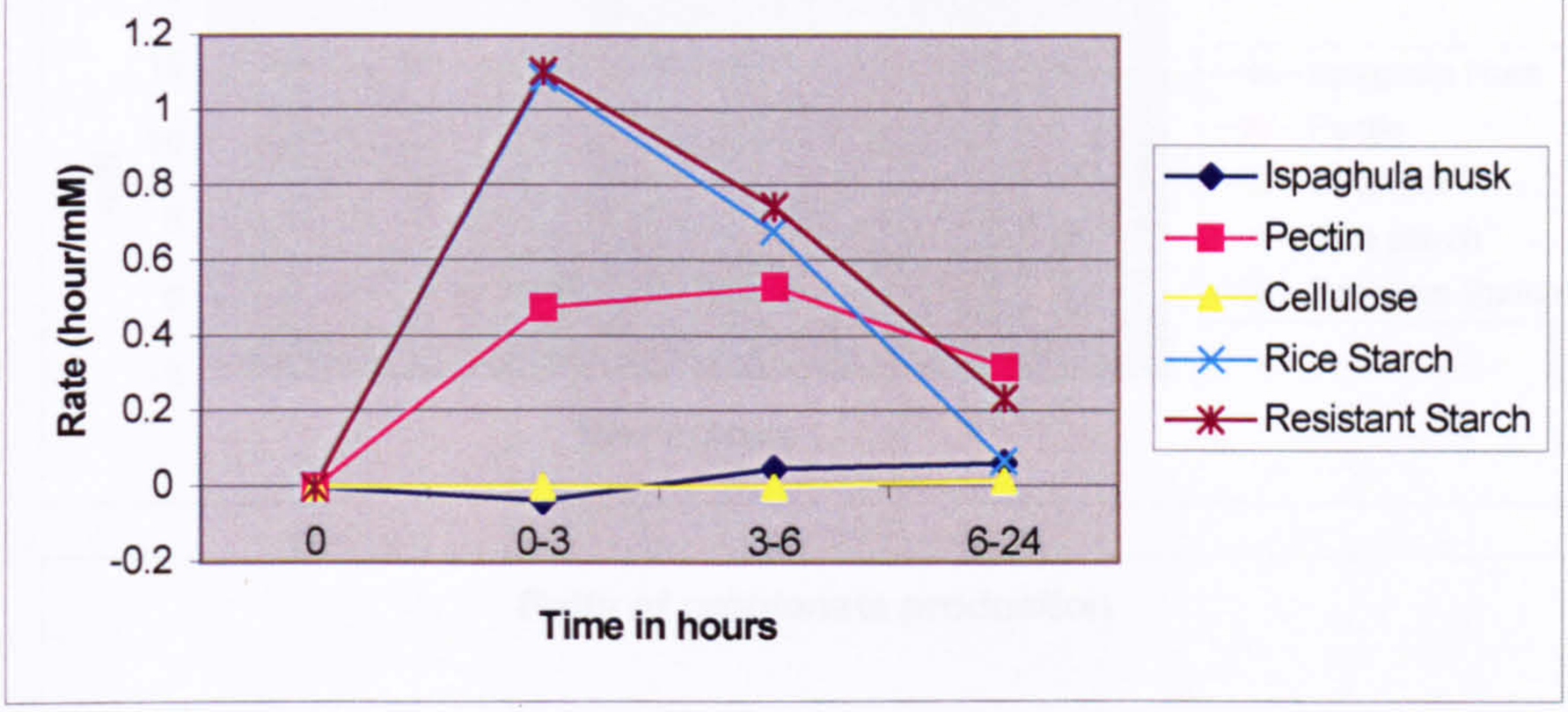
a) Total butyrate production (b) Ratio of butyrate to acetate production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the level of butyrate determined using GC analysis. (a) Both cellulose and ispaghula husk produced very low levels of butyrate. Resistant starch produced the largest amount of butyrate and at the fastest rate followed by rice starch then pectin, although at 24 hours pectins butyrate production exceeds rice starch. (b) Proportionally the largest producer of butyrate was resistant starch along with rice starch (up until 6 hours) the level decreased below that of pectin and cellulose, which both increased their butyrate production with time. Ispaghula husk proportionally produced very low levels.

Fig. 3.7(a) shows that both cellulose and ispaghula husk produced very low levels of butyrate throughout (0.25mM and 1.41mM at 24 hours, respectively). Resistant starch produced the largest amount of butyrate, 10.25mM at 24 hours. Rice starch initially produced high levels of butyrate up until 6 hours but at 24 hours levels decreased. Pectin produced low levels of butyrate initially but over time this appears to increase to levels higher than that found for rice starch. This was also reflected by the rate of production represented in figure 3.8. Ispaghula husk had a very slow rate of butyrate production, which was fairly constant accompanied by a very slight gradual increase to 0.06mM/hour. Cellulose also produces very low levels of butyrate at a very slow rate of 0.01mM/hour. Pectin produced butyrate at a fairly constant rate of 0.48mM/h, 0.53mM/h and 0.32mM/hour, although there was a gradual decrease over time, even so pectin was the greatest producer of butyrate at 24 hours when compared to the other fibres. Pectin, as discussed previously, is mainly made up of galacturonic acids, which have been shown to produce butyrate when fermented (Reviewed by Toole and Toole, 1991), which may provide an explanation as to why pectin produced fairly high levels of butyrate. Both rice and resistant starch initially produced butyrate very rapidly, at a rate of 1.09mM/h and 1.11mM/h respectively after 3 hours incubation. The production of butyrate gradually decreases over time particularly for rice starch with levels as low as 0.07mM/hr at 24 hours. Figure 3.7(b) shows that proportionally the highest producer of butyrate is resistant starch. Up until 24 hours rice starch proportionally produced higher levels of butyrate, but at 24 hours the level decreased to below that of pectin, which increased the proportion of their butyrate production with time. Ispaghula husk is shown to produce very low levels.

3.5.3.5 *Propionate production*

Propionate production is also very important for many physiological reasons. Fig 3.9 represents the (a) total propionate production (b) ratio of propionate to acetate production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch; by faecal homogenates (n=3).

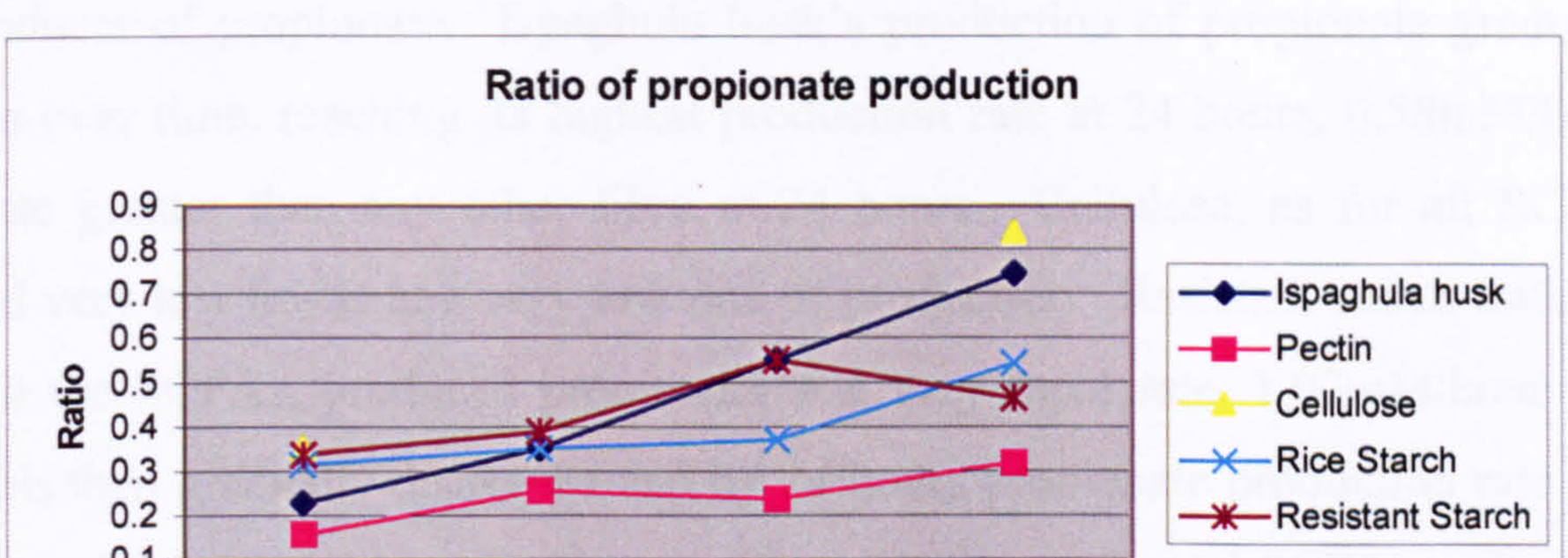
Figure 3.8 Rate of butyrate production by the fibres ispaghula husk, pectin, cellulose, rice starch and resistant starch.



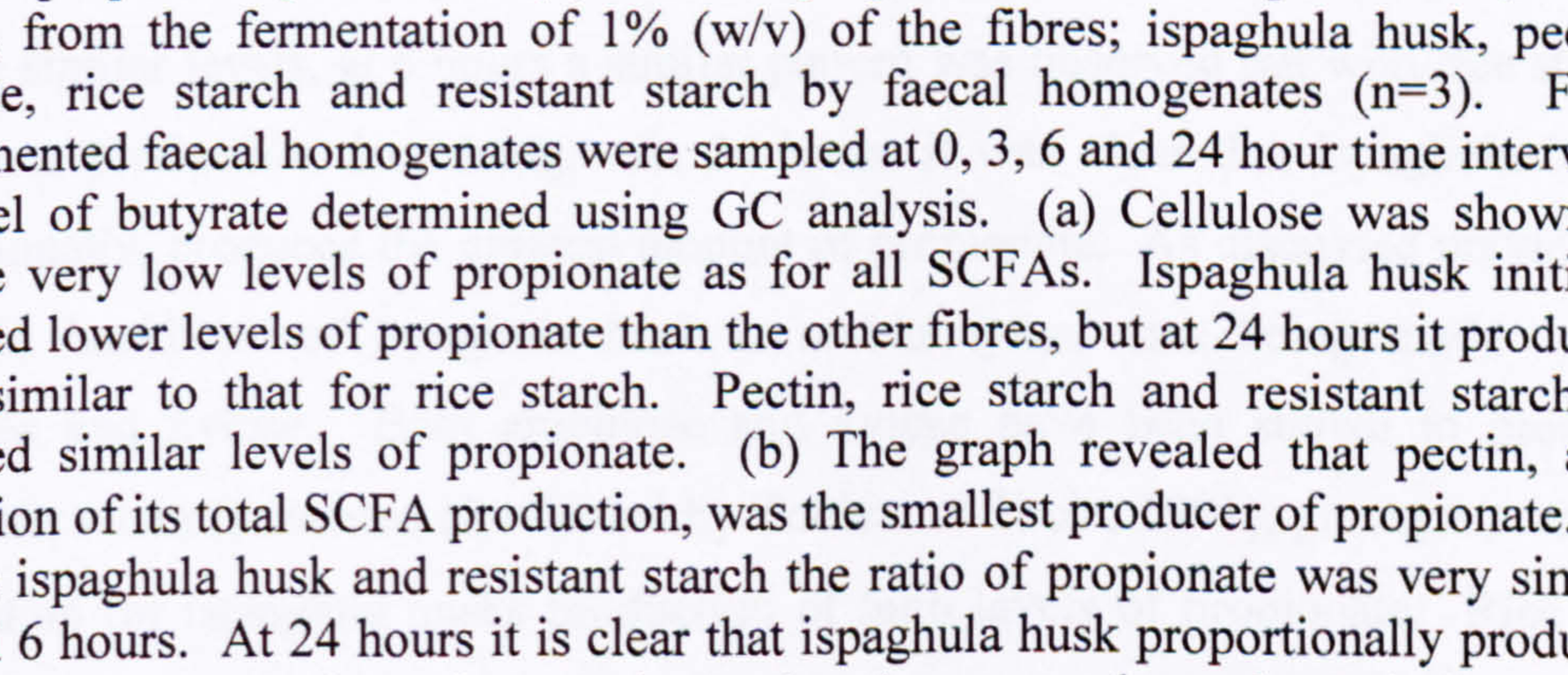
The rate of butyrate production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the levels of SCFAs were determined using GC analysis (Hewlett Packard). The graph revealed that initially the greatest rate of butyrate production was for resistant and rice starch. The rate of butyrate production by these two fibres then decreased steadily over time. Pectins rate of butyrate production steadily increased up until 6 hours and then decreased after 24 hours incubation. Ispaghula husks rate of butyrate production increased over time but butyrate was produced at a much slower rate than the other fibres. Cellulose rate of butyrate production was very slow mimicking that of its rate of fermentation.

Figure 3.9a) Total propionate production (b) Ratio of propionate:acetate production from the fermentation of dietary fibres; ispaghula Husk, pectin, cellulose, rice and resistant starch.

a)



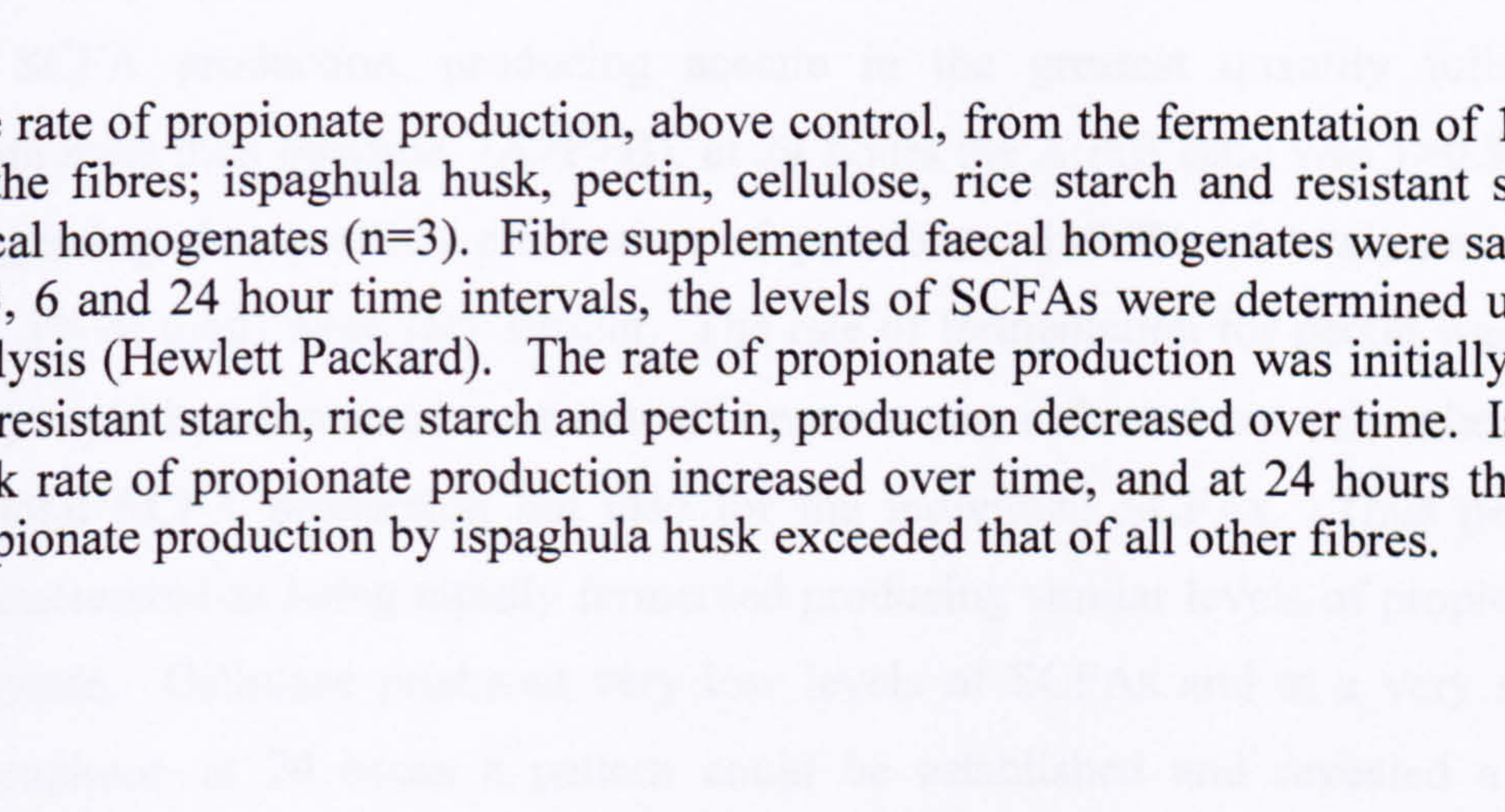
b)



(a) Total propionate production (b) Ratio of propionate to acetate production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the level of butyrate determined using GC analysis. (a) Cellulose was shown to produce very low levels of propionate as for all SCFAs. Ispaghula husk initially produced lower levels of propionate than the other fibres, but at 24 hours it produced levels similar to that for rice starch. Pectin, rice starch and resistant starch all produced similar levels of propionate. (b) The graph revealed that pectin, as a proportion of its total SCFA production, was the smallest producer of propionate. At 3 hours ispaghula husk and resistant starch the ratio of propionate was very similar up until 6 hours. At 24 hours it is clear that ispaghula husk proportionally produced the greatest amount of propionate, rice and resistant starch produce similar levels with cellulose also proportionally produces high levels of propionate.

Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the level of propionate determined using GC analysis. Figure 3.9(a) revealed that cellulose produced very low levels of propionate (0.51mM at 24 hours) as for all SCFAs. Ispaghula husk initially produced lower levels of propionate than the other fibres, 1.14mM and 2.07mM at 3 and 6 hours respectively, but at 24 hours it produces levels similar to that of rice starch (12.58mM and 13.64mM respectively). Pectin, rice starch and resistant starch all produced similar levels of propionate (4.44mM, 4.89mM and 5.77mM respectively at 6 hours) up until 24 hours where rice starch was found to produce higher levels. This can also be seen when examining the rate of production, shown in figure 3.10, as when the slower rate of ispaghula fermentation is taken into consideration it shows that ispaghula husk is a high producer of propionate. Ispaghula husk's production of propionate gradually increases over time, reaching its highest production rate at 24 hours, 0.58mM/hour, and a rate greater than any other fibre at 24 hours. Cellulose, as for all SCFAs produced very low levels at a very low rate of production. Resistant starch initially, as for all the SCFAs, produced propionate at a very rapid rate, 1.02mM/hour at 3 hours, this then gradually decreased and by 24 hours propionate production rate was very low and this is a pattern consistent with resistant starch total SCFA production. Pectin and rice starch initially produced propionate at a similar rate; this rate of production then reduces for both fibres over time, but at a greater rate for pectin. Figure 3.9(b) revealed that proportionally, pectin was the lowest producer of propionate. At 3 hours ispaghula husk, rice starch and resistant starch proportionally produce similar levels, at 6 hours a similar pattern was observed but with rice starch propionate production decreasing. At 24 hours it was clear that ispaghula husk, proportionally, produced the greatest amount of propionate. As discussed previously the main backbone of ispaghula husk is arabinoxylan, thus being made up of arabinose and xylose. Both arabinose and xylose have been shown to produce propionate when fermented (Reviewed by Toole and Toole, 1991), giving a possible explanation for ispaghula husks production of high levels of propionate. Rice and resistant starch produced similar levels of propionate, the ratio of propionate production also suggested that cellulose produced high levels of propionate.

Figure 3.10 Rate of propionate production by the fibres ispaghula husk, pectin, cellulose, rice starch and resistant starch during fermentation in faecal homogenates.



The rate of propionate production, above control, from the fermentation of 1% (w/v) of the fibres; ispaghula husk, pectin, cellulose, rice starch and resistant starch by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the levels of SCFAs were determined using GC analysis (Hewlett Packard). The rate of propionate production was initially greatest for resistant starch, rice starch and pectin, production decreased over time. Ispaghula husk rate of propionate production increased over time, and at 24 hours the rate of propionate production by ispaghula husk exceeded that of all other fibres.

3.5.4 SCFA profiles for the fibres; Ispaghula husk, Pectin, Cellulose, Rice starch and Resistant Starch.

As all fibres produced high levels of acetate, ratios of butyrate (fig 3.6b) and propionate (Fig 3.8b) were determined, along with their rates of production (Fig 3.7 and 3.9). Ispaghula husk resulted in an increase in SCFA levels over time and was shown to produce a consistent pattern of SCFA production with acetate production exceeding propionate production, which exceeds butyrate production. At 24 hours the acetate:propionate:butyrate ratio (A:P:B) was 1>0.75>0.08 respectively, which clearly shows that ispaghula husk proportionally produced high levels of propionate (40.8% of total) but very low levels of butyrate (4.6% of total) when fermented by the faecal homogenates. The rate of fermentation was fairly slow and consistent, although the rate of propionate production appeared to increase over time. Thus ispaghula husk was characterized as being fermented slowly and by producing high levels of propionate at 24 hours. Pectin also showed a consistent pattern in the ratio of SCFA production, producing acetate in the greatest quantity followed by propionate then butyrate (A>P>B), at 24 hours the A:P:B ratio was 1>0.32>0.299, suggesting that pectin's production of propionate (19.9% of total) and butyrate (18.5% of total) were very similar. The rate of fermentation for pectin was initially very rapid but decreased over time this pattern was reflected not only when looking at total SCFA production but also for the individual SCFAs. Thus pectin was characterized as being rapidly fermented producing similar levels of propionate and butyrate. Cellulose produced very low levels of SCFAs and at a very slow rate throughout, at 24 hours a pattern could be established and revealed a ratio of 1>0.84>0.41 for A:P:B, suggesting that cellulose proportionally produces high levels of propionate (38.3% of total) with lower levels of butyrate (18.3% of total). Thus cellulose was characterized as being a very slowly fermented fibre, producing high levels of propionate. Rice starch was shown to initially produce a ratio pattern of A>B>P, but at 24 hours propionate production exceeded butyrate production. Thus at 6 hours the A:P:B ratio was 1:0.37:0.45 respectively, but at 24 hours the ratio changed to 1>0.55>0.28, suggesting that rice starch produced high levels of propionate (29.8% of total) and lower levels of butyrate (15.5% of total). The rate of rice starch fermentation appeared to also have a consistent pattern, initially very rapid and then gradually falling over time with the greatest fall in rate of production

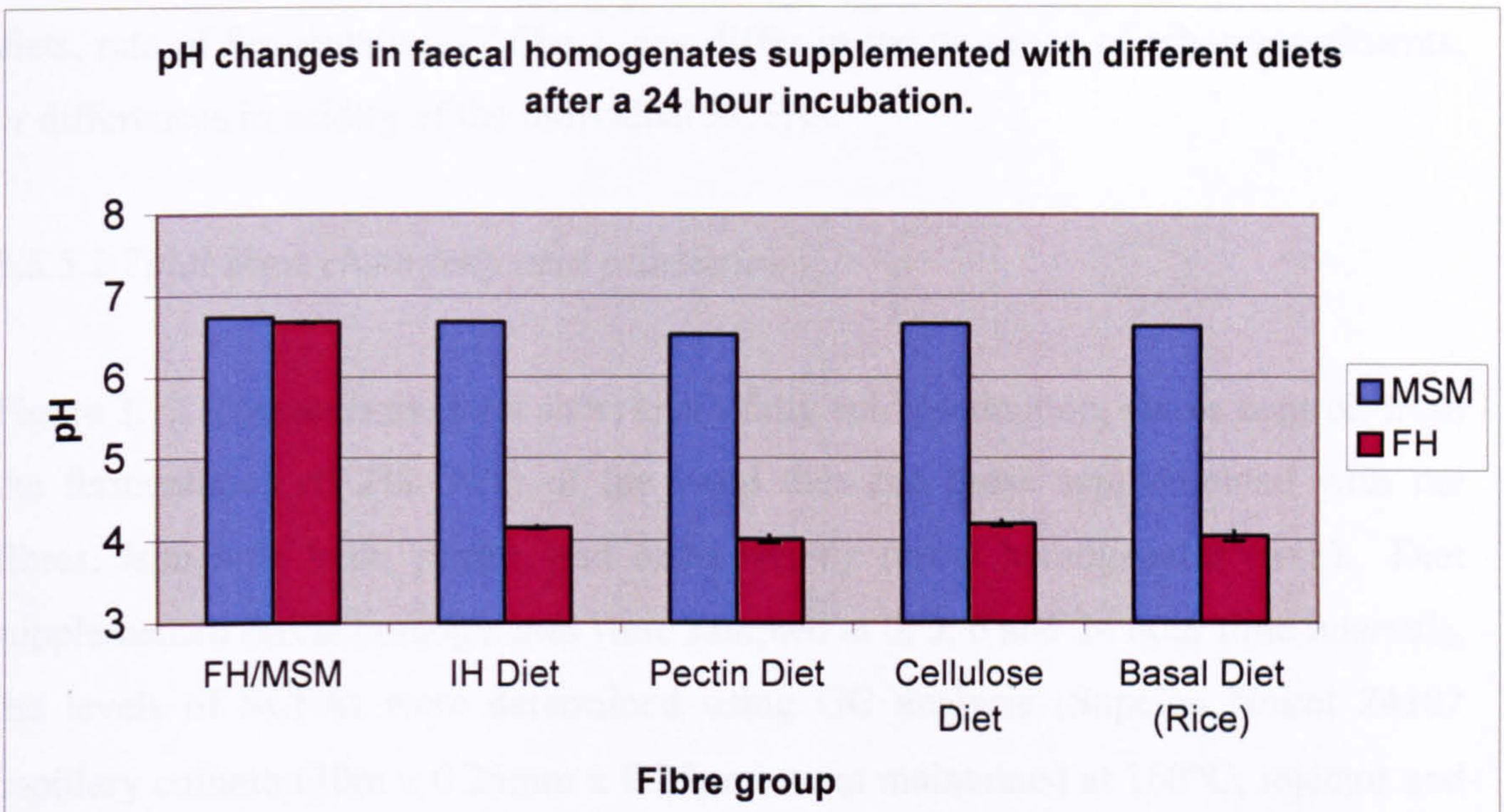
being for butyrate, as reflected in the changes in ratios. Thus rice starch was characterized as being rapidly fermented, initially producing high levels of butyrate but with propionate production exceeding butyrate production at later stages. Resistant starch was shown to produce an A:P:B ratio of 1:0.47:0.52 at 24 hours, suggesting that although propionate and butyrate levels were very similar, it produced more butyrate (26.25% of total) than propionate (23.5% of total). The rate of fermentation for resistant starch also appeared to have a fairly consistent pattern, again being fermented very quickly initially but then gradually decreasing over time. Thus resistant starch was characterized as being rapidly fermented, producing high levels of butyrate.

3.5.5 Fermentation characteristics of the basal and fibre supplemented diets Ispaghula husk, Pectin, Cellulose.

3.5.5.1 *pH changes*

Fig 3.11 Represents the pH changes found within human faecal homogenates (16.67% w/v) supplemented with 2% (w/v) of basal diets consisting of one of the following fibres; ispaghula husk, pectin, and cellulose, after 24 hours incubation under anaerobic conditions (80% N2, 10% CO2 and 10% H2) at 37°C using a MACS VA500 microaerophilic cabinet (Don Whitley Scientific Ltd, Shipley, West Yorks). pH was used to determine whether fermentation had been successful, as seen by a decrease in pH, and to detect any unwanted contamination. The graph clearly showed that for all diets the controls (MSM plus fibre, n=1) were free from contamination with faecal bacteria, the slight decrease in the pectin control was probably due to its own acidity. pH also revealed the extent of fermentation of the fibre supplemented diets within the faecal homogenate (n=3) after 24 hours. All diets resulted in a similar decrease in pH, an increase in acidity, suggesting they were all readily fermented, this fermentation pattern was probably the result of the sucrose and rice starch content. The graph showed a very subtle difference between the different groups, with the basal and pectin supplemented diet resulting in the lowest

Fig 3.11 pH changes within human faecal homogenates supplemented with the basal diet and the basal diet supplemented with the dietary fibres ispaghula husk, pectin and cellulose.



Key: MSM = Mineral salts media supplemented with fibre, FH = Faecal homogenate supplemented with fibre.

pH changes in human faecal homogenates containing 2% (w/v) of diets supplemented with the following fibres; Basal, Ispaghula husk, Pectin, and Cellulose, after 24 hours incubation under anaerobic conditions (80% N2, 10% CO2 and 10% H2) at 37°C using a MACS VA500 microaerophilic cabinet (Don Whitley Scientific Ltd, Shipley, West Yorks). This graphic representation showed that for all diets the controls (MSM plus fibre, n=1) were free from contamination. All fibre supplemented diets and the basal diet (n=3) resulted in a decrease in pH after 24 hours. This fermentation pattern was probably due to the sucrose and rice starch (See Table 3.6a, Table 3.7 and Fig 3.2) content. The graph showed a very subtle difference between the groups, with the basal and pectin supplemented diets resulting in the largest decrease in pH, followed by ispaghula husk and cellulose, a pattern which is consistent with the fibre fermentation (Fig 3.2).

pH, 4.05 and 4.02 respectively, followed by ispaghula husk, pH 4.16, and cellulose, pH4.2. The largest decrease in pH was found to be basal diet (pH decreased by 2.58), followed by ispaghula husk (2.52), pectin (2.51) and cellulose (2.46). This could have been due to a number of factors including non-homogenous mixing of the diets, rate of fermentation of fibres may differ in the presence of other constituents, or differences in acidity of the individual SCFAs.

3.5.5.2 *Total short chain fatty acid production*

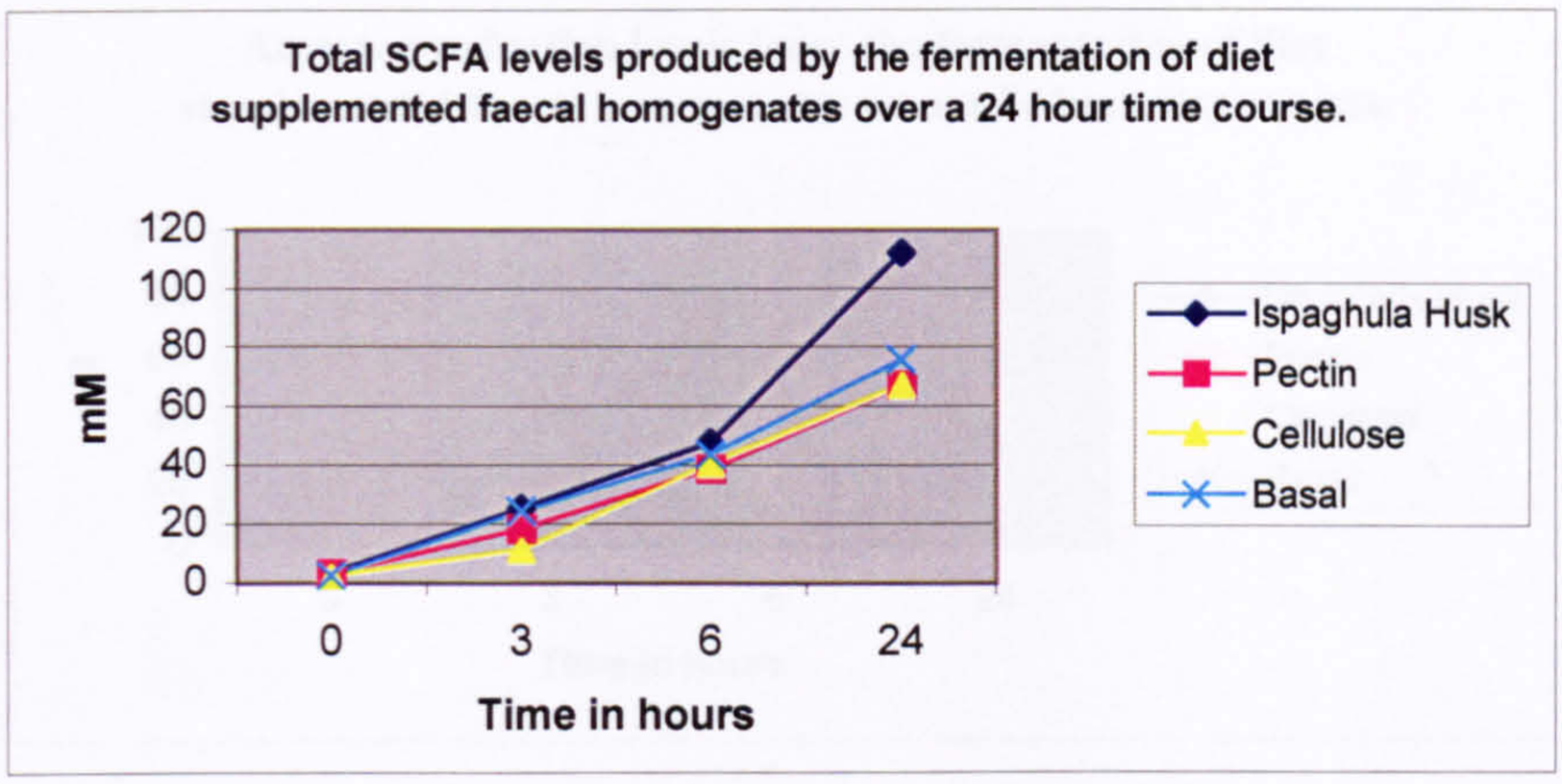
Figure 3.12 represents the total short chain fatty acid production, above control, from the fermentation of 2% (w/v) of the basal diet and those supplemented with the fibres; Ispaghula husk, pectin, and cellulose; by faecal homogenates (n=3). Diet supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the levels of SCFAs were determined using GC analysis (Supelco Nukol 24107 capillary column (30m x 0.25mm x 0.25μm) oven maintained at 160°C, injector and detector maintained at 250°C, with injection volume of 1μl. Carrier gas (helium at 16.6psi) flow rate of 8.2ml/min. (Hewlett Packard instrumentation) coupled with external standard solutions (5mM concentration). The graph revealed that the diets, up to 6 hours of incubation in the faecal homogenates, were fermented to a similar extent. At 24 hours there were differences in the final SCFA levels in both experiments not giving consistent findings. In experiment one the largest SCFA producer was ispaghula husk whereas in the second experiment it fell to cellulose. This could be the result of problems occurring within the internal standards for acetate levels within experiment 2 suggesting that the production of butyrate and propionate may provide clearer indicators of fermentation.

3.5.5.3 *Acetate production*

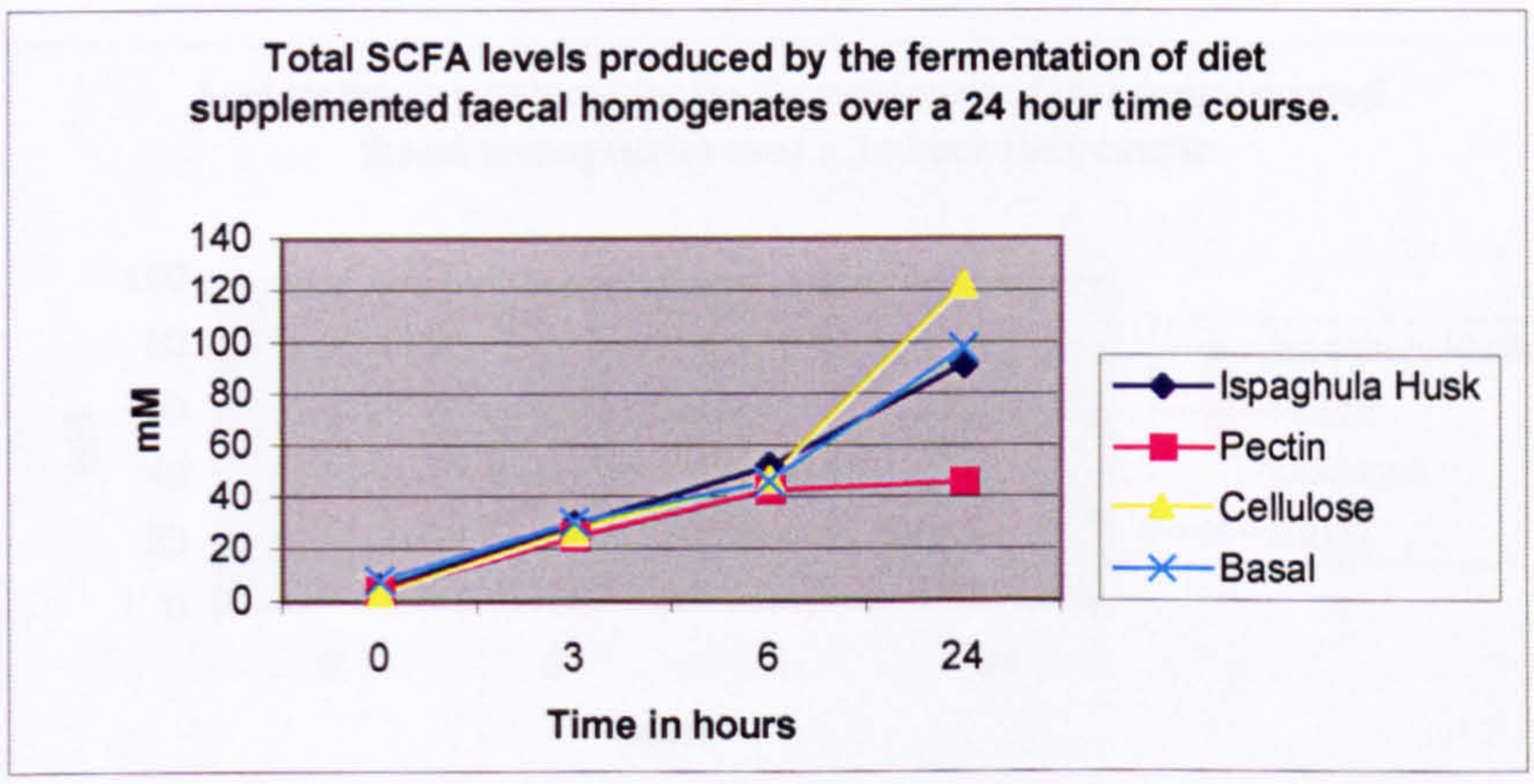
Figure 3.13 represent the total acetate production, above control, for the fermentation of 2% (w/v) of the basal and fibre supplemented diets; ispaghula husk, pectin, cellulose; by faecal homogenates (n=3). Fibre supplemented faecal homogenates

Figure 3.12 Total short chain fatty acid production from the fermentation of the fibre supplemented diets.

(a)Experiment 1



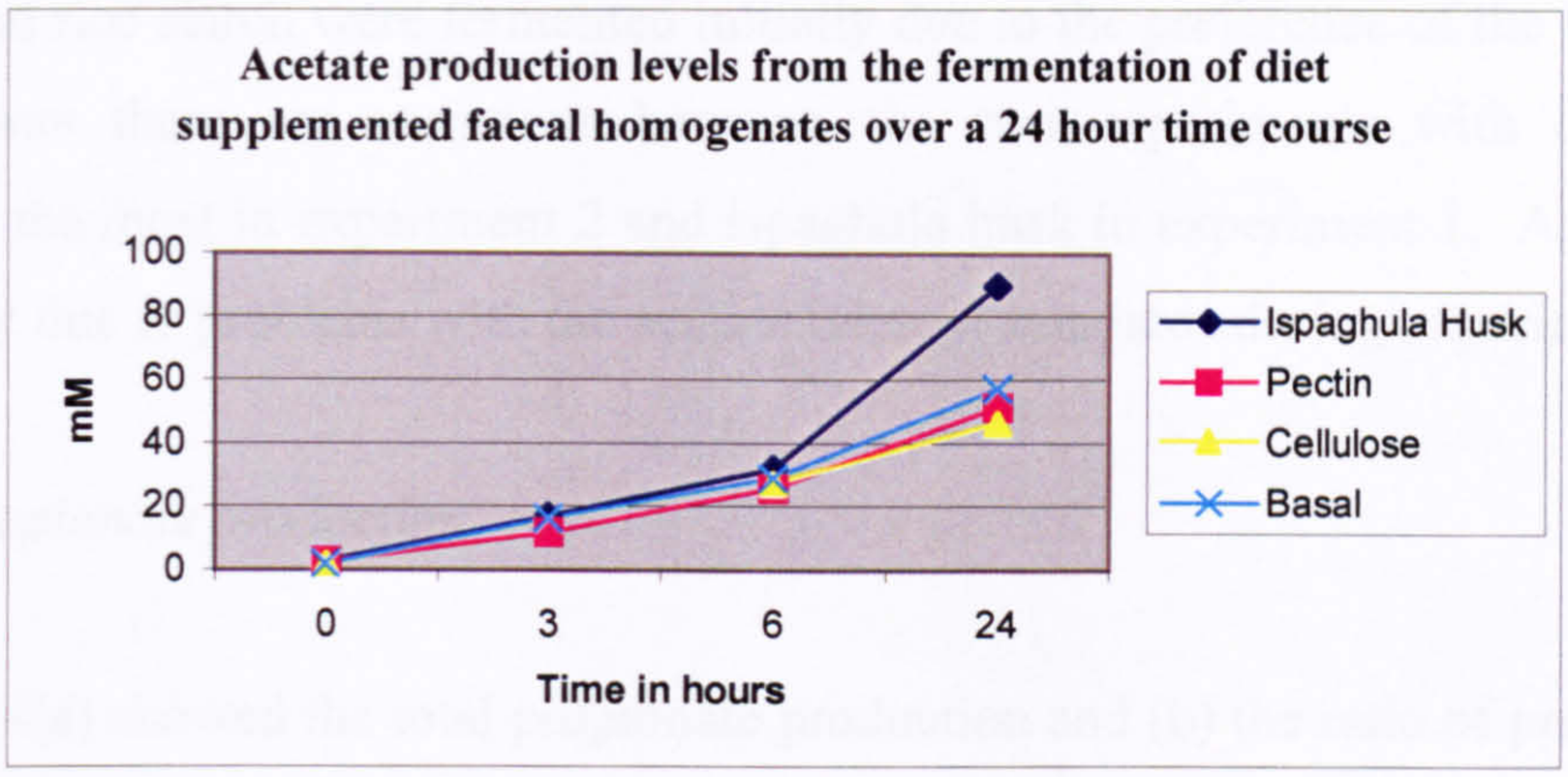
(b)Experiment 2



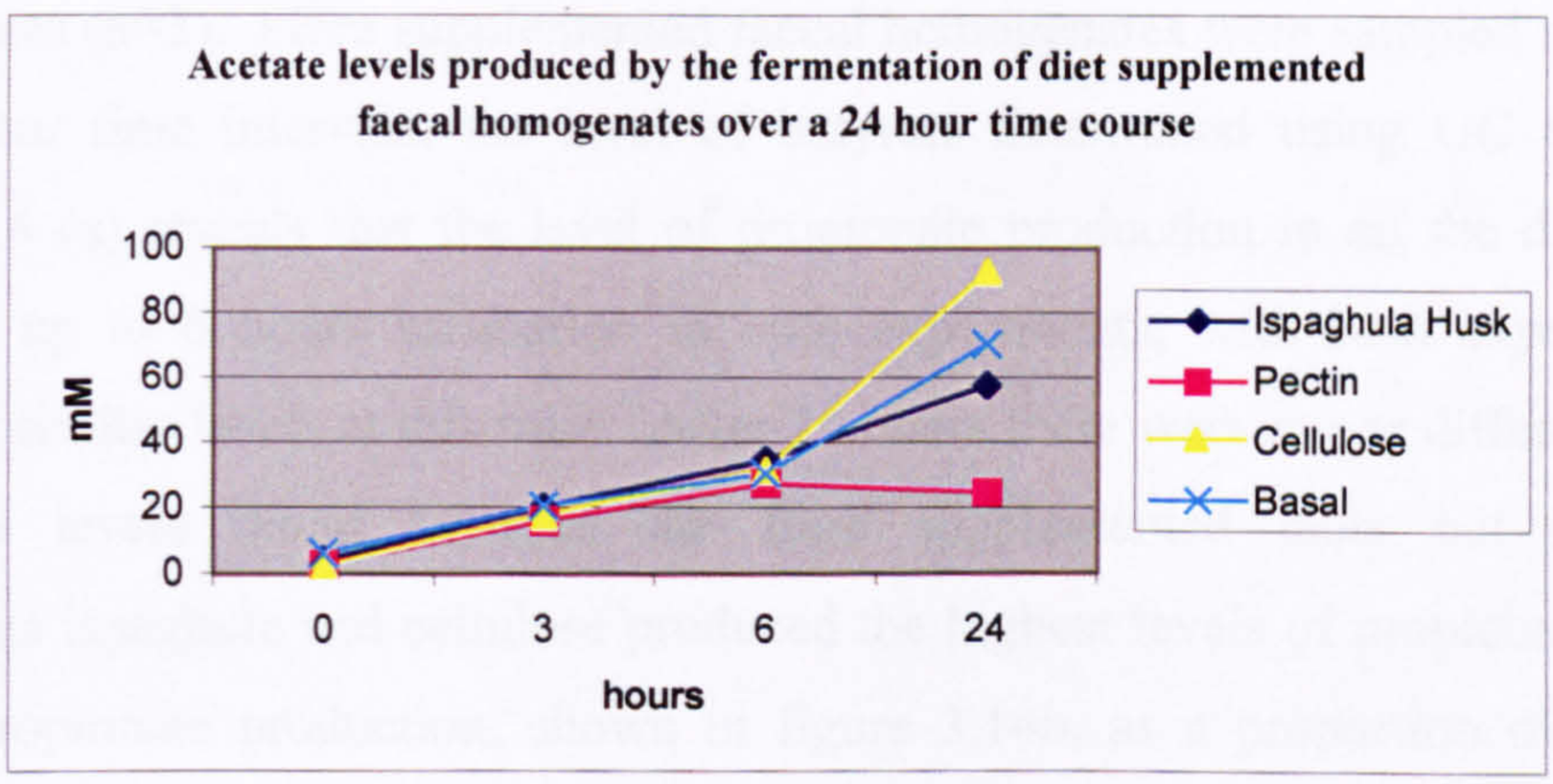
Total short chain fatty acid production, above control, from the fermentation of 2% (w/v) of the fibre supplemented diets; ispaghula husk, pectin, cellulose and basal diet (no fibre added) by faecal homogenates (n=3). Diet supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the levels of SCFAs were determined using GC analysis (Hewlett Packard). The graph showed that the diets, up until 6 hours were fermented to a similar extent. At 24 hours there were differences in the final SCFA levels with both experiments giving inconsistent findings, in experiment one the largest SCFA producer was ispaghula husk whereas in the second experiment it falls to cellulose. Thus could have been due to problems in the internal standards for acetate levels in experiment 2.

Figure 3.13 Total acetate production from the fermentation of fibre supplemented diets.

(a) Experiment 1



(b) Experiment 2



Total acetate production, above control, from the fermentation of 2% (w/v) of the fibre supplemented diets; Ispaghula husk, pectin, cellulose and basal diets by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the level of acetate determined using GC analysis. The graph revealed that all fibres produced acetate at similar levels up until 6 hours. At 24 hours there were variations between the two experiments, as for total SCFA production, with cellulose producing the greatest level of acetate in experiment 2 and ispaghula husk in experiment 1.

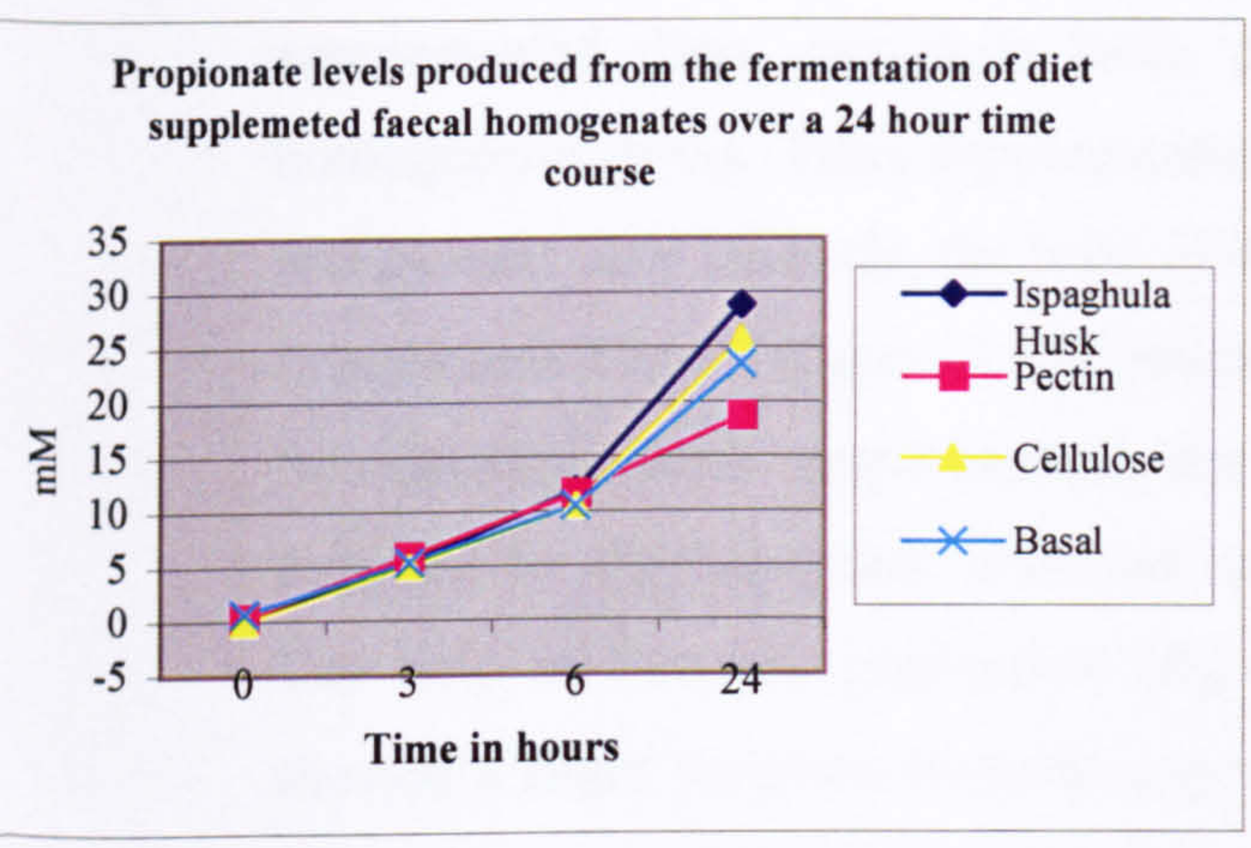
were sampled at 0, 3, 6 and 24 hour time intervals, the level of acetate determined using GC analysis. The graph showed that all fibres produced acetate at similar levels after 6 hours incubation, this may suggest that the common constituents, sucrose and rice starch were fermented initially due to the preference of the bacteria. At 24 hours there are variations between the two experiments with cellulose producing the most in experiment 2 and ispaghula husk in experiment 1. Again this is probably due to problems with the acetate internal standards during experiment 2.

3.5.5.4 *Propionate production*

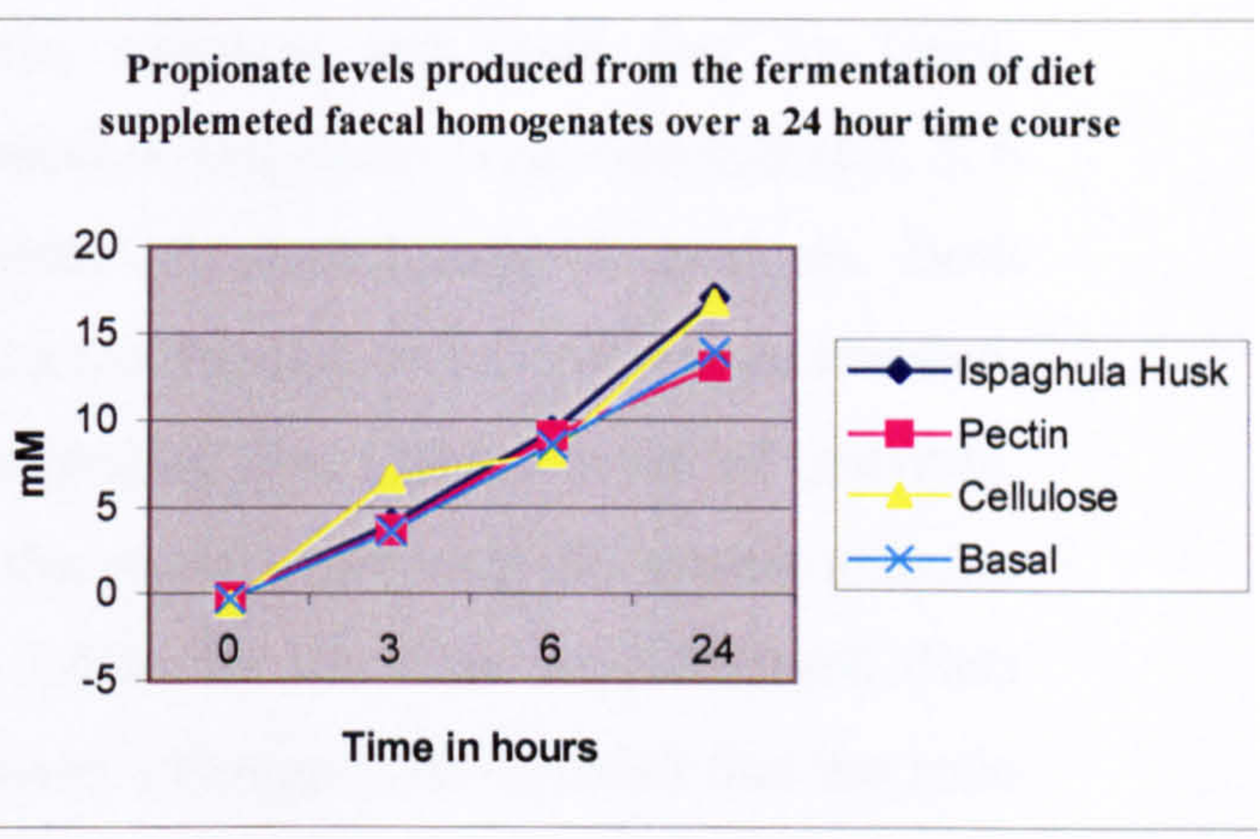
Figure 3.14(a) showed the total propionate production and (b) the ratio of propionate to acetate production, above control, from the fermentation of 2% (w/v) of the fibre supplemented diets; ispaghula husk, pectin, cellulose and basal diet; by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the level of butyrate determined using GC analysis. Figure 3.14 (a) reveals that the level of propionate production in all the diets was consistent up to 6 hours incubation in both experiments, with both experiments producing similar levels at this time. After 24 hours there were minor differences in propionate levels found between the fibre supplemented diets but in both experiments ispaghula and cellulose produced the highest levels of propionate. The ratio of propionate production, shown in figure 3.14b, as a proportion of acetate production revealed that in both experiments the pectin diet proportionally produced the highest level of propionate. This was observed up until 6 hours in experiment 1 and 24 hours within experiment 2 with cellulose being the greatest producer in experiment 1. Within all the fibre supplemented diets propionate production was proportionally higher than butyrate, with each diet having a profile of acetate>propionate>butyrate, for example the A:P:B ratio for pectin was 1:0.75:0.1 after a 24 hour incubation.

Figure 3.14a) Total propionate production (b) Ratio of propionate:acetate production from the fermentation of fibre supplemented diets.

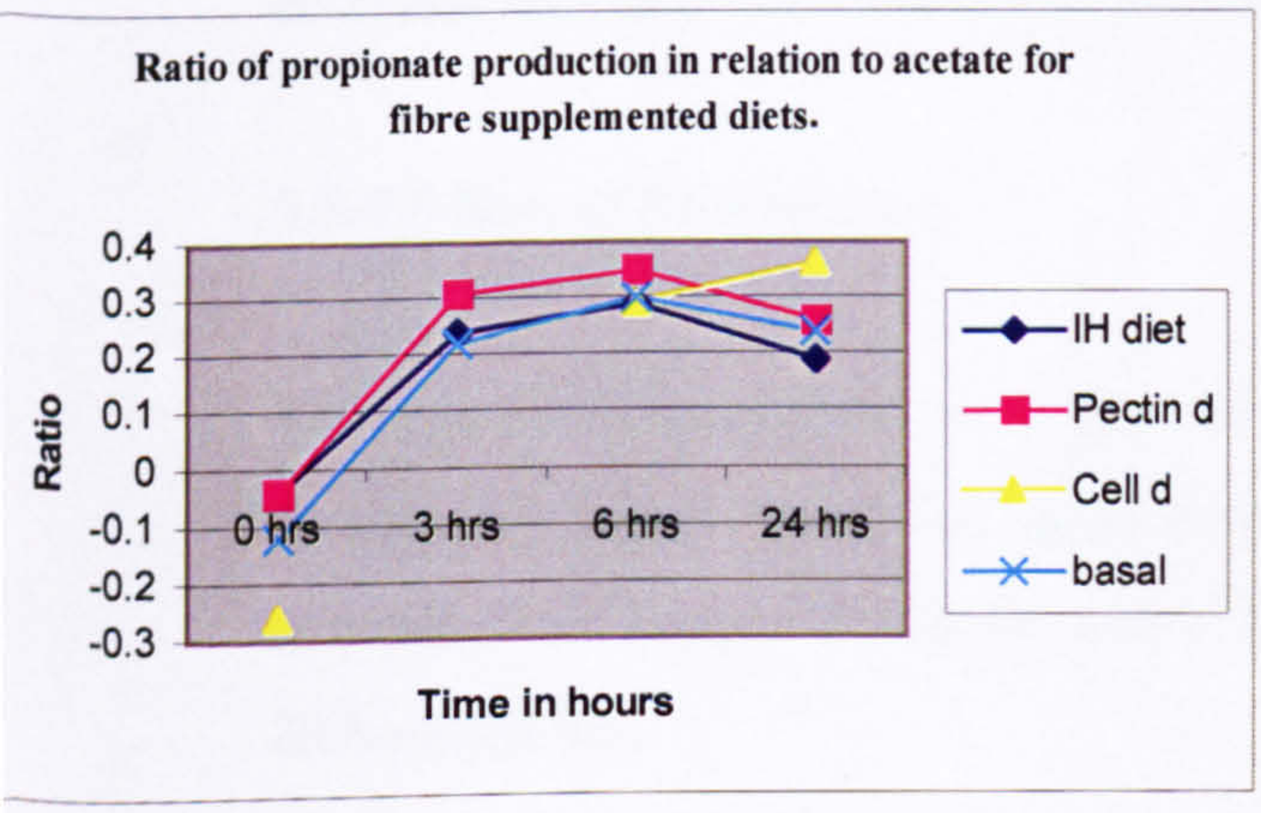
(a) Experiment 1



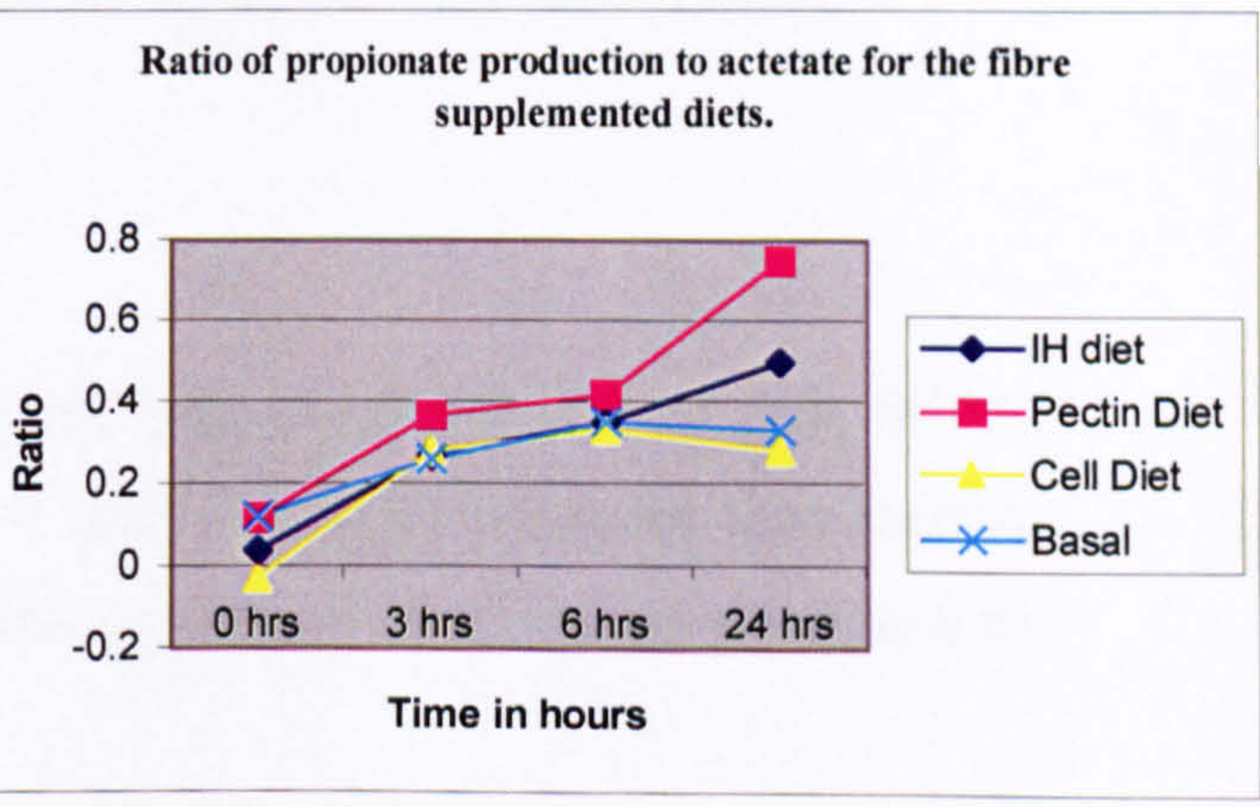
Experiment 2



(b) Experiment 1



Experiment 2



a) Total propionate production (b) Ratio of propionate to acetate production, above control, from the fermentation of 2% (w/v) of the fibre supplemented diets; ispaghula husk (IH), pectin, cellulose (cell) and basal diet; by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the level of butyrate determined using GC analysis. (a) The level of propionate production in all the diets was consistent up to 6 hours in both experiments, with both experiments revealing similar patterns. After 24 hours there were minor differences found between the fibre supplemented diets, in both experiments ispaghula husk and cellulose being the greatest producers. (b) The ratio of propionate production as a proportion of acetate production revealed, in both experiments, to be greatest in the pectin diet up until 6 hours it continued to be the highest in experiment 2, although cellulose was the highest in experiment 1.

3.5.5.5 *Butyrate production*

Figure 3.15 revealed (a) total butyrate production and (b) the ratio of butyrate to acetate production, above control, from the fermentation of 2% (w/v) of the fibre supplemented diets; ispaghula husk, pectin, cellulose and basal diet; by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the level of butyrate determined using GC analysis. Both experiments 1 and 2 (figure 3.15a) revealed a similar pattern for butyrate production, the ispaghula husk supplemented diet producing the highest level of butyrate, followed by the basal diet, cellulose and the pectin producing the lowest amount. The ratio of butyrate production (figure 3.15b) by the fibre supplemented diets showed a slight variation between experiments although both revealed that the ratio of butyrate production decreased over time for all diets. Butyrate production was proportionally the lowest for all fibre supplemented diets after 3,6 and 24 hours, with each diet having a ratio of acetate>propionate>butyrate for these time periods.

3.5.5.6 *Rate of fermentation*

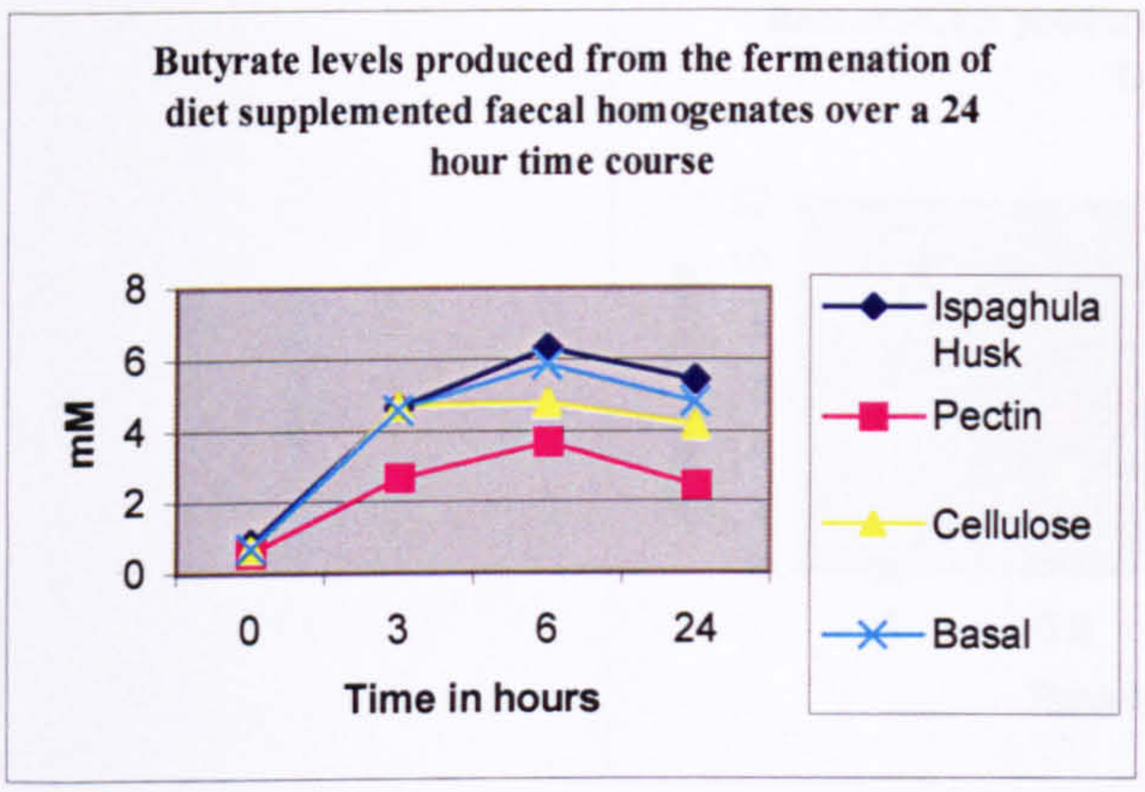
The rate of fermentation, as suggested by the rate calculated for the total SCFA production (figure 3.16) for experiments 1 and 2 revealed different time trends, although both suggested that the rate of SCFA production slows rapidly over the 6 to 24 hour period.

3.5.6 Comparison of fermentation characteristics of the fibres; Ispaghula husk, Pectin, Cellulose, Rice and Resistant Starch and their associated diets.

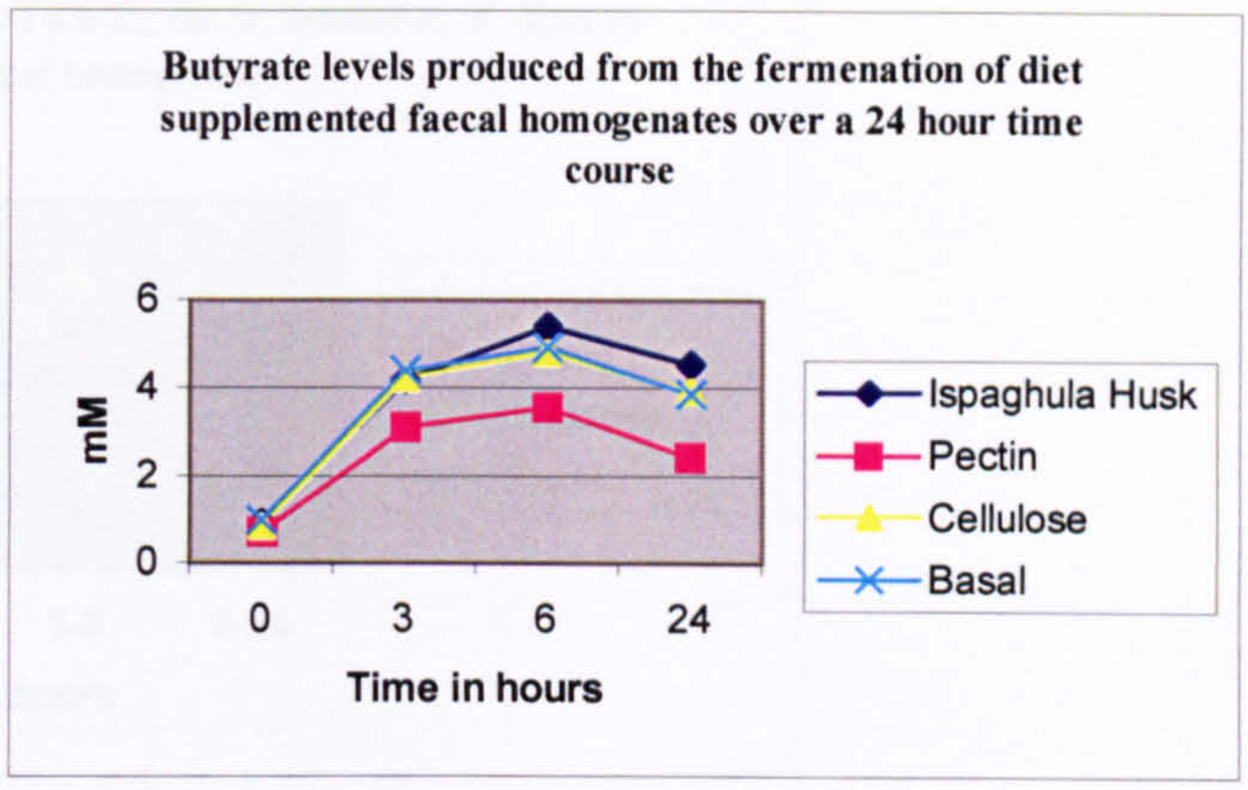
The fibre supplemented diets were fermented to a similar extent as indicated by their total SCFA levels (Figure 3.12) after 6 hours of incubation within the faecal homogenate. This may be due to all the fibre supplemented diets containing the same level of rice starch and sucrose, although the basal diet had higher levels of these to replace the lack of a fibre but this does not appear to effect the rate of SCFA

Figure 3.15a) Total butyrate production (b) Ratio of butyrate:acetate production from the fermentation of fibre supplemented diets.

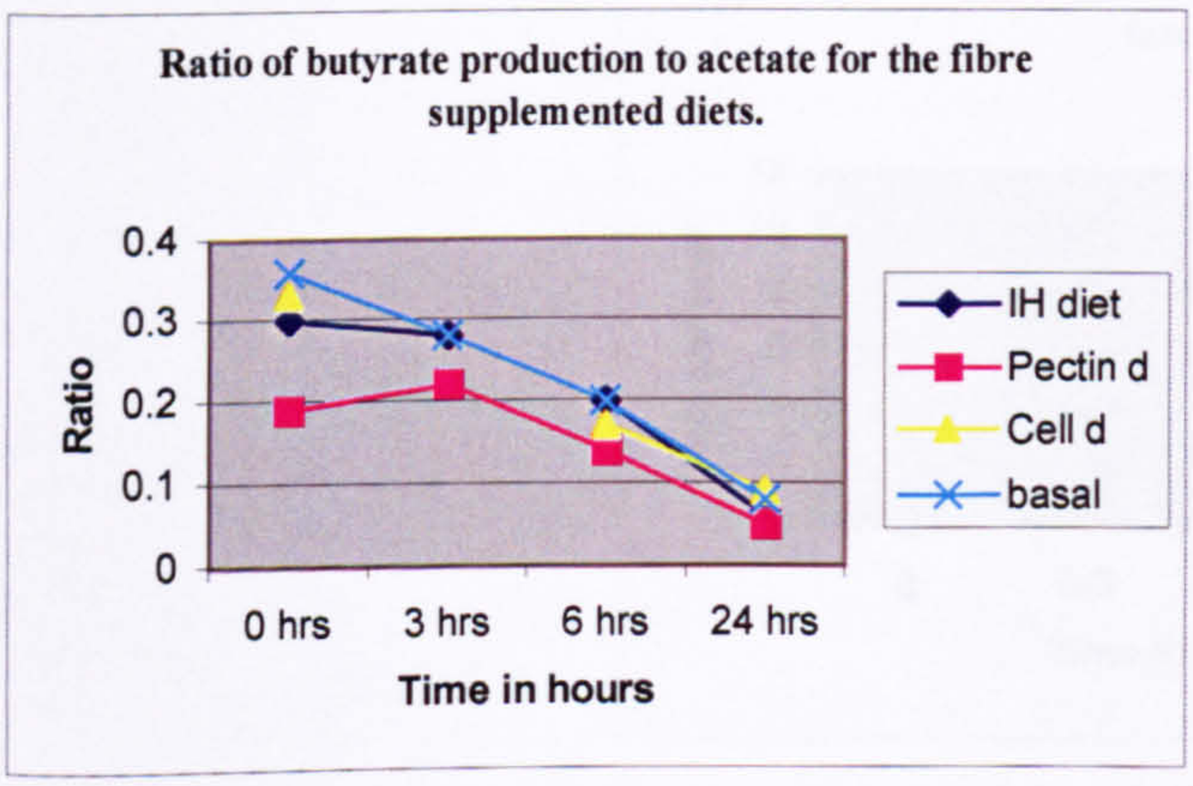
(a) Experiment 1



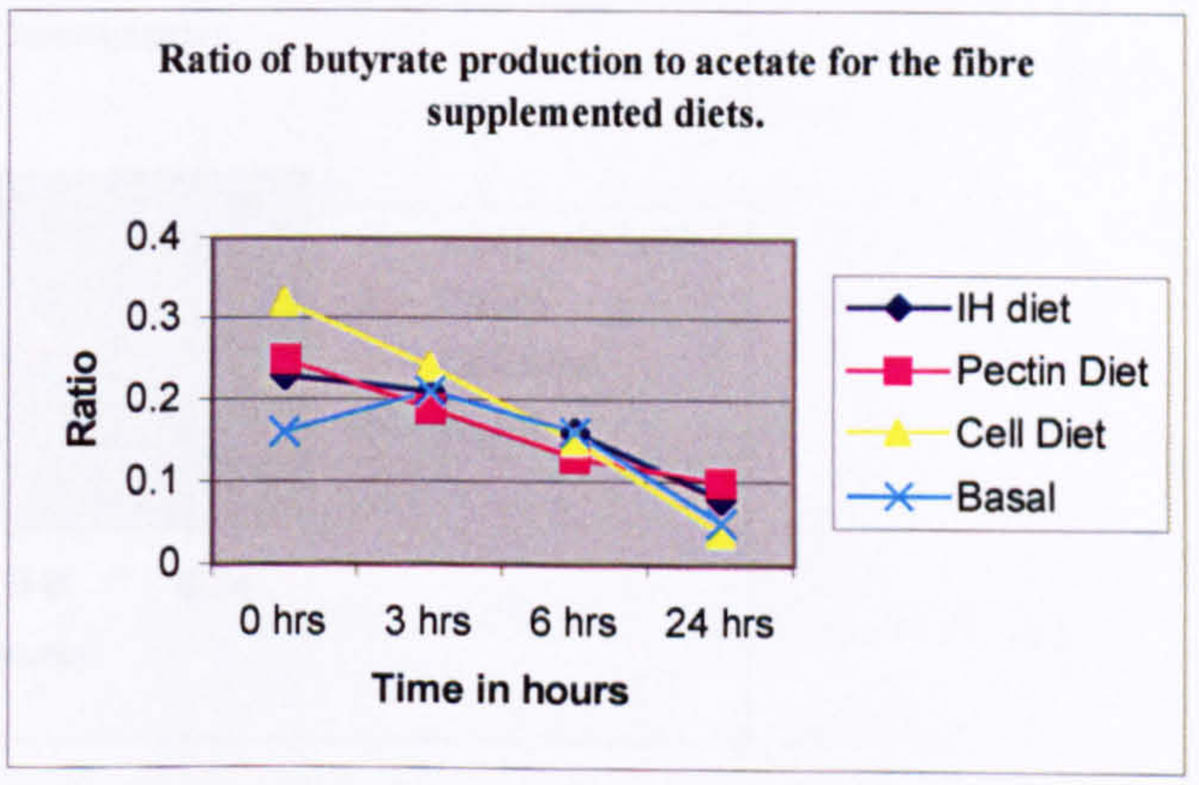
Experiment 2



(b) Experiment 1



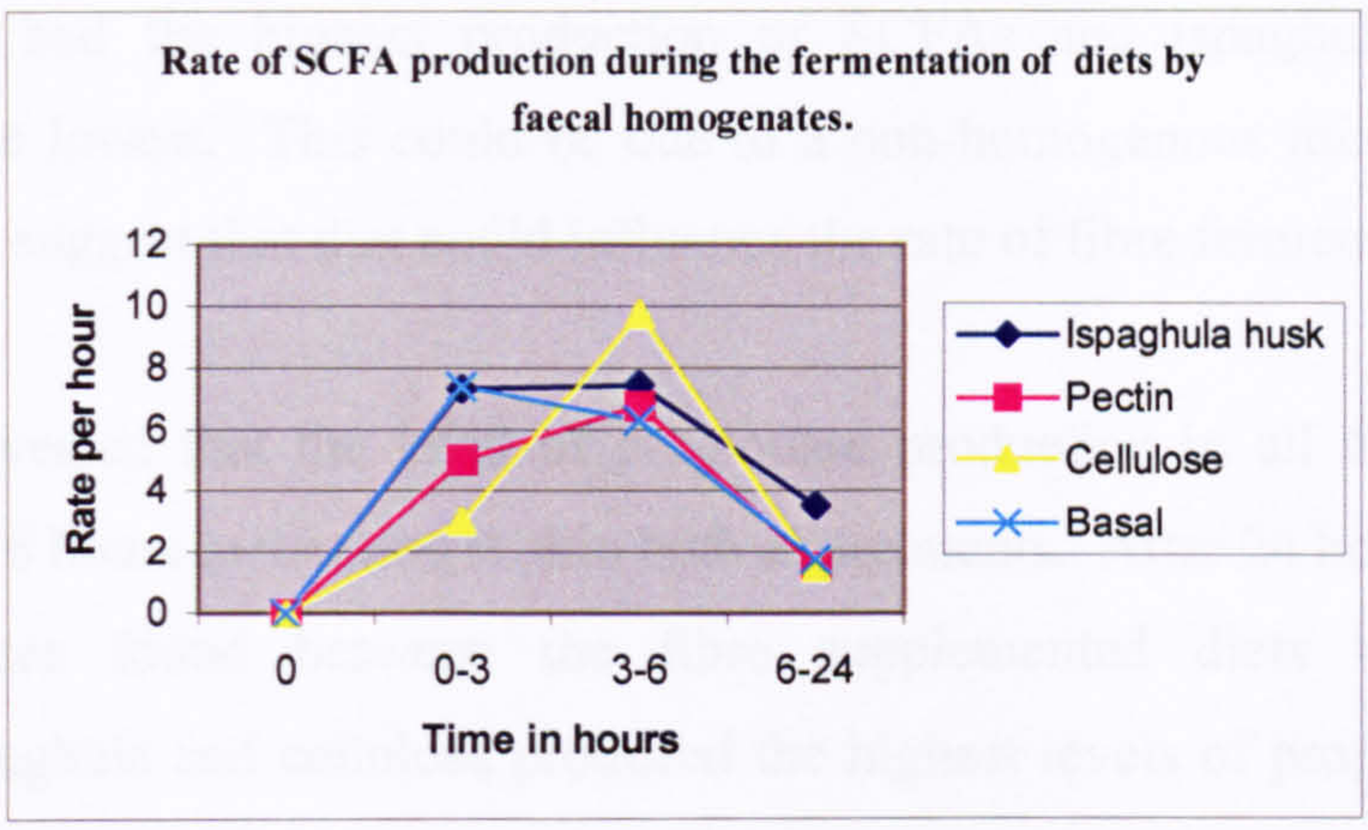
Experiment 2



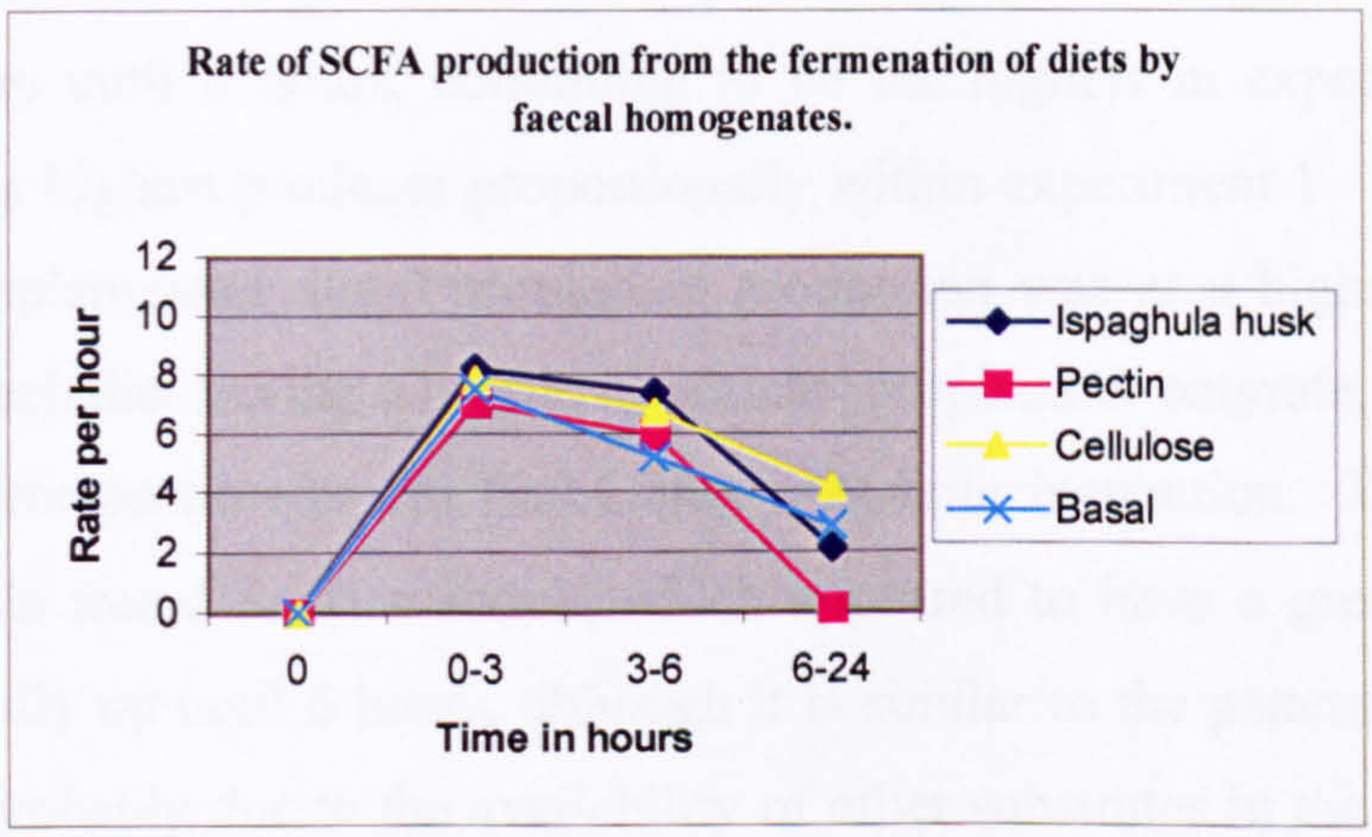
(a) Total butyrate production (b) Ratio of butyrate to acetate production, above control, from the fermentation of 2% (w/v) of the fibre supplemented diets; ispaghula husk (IH), pectin, cellulose (cell) and basal diet by faecal homogenates (n=3). Fibre supplemented faecal homogenates were sampled at 0, 3, 6 and 24 hour time intervals, the level of butyrate determined using GC analysis. (a) Both experiments 1 and 2 show a similar pattern for butyrate production with the ispaghula husk diet producing the highest level of butyrate, followed by the basal diet, cellulose and the pectin producing the lowest amount. (b) The ratio of butyrate production by the diets varied slightly between experiments although both show that the ratio of butyrate production decreases over time for all diets.

Figure 3.16 Rate of SCFA production from the fermentation of fibre supplemented diets within faecal homogenates.

(a) Experiment 1



Experiment 2



Experiments 1 and 2 show different time trends for fermentation initially, although both suggest that the rate of SCFA production slows rapidly over the 6 to 24 hour period.

production. At 24 hours there were differences in the final SCFA levels with both experiments not giving consistent findings. In experiment one the largest SCFA producer was ispaghula husk whereas in the second experiment it fell to cellulose. This is again in contrast to the findings for the fibres where pectin, rice starch and resistant starch had the highest production of SCFAs and ispaghula husk and cellulose had the lowest. This could be due to a non-homogenous mix of the diet, but it could also suggest that diet could influence the rate of fibre fermentation.

Figure 3.14a revealed that the level of propionate production in all the diets was consistent up to 6 hours incubation within both experiments. After 24 hours there are minor differences found between the fibre supplemented diets but in both experiments ispaghula and cellulose produced the highest levels of propionate. The ratio of propionate production as a proportion of acetate production (figure 3.14) revealed that in both experiments the pectin diet to be greatest producer proportionally up until 6 hours, continuing to be the highest in experiment 2 but cellulose was the highest producer proportionally within experiment 1. Interestingly all the fibre supplemented diets' propionate production was at a higher ratio than butyrate, with each diet having a profile of acetate>propionate>butyrate, for example the A:P:B ratio for pectin was 1:0.75:0.1 after a 24-hour incubation. This does not reflect the profile found for rice starch, which appeared to have a greater butyrate production initially up until 6 hours, although it is similar to the pattern found at 24 hours. This is probably due to the availability of other substrates in the diets for the bacteria within the faecal homogenate.

Both experiments 1 and 2 in figure 3.15a show a similar pattern for butyrate production, the ispaghula husk supplemented diet produced the highest level of butyrate, followed by the basal diet, cellulose and the pectin producing the lowest amount. This is in complete contrast to the individual fibres as ispaghula husk was found to be one of the lowest producers with pectin being one of the highest. This could be for a number of reasons, including a non-homogenous mix of the diets being examined, possibly the pectin diet had little pectin, rice starch or sucrose available. It could also suggest that other dietary constituents affect the fermentation of fibres and their SCFA production possibly by selecting for different bacteria. The ratio of butyrate production, as shown in figure 3.15(b), by the fibre supplemented

diets showed a slight variation between experiments although both showed that the ratio of butyrate production decreases over time for all diets. Butyrate production was proportionally the lowest for all fibre supplemented diets after 3,6 and 24 hours, with each diet having a ratio of acetate>propionate>butyrate for these time periods. This was true for all fibres, except resistant starch, which would only be found at trace levels, with rice starch butyrate production falling rapidly at 24 hours, which may reflect why this occurred in all the fibre supplemented diets, with rice starch being one of the main constituents.

The rate of fermentation, as suggested by the rate calculated for the total SCFA production shown in figure 3.16, in experiments 1 and 2 show different time trends, although both suggest that the rate of SCFA production reduced rapidly over the 6 to 24 hour period. Interestingly the rate of SCFA production decreased after 24 hours incubation, this mimics the findings of rice starch, one of the main constituents.

The differences and similarities found between the individual fibres ispaghula husk, pectin, cellulose, rice starch and resistant starch to the fibre supplemented basal diets and the basal diets itself could simply be attributed to a non-homogenous mixture of dietary constituents giving unequal measures of fibre, rice starch and sucrose. Other constituents within the diet may also interfere with the fibres digestion, for example other constituents may be more readily fermented or may actually aid the fermentation of the fibre.

3.6 Discussion

Dietary fibre (Section 1.5) is generally considered to be a complex mix of cellulose, hemicellulose, pectic substances, gums and mucilages, lignins, algal polysaccharides and starch (Reviewed by Dreher, 1995; Reviewed by Lembo, 1998). Dietary fibre is heterogeneous in both structure and function with a wide variety of chemical and physiological properties. The physiological effects of fibres are partly predictable on the basis of their physiochemical properties (Reviewed by Blackwood *et al.*, 2000). These properties are dependent on a number of factors intrinsic to the fibre, the host and other extrinsic factors (refer to section 1.5).

The dietary fibres intrinsic properties were examined, this included their NSP and monosaccharide content, waterholding capacity via solubility (Weisburger *et al*, 1977; Reviewed by Blackwood *et al.*, 2000; Reviewed by Morris, 2001; Reviewed by Kritchevsky, 2001) and those factors intrinsic to the fibre and host via fermentation. The interactions of the fibres with other components of the digesta were examined by profiling the diets using the Englyst technique and a fermentation study being representative of intrinsic factors of the fibre and host.

Fermentation is a very important parameter of dietary fibre characterisation, not only in terms of products produced during fermentation but also the rate and site of fermentation, giving some indication of its degradation during transit through the large intestine. The site, rate and extent to which they are absorbed or fermented in the colon and the type of products produced during fermentation all affect the physiological properties of the fibre (Reviewed by Chaplin, 1999; Reviewed by Blackwood *et al.*, 2000).

Acquisition of the microflora itself influences host biochemistry, such as enzymatic activity of the intestinal contents (e.g. β-D-Glucuronidase, azoreductase, bile salt hydrolyase), SCFA concentrations, oxidation-reduction potential, host physiology (e.g. rate of replacement of enterocytes), immunology (stimulation of reticuloendothelial tissues) and the modification of host-synthesized molecules (e.g. reduction of cholesterol to coprostanol, dehydroxylation of primary bile acids to

form secondary bile acids, mucin degradation) (Reviewed by Tannock, 1994). The colonic microflora also influences colonic mucosal architecture, mucosal cell turnover and intestinal motility, the precise mechanisms involved are unknown but could be due to SCFA production (Reviewed Salyers, 1995).

The ratio of SCFA production is thus very important (Reviewed Eastwood, 1995), with different fibres producing different levels of these SCFAs (Wang and Friedman, 1998; Reckitt and Colman personal communication). A number of structural properties of the fibre; degree of stiffness and regularity of the polysaccharide chains, esterification, side chains and monosaccharide composition all affect the rate and extent of their fermentation (Reviewed by Blackwood *et al.*, 2000), along with a number of intrinsic factors of the host including their type of microflora. Overall soluble fibres are more readily fermented and earlier in the colon, than insoluble fibres (Reviewed by Blackwood *et al.*, 2000). The metabolism of the three main SCFAs: acetate, propionate and butyrate, are very different (Reviewed by Rombeau and Roth, 1995) and have a number of biological effects profoundly altering the milieu of the colon (Refer to section 1.5.4). The physiological effects of SCFA production include the enhancement of sodium absorption, stimulation of blood flow improving peripheral artery dilation (mainly by acetate), and regulation of carbohydrate and lipid metabolism (Reviewed by Rombeau and Roth, 1995) and water retention in faecal matter (Reviewed by Brydon, 1995; Reviewed by Lembo, 1998).

3.6.1 The physiochemical properties of the dietary fibres

3.6.1.1 *Ispaghula husk*

Ispaghula husk is a rich source of non-starch polysaccharides (dietary fibre) comprising 80-85% NSP, as determined by the Englyst method (Reviewed by Dettmar, 1996). Using the Englyst technique our ispaghula husk was shown to have 81.43% NSP content which lies within this range. Ispaghula husk is an arabinoxylan, having a xylose backbone with arabinose side chains (Kennedy *et al.*, 1979; Sandhu *et al.*, 1981) with other sugar side chains associated. Arabinoxylans, such as ispaghula husk, consist of β-xylosyl chains forming a backbone with

arabinose side-chains. This structure results in a relatively stiff molecule with short branch points (Kennedy *et al.*, 1979; Sandhu *et al.*, 1981). Arabinoxylans form extended conformations; exhibit a high viscosity and can vary in flexibility and stiffness, which can also be affected by the level of arabinosylation (Reviewed by Blackwood *et al.*, 2000). Of the 81.43% NSP content for our ispaghula husk, 88.6% was soluble and comprised of xylose, arabinose and uronic acids, 11.4% of which is insoluble made up primarily of glucose and arabinose, which reflect its structure. Our ispaghula husk was shown to be primarily soluble with a solubility ratio of 7.78:1. Observations using the image analyser confirmed the solubility of ispaghula husk, with the soluble fraction being gel-like in nature and attached to an insoluble cytoskeleton. This is consistent with previous findings where isphagula husk was shown to be partially soluble (forming a mucilage/gel) (Kennedy *et al.*, 1979; Sandhu *et al.*, 1981) having a high water holding capacity giving it a viscous nature (Kennedy *et al.*, 1979; Sandhu *et al.*, 1981; Reviewed by Blackwood *et al.*, 2000).

The ispaghula husk used in this study was found to be partially fermented at a fairly consistent rate which steadily increased over a 24 hour period, producing a SCFA profile of acetate>propionate>butyrate (A>P>B), where acetate was the main SCFA produced but it also produced high levels of propionate when compared to the other fibres. McBurney (1991) showed that different fibres produced different levels of SCFAs with psyllium producing all three of the main SCFAs (McBurney, 1991; Reviewed by Brydon, 1995), but was noted for particularly producing propionate (Reviewed by Blackwood *et al.*, 2000). Ispaghula husk has previously been shown to ferment relatively slowly retaining its structure (Marteau *et al.*, 1994) throughout the colon (Reviewed by BeMiller, 1993). A study of the ability of *Bacteriodes* from the human colon in degrading ispaghula husk found that the ability of bacteria to degrade psyllium (Ispaghula husk) was uncommon (Salyers *et al.*, 1978). Observations of ispaghula husk after 24 hours of fermentation in a faecal slurry suggested that ispaghula husk was not only partially fermented but also maintained its viscosity throughout. This compares the findings by others where with psyllium, once formed, the gels are relatively unaffected by temperature and pH maintaining its viscosity even when subjected to artificial gastric and intestinal juices suggesting that it maintains its viscosity within the colon (Reviewed by BeMiller, 1993). Hydrolysis of ispaghula husk within the stomach has been shown to be only partial (1-6%) in

human volunteers (Andersen *et al.*, 1988). This suggests that ispaghula husk is fermented throughout the colon releasing a steady stream of SCFAs throughout the colon. The viscous nature could also result in microflora adhering to the fibre allowing for its fermentation to continue, the viscosity and maintenance of its structure also allows for extra bulking of the faeces (Marteau *et al.*, 1994).

3.6.1.2 *Pectin*

Over half of the pectin used in this study was made up of NSP (60.48%) 99.7% of which was soluble and primarily consisted of uronic acids with low levels of galactose, 0.3%. The insoluble fraction of the pectin comprised of arabinose, rhamanose and mannose. Thus pectin is primarily soluble with a solubility ratio of 344.6:1. This reflects previous findings for pectin, citrus peel, being virtually completely soluble (forms a weak gel), fermented very quickly (completely in the caecum) (Reviewed by May, 1999). Pectin is heterogeneous in nature (Reviewed by Reiser, 1987; Reviewed by May, 1999) but is primarily made up of poly-α-D galactouronic acid (Reviewed by May, 1999; Reviewed by Morris, 2001). The polygalacturonic acid regions are partially esterified with methanol, linked to complex regions based on a galactouronic acid-rhamanose copolymer bearing extensive side chains (Reviewed by Reiser, 1987). The level of esterification of pectin affects the rate of its degradation (Dongowski *et al.*, 2000). These 'hairy regions' have arabinogalactans attached tending to expand the structure and flexibility of pectin (Reviewed by May, 1999; Reviewed by Blackwood *et al.*, 2000). Pectin can also have regions of rhamnogalaturonosyl within its backbone (Reviewed by Morris, 2001), which may explain rhamanous being found within the insoluble fraction.

Pectins are very soluble in water giving a viscous solution and are easily degraded, although not by human gut enzymes (Reviewed by May, 1999), being fermented rapidly in the caecum/colon. Polysaccharide chains are fermented at the terminal region first and carbohydrates containing α-arabinose (pectin/ispaghula husk) and α-galacturonic acid (pectin) residues are generally more susceptible to fermentation. Pectins flexible extended conformations are completely degraded in the large

intestine. Pectin initially has a high water holding capacity (WHC) but because it is completely fermented in the colon it has little effect on transit time (Reviewed by Blackwood *et al.*, 2000). The pectin used in this study was rapidly and fully fermented throughout the 24 hours experimental period slowing at the 24 hour period which could be due to lack of substrate, thus reflecting that seen for a typical pectin. The pectin also produced a SCFA profile of A>P>B, as for all fibres acetate was produced in the largest quantity and this was accompanied by the production of propionate and butyrate at similar levels. This suggests that with pectin being readily fermented by the faecal slurries, that our pectin, as for all other pectins, would be fermented very quickly and within the caecum. This would result in the SCFAs having little effect on the distal colon and possibly minimal effect on the proximal colon and possibly enlarged caecum. Armstrong *et al.* (1990) found that in the rat, pectin increased free water in both the caecal content and faeces (Reviewed by Brydon, 1995). This was also accompanied with the finding that pectin had the highest SCFA production when compared to a number of other fibres including carboxymethylcellulose (CMC) and Bran, when using faecal cultures (Reviewed by Brydon, 1995). Pectin has previously been shown to result in an increase in cell turnover (Reviewed by Cassidy and Fitzpatrick, 1995) this could be due to the high levels of SCFAs being produced due to its fermentation. Pectin is affected by temperature and pH, it is stable under acid conditions but is less stable as pH increases, especially at higher temperatures (Reviewed by May, 1999).

3.6.1.3 *Cellulose*

The purified cellulose used in this study was found to comprise of NSP (108.35%), which compares to findings where cellulose was shown to have a total dietary fibre content of 99% (Reviewed by Dreher, 1995). Cellulose varies with purity depending on its origin, acid hydrolysis found almost 96% glucose (Reviewed by Aspinall *et al.*, 1967). From the purified cellulose 7.8% was found to be soluble and made up of glucose, 92.2% of which is insoluble and made up of glucose, thus cellulose is primarily insoluble with a solubility ratio of 0.09:1. Cellulose is made up of poly-β(1-4)-D-anhydroglucose (pyranose) glycosidically linked forming microfibrils (parallel ribbons), which confers with our findings of it being made up primarily of

insoluble glucose. Being a neutral polysaccharide that tends to self-associate (Reviewed by Morris, 2001) as a result it has a very low solubility (low water holding capacity) (Reviewed by Aspinall *et al.*, 1967; Reviewed by Zecher and Gerrish, 1999). This was shown for our purified cellulose using the Englyst method and image analysis. Image analysis revealed that after one hours incubation of cellulose in water there were no solubilisation of the cellulose fibres, suggesting the cellulose fibres are insoluble.

Cellulose is only partially digested by the human body (Reviewed by Zecher and Gerrish, 1999) and is fermented very slowly throughout the colon. The purified cellulose in this study was virtually unfermented and the rate of fermentation was very slow during the 24 hour fermentation period. Cellulose produced very low levels of SCFAs with a SCFA profile of A>P>B, thus in this case producing proportionally more propionate than butyrate. Previously cellulose was shown to ferment under anaerobic conditions by bacteria to produce methane and fatty acids (Reviewed by Aspinall *et al.*, 1967). Purified cellulose has been shown to be virtually indigestible, although cellulose within whole foods is digested more readily within the gastrointestinal tract (Reviewed by Wolever 1995). The ordered packing of cellulose confers resistance to enzymatic attack thus limiting degredation/fermentation via colonic microflora (Reviewed by Aspinall *et al.*, 1967; Reviewed by Alberts *et al.*, 1994; Reviewed by Zecher and Gerrish, 1999; Reviewed by Morris, 2001). Thus the level of SCFAs produced would be minimal, although would probably be consistently produced throughout the proximal and distal colon. The lack of SCFAs may explain why rats fed on cellulose or fibre deficient diet had evidence of little villus maturation, i.e. no ridged morphology, with cellulose found to have a lower cell turnover and migration up the crypt (Reviewed by Cassidy and Fitzpatrick, 1995). The angles within these pyranose rings affect the fibres conformation, which in turn affects fermentation (Reviewed by Morris, 2001). The differences in the fermentation of cellulose poly-β-D-Glucose and resistant starch poly-α-D-Glucose is its conformation, i.e. axial versus equatorial, and due to its packing, for example cellulose is more resistant to enzymatic attack resulting in limited degradation during fermentation because of its ordered packing (Reviewed by Morris, 2001). Cellulose has a low WHC initially, but because it is only partially

fermented, it has been shown to increase stool weight, decreasing transit time, by its ability to retain water. Cellulose has previously been shown to produce proportionally higher levels of butyrate (Reviewed by Blackwood *et al.*, 2000), which have a number of potential health benefits but this was not shown in this study.

3.6.1.4 *Starch*

Starch is the reserve food material of most plants and is one of the principal components of the diet (Reviewed by Aspinall *et al.*, 1967). Starch is made up of poly-α-(1-4)-D-Glucose (or α-(1-3)-D-Glucose the branches in amylopectin) (pyranose rings), which forms non-parallel buckled ribbons forming coaxial double helices (Reviewed by Aspinall *et al.*, 1967; Reviewed by Morris, 2001). It is a neutral polysaccharide that tends to self-associate (Reviewed by Morris, 2001) making it insoluble. Starch is made up of glucose either amylose or amylopectin in origin, and proportions of these may vary (Reviewed by Aspinall *et al.*, 1967). Starch granules contain densely packed assemblies of ordered (double helical) amylopectin, together with disordered amylose (Reviewed by Morris, 2001). Starch in the form of amylose, a storage polysaccharide, tends to adopt a wide helical conformation (Reviewed by Blackwood *et al.*, 2000). Our resistant starch was of amylose origin and shown to have a very low level (0.715%) of NSP as expected, 63.5% of which is soluble glucose, 36.5% of which is insoluble glucose. This glucose could be residual resistant starch after incomplete degradation during the Englyst procedure. Thus resistant starch residue was primarily soluble with a solubility ratio of 1.75:1.

The degradation of starch within the gastrointestinal tract depends on its chain conformation and packing which is dependent on any pre-processing that it may have been subjected too (Reviewed by Morris, 2001). Amylose is more readily digested (Reviewed by Aspinall *et al.*, 1967). Starch can be categorised into three main categories; the first is the rapidly digested starch, which is digested in the small intestine, the second group is the starch, which is digested slowly in the small intestine, the third group are the resistant starches. Our resistant starch, which was

composed of amylose granules (thus probably RS2 type), was rapidly fermented with fermentation slowing at 24 hours and producing a ratio of SCFA production of A>B>P, thus resistant starch was shown to be a large producer of butyrate. Rice starch (the main constituent of the basal diet) was also rapidly fermented with fermentation slowing at 24 hours. Initially the rice starch was shown to produce high levels of butyrate giving a ratio of A>B>P, but after 24 hours butyrate production dropped giving a ratio of A>P>B, although proportionally a high producer of butyrate compared to ispaghula husk. The majority of starch reaching the colon is fermented in the colon, the extent varying on the fermentative capacity of the colonic microflora (Reviewed by Jenkins and Kendall, 2000), as for the other fibres. Prolonged feeding of rats with potato starch has been shown to result in an increase in butyrate within the caecum and towards the distal colon, and levels increased over time revealing an adaptive process of SCFA production by the colonic mucosa (Le Blay *et al.*, 1999).

3.6.2 The health benefits of dietary fibres and their use in disease states.

Dietary fibre is thought to have a wide range of properties that influence a number of prevailing western diseases. Diet gives clues to the aetiology of many of the life style diseases, such as colon cancer and heart disease (Reviewed by Kritchevsky, 2001). Metabolic affects of fibres include reducing obesity (bringing about quicker satiety and changing nutrient absorption), gallstones (through reducing cholesterol saturation and possibly affecting the bile acid pool), diabetes (work carried out by Jenkins and Anderson found fibres to help alleviate symptoms by normalising glucose tolerance and plasma insulin levels), coronary heart disease and colon cancer (Reviewed by Kritchevsky, 2001).

3.6.2.1 *Weight management.*

Dietary fibre has a number of benefits to health including weight management (Reviewed by Stephen, 1995). Dietary fibre helps bring about quicker satiety, delayed gastric emptying, changing nutrient absorption (Harmuth-Hoene and Schelenz, 1980), gastrointestinal hormones, ileal break and caloric excretion (Reviewed by Stephen, 1995). Fibres, especially bulking fibres such as ispaghula

husk, help with weight management due to a quicker satiety being reached along with the fact that they are low calorie foods. Determinants of obesity include calorie intake and calorie expenditure (including physical activity) with energy balance being relevant to a number of disease processes including colorectal cancer. High intake of calorific foods (inc starches and refined cereals) may favour hyperinsulinemia and insulin resistance, with a possible increase in activity of IGF-1 (Reviewed by Gerber and Corpet, 1999). Thus weight management will also indirectly counteract a number of other conditions.

3.6.2.2 *Cardiovascular Disease - Cholesterol metabolism.*

It is now established that insoluble fibres, such as cellulose, do not influence serum lipids, whereas soluble fibres, pectin and psyllium (ispaghula husk), exert a hypocholesterolaemic effect (Reviewed by Reiser, 1987; Reviewed by Anderson, 1995; Gallaher and Hassel, 1995; Reviewed by Graham, 1996; Reviewed by Kritchevsky, 2001). Bile is produced in the liver, stored by the gallbladder, secreted into the small intestine via bile ducts when needed for digestion (Reviewed by Miller and Keane, 1987). Bile helps in alkalinising the intestinal contents and plays a role in the emulsification, absorption, and digestion of fat. The chief constituents are conjugated bile salts, cholesterol, phospholipid, bilirubin, and electrolytes (Reviewed by Miller and Keane, 1987). Bile acids are steroid acids derived from cholesterol; they are classified as primary, those synthesised by the liver, e.g. cholic and chenodeoxycholic acid, or secondary, those produced from primary bile acids by intestinal bacteria and returned to the liver by the enterohepatic circulation, e.g. deoxycholic and lithocholic acid (Reviewed by Miller and Keane, 1987). These bile acids affect a number of other disease processes including gallstones and colorectal cancer development.

Low glycaemic foods are predictors of cardiovascular disease, two studies have shown that the lower the glycaemic index of the food the higher the high density lipoprotein (HDL) (transports cholesterol out of the body) cholesterol suggesting they are protective (Reviewed by Wolever, 1995; Reviewed by Jenkins and Kendall, 2000). The mechanisms by which soluble fibres exert their effects is due to the increased viscosity of both stomach and small bowel contents thus reducing

absorption of cholesterol and possibly by influencing bile acid metabolism (Reviewed by Reiser, 1987; Gallaher and Hassel, 1995; Reviewed by Kritchevsky, 2001). The data suggests that soluble fibres may lower serum lipid levels, but a high-fibre diet itself may afford protection against cardiovascular heart disease (Reviewed by Kritchevsky, 2001). There appears to be a positive association between slow releasing carbohydrates, reduced insulin levels, higher HDL cholesterol concentrations, and reduced diabetes and cardiovascular disease risks (Reviewed by Jenkins and Kendall, 2000) and resistant starch has been shown to achieve this. Fibres have been shown to bind cholesterol and bile acids with differing affinities, for example cellulose has been shown to be a poor binder of bile acids, whereas ispaghula husk has been shown to bind certain bile acids (Reviewed by Kritchevsky, 1995; Reviewed by Blackwood *et al.*, 2000) and increase the level of lipid output within faeces, when compared to wheat bran (Nyman and Asp, 1985). Altogether fibres reduce the appearance of cholesterol in the blood and tissues resulting in the increased excretion of neutral and acidic steroids (Reviewed by Kritchevsky, 1995). The conversion of cholesterol to bile acids occurs when hydroxylation is at the 7-α position, which is interrelated to the activity of HMG-CoA reductase, a key step in cholesterol biosynthesis (Reviewed by Kritchevsky, 1995). Fibres have been shown to affect the activity of these enzymes, in a rat experimental model cellulose resulted in elevation of 7α-hydroxylation and the reduction of HMG-CoA (3-hydroxy-3methylglutaryl coenzyme A) reductase activity (Reviewed by Kritchevsky, 1995). There is an important physiological relationship between dietary fibre, daily bile acid excretion, faecal bile acid concentrations and cardiovascular disease and colon cancer (Reviewed by Story, 1995). Some dietary fibres lower cholesterol levels and result in an increase in bile acid excretion but not all do both like ispaghula husk (Reviewed Story, 1995). Ispaghula husk has been proposed as having potentially wide use as a prophylactic in the treatment of coronary heart disease, with oral intake of ispaghula husk resulting in the lowering of total serum cholesterol and serum low density lipoproteins (LDL) in humans (Reviewed by Dettmar, 1996). Pectin's ability to lower cholesterol is thought to occur by a number of mechanisms including viscosity (Gallaher and Hassel, 1995) decreasing cholesterol absorption, its ability to increase HDL levels accompanied by a decrease in LDL levels and propionate production (Reviewed by Kritchevsky,

2001). The production of SCFAs (particularly propionate and butyrate) may also influence hepatic cholesterol synthesis (Reviewed Behall, 1997) and bile acid metabolism (Reviewed by Ink and Hurt, 1987; Reviewed by Kritchevsky, 2001). Pectin binds strongly to a number of cations due to its negative charge using electrostatic forces and also to a number of other particles non-specifically including bile acids and proteins (Reviewed by Eastwood, 1995). Thus pectin exerts beneficial effects on cholesterol metabolism and lipoprotein levels (Reviewed by May, 1999). All viscous polysaccharides have been shown to decrease small bowel transit and thus slow down nutrient absorption (Reviewed by Blackwood *et al.*, 2000), including ispaghula husk. Thus a number of dietary fibres may help in the prevention of cardiovascular disease. Insulin appears to also affect cholesterol metabolism, Ludwig (1999) study (Reviewed by Kritchevsky, 2001) concluded that high fibre diets might protect against obesity and cardiovascular disease by lowering insulin levels (Reviewed by Jenkins and Kendall, 2000; Reviewed by Kritchevsky, 2001). Other diet constituents, such as Vitamin E, are inversely related to the risk of death from coronary heart disease (Reviewed by Rodgers, 1997).

3.6.2.3 *Diabetes - Glucose metabolism and Insulin response.*

Insulin is a major fuel regulating hormone, secreted into the blood in response to a rise in concentration of blood glucose and amino acids. Insulin is secreted not only in the presence of glucose and amino acids but also in response to the gut hormones secretin, pancreozymin and gastrin, to help maintain glucose levels. Insulin's main function is through the regulation of glycaemic control; regulating postpandrial glucose levels possibly by delayed gastric emptying and delayed glucose absorption (Reviewed by Miller and Keane, 1987).

Low glycaemic foods have been shown to reduce the risk of type 2 diabetes (Reviewed by Jenkins and Kendall, 2000). Type 2 (95%) or non-insulin-dependent diabetes mellitus (NIDDM) is the result of inappropriate insulin secretion, such as a delayed response to glucose load or an insufficient quantity (Reviewed by Miller and Keane, 1987). There is a strong association between obesity and NIDDM, 80% NIDDM are obese leading a sedentary (lack of physical exercise) and high calorie life (Reviewed by Miller and Keane, 1987). Increasing physical exercise activity can

help normalise blood glucose and lipid levels, possibly a mechanism by which physical exercise protects against colorectal cancer as type 2 diabetes increases the risk of developing colorectal cancer (Reviewed by Bruce *et al.*, 2000). Diet is a part of the control of diabetes and intake is adjusted to make sure blood glucose and serum cholesterol are kept within acceptable limits. The introduction of certain carbohydrates to maintain blood glucose response, delaying postprandial rise in glucose, with dietary fibre reducing carbohydrate absorption and slowing gastric emptying (Reviewed by Reiser, 1987; Reviewed by Spiller, 1999). High fibre foods are thought to influence glucose levels in two ways; firstly some fibres result in a reduction in the rate of absorption, which in turn affects systemic metabolism; secondly also provides a carbohydrate source for fermentation by intestinal bacteria (Reviewed by Reiser, 1987; Reviewed by Wolever 1995). The viscous polysaccharide pectin has been shown to reduce glucose absorption helping in the management of type II diabetes mellitus (DM) (Reviewed by Reiser, 1987; Reviewed by Wolever 1995). The reduction of blood glucose and lipid control in diabetics seem to depend on whether a fibre is viscous (Reviewed by Wolever 1995; Reviewed by Spiller, 1999), not only in water but also during transit through the gastrointestinal tract. There are fibres that maintain their viscosity throughout the gastrointestinal tract including ispaghula husk (Reviewed by Wolever 1995). High viscosity associated with soluble fibres is often given as the cause of their effect on glucose and lipid metabolism (Reviewed by Blackwood *et al.*, 2000). A stronger link with low glycaemic index foods and a reduction of blood glucose and lipids has been found (Reviewed by Wolever 1995). Soluble fibres such as ispaghula and pectin increase the viscosity of the intestinal contents, reducing the rate of absorption of bile acids, glucose and nutrients (Reviewed by Jenkins *et al*, 1995) thus allowing their absorption along a greater length of the small intestine (Reviewed by Blackwood *et al.*, 2000). Fibre supplemented diets have also been shown to result in a reduction in insulin levels within rats (Ghatei *et al.*, 1997). Insulin resistance has also been associated with increased risk of type 2 diabetes and colon cancer, with it providing a link between diet and colon cancer risk (Reviewed by Bruce *et al.*, 2000).

Ispaghula husk has potential as a prophylactic in the treatment of type II diabetes by reducing the rate of glucose uptake (Reviewed by Dettmar, 1996). Pectin, a low

glycaemic index food reduces the risk of developing diabetes by decreasing postprandial insulin levels. In the control of diabetes it has been suggested by Jenkins that gelling agents such as pectin form a matrix trapping the glucose preventing them from being absorbed in the small bowel (Reviewed by Kritchevsky, 2001). There also appears to be a positive association between slow releasing carbohydrates, reduced insulin levels, higher HDL cholesterol concentrations, and reduced diabetes (Type II) and cardiovascular disease risks. Physiological effects of resistant starch include their effects on postprandial glycaemic index in relation to diabetes (Reviewed by Jenkins and Kendall, 2000).

3.6.2.4 *Large Bowel Disorders.*

Burkitt was the first to propose the link between fibre intake and susceptibility to cancer of the colon and rectum (Reviewed by Kritchevsky, 2001). Vegetables have the highest fibre content per Kcal and in rural Uganda, where the fibre hypothesis was first developed by Burkitt and Trowell, vegetables contribute over 90% of fibre intake (Reviewed Wolever and Jenkins, 1997). A diet rich in plant foods contains a number of possible anti-carcinogenic phytochemicals which may augment fibre action, exert a specific action of their own, or even antagonize the action of other food components (Reviewed by Kritchevsky, 2001). Colon cancer is a disease of excess, over nutrition, which is a symptom of westernisation/industrialisation, resulting in an increase intake of refined carbohydrates but also fast foods, this accompanied by a decline in the level of physical exercise has attributed to an increase in obesity (loss of energy balance) and thus as a possible consequence colonic cancer development. The link between weight and cancer development has been shown through a number of studies where a clear association between obesity and an excess risk of cancer development was found (Reviewed by Hill, 1999a). Obesity (energy imbalance) is positively associated with colorectal adenomas and cancer risk, with several determinants of obesity being related to colorectal cancer risk (Reviewed by Stephen, 1995; Reviewed by Gerber and Corpet, 1999). Structural features of dietary fibre may influence binding of bile acids and inorganic ions and potential carcinogens (Reviewed by Blackwood *et al.*, 2000; Reviewed by Kritchevsky, 2001). Bulking may increase the excretion of those substances trapped in the faecal mass (Reviewed by Kritchevsky, 2001). Water immobilisation by fibres

also affects their properties; viscous polysaccharides have been shown to slow gastric emptying and retard the absorption in the jejunum and ileum (Reviewed Eastwood, 1995; Reviewed by Blackwood *et al.*, 2000; Reviewed by Kritchevsky, 2001), influencing enzyme activity, facilitates fermentation and influences stool weight. This will also affect intestinal and colonic muscle activity, metabolic turnover rate of absorbed nutrients and endocrine hormone activity (Reviewed Eastwood, 1995). Determinants of obesity include calorie intake and calorie expenditure (including physical activity) with the proposal that an energy balance is more relevant to colon carcinogenesis than calorie intake itself (Reviewed by Gerber and Corpet, 1999). Type II diabetes and cardiovascular disease in turn also affects colorectal cancer risk (Reviewed by Jenkins and Kendall, 2000). Low glycaemic foods, such as certain fibres, have been shown to reduce the risk of type 2 diabetes. Type 2 diabetes has in turn been related to a higher risk of colon cancer especially colon cancer deaths. Refer to section 1.4 for more detailed information on the role of fibre in colorectal cancer development.

The majority of diseases that occur in the human large intestine are of unknown aetiology, but diet, especially dietary fibre has been implicated in having a role. The mechanical action of dietary fibre includes influencing stool weight resulting in the relief of constipation and diverticular disease (Reviewed by Cummings 1994; Reviewed by Kritchevsky, 2001). Painter and Burkitt proposed that an absence of dietary fibre was actually a cause of diverticulosis and considerable supporting data has accumulated suggesting fibre therapy improved symptoms (Reviewed by Lembo, 1998). Mechanical action of dietary fibre by influencing stool weight results in the relief of constipation and diverticular disease, the effects on diverticular disease shown by Painter (Reviewed by Dwyer *et al.*, 1978; Reviewed by Cummings, 1994; Reviewed by Kritchevsky, 2001). Constipation is an over working of faecal matter resulting in decreased water content and hardening of stool, common causes include a lack of fluids, lack of physical activity and disease processes. Mild constipation can be easily relieved with fibre (Reviewed by Lembo, 1998). Dietary fibre relieves constipation by softening stools, regulating and shortening transit time, increasing bulk and water holding capacity; increased propulsion resulting in removal of the blockage. Diarrhoea is the formation of watery stools usually as a result of an Na/K exchange imbalance or infection. Dietary fibre helps absorb excess water and

electrolytes, increases bacterial mass re-establishing the microflora, provides a structure (especially those not fermented, generally the insoluble fibres) and the production of SCFAs through fermentation also help regulate water and electrolyte absorption (Reviewed by Lembo, 1998). Butyrate intracolonic infusions have been shown to have beneficial effects on diversion colitis enhancing morphology and healing, with similar effects being shown in distal ulcerative colitis (Reviewed by Rombeau and Roth, 1995). Diabetes Mellitus itself has been shown to affect the peripheral nerves of the lower extremities and autonomic nervous system resulting in delayed gastric emptying, diarrhoea and constipation (Reviewed by Miller and Keane, 1987).

The majority of diseases that occur in the human large intestine are of unknown aetiology, but bacteria have been implicated either as causative agents or maintenance factors involved in many colonic disorders. Antibiotic associated colitis, inflammatory bowel disease (IBD) and colon cancer are all been thought to have an aetiology connected with the activities of the gut flora (Reviewed by Gibson and Macfarlane, 1994). Studies within patients with diarrhoea prominent irritable bowel syndrome have shown that the colonic bacterial flora produce different SCFA patterns (Treem *et al.*, 1996). Epidemiological studies have also found an association with colorectal cancer with lower faecal pH being associated with reduced risk (Reviewed by Lupton, 1995). Bacteria can also have a beneficial role with probiotics being shown to be an effective treatment of ulcerative colitis via SCFA production particularly butyrate (Reviewed by Gibson and Macfarlane, 1994). Both food and insulin have been shown to stimulate ornithine decarboxylase (ODC) intestinal activity, which is one of the earliest biochemical parameters associated with cellular turnover and development (Reviewed by Cassidy and Fitzpatrick, 1995). Diet has been shown to influence colonic morphology in rats, resulting in a physiological adaptation of the colonic epithelium (Reviewed by Cassidy and Fitzpatrick, 1995; Reviewed by Behall, 1997).

Ispaghula husk has traditionally been used in India for the treatment of chronic constipation, dysentery, and inflammation in the gastrointestinal and urinogenital tracts. Ispaghula husk has many therapeutic actions and is a commonly used prophylatic in a number of large bowel disorders (Kennedy *et al.*, 1979; Sandhu *et*

al., 1981; Reviewed by Dettmar, 1996). Ispaghula husk is an effective stool bulking treatment for constipation and diaohrrea (Fybogel® and Metamucil®) (Reviewed by BeMiller, 1993; Reviewed by Dettmar, 1996; Reviewed by Blackwood *et al.*, 2000) and in the treatment of diverticular disease (Kennedy *et al.*, 1979; Sandhu *et al.*, 1981; Thorburn *et al.*, 1992). Davies *et al.*, 1998 showed that ispaghula husk treatment helped relieve premenstrual constipation type symptoms. This is due to its water holding capacity and subsequent viscous nature providing a laxative effect (Sandhu *et al.*, 1981; Reviewed by Blackwood *et al.*, 2000). Psyllium gel can inhibit pancreatic amylase activity; with no effect on itself and it may also inhibit lipase in duodenal juice, but not phospholipase. It can also bind ferrous ions, thus lowering iron absorption (Reviewed by BeMiller, 1993) and can bind bile acids resulting in an increase in the excretion of bile salts (Reviewed by BeMiller, 1993; Reviewed by Blackwood *et al.*, 2000; Reviewed by Kritchevsky, 2001). The increased viscosity of both stomach and small bowel contents may also influence bile acid metabolism (Reviewed by Kritchevsky, 2001). Ispaghula is an effective treatment in diverticulitis disease (Sandhu *et al.*, 1981); combined with mebevrin (an antispasmodic) it is an effective treatment of irritable bowel syndrome (M.Havler, ReckittBenckiser, personal communication; Reviewed by Dettmar, 1996). The dietary fibre ispaghula husk has also been implicated in relieving IBS symptoms, although maybe due to a placebo effect (Longstreth *et al.*, 1981). It has been proposed as a possible prebiotic, protecting against the development of colorectal cancer although ispaghula husk has been shown to increase the number of DMH tumours, but only in male mice (Toth, 1984). Fybogel® comes in a variety of types, the most promising being Fybogel® lemon, as it provides both a fibre but also contain curcumin. Ispaghula may also cause epithelial cell loss and muscle hypertrophy in the jejunum and ileum and thinning the colonic wall after prolonged feeding (Reviewed by BeMiller, 1993). Ispaghula has also been shown to affect the enzyme activity of β-galactosidase.

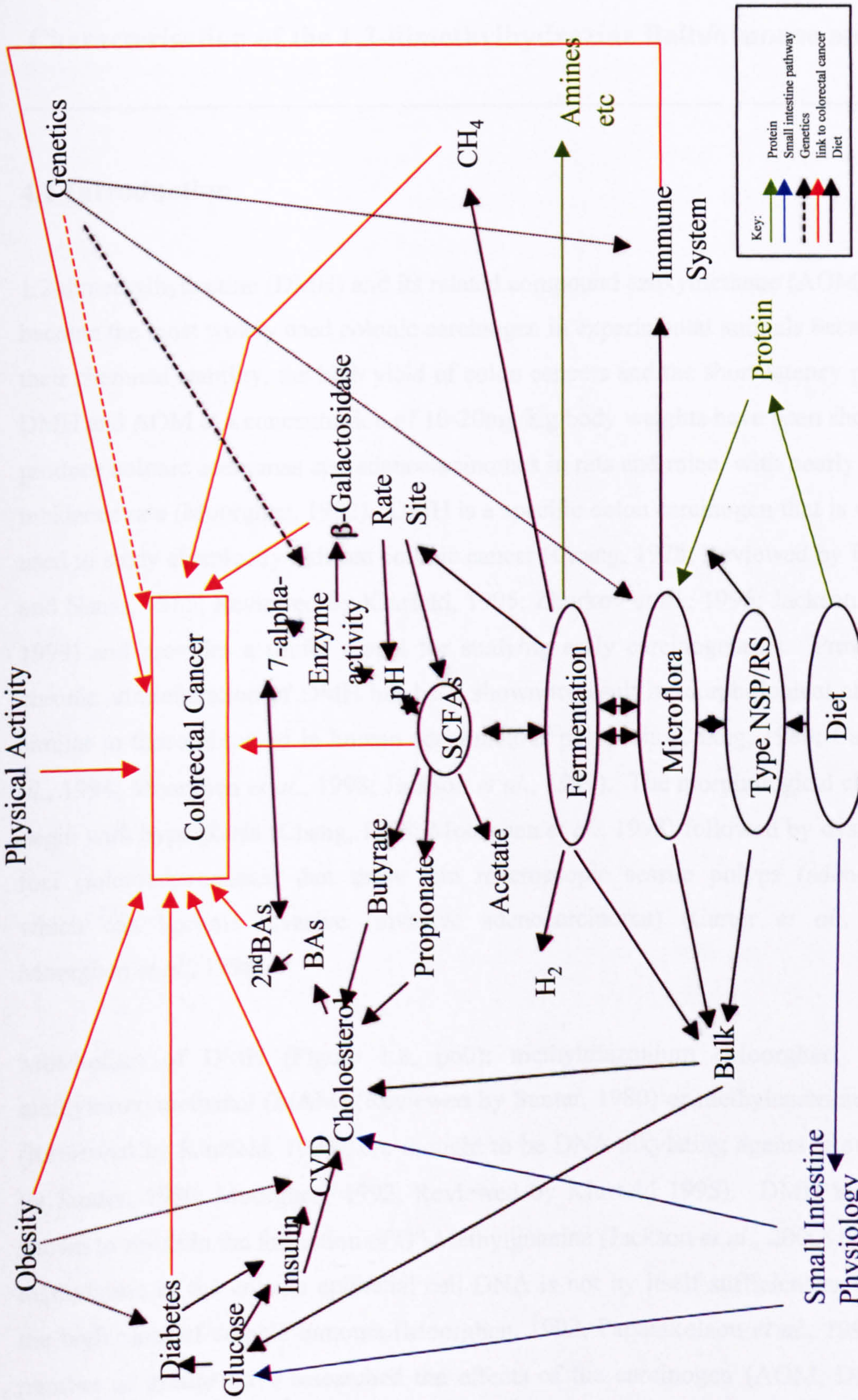
Pectin's form zig-zag chains bearing a negative charge and are found in association with cations such as Ca^{2+} (Reviewed by May, 1999; Reviewed by Morris, 2001) with some pectins being calcium sensitive (Reviewed by May, 1999). Ca^{2+} has been proposed to have beneficial effects on the development of colorectal cancer. Pectins

negative charge means it is able to complex with toxic heavy metals and has been used to promote wound healing (Reviewed by May, 1999). Cellulose has previously been shown to result in damage to colonic folds, as well as a lack of maturation of the colonic mucosa, and a corresponding decrease in proliferation and increase in cell turnover time (hypoplastic state). Fibre deficient diets have also been shown to result in a state of hypoplasia in the colon but a possible hyperplastic state in the small intestine. It has been proposed that cellulose results in a decrease in cell turnover, whereas pectin has previously been shown to result in an increase in cell turnover, thus in fruit and vegetables a balance may be achieved with them containing both cellulose and pectin (Reviewed by Cassidy and Fitzpatrick, 1995).

Large amounts of resistant starch in the diet may also impair the colonic digestion of NSP (Reviewed by Hill, 1997b). Fermentation of resistant starch not only produces more butyric acid but also lowers faecal pH and increases faecal bulk (Reviewed by Jenkins and Kendall, 2000; Ranhotra 2001), although is less effective at stool bulking than NSP (Reviewed by Topping and Clifton, 2001). Resistant starch has been shown to increase stool bulk, to increase the molar ratio of butyrate and dilute faecal bile acids (Reviewed by Jenkins and Kendall, 2000). High levels of resistant starch has a positive association with faecal NH_3 concentrations along with reduced bile acid output and a decrease in bile acid concentration (Reviewed by Jenkins and Kendall, 2000). A number of studies have reported no physiological benefits and a mouse study using APC 1638N mice found resistant starch resulted in an increase in tumours (Reviewed by Jenkins and Kendall, 2000).

Figure 3.17 provides a schematic representation of the role of diet, particularly dietary fibre in health and disease, referred to in the above literature.

Figure 3.17 A proposed model for the interactions of diet within the lower Gastrointestinal tract in health and disease



Chapter Four

Characterisation of the 1,2-dimethylhydrazine Balb/c mouse model.

4.1 Introduction.

1,2-dimethylhydrazine (DMH) and its related compound azoxymethane (AOM) have become the most widely used colonic carcinogen in experimental animals because of their chemical stability, the high yield of colon cancers and the short latency period. DMH and AOM at a concentration of 10-20mg/Kg body weights have been shown to produce colonic adenomas and adenocarcinomas in rats and mice, with nearly 100% incidence rate (Moorghen, 1992). DMH is a specific colon carcinogen that is widely used to study chemically induced colonic cancer (Chang, 1978; Reviewed by Rogers and Nauss, 1985; Reviewed by Klurfeld, 1995; Zhurkov *et al.*, 1996; Jackson *et al.*, 1999) and provides a useful model for studying early carcinogenesis. Previously chronic administration of DMH has been shown to result in morphological changes similar to those observed in human adenomatous polyposis (Chang, 1980; Carter *et al.*, 1994; Moorghen *et al.*, 1998; Jackson *et al.*, 1999). The morphological changes begin with hyperplasia (Chang, 1978; Moorghen *et al.*, 1998) followed by dysplastic foci (microadenomatas) that grow into macroscopic sessile polyps (adenomas), which can become invasive (invasive adenocarcinoma) (Carter *et al.*, 1994; Moorghen *et al.*, 1998).

Metabolites of DMH (Figure 1.8, p60); methyldiazonium (Moorghen, 1992), methylazoxymethanol (MAM) (Reviewed by Sunter, 1980) or methylcarbonium ion (Reviewed by Klurfeld, 1995), are thought to be DNA alkylating agents (Reviewed by Sunter, 1980; Moorghen, 1992; Reviewed by Klurfeld 1995). DMH has been shown to result in the formation of O^6-Methylguanine (Jackson *et al.*, 2000) however methylation of the colonic epithelial cell DNA is not by itself sufficient to explain the high yield of colonic tumours (Moorghen, 1992; Papanikolaou *et al.*, 1998). A number of groups have researched the effects of the carcinogen (AOM, DMH or MAM) on various genes and proteins that have been found to be mutated or their

expression altered within human colorectal cancer (Jacoby *et al.*, 1991; Maltzman *et al.*, 1997; Rudzki *et al.*, 1997; Sheng *et al.*, 1998; Takahashi *et al.*, 1998; Jackson *et al.*, 1999; Takahashi *et al.*, 2000a; Takahashi *et al.*, 2000b; Endo *et al.*, 2001; Koesters *et al.*, 2001; Suzui *et al.*, 2001; Kishimoto *et al.*, 2002; Yamada *et al.*, 2003). The genetic changes found during human colorectal cancer development are shown in Figure 1.4 (p9) (Reviewed by Kinzler and Vogelstein, 1996). The first genetic change involves the tumour suppressor gene APC, which is found mutated in 80-85% of all colorectal cancers (Reviewed by Nakamura, 1997). APC binds β-catenin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993) and functions to regulate β-catenin levels within the cell by targeting it for degradation, when in a complex with GSK-3β and Axin (Fagotto *et al.*, 1991; Reviewed by Moon and Miller, 1997; Reviewed by Peifer, 1997; Reviewed by Ben Ze've, 1997; Reviewed by Gordon, 1998; Ikeda *et al.*, 1998; Kishida *et al.*, 1998; Spink *et al.*, 2000). Interestingly, β-catenin mutations are also found within colorectal tumours with the most common mutation found within the human gene affects the protein's serine and threonine residues that are targeted by GSK-3β (Ikeda *et al.*, 1998; Kishida *et al.*, 1998; Müller *et al.*, 1998). β-catenin, γ-catenin, $p120^{(ctn)}$ and α-catenin are involved in a number of cellular process namely cell adhesion and cell signaling (Reviewed by Barth *et al.*, 1997b). β-catenin, γ-catenin and $p120^{(ctn)}$ all bind to E-cadherin with α-catenin linking this complex to the actin cytoskeleton to form functional adhesive complexes (Peifer *et al.*, 1992; Nagafuchi *et al.*, 1994; Reviewed by Jawhari and Pignatelli, 1997; Reviewed by Lewis *et al.*, 1997; Reviewed by Pignatelli, 1998). β-catenin and γ-catenin have also been shown to be involved in the Wnt-1 signaling pathway (Reviewed by Miller and Moon, 1996; Reviewed by Moon and Miller, 1997; Morin *et al.*, 1997a; Reviewed by Nusse, 1997). Activating mutations in β-catenin, loss of function mutations in APC or activation of the WNT-1 pathway results in cytoplasmic accumulation and nuclear translocation of β-catenin where it associates with the transcription factors LEF and TCF (Behrens *et al.*, 1996; Korinek *et al.*, 1997) activating β-catenin-Tcf signaling (Morin *et al.*, 1997a). Previous studies revealed AOM, a metabolite of DMH, resulted in a mutation of the GSK-3β consensus motif of β-catenin rats and ICR mice (Takahashi *et al.*, 1998; Takahashi *et al.*, 2000b). Other studies have also found over-expression and nuclear localization of β-catenin within AOM induced rat colonic tumours (Sheng *et al.*, 1998). Studies

have also revealed a reduction in wild type APC expression within AOM induced mouse colonic tumours (Maltzman *et al.*, 1997), AOM was also found to reduce *APC* mRNA expression levels in the rat colon (Kishimoto *et al.*, 2002). Investigations using DMH induced tumours in the rat colon found *APC* mutations in the region homologous to the human mutational cluster region in 6% of tumours (Endo *et al.*, 2001). The colon has a rapid cell turnover and a disruption in its natural homeostasis can result in cancer development (Reviewed by Kinzler and Vogelstein, 1996; Reviewed Wasan and Goodlad, 1996; Reviewed by Jawhari *et al.*, 1997a). E-cadherin, β-catenin and APC all play a fundamental role in the regulation of the normal colon with disruptions sufficient for colorectal cancer development (Reviewed by Ilyas and Thomlinson, 1997).

4.2 Aim.

To characterise the expression of the E-cadherin/catenin complex within Balb/c mice colonic tissues treated with 1,2-dimethylhydrazine, a colonic carcinogen.

4.3 Objectives.

1. To examine the expression and cellular localisation of the adhesion molecules E-cadherin, α-catenin, β- catenin, γ-catenin and p120 in the non-dysplastic and dysplastic tissues of Balb/c mice exposed to the carcinogen 1,2-dimethylhydrazine.
2. To carry out mutational analysis on exon 3 of the β-catenin gene, which contains the GSK-3β consensus sequence.

4.4 Methodology.

1,2-dimethylhydrazine (DMH) was subcutaneously injected into rodents, female Balb/c mice were chosen in this study due to their resilience to liver toxicity caused by DMH administration whilst being susceptible to its carcinogenic effects, producing distal colonic tumours (Moorghen *et al.*, 1998). After 17 weeks of exposure to the carcinogen, as discussed in section 2.2, the mice were sacrificed, their colons removed/excised, fixed in carnoys for 3 hours followed by formalin fixation. The colons were pinned out on corkboard serosal side down before fixation to ease visualisation of nodules. Using a dissecting microscope the nodules along with the surrounding tissue were removed by Dr M. Moorghen, Jyoti Gupta and Emma Tucker and placed in formalin for paraffin embedding carried out by Jenny Baker and Joy Code. Four-micrometer transverse sections were stained with haematoxylin and eosin (H&E), with 3-6 sections per slide. Following H&E confirmation of tumours, 40 tumours for immunohistochemical analysis were selected, 10 from each feeding study group (refer to section 5). Tumours were stained for E-cadherin and the catenins (α-,β-, γ-) using indirect immunohistochemical methods, described in section 2.3. The tissues were then scored for cytoplasmic and membranous immunoreactivity using the following scoring system:- 3 = greater than 90% of epithelial cells showed positive expression, 2 = 90-50% of epithelial cells were positive, 1 = less than 50% of epithelial cells were positive, with nuclear localisation marked as present or absent.

Preliminary studies carried out using archival 1,2-dimethylhydrazine tissues revealed nuclear localisation of β-catenin suggesting a mutation in either *β-catenin* or *APC* gene or activation of the Wnt-1 pathway. Takahashi *et al.* (1998, 2000a) showed that the metabolite of DMH, AOM results in a mutation in the GSK-3β consensus sequence of β-catenin in a rat model, thus mutational analysis of β-catenin was carried out. PCR was used to amplify up DNA extracted from the DMH mouse colonic tissues, incorporating primers designed by Takahashi *et al.* (1998, 2000a). Attempts were made at amplifying DNA from both paraffin and frozen tissues. Paraffin embedded tumours were firstly stained for β-catenin to determine the presence or absence of nuclear localisation. Those with nuclear staining were chosen

for mutational analysis along with a control group that were not exposed to the carcinogen DMH. Tissue lysates were made using protocol described in section 2.4.2. Frozen tissues were also retained from the experiment, 1 mouse from each group plus a control non-DMH treated mouse. For the frozen tissues, sections from the distal colon were removed, as nodules were not easily visible in frozen tissues the lysates were made from sections of the distal colon using protocol described in section 2.4.2. A range of conditions were applied to amplify the DNA involving altering the number of cycles (40 to 50 cycles), varying the annealing temperature from 50°C to 58°C, and applying the conditions used successfully by Takahashi *et al.* (2000a). This was unsuccessful in the paraffin embedded tissues but achieved in the frozen tissues (Fig 4.7). DNA from the frozen tissues was then amplified using the protocol described in section 2.4. Mutation detection was carried out using single strand conformational polymorphism (SSCP) techniques (section 2.4) and sequence analysis was used to confirm and reveal the type of mutations. Sequence data was analysed using websites (Blast search Genebank data base at ncbi, www.ncbi.nlm.nih.gov/genebank) and restriction maps (Bioedit sequence alignment web cutter).

4.5 Results.

4.5.1 Examination of the expression and cellular localisation of the E-cadherin and catenin complex by immunohistochemistry.

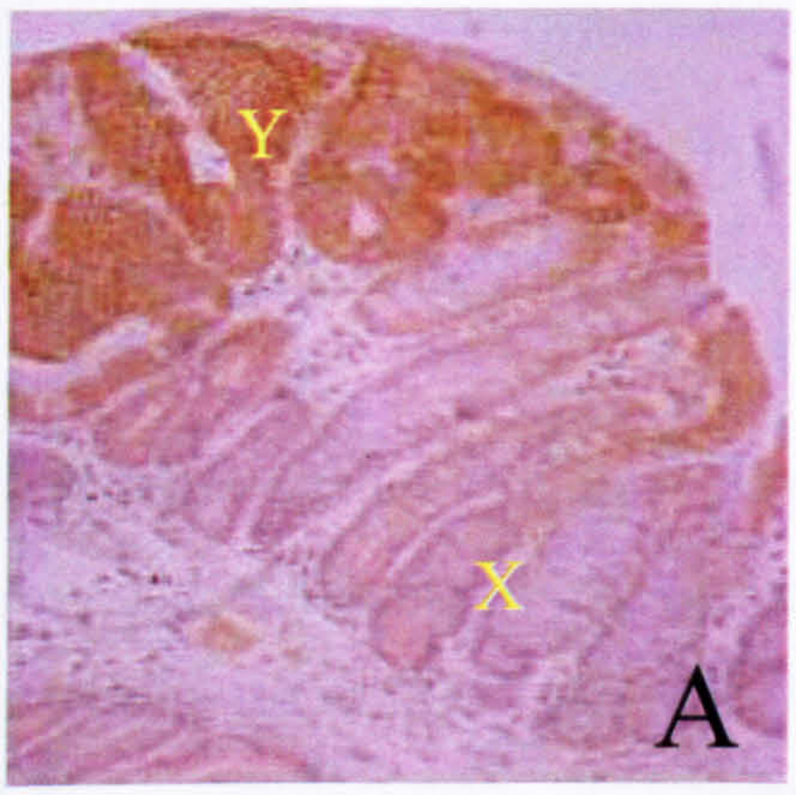
4.5.1.1 *Increase in cytoplasmic expression and nuclear localisation of β-catenin in colonic dysplastic mucosa of Balb/c mice administered with DMH.*

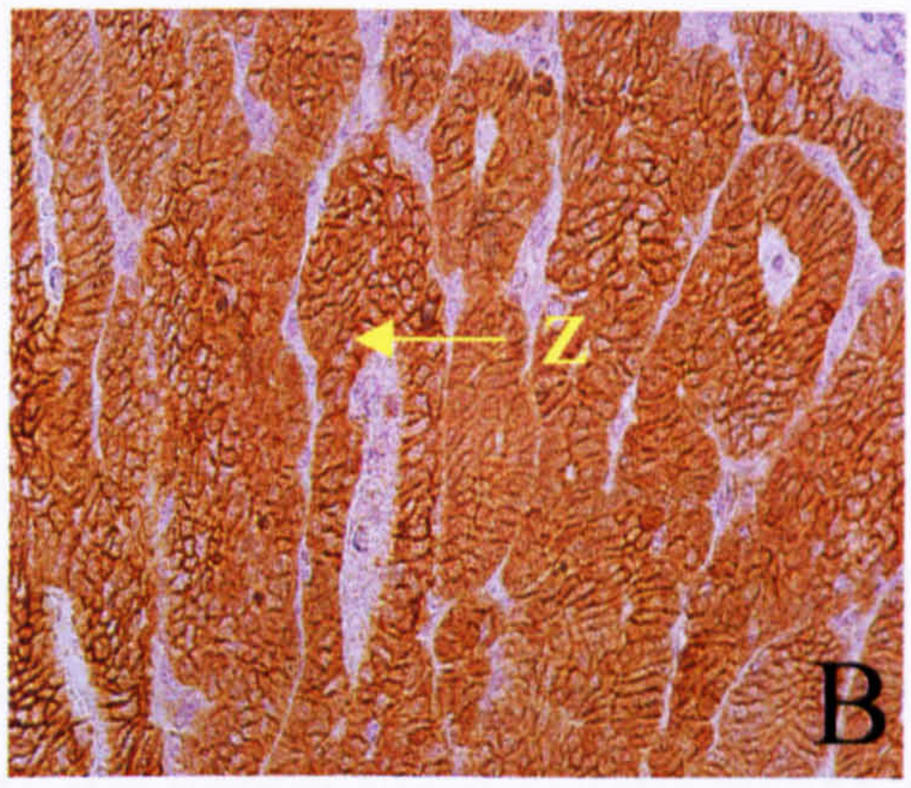
Within the non-dysplastic tissues over 90% of the colonic epithelial cells were positive for β-catenin expression at the cell membrane within 33 of the 41 colonic tissues examined (Figure 4.1 and 4.5). The non-dysplastic tissues also revealed areas of cytoplasmic β-catenin expression, although this expression was found to be heterogeneous with 24 out of the 41 tissues having areas of less than 50% of the epithelial cells being positive. No nuclear expression for β-catenin was observed within the non-dysplastic tissues. The dysplastic tissues revealed increased membranous and cytoplasmic β-catenin immunoreactivity with over 90% of epithelial cells being positive for β catenin in the majority of tissues (39/41 and 38/41 scored 3, respectively). Within these dysplastic tissues focal and scattered nuclear expression was also observed in 14/41 tissues. Thus when comparing the non-dysplastic tissues with the dysplastic tissues there was an increase in cytoplasmic and focal nuclear expression for β-catenin within the dysplastic areas, an increase in immunoreactivity was also noted (Fig. 4.1) but not quantified.

4.5.1.2 *Cytoplasmic E-cadherin cellular localisation is not associated with reduced membranous expression in colonic dysplastic mucosa from mice administered with DMH.*

Within the non-dysplastic tissues over 90% of epithelial cells were positive for E-cadherin membrane expression within the majority of the tissues (39 out of 41 tissues scored 3) (Fig. 4.2 and 4.5). Cytoplasmic expression showed heterogeneity with over

Figure 4.1A. Representative 1,2-dimethylhydrazine mouse colonic tissue section showing β-catenin localisation and expression in non-dysplastic (X) and dysplastic (Y) tissues (x20 Obj). B. Area of dysplasia with increased membranous, cytoplasmic and focal nuclear localisation (Z) (x40 Obj).





Non-dysplastic tissues revealed **β-catenin** expression at the membrane with areas of cytoplasmic expression but no nuclear expression. Dysplastic tissues revealed increased membranous and cytoplasmic β-catenin immunoreactivity with focal nuclear localisation. Thus within the dysplastic tissues there was an increase in cytoplasmic and focal nuclear localisation for β-catenin when compared to the non-dysplastic tissues. An apparent increase in β-catenin immunoreactivity was also noted.

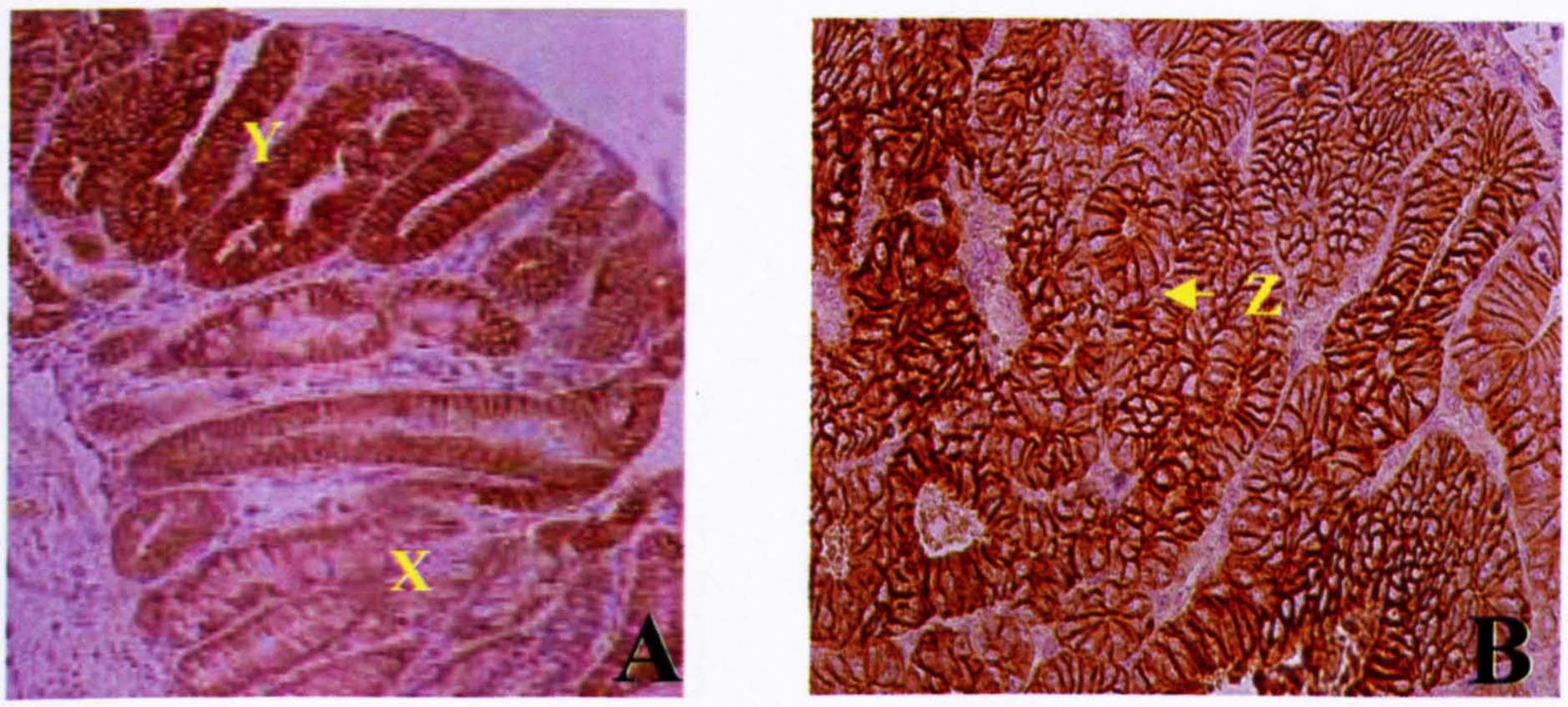
50% of the epithelial cells being positive for E-cadherin in some tissues (23/41 scored 2) the remainder of tissues having lower expression levels. There was no nuclear E-cadherin expression within the non-dysplastic tissues (0/41). Over 90% of epithelial cells were positive for E-cadherin membrane expression within the dysplastic tissues suggesting membrane expression was maintained (39/41 scored 3). This was accompanied by an increase in cytoplasmic expression with 90% of the epithelial cells being positive in the majority of tissues (32/41 scored 3) but no nuclear localisation (0/41) was observed. Thus the results show an increase in cytoplasmic expression for E-Cadherin within the dysplastic tissues (Fig. 4.2).

4.5.1.3 *Increase in α-catenin and p120 cytoplasmic expression in dysplastic colonic mucosa is not associated with nuclear localisation.*

Expression of α-catenin (Figure 4.3 and 4.5) within the non-dysplastic colonic tissues revealed membranous expression in over 90% of all colonic epithelial cells within all tissue examined (40/40 scored 3). Over 50% of epithelial cells were positive for α-catenin within the cytoplasm in the majority of these tissues (35/40 scored 2) and no nuclear expression 0/41 was observed. Within the dysplastic areas of these colonic tissues there was maintenance of membrane expression, with 90% of epithelial cells being positive for membrane expression in all tissues examined (40/40 scored 3). This was accompanied by an increase in cytoplasmic localisation with 90% of the cells positively expressing α-catenin in the majority of tissues (29/40 scored 3), although there was some heterogeneity. There was no nuclear expression of α-catenin within these dysplastic tissues. Thus within the dysplastic tissues α-catenin expression was shown to be maintained at the membrane and increase within the cytoplasm.

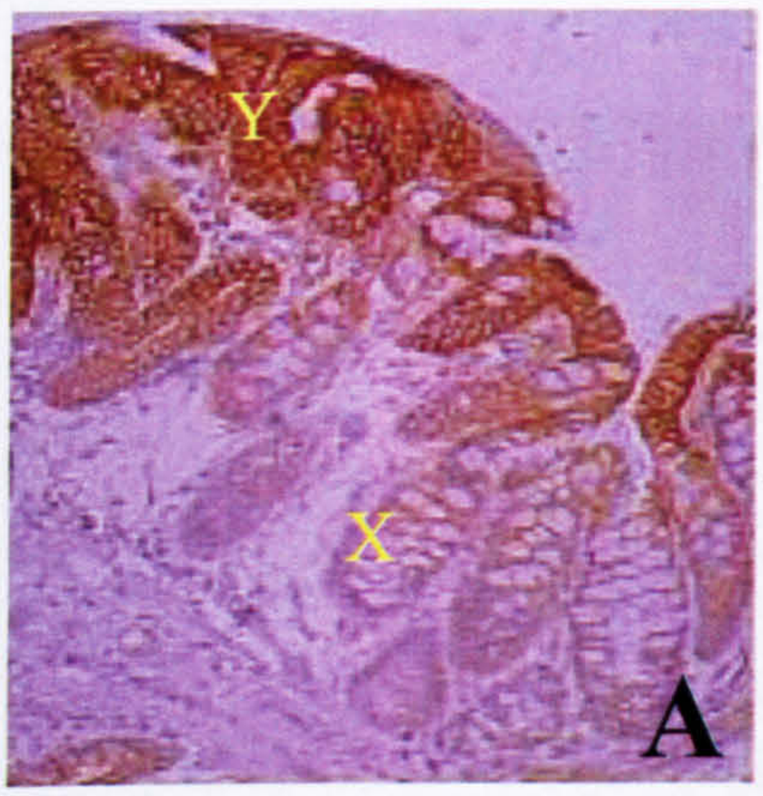
Expression of p120 (Figure 4.4 and 4.5) within the non-dysplastic areas of the colonic tissues revealed membranous expression in 90% of epithelial cells for the majority of tissues (39/41 scored 3), expression was throughout the crypt, although greatest at the mid to top point of the crypt. Some cytoplasmic expression was observed with 23/41 of

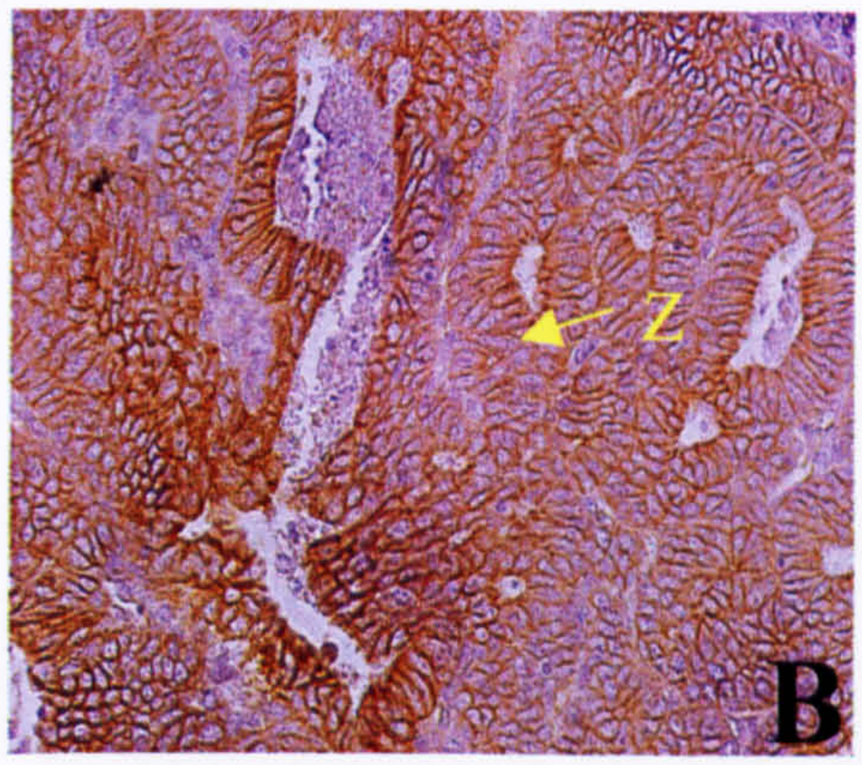
Figure 4.2A. Representative 1,2-dimethylhydrazine mouse colonic tissue section showing E-cadherin localisation and expression in non-dysplastic (X) and dysplastic (Y) tissues (x20 Obj). B. Area of dysplasia with increased membranous, cytoplasmic but no focal nuclear localisation (Z) (x40 Obj).



Non-dysplastic tissues showed that for **E-cadherin** membranous expression was over 90% of the majority of tissues with evidence of cytoplasmic expression in areas but there was no nuclear expression. Within the Dysplastic tissues membrane expression was maintained accompanied by an increase in cytoplasmic localisation but with no nuclear localisation. The results show an increase in cytoplasmic localisation of E-Cadherin in the dysplastic tissues. Increased immunoreactivity within dysplastic tissue for E-cadherin was also noted.

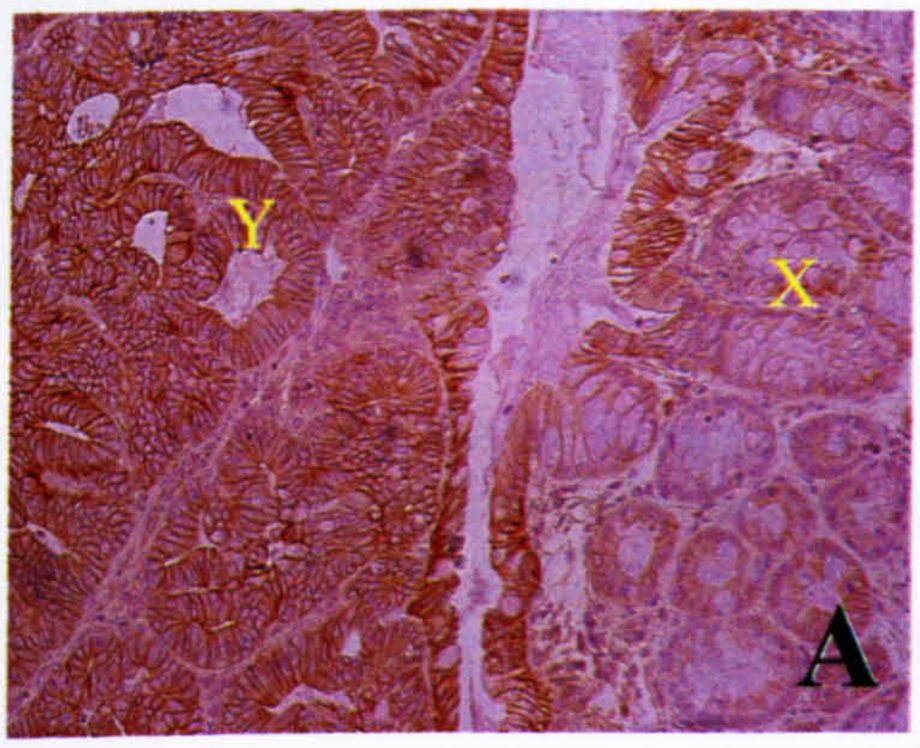
Figure 4.3A. Representative 1,2-dimethylhydrazine treated mouse colonic tissue section showing α-catenin localisation and expression in non-dysplastic (X) and dysplastic (Y) tissues (x20 Obj). B. Area of dysplasia with increased membranous, cytoplasmic but no nuclear expression (x40 Obj).

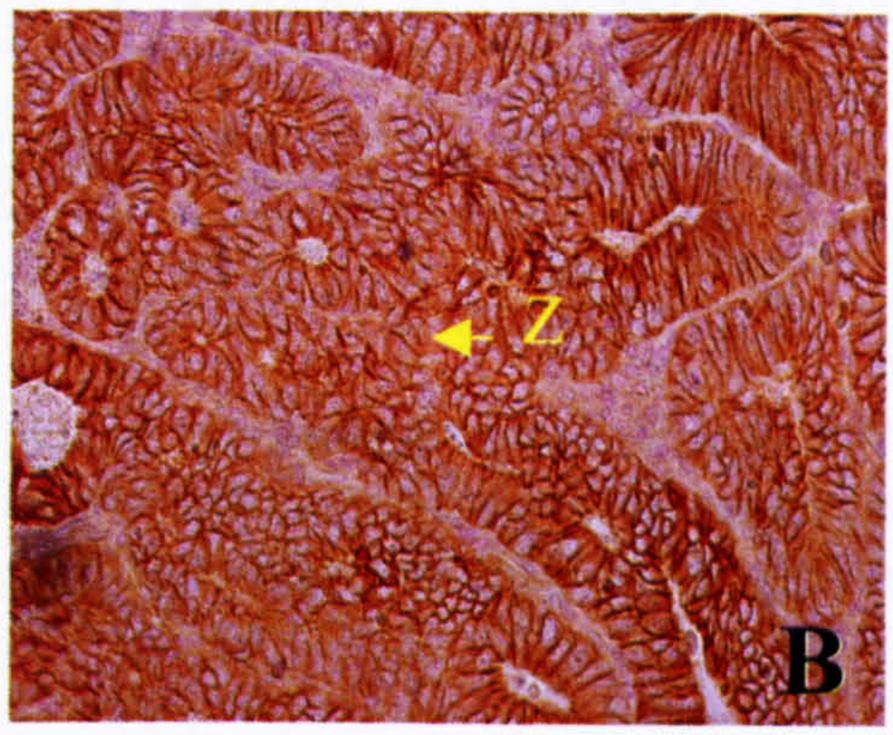




Alpha catenin expression within the non-dysplastic tissues revealed membranous expression in all tissue with cytoplasmic localisation found in some of these tissues with no nuclear expression. Dysplastic tissues revealed membranous expression in over 90% of all colonocytes within the dysplastic tissues with an increase in cytoplasmic localisation but with no nuclear localisation. Thus within the dysplastic tissues α-catenin localisation was shown to increase within the cytoplasm.

Figure 4.4A. Representative 1,2-dimethylhydrazine treated mouse colonic tissue section showing p120 localisation and expression in non-dysplastic (X) and dysplastic (Y) tissues (x20 Obj). B. Area of dysplasia with increased membranous, cytoplasmic but no nuclear expression (x40 Obj).





p120 localisation within the non-dysplastic tissues revealed membranous expression with some cytoplasmic localisation but with no nuclear localisation. Within the dysplastic tissues there was maintenance of membrane expression with an increase in cytoplasmic localisation but with no nuclear expression. Thus in the dysplastic tissues p120 localisation was shown to increase within the cytoplasm.

Figure 4.5 Expression of α-, β-catenins, p120$^{(ctn)}$ and E-cadherin within the 1,2-dimethyl -hydrazine mouse colonic tissues as determined by immunohistochemistry.

	Non-dysplastic tissue						Dysplastic tissue					
	Membrane		Cytoplasm		Nucleus		Membrane		Cytoplas		Nucleus	
	S	No.	S	No.	S	No.	S	No.	S	No.	S	No.
Beta-catenin	**3**	33/41	**1**	24/41	**0**	41/41	**3**	39/41	**3**	38/41	**F**	14/41
E-cadherin	**3**	39/41	**2**	23/41	**0**	41/41	**3**	39/41	**3**	32/41	**0**	0/41
Alpha-caten	**3**	40/40	**2**	35/40	**0**	41/41	**3**	40/40	**3**	29/40	**0**	0/41
p120(ctn)	**3**	39/40	**2**	23/41	**0**	41/41	**3**	39/41	**3**	28/41	**0**	0/41

Key:- **S,** Score; **No.**, number of tissues with the score shown. Scoring system used 3 = greater than 90% of epithelial cells showed positive expression, 2 = 90-50% of epithelial cells were positive, 1 = less than 50% of epithelial cells were positive, with nuclear localisation marked as present (**F**) or absent (**0**).

The average scores from the immunohistochemical analysis of the 1,2 dimethylhydrazine mouse colonic tissues revealed an interesting pattern for the catenins and E-cadherin with the dysplastic tissues being associated with an increase in cytoplasmic localisation. The tissues were scored as outlined in section 4.4. The Non-dysplastic tissues revealed that for β-catenin, α-catenin, p120 and E-cadherin there was expression at the cell membrane within over 90% of the colonic epithelial cells examined (33/41, 40/40, 39/40 and 39/41 scored 3 respectively) with heterogenous cytoplasmic localisation. There was no nuclear expression for β-catenin, α-catenin, p120 and E-cadherin. The dysplastic tissues revealed an increase in membranous β-catenin localisation (39/41 scored 3) accompanied with the maintenance of α-catenin, p120 and E-cadherin expression at the cell membrane. Significantly there was an increase in cytoplasmic localisation for β-catenin, α-catenin, p120 and E-cadherin, with over 90% of the epithelial cells expressing cytoplasmic catenins and cadherin. For β-catenin this increase in cytoplasmic expression was also accompanied by focal nuclear localisation within 14 of 41 dysplastic tissues. For α-catenin, p120 and E-cadherin nuclear localisation was not observed within the dysplastic tissues.

tissues having over 50% (but less than 90%) epithelial cells positive for p120. As for α-catenin and E-cadherin there was no nuclear expression (0/41) within the non-dysplastic tissues. Within the dysplastic colonic tissues there was maintenance of p120 membrane expression (39/41 scored 3), as for α-catenin, accompanied by an increase in cytoplasmic expression with over 90% of epithelial cells being positive for $p120^{(ctn)}$ within 28 out of 41 tissues but with no nuclear expression (0/41), as for α-catenin and E-cadherin. Thus in the dysplastic areas of the DMH mouse tissues p120 expression was shown to increase within the cytoplasm.

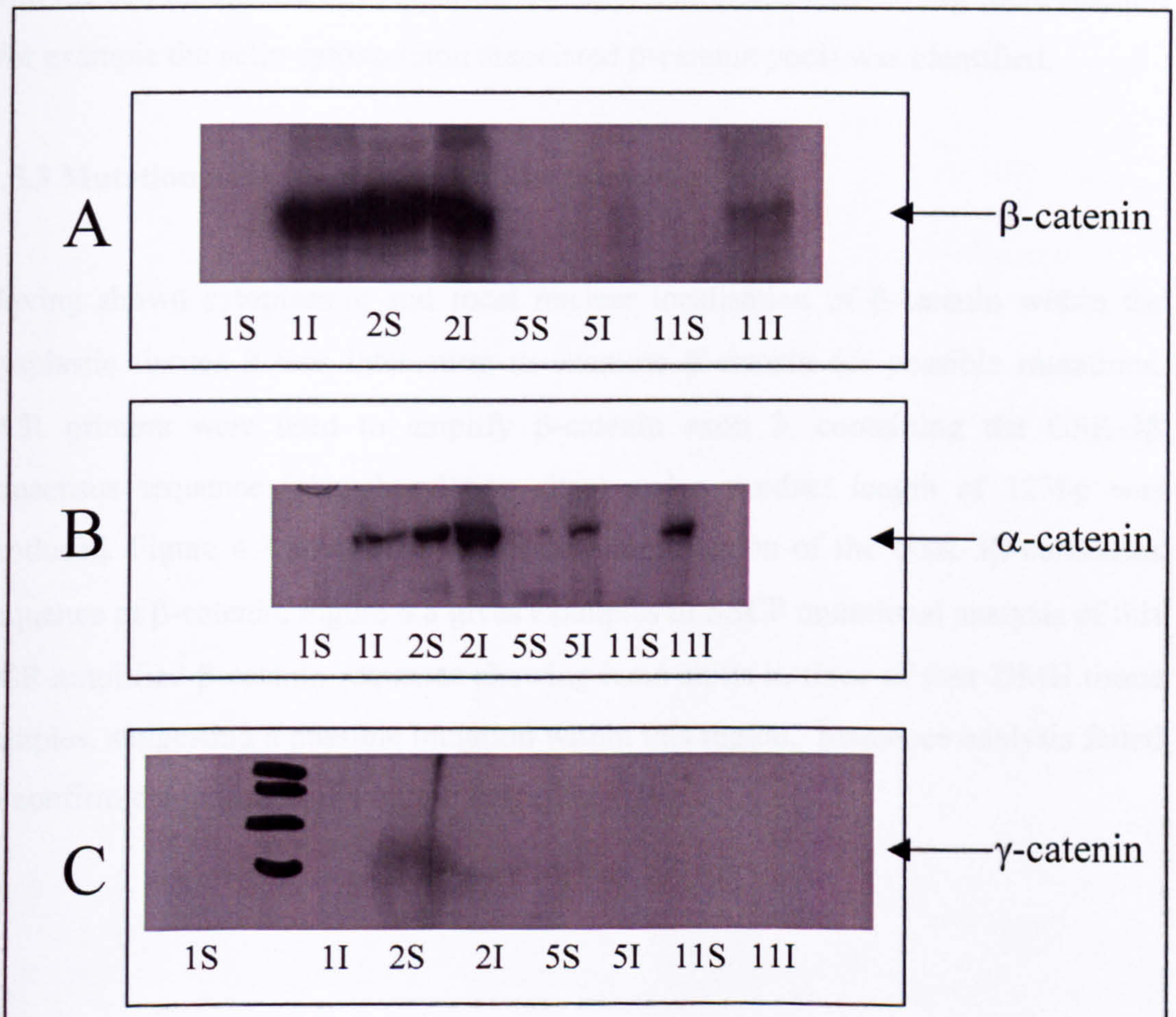
4.5.1.4 *γ-catenin expression in the intestinal mucosa of Balb/c mice exposed to the carcinogen 1,2-dimethylhydrazine.*

The expression of γ-catenin within the colonic epithelial tissues was very heterogeneous in nature. Tissues ranged from being completely negative in both the non-dysplastic and dysplastic tissues (9 out of 39) to having heterogeneous expression within either the non-dysplastic, dysplastic tissues or both. There was no consistent pattern of expression observed except that in all cases epithelial cells expressed very low levels of γ-catenin. Only a few areas of focal expression were observed including membranous or diffused cytoplasmic expression. For all tissue samples there was no nuclear γ-catenin expression within both the non-dysplastic or dysplastic epithelium (0/41).

4.5.2 *Expression of β-catenin and α-catenin by western blotting.*

Figure 4.6 confirmed that the antibodies β- and α-catenin used in this study were specific for the proteins being examined. It also confirmed that there is low coexpression of γ-catenin in wild type or DMH treated colonic mouse tissue. The aim of this experiment was to confirm the β-catenin expression observed by the immunohistochemical technique. This proved complex due to the difficulties in identifying dysplastic regions from frozen tissue sections stained with haematoxylin and

Figure 4.6 Expression of β- and α-catenin by western blotting.



(A) β-catenin (N=4), (B) α-catenin (N=1) and (C) γ-catenin (N=1) expression in wild-type Balb/c mice (1) and DMH treated Balb/c mice (2, 5 and 11) examining both the soluble (S) and insoluble (I) fractions. The western blots (A and B) show that the antibodies used were specific for their target proteins β -catenin and α-catenin in the DMH treated Balb/c mouse colonic tissues in both the soluble and insoluble fractions. Western blot C reveals no significant levels of γ-catenin expression within the mouse colonic tissue.

eosin. However, the western blotting results suggest that total β-catenin expression levels maybe increased in the tissues from the DMH treated mice when compared to the wild type Balb/c mice. The increase in β-catenin expression appeared to be localised within the soluble fraction, suggesting an increase in β-catenin associated with the cell membrane or cytoplasm. No apparent increase in the insoluble fraction (for example the actin cytoskeleton associated β-catenin pool) was identified.

4.5.3 Mutation analysis of exon 3 of the β-catenin gene.

Having shown cytoplasmic and focal nuclear localisation of β-catenin within the dysplastic tissues it was interesting to examine β-catenin for possible mutations. PCR primers were used to amplify β-catenin exon 3, containing the GSK-3β consensus sequence (phosphorylation sites) and a product length of 123bp was produced. Figure 4.7 shows the successful amplification of the GSK-3β consensus sequence of β-catenin. Figure 4.8 gives examples of SSCP mutational analysis of this PCR amplified β-catenin sequence showing band shifts in three of four DMH tissue samples, suggesting a possible mutation within this region. Sequence analysis failed to confirm the presence of a mutation (figure 4.10).

Figure 4.7 PCR amplification of beta-catenin from frozen distal colonic tissue samples of DMH treated Balb/c mice.

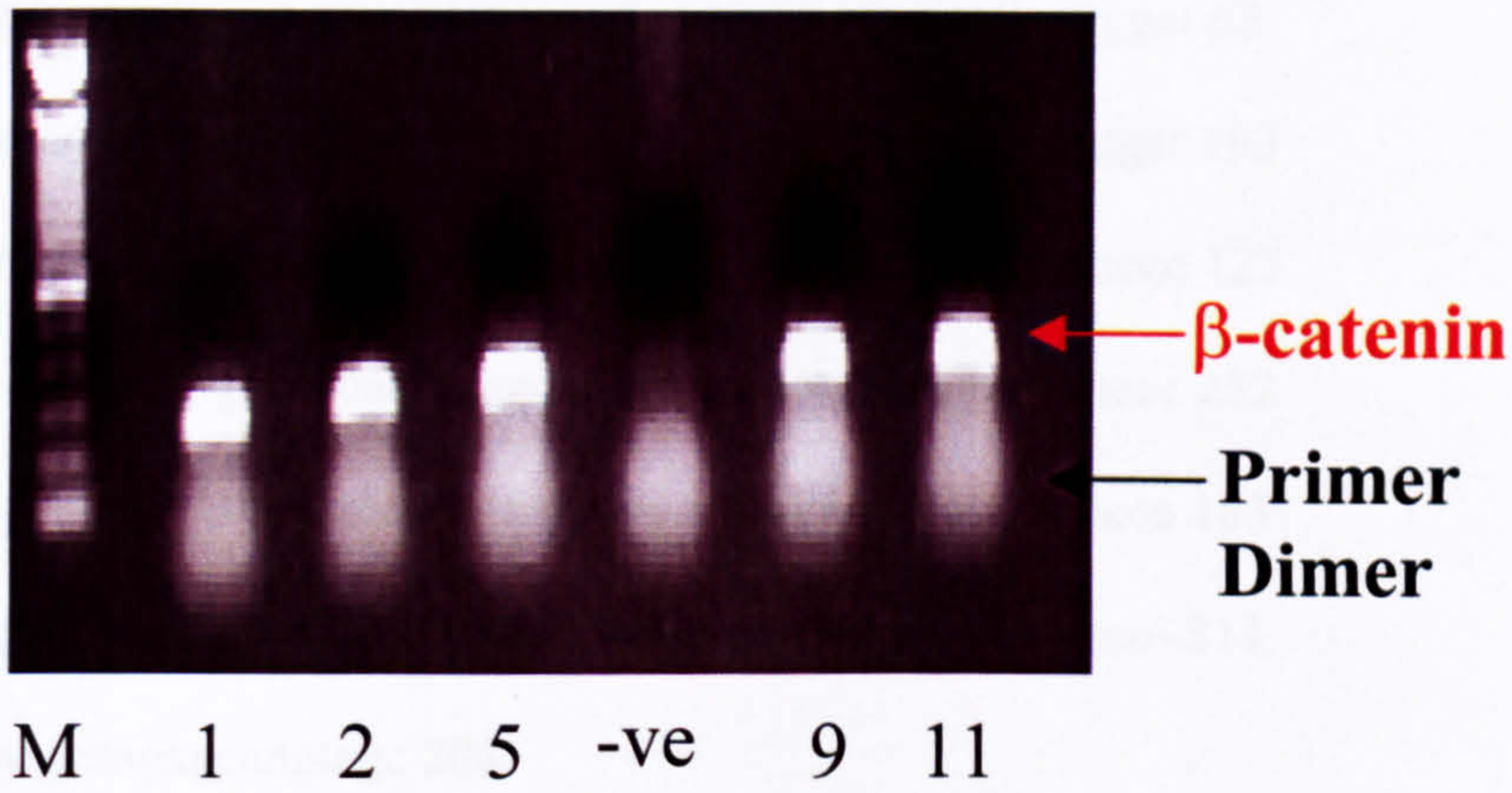
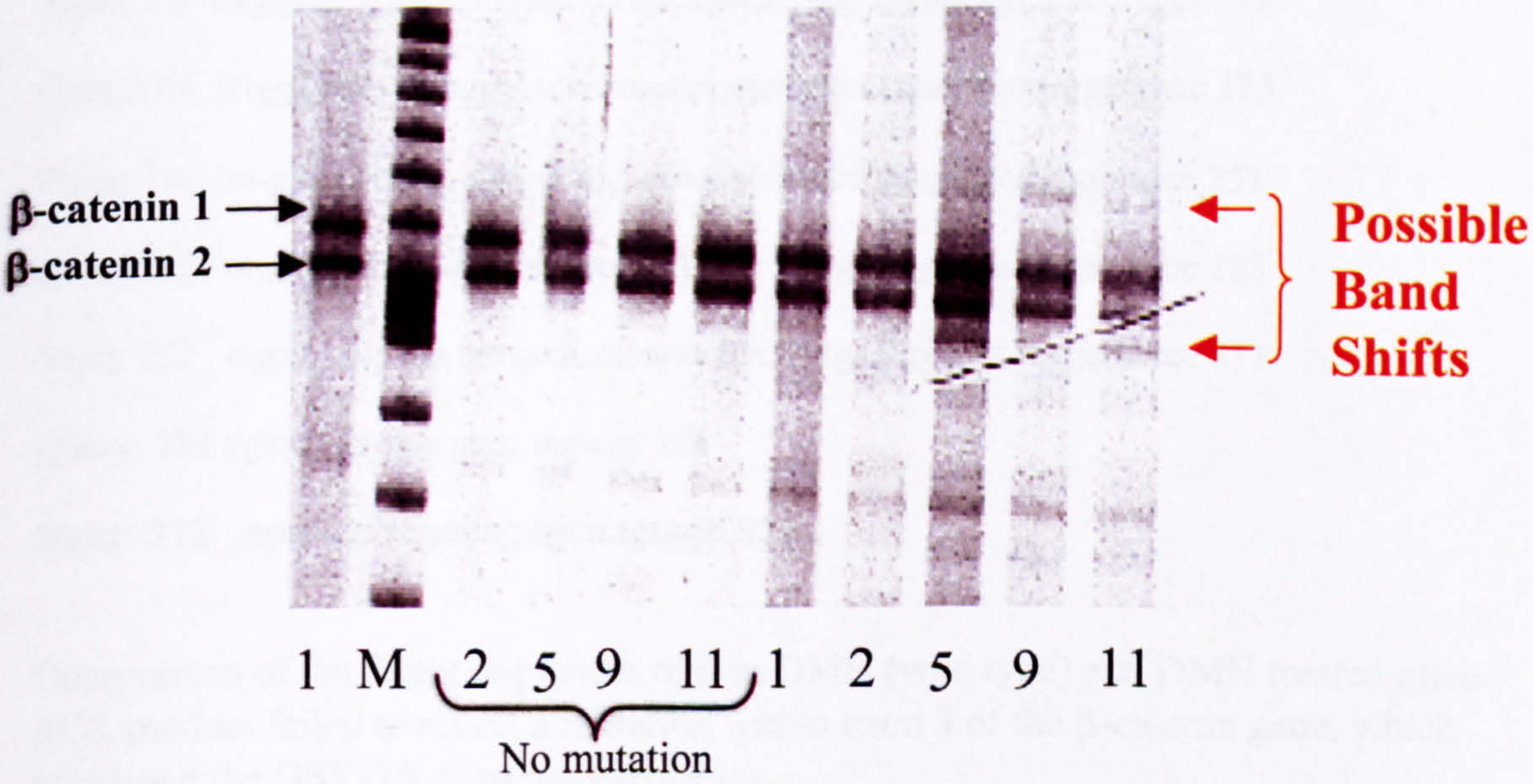


Figure 4.8 SSCP revealing possible beta-catenin mutations in 3 of the 4 DMH treated Balb/c mice.



Key:- 1 = Group 1 control (non-DMH), 2= Group 2 IH/DMH, 5= Group 5 pectin/DMH, 9= Group 9 Cellulose/DMH,, 11 = Group 11 Basal/DMH.

Fig 4.9 DNA sequences from wild-type and DMH-treated mouse colonic tissue for exon 3 of the *β-catenin* gene.

A. Wild type (Group 1) Forward primer.

Query: 5 catggagccgggacagaaaa-ctgctgtcagccactgggnagcagcagtcttacttggat 63

Sbjct: 136 catggagcc-ggacagaaaagctgctgtcagccact-ggcagcagcagtcttacttggat 193

Query: 64 tctgggaatccattctggtgccaccaccacagctccttccctgagtggcaagggcaaccc 123

Sbjct: 194 tct-ggaatccattctggtgccaccaccacagctccttccctgagtggcaagggcaaccc 252

Query: 124 tgaggaagaagatgttgacacctcccaagtcctttatgaatgggagcaaggcttttccca 183

Sbjct: 253 tgaggaagaagatgttgacacctcccaagtcctttatgaatgggagcaaggcttttccc- 311

Query: 184 agtccttcacgcaagagcangtagc 208

Sbjct: 312 agtccttcacgcaagagcaagtagc 336

B. DMH treated (Group 5) Forward primer.

Query: 5 catngagcgggacagaaaa-ctgctgtcagncactgggnagcagcagtcttacttgggat 63

Sbjct: 136 catggagccggacagaaaagctgctgtcagccact-ggcagcagcagtcttactt-ggat 193

Query: 64 tctgggaatccattctgggtgccaccaccacagctccttccctgagtggcaagggcaacc 123

Sbjct: 194 tct-ggaatccattct-ggtgccaccaccacagctccttccctgagtggcaagggcaacc 251

Query: 124 ctgaggaagaagatgttgacacctcccaagtcctttatgaatgggagcaaggcttttccc 183

Sbjct: 252 ctgaggaagaagatgttgacacctcccaagtcctttatgaatgggagcaaggcttttccc 311

Query: 184 agtccttcacgcaagagcangtagc 208

Sbjct: 312 agtccttcacgcaagagcaagtagc 336

Comparison of the direct sequences of non-DMH (wild type) and DMH treated mice PCR product failed to reveal a mutation within exon 3 of the β-catenin gene, which contained the GSK-3β consensus sequence.

4.6 Discussion.

In humans the development of colorectal cancer has been well studied, and the genetic steps involved in the adenoma to carcinoma sequence have been elucidated (Reviewed by Fearon and Vogelstein, 1990; Reviewed by White, 1998). The first genetic alteration (as discussed in section 1.2) in the multistep process of colorectal cancer, in inherited (familial adenomatous polyposis coli) and sporadic tumours, is the loss of APC (Reviewed by Fearon and Vogelstein, 1990; Reviewed by Kinzler and Vogelstein, 1996; Reviewed by White, 1998; Reviewed by Fearnhead *et al.*, 2001) with 80-85% of adenomas carrying an APC mutation (Reviewed by Nakamura, 1997). The APC gene is located on chromosome 5q21-22 and codes for a 310 Kd protein (Reviewed by Moon and Miller, 1997; Mulkens *et al.*, 1998), being described as a `gatekeeper' gene directly regulating the growth of tumours by inhibiting growth and/or promoting death (Reviewed by Kinzler and Vogelstein, 1996; Reviewed by Morin *et al.*, 1997b; Morin *et al.*, 1997a; Reviewed by Kinzler and Vogelstein, 1998; Mulkens *et al.*, 1998). APC is a cytoplasmic protein with multiple functional domains (Reviewed by Moon and Miller, 1997) and is found in association with both γ-catenin and β-catenin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993; Reviewed by Barth *et al*, 1997b; Reviewed by Jawhari *et al.*, 1997a). APC is thought to function by controlling the cytoplasmic levels of β-catenin in association with GSK-3β and axin (Reviewed by Moon and Miller, 1997; Reviewed by Peifer, 1997; Reviewed by Ben Ze've, 1997; Reviewed by Gordon, 1998; Ikeda *et al.*, 1998; Kishida *et al.*, 1998; Fagotto *et al.*, 1999; Spink *et al.*, 2000). Interestingly Samowitz *et al.* (1999) have reported that *β-catenin* mutations are more frequently found in small colorectal adenoma than in larger adenomas and invasive carcinomas. In contrast *APC* mutations within the mutational cluster region (MCR) has been associated with a greater number of adenoma development and tend to be associated with larger adenomas (Mulkens *et al.*, 1998). This suggests that *APC* and *β-catenin* mutations are not functionally equivalent and that the *APC* gene may have other functions inhibiting tumourigenesis.

The disruption of catenin functions have been shown to occur through a number of genetic and epigenetic factors along with changes in associated proteins. Mutations

involving *APC* resulting in a truncated protein that can no longer degrade β-catenin (Rubinfeld et al., 1996; Reviewed by Moon and Miller, 1997; Reviewed Fearnhead *et al.*, 2001), leading to an increase in cytoplasmic β-catenin levels and subsequent nuclear translocation. Stabilization and accumulation of cytoplasmic β-catenin as a result of mutations in either the *APC* (loss of function) or *β-catenin* (*CTNNBI*, gain of function) genes are causatively associated with colon carcinogenesis (Rubinfeld *et al.*, 1996; Takahashi *et al.*, 1998; Reviewed by Moon and Miller, 1997; Akiyama *et al.*, 2000; Reviewed Fearnhead *et al.*, 2001). The characterisation of mutations, outside of those within *APC* are not well defined and are relatively rare. Changes in cellular expression of the catenins, largely a reduction in membranous staining associated with increased cytoplasmic and nuclear staining (for β-catenin) suggests that they play a fundamental role in cancer development (Hao *et al.*, 1997). These factors all suggest that loss of catenin function either directly or indirectly results in cancer development.

In humans β-catenin expression in the normal non-dysplastic tissue is associated with the cell membranes, but in adenoma and carcinoma tissues there is reduced β-catenin membrane expression, which is accompanied by an increase in cytoplasmic and nuclear expression correlating with the progression from adenomas to carcinomas (Takayama *et al.*, 1996; Hao *et al.*, 1997). Activating mutations in the β-catenin gene are thought to be responsible for the excessive β-catenin signaling involved in the majority of carcinogen induced colonic carcinomas (Yamada *et al.*, 2000). Yamada *et al.* (2000) found that β-catenin signaling may be involved in the initial stage, aberrant crypt foci, of colon carcinogenesis in azoxymethane treated male F344 rats. Analysis of aberrant crypt foci (ACF) within DMH-induced colon carcinogenesis in Fischer 344 rats found that in about 9% of the ACF, β-catenin was overexpressed not only on the cell membrane but also in the cytoplasm (Furihata *et al.*, 2002). These findings suggest that immunohistochemical staining of ACF for β-catenin can evaluate the malignant potential of ACF (Furihata *et al.*, 2002). A further study revealed that crypts where β-catenin has accumulated are truly premalignant lesions of colon cancer, and are independent of aberrant crypt foci (ACF) (Yamada *et al.*, 2001a). In all of the cancer cells induced by DMH in rats, β-catenin was overexpressed in the nucleus (Furihata *et al.*, 2002). In the

azoxymethane (AOM) treated rats, overexpression and nuclear localization of β-catenin was observed in all adenomas (Sheng *et al.*, 1998). β-catenin cellular localization within azoxymethane (AOM) treated rats and mice revealed alterations in the cellular localization of β-catenin, within the dysplastic tissues β-catenin was expressed within the cytoplasm and nucleus, whereas in normal tissues it was expressed at the cell membrane (Takahashi *et al.*, 1998 and 2000a). In our DMH mouse model we found similar β-catenin expression within the non-dysplastic tissues when compared to the cellular localisation in human colonic mucosa. Within the dysplastic tissues there were also similarities with an increase in cytoplasmic expression and focal nuclear staining for β-catenin, but this was accompanied by maintenance of membrane expression, suggesting a possible difference in β-catenin activation as it does not appear to interfere with E-cadherin/β-catenin interactions. The focal nuclear expression shown for β-catenin could possibly be the result of a number of possible genetic (*APC* or *β-catenin* mutation) and epigenetic factors (tyrosine phosphorylation). Mutations within the GSK-3β consensus sequence allows β-catenin to escape from the proteosomal degradation with subsequent accumulation in the cytoplasm and translocation into the nucleus. Mutations in *APC* or WNT-1 activation would also result in this cytoplasmic accumulation, APC no longer being able to target it for degradation, and inappropriate activation of WNT would result in the inactivation of GSK-3β. The nuclear localization of β-catenin is a common event in colorectal tumourigenesis (Sheng *et al.*, 1998). SSCP mutational analysis of this DMH mouse model revealed a possible mutation within the GSK-3β consensus sequence of *β-catenin*. Sequence analysis failed to confirm a mutation, but this could be due to the differences in sensitivities of the different procedures, with SSCP being highly sensitive, or inaccuracies during PCR amplification. Takahashi *et al.* (2000a) revealed a β-catenin mutation in mice using the carcinogen AOM, a metabolite of DMH subsequent examinations of the colonic tissues induced by AOM in mice and rats revealed a mutation in the GSK-3β consensus motif (Takahashi *et al.*, 1998 and 2000a). Yamada *et al.* (2003) used Male F344 rats receiving subcutaneous injections of azoxymethane (15 mg/kg body weight) mutations in the exon 3 of *β-catenin* gene were detected in 22 of 56 early lesions (39.3%) (16 weeks old) and 21 of 37 colon cancers (56.8%) (46 weeks old). The

results of this study (Yamada *et al.*, 2003) provided evidence for the first time that a *β-catenin* mutation was selected for during colon carcinogenesis (Yamada *et al.*, 2003).

DMH induced colonic tumors in the rat contain mutations in β-catenin but the pattern of mutation differs from that found in human colon cancers (Blum *et al.*, 2001; Koesters *et al.*, 2001). In both humans and rodents, mutations affect the glycogen synthase kinase-3β consensus region of β-catenin, but within different codons (Blum *et al.*; 2001; Koesters *et al.*, 2001). The majority of studies tend to use relatively short-term treatments with carcinogen thus Koesters *et al.* (2001) investigated the mutational spectrum of the β-catenin gene in rat colon tumors induced by long-term (20 weeks) DMH-treatment. There were β-catenin mutations in 12 of 33 (36%) tumours with only one of the β-catenin mutations found to affect the previously implicated codon 33 cluster region, whereas 11 of 12 (>90%) mutations within codon 41, resulting in a similar mutation pattern to that observed in humans (Koesters *et al.*, 2001).

The role of APC in azoxymethane (AOM) induced colorectal tumors has been investigated in mice (Maltzman *et al.*, 1997). Using an antibody that recognizes the carboxy terminus of APC studies revealed that AOM-induced adenomas and carcinomas were negative for APC, suggesting the loss of wild type APC. This reflected the observations within MIN mouse colonic adenomas, which are known to have a APC mutation. Thus within the AOM-induced murine model of colorectal cancer there is a loss of wild-type APC protein within the colonic tumours, suggesting that alterations in expression of the tumor suppressor gene commonly mutated in human colon cancer, is also involved in this animal model of colon cancer (Maltzman *et al.*, 1997). APC mutations where found in the region homologous to the human mutation cluster region, but these were observed in only 6% of tumors induced in the rat colon by the administration of DMH (Endo *et al.*, 2001). Previous studies have indicated the incidence of APC mutations amongst AOM-induced tumors to be 15% or less (Sheng *et al.*, 1998). APC has been implicated in cell signaling (Vleminckx *et al.*, 1997) by its regulation of β-catenin levels within the cell. Investigations have shown that APC contains highly conserved nuclear export

signals that enable it to exit from the nucleus (Rosin-Arbesfeld *et al.*, 2000). Cancer cells containing a mutant APC results in β-catenin accumulating within the nucleus (Rosin-Arbesfeld *et al.*, 2000). The APC export signals confer efficient nuclear export and are critical for the function of APC in reducing the transcriptional activity of β-catenin (Rosin-Arbesfeld *et al.*, 2003).

Mutation of either the *APC* or the *β-catenin* (*CTNNBI*) genes results in nuclear accumulation of β-catenin and leads to activation of Tcf-4/Lef-1 dependent transcription and upregulation of target genes including c-myc (He *et al.*, 1998), cyclin D1 (Shtutman *et al.*, 1999), PPARδ (He *et al.*, 1999), matrix metalloproteinases (MMP) (Crawford *et al.*, 1999), CD44 (Wielenga *et al.*, 1999), uPAR (Mann *et al.*, 1999) and cox-2 (Howe *et al.*, 1999) which are important in tumourigenesis. Activating mutations in the *β-catenin* gene are thought to be responsible for the excessive β-catenin signaling involved in the majority of carcinogen induced colonic carcinomas. Rats exposed to AOM for 4 weeks resulted in an increase in COX-2 and c-myc mRNA expression along with a decrease in APC mRNA expression within the rats treated with AOM, along with a K-ras mutation within ACF consisting of 4 or more crypts (Kishimoto *et al.*, 2002). Previous studies carried out by Jackson *et al.* (1999) failed to detect any K-ras gene mutations within DMH induced tumours in SWR mice. DMH and 0.001% chlorophyllin induced tumours containing β-catenin mutations that also had marked over-expression of cyclin D1, c-myc and c-jun mRNA and c-Myc and c-Jun proteins (β-catenin/TCF target genes) were strongly elevated compared with tumors containing wild-type β-catenin (Blum *et al.*, 2001). Thus the majority of tumours induced by DMH and AOM select for β-catenin mutations, having a low level of APC mutations, but both could result in the same end with an increase in β-catenin nuclear localization and β-catenin/Tcf-4 target gene transcription.

In normal appearing colonic mucosa AOM has been shown to induce apoptosis, possibly to eliminate cells damaged by AOM administration and this was accompanied by an elevation in cell proliferation (Kishimoto *et al.*, 2002). The PCNA-LI in the ACF showing normal β-catenin expression pattern with that in the ACF showing abnormal β-catenin expression pattern was examined; there was no

significant difference of the PCNA-LI in these two different types of ACF (Furihata *et al.*, 2002), suggesting β-catenin overexpression does not play a role in proliferation. These results also suggest that the activation of β-catenin signaling pathway is not only an initiating event, but also plays a pivotal role in the promotion stage of colorectal carcinogenesis (Yamada *et al.*, 2003).

Loss of E-cadherin-mediated adhesion appears to be a fundamental aspect of the neoplastic process allowing cells to escape normal growth control signals resulting in increased cell proliferation, loss of differentiation and an increase in invasion (Frixen *et al.*, 1991; Nigam *et al.*, 1993; Reviewed by Barth *et al.*, 1997b; Reviewed by Jankowski *et al.*, 1997; Reviewed by Jawhari *et al.*, 1997a; Guilford *et al.*, 1998; Reviewed Semb and Christofori, 1998; Gofuku *et al.*, 1999; Jawhari *et al.*, 1999). Mutations in E-cadherin, β-catenin, α-catenin and γ- catenin and the activation of several receptor tyrosine kinases (EGFR and c-MET, resulting in catenin phosphorylation) have all been shown to result in the perturbation of cellular adhesion (Behrens *et al.*, 1993; Reviewed by Jankowski *et al.*, 1997; Guilford *et al.*, 1998). E-cadherin has also been found to localize in the cytoplasm of intestinal tumours derived from transgenic APC mice and FAP patients (Sloncova *et al.*, 2001). α-catenin has been shown to be reduced or absent in a number of primary tumours of the oesophagus, stomach and colon, with E-Cadherin mediated adhesion being abrogated by down-regulation of α-catenin (Shiozaki *et al.*, 1994; Reviewed Shiozaki *et al.*, 1996; Ghadimi *et al.*, 1999). Reduced p120 expression has also been observed in 20% of adenomatous polyps, with loss of membranous p120 expression correlating with reduced E-Cadherin expression. Decreased expression of p120 was also found to correlate with the larger tumour size (Skoudy *et al.*, 1996).

In human normal colorectal epithelial cells E-cadherin results in membranous staining at the adherens junctions, with changes in immunoreactivity and cellular localization within adenomas and carcinomas (Sloncova *et al.*, 2001). A decrease in E-cadherin expression has been found associated with poorly differentiated adenocarcinomas (Nigam *et al.*, 1993) and adenomas (Valizadeh *et al.*, 1997). These changes in E-cadherin expression correlated to tumour size, histopathology, growth patterns and the degree of dysplasia (Dorudi *et al.*, 1993; Gagliardi *et al.*, 1995).

Loss of E-cadherin expression was not observed within the DMH mouse model but this may be explained by the fact that this is an early stage model with low levels of invasive adenocarcinomas, the majority of tissues being examined being adenomas.

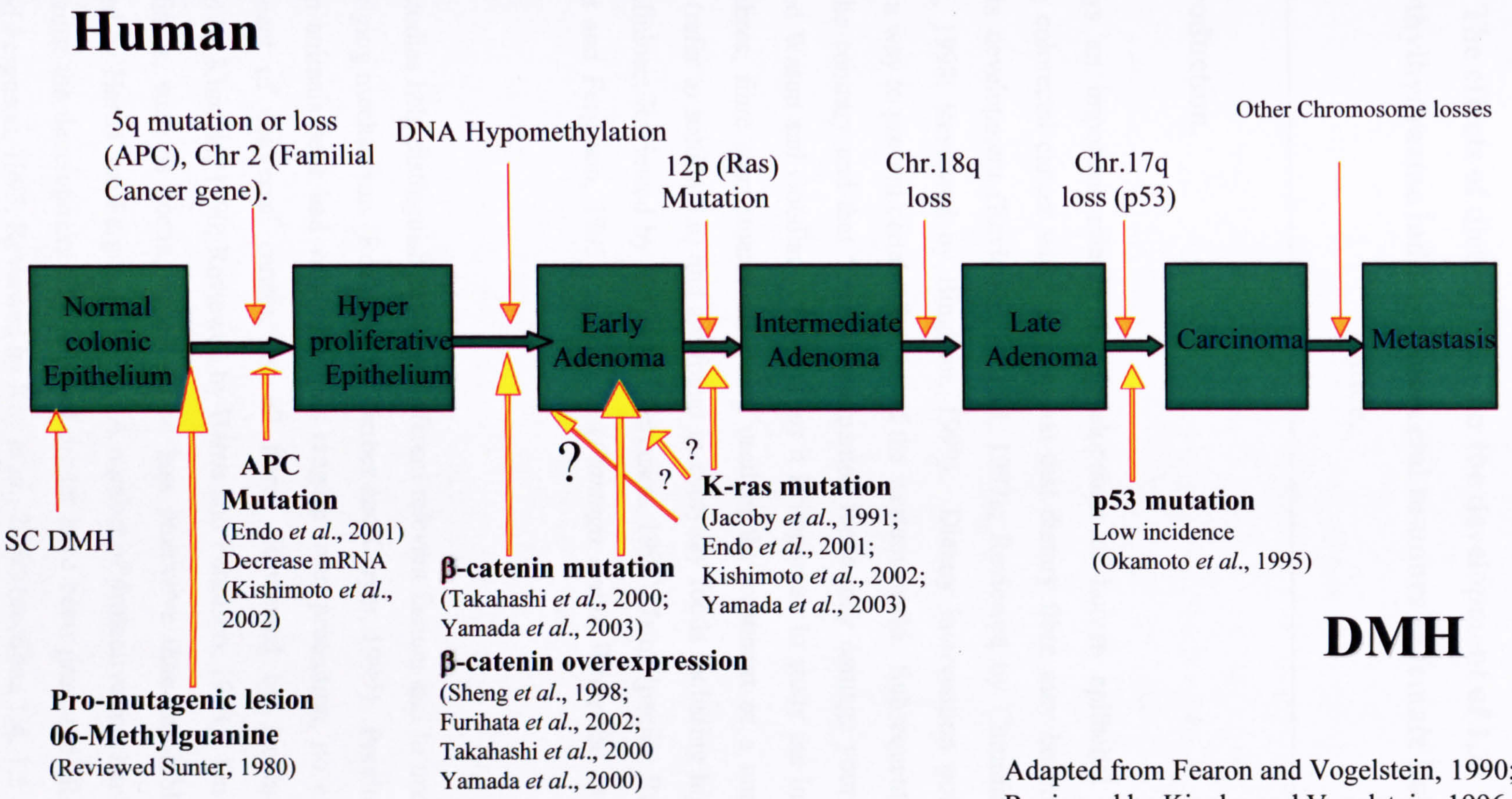
In normal human epithelium α-catenin is strongly expressed and is required for stable E-cadherin mediated cell adhesion (Shiozaki *et al.*, 1994; Imamura *et al.*, 1999). In normal human epithelium α-catenin is strongly expressed but is reduced or absent in a number of primary tumours of the oesophagus, stomach and colon (Shiozaki *et al.*, 1994). Skoudy *et al.* (1996) also reported altered α-catenin expression in human colorectal cancer. Within the DMH mouse model there were similarities with human α-catenin expression within the non-dysplastic tissues but differences within the dysplastic tissues as membrane expression was maintained and cytoplasmic localisation was increased not reduced as for humans. This could be due to the stage reached within the adenoma to carcinoma sequence, with the DMH mouse model being an early model of colon cancer development, and the fact that E-cadherin expression is maintained.

In human normal colon p120-catenin is present in the crypt and surface epithelium with cells showing reactivity in both the membrane and cytosol (Skoudy *et al.*, 1996), with staining intensity being greatest in proliferating crypt cells (Valizadeh *et al.*, 1997). Reduced expression of p120 was observed in 20% of adenomatous polyps, with loss of membranous p120 expression correlating with reduced E-cadherin expression. Decreased expression of p120 was found to correlate with the larger tumours (Skoudy *et al.*, 1996). In the DMH mouse model there are similarities in expression, the main difference being that expression is maintained within the dysplastic tissues, this could be due to the fact that E-cadherin expression is maintained within this model. Although in our study we found an increased cytoplasmic localisation of p120 in dysplastic mucosa the overall expression and the association with E-cadherin was maintained.

4.7 Conclusion

The increased cytoplasmic expression for the catenins and E-cadherin within the dysplastic tissues suggests disruption in catenin regulation. β-catenin was found to be localised within the cytoplasm and the nucleus, along with maintenance at the cell membrane, within the dysplastic tissues. Within the non-dysplastic epithelium it was found to be primarily expressed at the epithelial cell membranes. Expression patterns for α-catenin, p120, and E-cadherin were similar to β-catenin with increased membranous and cytoplasmic immunoreactivity in dysplastic tissues, although no nuclear staining was observed. Alterations in β-catenin expression and localisation in DMH mice are similar to those changes seen in human sporadic, familial and HNPCC derived colorectal tumours (Herter *et al.*, 1999; Miyaki *et al.*, 1999; Akiyama *et al.*, 2000; Fukushima *et al.*, 2001) and the MIN mouse (McEntee *et al.*, 1999) (Figure 4.10). Further observations in the mouse have revealed that the changes observed have many similarities with the morphological changes observed in human adenomatous polyposis (Reviewed by Sunter, 1980; Reviewed by Rogers and Nauss, 1985; Carter *et al.*, 1994; Moorghen *et al.*, 1998) and has been proposed as a good model for studying early changes during tumourigenesis (Chang *et al.*, 1980). Therefore this chemically induced model of carcinogenesis appears to be useful for studying the abnormalities of the cadherin/catenins pathway at the early stages of tumour development. Brown *et al.* (2001) has used this DMH model to examine the effects of non-steriodal anti-inflammatory drugs (NSAIDs) on β-catenin expression and found that NSAIDs resulted in an inhibition of β-catenin translocation.

Figure 4.10 Proposed 1,2-dimethlyhydrazine adenoma to carcinoma sequence.



Adapted from Fearon and Vogelstein, 1990;
Reviewed by Kinzler and Vogelstein, 1996

Chapter Five

The effects of dietary fibre on the development of 1,2-dimethylhydrazine induced colorectal tumours in female Balb/c mice.

5.1 Introduction.

Diet plays an important role in the development of human epithelial cancers including colorectal cancer with the proposal that dietary fibre may be protective against its development (Reviewed by Hill, 1997a; Reviewed by Cummings and Bingham, 1998; Reviewed by Bingham, 1999). Dietary intervention potentially provides a way to prevent certain diseases of the western world. Subsequent reports suggest the contrary and that 'fibre supplemented foods may damage your health' (Reviewed Wasan and Goodlad, 1996). Thus it is important to study the impact of dietary fibres; fibre supplements are being used in the treatment of a number of ailments (refer to section 3.6) and are found in everyday foods including high fibre bread (cellulose; Reviewed by Harris and Ferguson, 1993), Jams (pectin; Reviewed by Harris and Ferguson, 1993) and cereals (*Plantago ovata*; Reviewed by Leeds, 1999).

Animal studies help distinguish between different relevant factors and to understand the underlying mechanisms (Reviewed by Gerber and Corpet, 1999). Previous fibre studies in animals have had varying results ranging from protection, no effect, to enhancement of colorectal cancer, for all fibres (Reviewed by Jacobs, 1990; Reviewed by Klurfeld, 1990; Reviewed by Harris and Ferguson, 1993). On balance soluble fibres, such as pectin, appear to be less protective than insoluble fibres (Reviewed by Harris and Ferguson, 1993). A number of protective mechanisms for fibres against the development of colorectal cancer have been proposed (Reviewed Harris and Ferguson, 1993; Reviewed by Key *et al.*, 2002) (sections 1.4, 1.5 and 3.6) including stool bulking, SCFA production and changes in microflora. Although

dietary fibres are not consumed in isolation or as individual fibres it is necessary to isolate the potential effects of individual fibres.

To determine the biological effects of the fibres a number of parameters were explored. In this study colonic epithelial cell homeostasis, keeping a balance between proliferation and apoptosis was investigated by examining the effects of fibres on cell proliferation within the colonic crypt. As luminal contents are in contact with the colonic epithelium it could have profound effects on these parameters, thus affecting patterns of growth, differentiation and apoptosis (Hague *et al.*, 1995). The expression of the adhesion molecules, E-cadherin and the catenins (α-,β-,γ-) and p120 (section 1.3) were examined due to their role in growth, differentiation and apoptosis of colonic epithelium and disruption in their functions leads to colorectal cancer development. β-catenin regulation is altered in a large number of colorectal tumours, as a result of loss of function mutations in APC, gain of function mutations within β-catenin and activation of Wnt-1 (section 1.3).

5.2 Aim.

The aim of this study was to determine the effects of the dietary fibres: Ispaghula husk, pectin and cellulose on the development of 1,2-dimethylhydrazine induced colorectal tumours in female Balb/c mice.

5.3 Objectives

This model examined the effects of fibre supplements on the development of chemically (1,2-dimethylhydrazine) induced colorectal cancer.

1. The mice were examined for macroscopic nodule formation, followed by excision of these nodules.
2. Microscopic confirmation of nodules as adenoma/adenocarcinomas using histological examination with further microscopic examination for microadenoma numbers (adenoma prelesions).
3. Tumour number, size and distribution were determined.

The following effects of these dietary fibres on epithelial cells within the colonic tissues were examined.

1. Proliferation was examined using flash-labelling with bromodeoxyuridine (BrdU), and
2. the expression of E-cadherin/catenin complex was examined within the non-dysplastic and dysplastic tissues of the colon by immunohistochemistry.

5.4 Study design.

The 1,2-dimethylhydrazine model using female Balb/c mice examined the effects of fibre supplements on tumour development. The mice were received at two weeks of age and acclimatised for 1 week on normal RM 1 diet (section 2.2). The mice were fed on sample diets for 1 week prior to administration of 1,2-dimethylhydrazine injections. The experimental diets were initially fed in pellet form but were subsequently abandoned for a powdered form as pelletting of these diets made them inedible. New ideas have recently come to light involving making biscuits so that food intake can be monitored (Iain Brownlee, Newcastle University, Personal communication). The mice were continuously monitored for weight changes (Fig 5.1) and initially for water and food intake. Food intake monitoring was abandoned due to the nature of the food, thus the mice were allowed access *ad libitum*.

Tissues were collected, fixed and examined for nodule number, size and distribution (section 2.2.2). Nodules (Fig 2.2 section 2.2), were excised along with surrounding non-dysplastic tissue and placed in fresh formalin, performed by Dr M. Moorghen, Dr J. Gupta and E. Tucker. These tissues were then processed, dehydrated in alcohol and histoclear and embedded in paraffin by J. Baker, J. Code and E. Tucker. J. Baker and J. Code then stained serial sections with haematoxylin and eosin for histological examination of the macroscopic nodules carried out by E. Tucker and Professor M. Pignatelli, E. Tucker and Dr J. Gupta performed microadenoma analysis.

E-cadherin and catenin expression was determined in 20 tumours from each of the fibre supplemented groups basal, ispaghula husk, pectin and cellulose diets. These sections were scored (2.3.2) and the four groups compared.

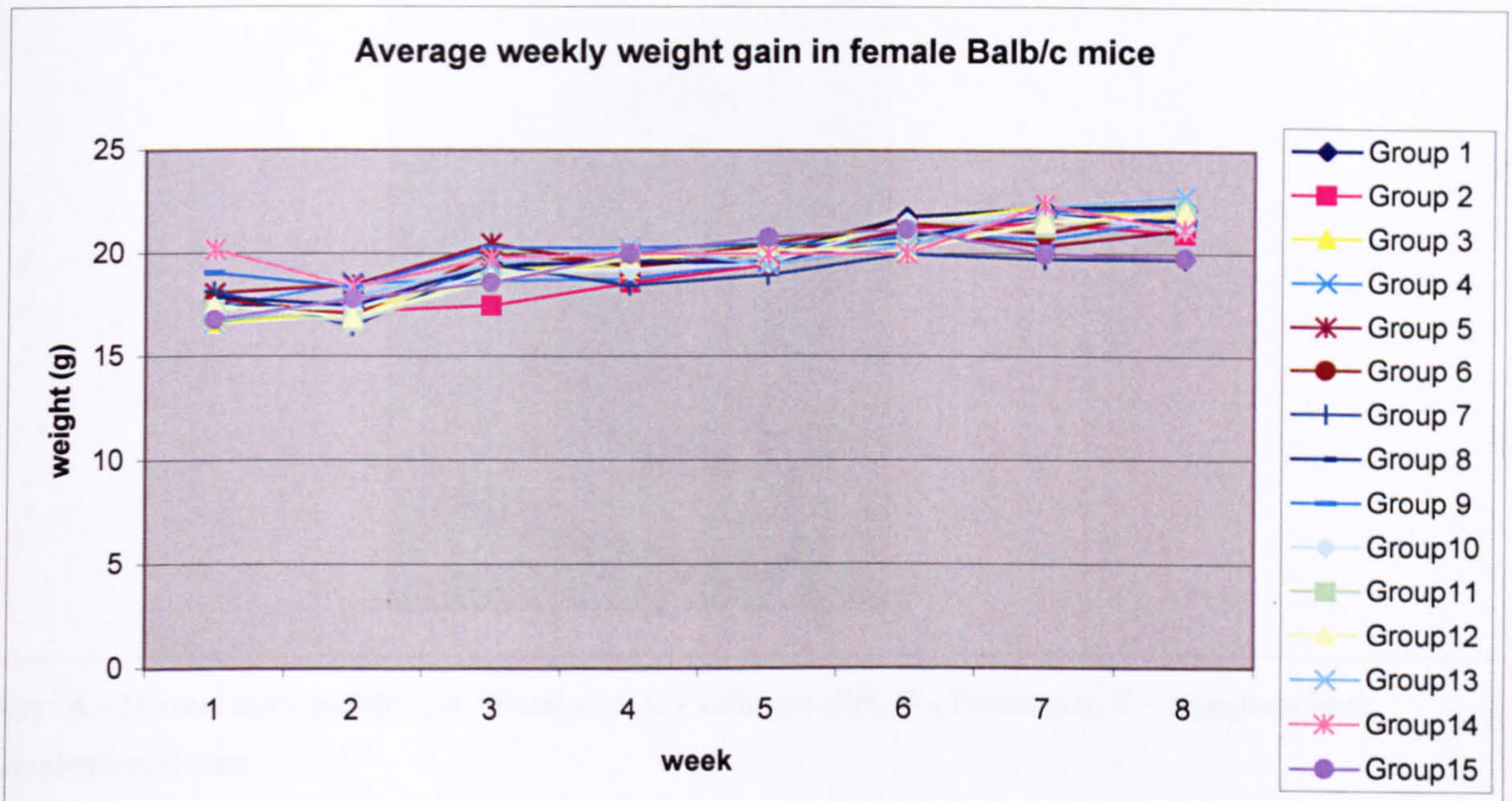
Bromodeoxyuridine (BrdU) detection via immunohistochemical methods is a powerful tool to study the cytokinetics of normal and neoplastic cells. Bromodeoxyuridine (BrdU) is a thymidine analogue that is specifically incorporated into DNA during S-phase of cell cycle and thus is expressed in the nuclei of proliferating cells (Coulter website; Chemicon International website) and at low concentrations will not block cell division (Ulrike Heberlein website). *In vivo* labelling of tumour cells with BrdU and its subsequent detection of incorporation with specific anti-BrdU monoclonal antibodies provides an accurate and comprehensive method for quantifying DNA synthesis (Chemicon International website). Thus to study the effects of these fibres on the proliferation of the colonic epithelium, mice were injected with Bromodeoxyuridine (BrdU) intraperitoneal (IP) one hour prior to sacrifice. The BrdU labelling index was determined (section 2.2.4), to achieve this the left side of 50 (where possible) relatively normal hyperplastic crypts were counted for each mouse.

5.5 Results.

5.5.1 The feeding study was monitored in terms of weight, faecal matter and dietary constituents.

During the duration of the experiment the weight (Figure 5.1) was continuously monitored with food intake being allowed *ad libitum*. Observations of the effects of fibre on defecation (Figure 5.2) revealed that the basal diet (fibre deficient) resulted in faecal matter that were similar in colour to the control mice but the faecal matter appeared to be more solid and this was accompanied by reduction in volume (although this was not specifically measured). Cellulose resulted in faecal pellets that were well formed, very similar to the control, although were white in colour elucidating the role of crude fibre in forming faeces (cellulose is white). Pectin resulted in softer, darker and stickier faeces with ispaghula resulting in long string like faeces, which could have been the result of mucilage formation from the ispaghula husk. The level of fibre within the basal diet, along with the energy content was examined (Table 5.1) by special dietary services (SDS, Witham, Essex). The basal diet was found to have a very low level of crude fibre and NSP, so represented essentially a fibre free diet. The constituents of the basal diet and the fibre supplemented diets (Table 5.2) were also provided by special dietary services (SDS, Witham, Essex) and revealed that there were slight differences between the basal diets and the fibre supplemented diets. The basal diet contained a higher level of sucrose and rice starch than the fibre supplemented basal diets, this was to compensate for the lack of fibre within this diet. The basal diets supplemented with fibres were shown to consist of 14.5% of each fibre.

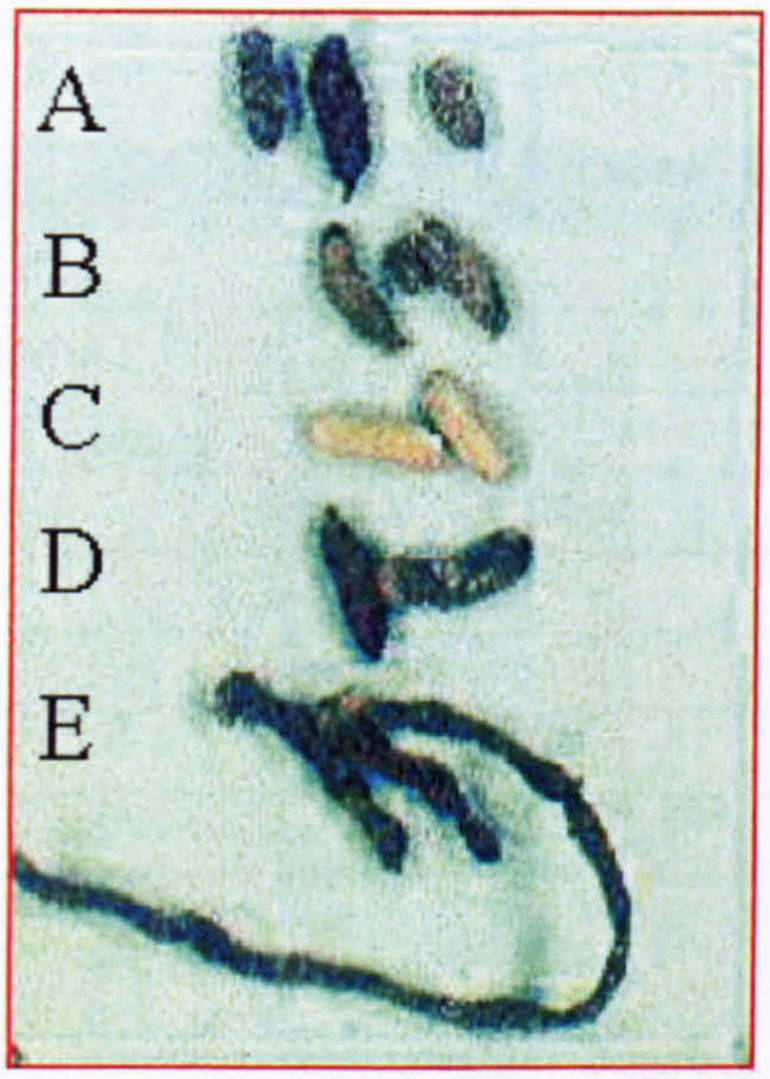
Fig 5.1 Average weight gain in female Balb/c mice fed various fibre supplemented diets and injected with 1,2-dimethylhydrazine mice over the first eight weeks.



Key: **Group 1** wild type mice fed on a basal diet, **Groups 2,3 and 4** DMH treated mice fed on a ispaghula husk supplemented diet, **Groups 5,6 and 7** and 15 DMH treated mice fed on a pectin supplemented diet, **Groups 8,9 and 10** DMH treated mice fed on a cellulose supplemented diet, **Groups 11,12 and 13** DMH treated mice fed on a basal diet and **Group 14** fed on RM1.

A graphic representation of the average increase in weight for the Balb/c mice groups fed on different fibre supplemented diets over the first eight week period. The graph revealed an initial decrease in weight at week two this was probably due to acclimatisation to the new surroundings and diets. The graph showed from week two the average weight of the mice generally increased over time.

Fig 5.2 Faecal matter obtained from female Balb/c mice fed on the different diets, basal and basal diet supplemented with ispaghula husk, pectin and cellulose.



Key: **A** - Normal mice on RM1, **B** - Basal diet, **C** - cellulose diet, **D** - Pectin diet, **E** - Ispaghula husk supplemented diets.

A pictorial representation of the changes observed in faecal material collected from the Balb/c mice fed on the diets, RM1, Basal, and the fibre supplemented basal diets, ispaghula husk, pectin and cellulose. The effects of the individual fibre on faecal defecation was clearly seen, those on basal diet (**B**) (fibre deficient) resulted in faecal matter that were similar in size and colour to the control mice fed on a standard RM1 diet (**A**), although appeared to be harder and less frequently produced, although this was not measured (SDS, Witham Essex). Cellulose (**C**) resulted in faecal matter similar in size and constitution to the control mice but reflected the white colour attributable to the cellulose. Pectin (**D**) resulted in darker faecal matter and from observations they were also sticky in nature with ispaghula husk (**E**) resulting in long string like faeces a possible result of the mucilage form of ispaghula husk when it is in contact with water.

Table 5.1 A) Fibre content B) Energy content of the Basal diet (Fibre Deficient diet) as determined by the manufacture special dietary services (SDS, Witham, Essex).

A.

Fibre content		%
Crude Fibre		0.38
Total Dietary Fibre		1.13
	Pectin	-
	Hemicellulose	-
	Cellulose	-
	Lignin	-
Starches		33.01
Sugars		35.55

B.

	MJ/Kg
Gross Energy	16.41
Digestible Energy	15.75
Metabolised Energy	14.32

Table **A** showed that the basal diet contained a very low level of crude fibre (lignin) and dietary fibre (NSP) but contained a high level of starch and sugar contents, 33.01% and 35.55% respectively. Table **B** represents the gross energy (total), digestible energy (gross energy of the food less the gross energy within the faecal matter) and the metabolised energy (digestible energy of the food less the energy used to consume and digest the food) in MJ where 1MJ is equivalent to 239.23 calories.

Table 5.2 Formulation of the different fibre supplemented diets; basal (FDD), ispaghula husk, pectin and cellulose; as determined by the manufacture special dietary services (SDS, Witham, Essex).

		Diet (%)			
	Ingredients	Basal(Fibre free)	Ispaghula	Pectin	Cellulose
Proteins	Ca caseinate	20	20	20	20
Energy	Sucrose	32.5	25.5	25.5	25.5
	Soya Oil	5	5	5	5
Carbohydrate	Rice Starch	37.5	32.5	32.5	32.5
Fibre	Ispaghula	0	14.5	0	0
	Pectin	0	0	14.5	0
	Cellulose	0	0	0	14.5
Supplement	Vit/Min	5	5	5	5

The diets manufactured by SDS varied in their sucrose, rice starch and fibre contents. Within the fibre supplemented diets the fibre supplements accounted for 14.5% of the diet. In the basal diet, where no fibre was added, there was an accompanied increase in both sucrose (from 25.5% to 32.5%) and rice starch (32.5% to 37.5%) levels. The protein, soya oil and vitamin and mineral supplement content were equal within the individual diets.

5.5.2 Macroscopic analysis of nodules induced by the administration of 1,2-dimethylhydrazine within the colonic tissues of Balb/c mice treated with various fibre supplemented diets.

Figures 5.3, 5.4, 5.5, and 5.6 represent the macroscopic findings from this study. The macroscopic results revealed that those mice fed on a basal diet supplemented with 14.5% pectin had the greatest macroscopic nodule incidence (fig.5.3), although the overall difference between diets was not statistically significantly different (*CHI-squared test p=0.203), nodule number (fig.5.4) and total nodule burden (fig.5.5). For all the fibre fed groups; ispaghula husk, pectin and cellulose, and the fibre deficient group, the majority of these nodules found were in the distal colon (fig.5.6). Interestingly, although cellulose did not result in an increase in nodule number, compared to the control, there was an increase in nodule size suggesting that cellulose enhanced the progression of nodule development, although there was no significant difference in tumour burden from the control. These macroscopic findings suggested that the addition of pectin to the basal diet increased nodule initiation, development and progression within the DMH mouse model.

5.5.3 Microscopic analysis of tumours induced by the administration of 1,2-dimethylhydrazine within the colonic tissues of Balb/c mice treated with various fibre supplemented diets.

Histological confirmation of nodules identified macroscopically was achieved using light microscopy of tissue sections stained with haematoxylin and eosin (H&E). The majority of nodules were confirmed as being dysplastic epithelium 67.6%, 75%, 87.9% and 59.6% for the basal, ispaghula, pectin and cellulose diets respectively (figure 5.7). The remainder were either lymphoid aggregates, mucosal folds or of an unknown origin (figure 5.8). The histological confirmation also revealed that the pectin supplemented

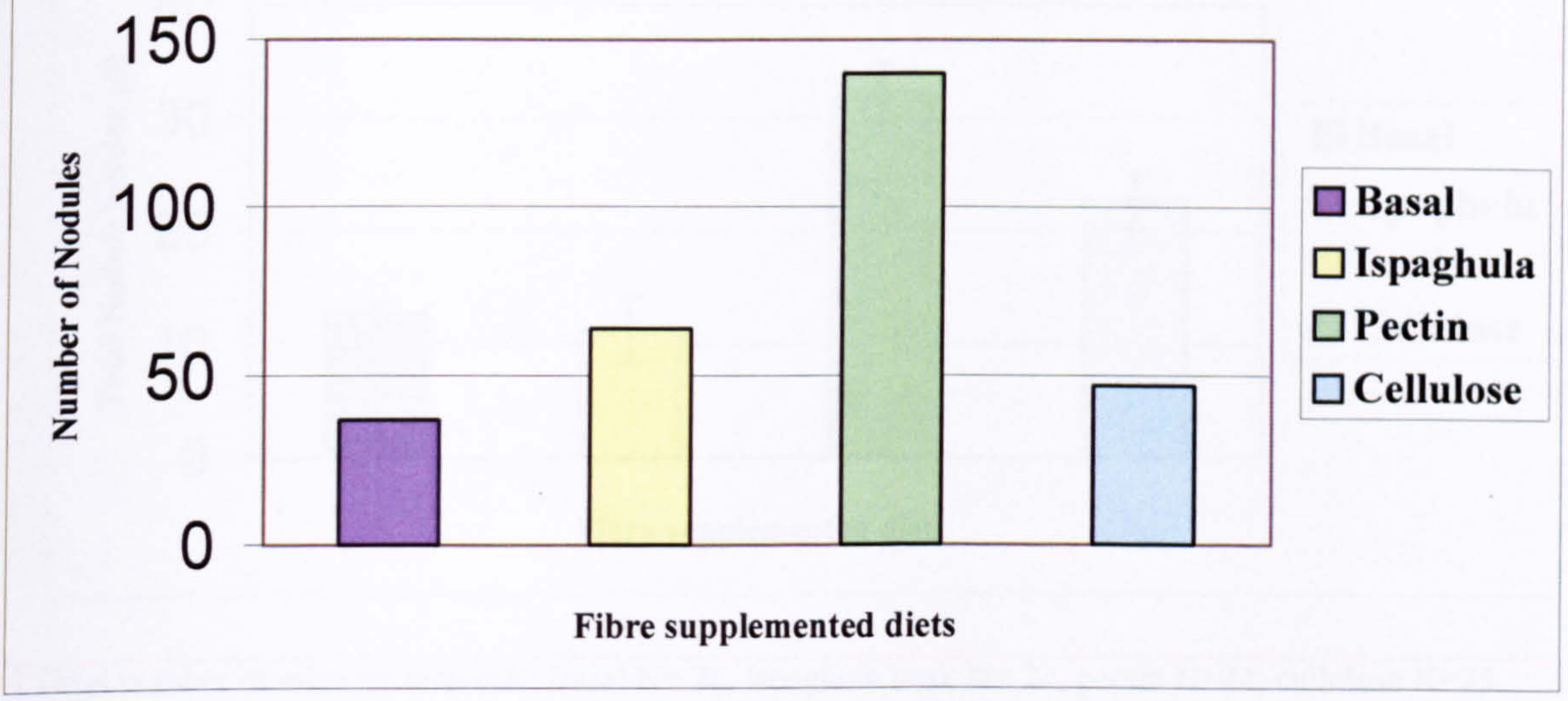
Figure 5.3 Incidence of nodule development in 1,2-dimethylhydrazine female Balb/c mice fed on various diets; basal and the basal diet supplemented either with ispaghula husk, pectin or cellulose.

No. of Mice	Basal	Ispaghula	Pectin	Cellulose
Nodule	16	16	21	17
No Nodule	10	8	3	8
Total No. of mice	26	24	24	25
% with Nodules	61.5	66.7	87.5	68

Percentage of female Balb/C mice that developed macroscopic 1,2-dimethylhydrazine induced nodules within the different dietary groups. A higher incidence of nodule development was observed within the pectin supplemented group when compared to the basal (control) group, although there was no statistically significant overall difference in nodule incidence between the diets (CHI-squared test p=0.203*).

* statistical analysis performed by J. Sykes, ReckittBenckiser Healthcare Limited, Hull.

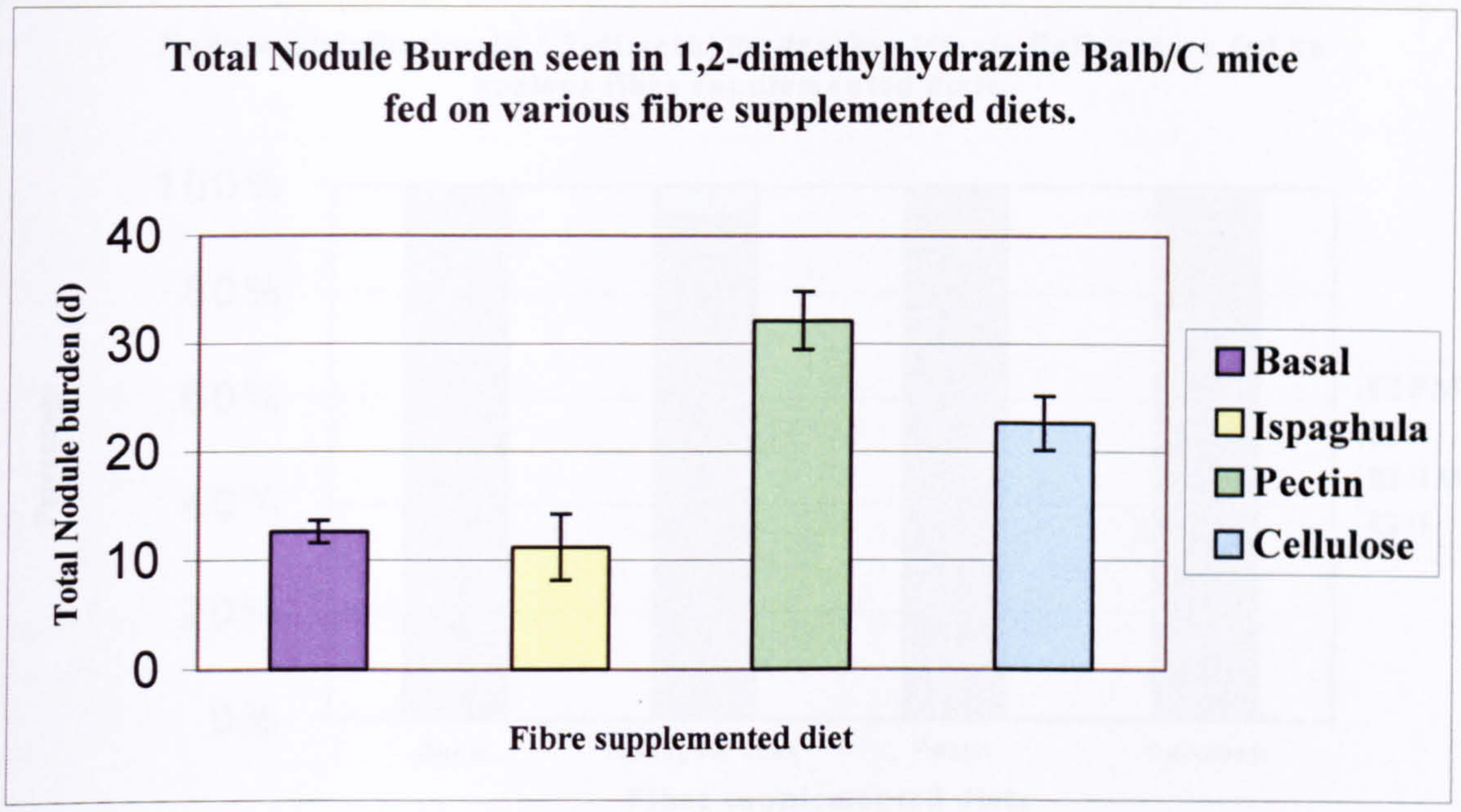
Fig 5.4 Total number of macroscopic nodules found within the colons of 1,2-dimethylhydrazine treated female Balb/c mice on various dietary fibre supplemented diets.



Total number of mice on each diet; Basal N= 26, ispaghula husk N= 24, pectin N=24, cellulose N=25.

This graphic representation of total nodule number revealed that the pectin supplemented basal diet resulted in an increase in the number of macroscopic nodules formed by the carcinogen 1,2-dimthylhydrazine in female Balb/c mice. The graph revealed ispaghula husk supplemented basal diet also resulted in a slight increase in the number of macroscopic nodules formed by 1,2-dimethylhydrazine in comparison to the diet supplemented with cellulose and the basal diet that contained no additional fibre. Thus the basal diet and the cellulose supplemented basal diet resulted in the lowest number of tumours. Thus the fibres appeared to influence nodule development to different extents, with the fibre free diet (basal) diet resulting in the lowest number of nodules.

Figure 5.5 Total macroscopic nodule burden formed within the colons of 1,2-dimethylhydrazine treated female Balb/c mice on various dietary fibre supplemented diets.

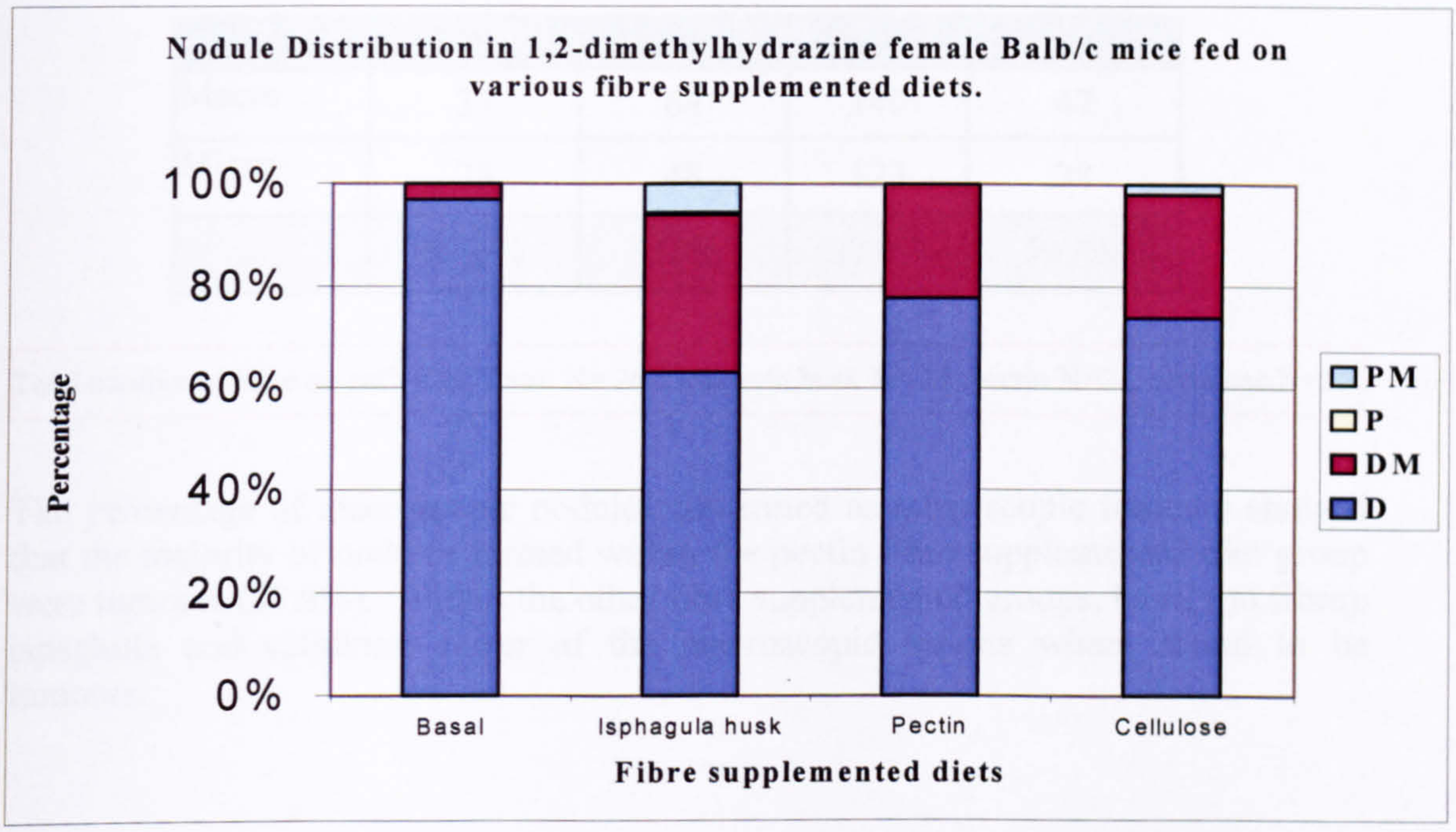


Total number of mice on each diet; Basal N= 26, ispaghula husk N= 24, pectin N=24, cellulose N=25.

The total nodule burden found in each mouse in each of the fibre-supplemented groups was calculated using the formula explained in section 2.4 (Page 14). The graph shows that both the pectin and cellulose fibre supplemented basal diets resulted in a greater tumour burden, with pectin being statistically significantly different to the control. (*Chi-square – $p < 0.001$ – evidence of overall difference between treatments; ANOVA – $p < 0.0001$ – evidence of an overall difference between treatments; Wilcoxon Rank Sums – $p = 0.0004$ again proving an overall difference). Cellulose, which was also shown to increase nodule burden was found not to be statistically significantly different from control.

* statistical analysis performed by J. Sykes, ReckittBenckiser Healthcare Limited, Hull.

Figure 5.6 Represents the nodule distribution within the colon in 1,2-dimethylhydrazine female Balb/c mice fed on various fibre-supplemented diets.



Total number of mice on each diet; Basal N= 26, ispaghula husk N= 24, pectin N=24, cellulose N=25.

Nodule distribution was determined, locating them in one of four quadrants; D = Distal, DM = Distal Middle, P = Proximal, PM = Proximal Middle (fig 2.1, section 2.2). The graph shows that for all groups the majority of nodules were found within the distal colon. Nodules of the proximal region were also found in the cellulose, pectin and ispaghula husk fibre groups.

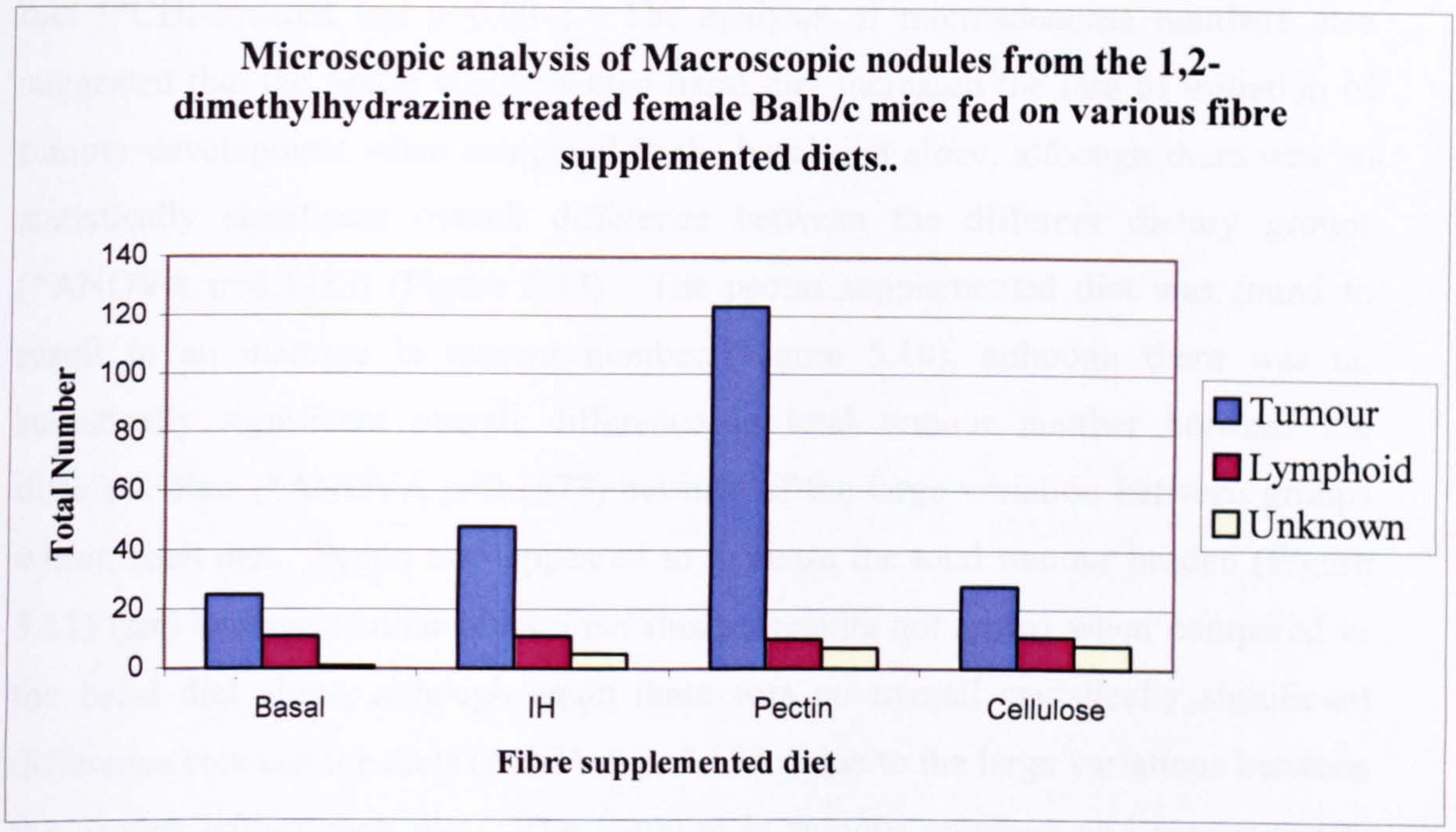
Figure 5.7 Percentage of macroscopic nodules confirmed as microscopic tumours within the 1,2-dimethylhydrazine female Balb/c mice fed on various fibre supplemented diets.

Total	Basal	Ispaghula	Pectin	Cellulose
Macro	37	64	140	47
Micro	25	48	123	28
	67.6%	75%	87.9%	59.6%

Total number of mice on each diet; Basal N= 26, ispaghula husk N= 24, pectin N=24, cellulose N=25.

The percentage of macroscopic nodules confirmed as microscopic tumours showed that the majority of nodules formed within the pectin fibre supplemented diet group were tumours (87.9%). Within the other fibre supplemented groups, basal (no fibre), ispaghula and cellulose, fewer of the macroscopic lesions where found to be tumours.

Figure 5.8 Classification of the macroscopic nodules formed in the 1,2-dimethylhydrazine female Balb/c mice on various fibre supplemented diets.



Total number of mice on each diet; Basal N= 26, ispaghula husk N= 24, pectin N=24, cellulose N=25.

Classification of macroscopic nodules confirmed that the majority were tumours in all fibre-supplemented groups, particularly the pectin-supplemented group. The remaining macroscopic nodules were found to be lymphoid aggregates or were of unknown origin possibly mucosal folds.

basal diet resulted in the greatest incidence of tumour development within the DMH mice (figure 5.9). Tumour incidence was statistically significantly higher for those mice fed on the diet supplemented pectin when compared with those fed on the basal diet (*CHI-squared test p=0.004). The analysis of microadenoma numbers also suggested that the pectin supplemented basal diet increased the rate of initiation of tumour development when compared to the basal diet alone, although there was no statistically significant overall difference between the different dietary groups (*ANOVA p=0.1483) (Figure 5.13). The pectin supplemented diet was found to result in an increase in tumour number (Figure 5.10), although there was no statistically significant overall difference in total tumour number between the different diets (*ANOVA p=0.1577) because of the large variation between groups within each diet. Pectin also appeared to increase the total tumour burden (Figure 5.11) (and average tumour burden per mouse, results not sown) when compared to the basal diet alone, although again there was no overall statistically significant difference between the diets (ANOVA p=0.1506) due to the large variations between the groups within each diet. The increase in tumour numbers and size suggests pectin also increased the rate of tumour development. The ispaghula husk supplemented basal diet was shown to result in an increase in tumour number, but this was not significantly different to the basal diet alone (figure 5.10). The ispaghula husk supplemented diet appeared to have a similar tumour burden to the basal diet (figure 5.11), thus it appeared that ispaghula husk resulted in a greater tumour initiation but that these tumours developed at a slower rate. The cellulose supplemented basal diet appeared to result in a similar number of tumours to the basal diet alone (figure 5.10), although the cellulose diet resulted in a larger total tumour burden (figure 5.11), suggesting that cellulose may have had little effect on tumour initiation but those tumours that did develop were larger in size. The majority of tumours were found in the distal colon (figure 5.12) only the pectin supplemented dietary group had any tumours confirmed in the proximal and proximal middle sections of the colon (figure 2.1, section 2.2). These results suggest that the type of fibre affected the rate of tumour initiation and development of 1,2-dimethylhydrazine induced colonic tumours.

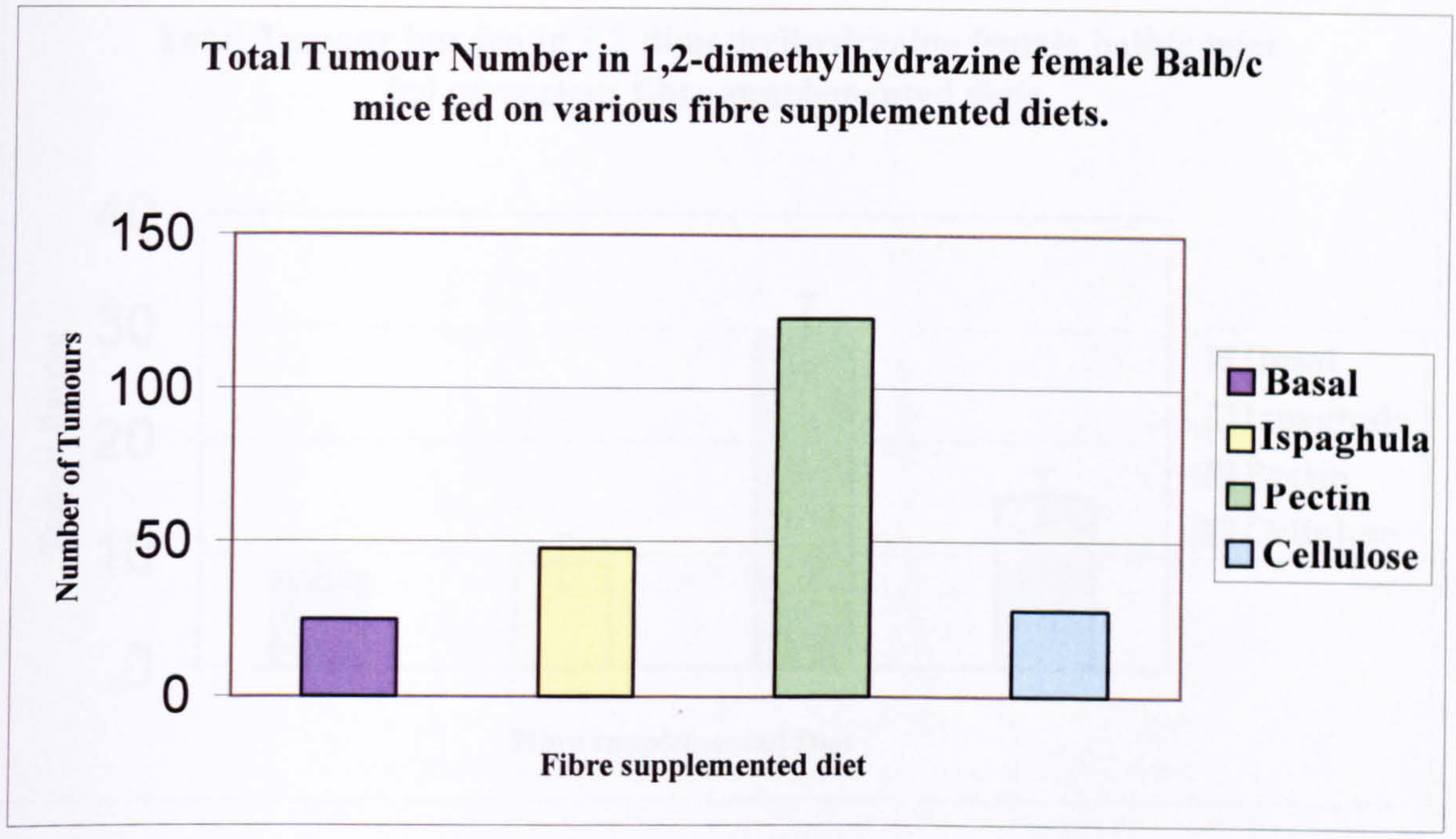
Figure 5.9 Percentage of female Balb/c mice that developed 1,2-dimethylhydrazine induced tumours fed on various fibre supplemented diets.

No. of mice	Basal	Ispaghula	Pectin	Cellulose
Tumours	10	15	19	12
No Tumours	16	9	5	13
Total	26	24	24	25
% with tumours	38.5	62.5	79.2	41.4

This table revealed that a higher incidence of tumour development occurred within the pectin supplemented group after induction by 1,2-dimethylhydrazine when compared to the basal (control) group (and the other fibre supplemented groups). There was a statistically significant overall difference in tumour incidence between the different fibre supplemented diets (*CHI-squared test p=0.023). Tumour incidence was statistically significantly higher for those mice fed on the diet supplemented pectin when compared with those fed on the basal diet (*CHI-squared test p=0.004). There was no statistically significant difference in tumour incidence between the ispaghula husk and basal diet (*CHI-squared test p=0.089). There was no statistically significant difference in tumour incidence between the cellulose and basal diet (*CHI-squared test p=0.492).

***Statistical analysis performed by J. Sykes, ReckittBenckiser Healthcare Ltd, Hull, UK**

Figure 5.10 Total number of tumours induced by 1,2-dimethylhydrazine in female Balb/c mice on various fibre-supplemented diets.

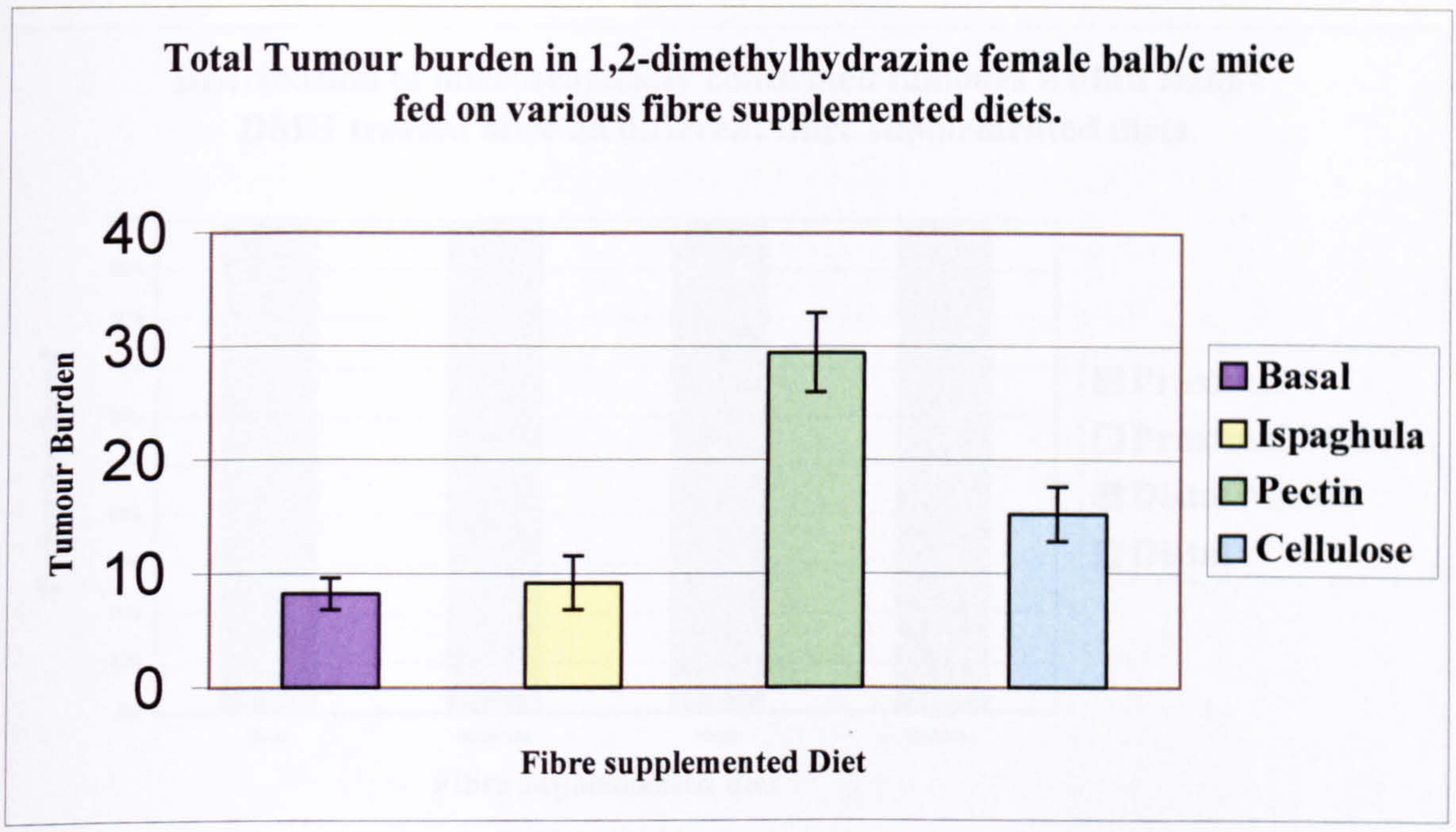


Total number of mice on each diet; Basal N= 26, ispaghula husk N= 24, pectin N=24, cellulose N=25.

The total number of tumours induced by 1,2-dimethylhydrazine in female Balb/c mice appeared to be greater in those mice fed a pectin supplemented basal diet. Ispaghula husk supplementation of the basal diet also appeared to promote tumour development, but to a lesser extent than that of pectin, when compared to the basal diet findings. Cellulose supplementation of the basal diet appeared to have little effect on tumour development, resulting in a similar number of tumours as the basal diet alone. There was no statistically significant overall difference in total tumour number between the different diets (*ANOVA p=0.1577) because of the large variation between groups treated with the same dietary fibre supplement diet.

*Statistical analysis performed by J. Sykes, ReckittBenckiser Healthcare Ltd, Hull, UK

Figure 5.11 Total tumour burden found in 1,2-dimethylhydrazine treated female Balb/c mice on various fibre-supplemented diets.

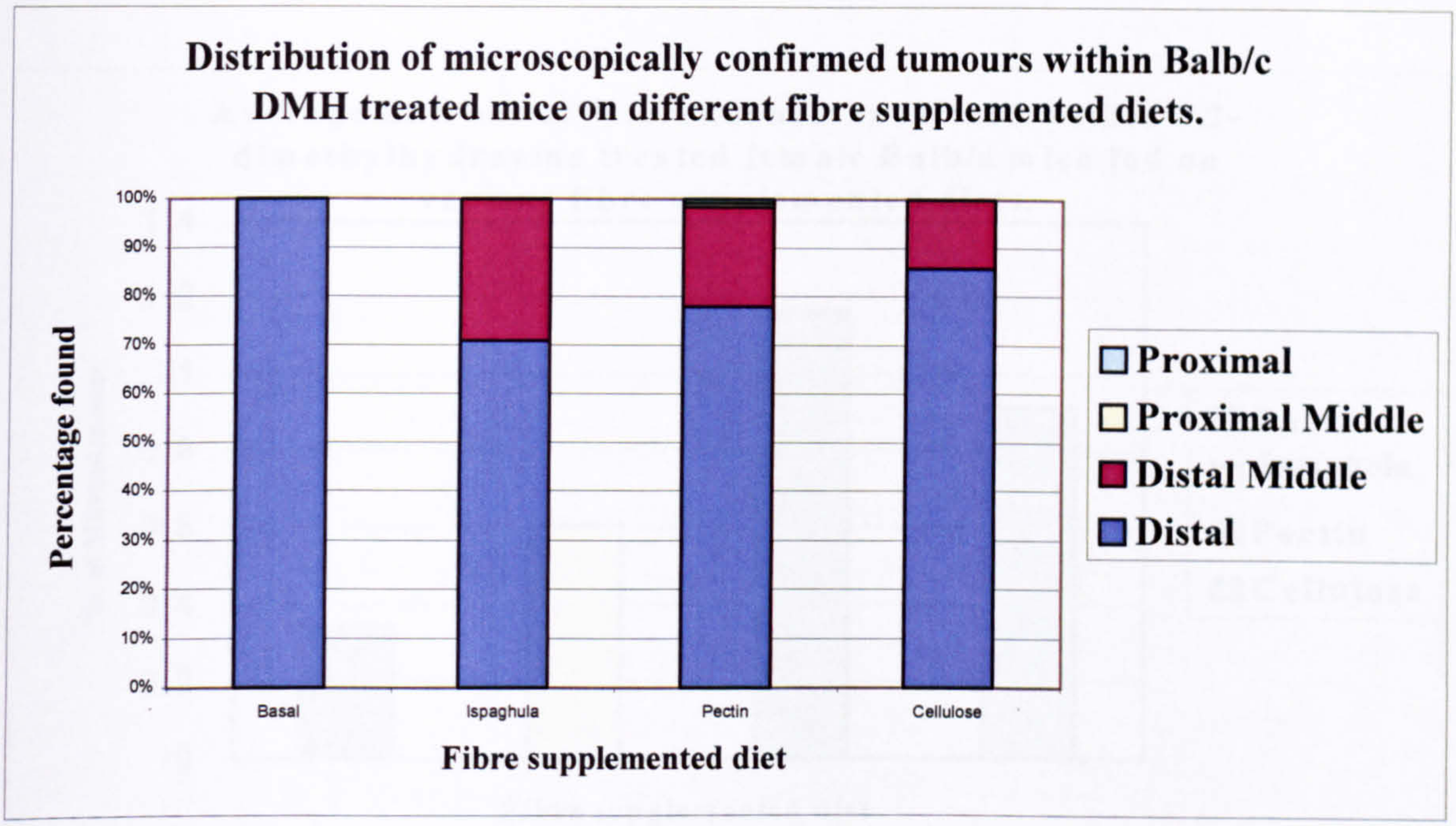


Total number of mice on each diet; Basal N= 26, ispaghula husk N= 24, pectin N=24, cellulose N=25.

The pectin supplemented basal diet increased tumour burden within the colon when compared to the other fibre groups. This suggested that pectin not only increased the number of tumours but also increased the size (as suggested by the average tumour burden per mouse, results not shown). Cellulose supplementation of the basal diet also revealed a greater tumour burden, this is interesting as the number of tumours formed were relatively small suggesting that although fewer tumours were formed they were bigger in size. There was no statistically significant overall difference in tumour burden between the different diets (*ANOVA p=0.1506) because of the large variation between groups treated with the same dietary fibre supplemented diet.

*Statistical analysis performed by J. Sykes, ReckittBenckiser Healthcare Ltd, Hull, UK

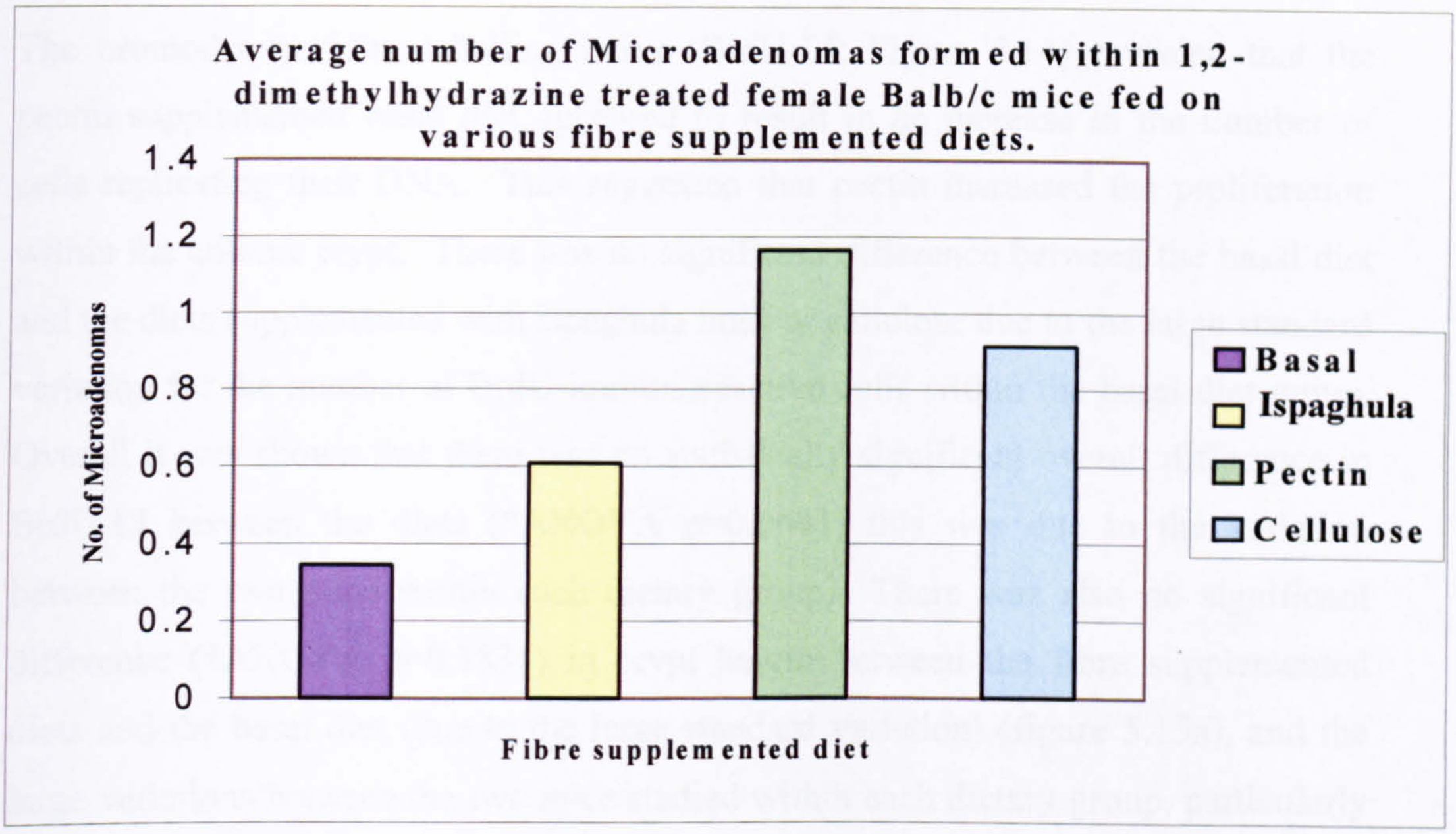
Figure 5.12 Tumour distribution within 1,2-dimethylhydrazine treated female Blab/c mice fed on various fibre-supplemented diets.



Total number of mice on each diet; Basal N= 26, ispaghula husk N= 24, pectin N=24, cellulose N=25.

Tumour distribution within 1,2-dimethylhydrazine treated mice revealed that the majority of the tumours formed, within all fibre supplemented groups, were primarily within the distal colon. Only those mice fed on the pectin supplemented basal diet had any tumours confirmed in the proximal or proximal middle sections of the colon.

Figure 5.13 Average microadenoma number in 1,2-dimethylhydrazine treated female Balb/c mice fed on different fibre supplemented diets.



Total number of mice on each diet; Basal N= 26, ispaghula husk N= 23, pectin N=24, cellulose N=24.

The average number of microadenomas formed, prelesions to adenoma development, was greatest within the pectin supplemented basal diet, followed by the cellulose supplemented basal diet, which was interesting as cellulose had the lowest number of tumours. The basal diet had the lowest number of microadenomas. There was no statistically significant overall difference in microadenoma number between the different diets (*ANOVA p=0.1483) because of the large variation between groups treated with the same dietary fibre supplemented diet.

*Statistical analysis performed by J. Sykes, ReckittBenckiser Healthcare Ltd, Hull, UK

5.5.4 Addition of dietary fibres to the basal diet increased the number of cells undergoing DNA replication suggesting an increase in cell proliferation within the crypt.

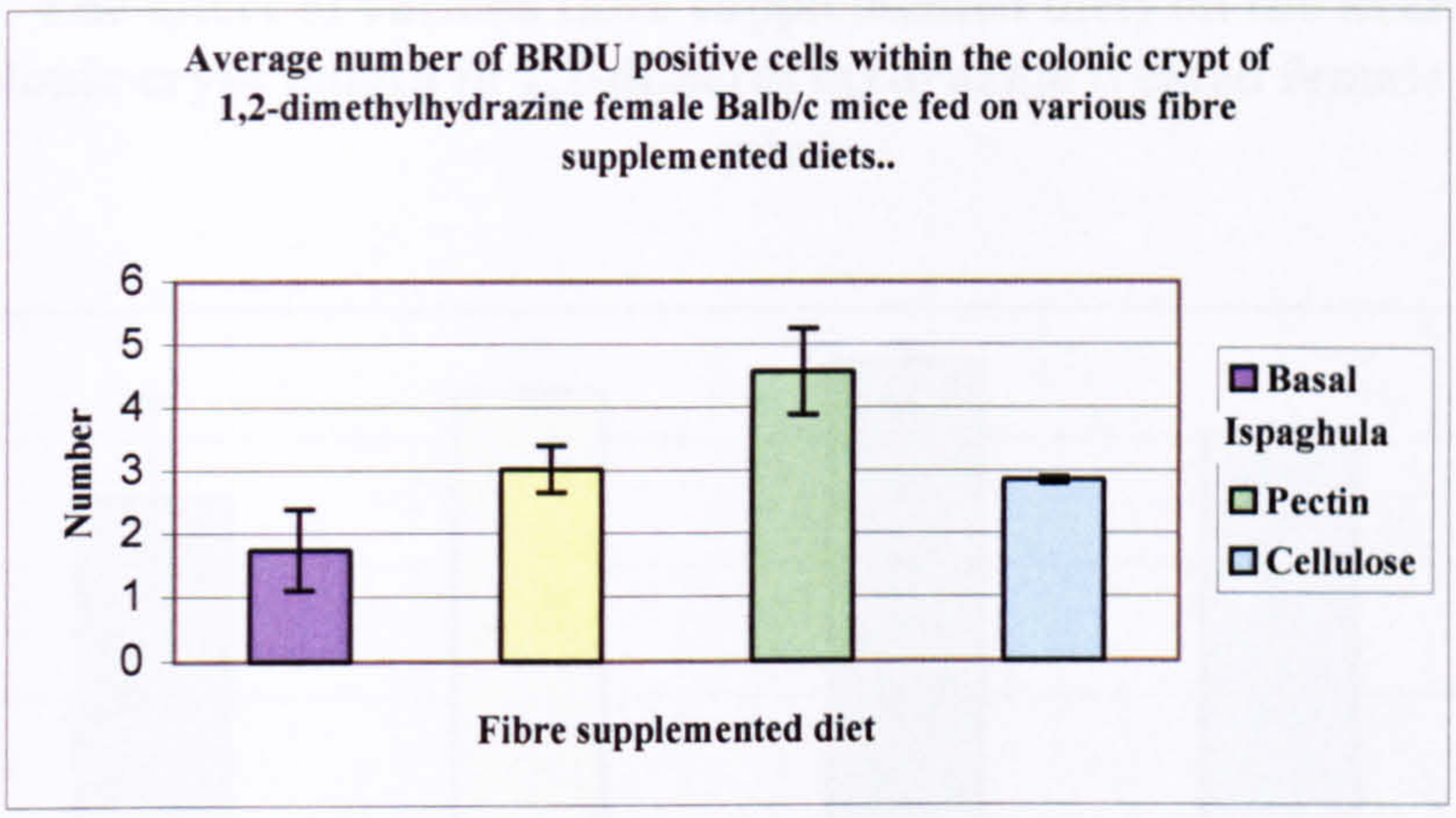
The bromodeoxyuridine-labelling index (BrdU-LI; Figure 5.14) revealed that the pectin supplemented basal diet appeared to result in an increase in the number of cells replicating their DNA. This suggested that pectin increased the proliferation within the colonic crypt. There was no significant difference between the basal diet and the diets supplemented with ispaghula husk or cellulose due to the large standard variation for the number of BrdU immunoreactive cells within the basal diet group. Overall it was shown that there was no statistically significant overall difference in BrdU-LI between the diets (*ANOVA p=0.0641) this was due to the variation between the two mice within each dietary group. There was also no significant difference (*ANOVA p=0.1835) in crypt length between the fibre supplemented diets and the basal diet (due to the large standard variation) (figure 5.15a), and the large variations between the two mice studied within each dietary group, particularly between those mice on the basal diet. The increase, although not significant, in proliferation by the pectin group together with the finding that there were no significant difference in crypt length may indicate that there is an alteration in the level of apoptosis within the crypt.

5.5.5 The basal diet that was deficient in fibre resulted in the lowest tumour incidence, tumour number and tumour burden induced by 1,2-dimethylhydrazine.

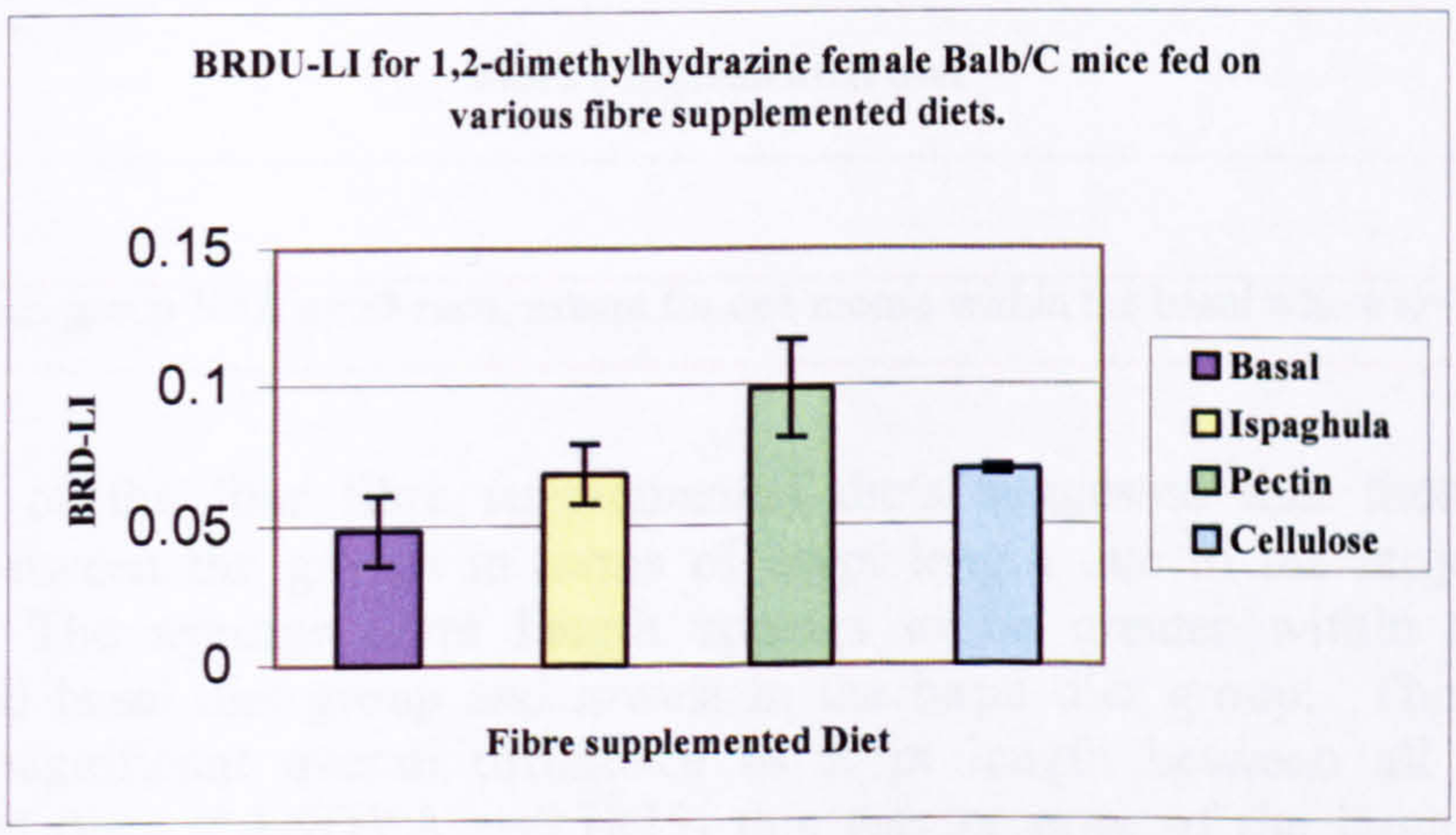
The DMH mice fed on the basal diet, a diet deficient of fibre, resulted in the lowest tumour incidence (figure 5.9 and figure 5.13), tumour number (figure 5.10) and total tumour burden (figure 5.11). There were some discrepancy as to whether the basal diet was strictly fibre deficient as the fibre was replaced with rice starch. Rice starch may contain levels of resistant starch although the level would be expected to be low.

Figure 5.14 Effects of various fibre supplemented diets on the number of colonic cells in S phase as determined by flash-labelling with BrdU within 1,2-dimethylhydrazine treated female Balb/c mice.

A. Average number of bromodeoxyuridine positive cells



B. Bromodeoxyuridine labelling index

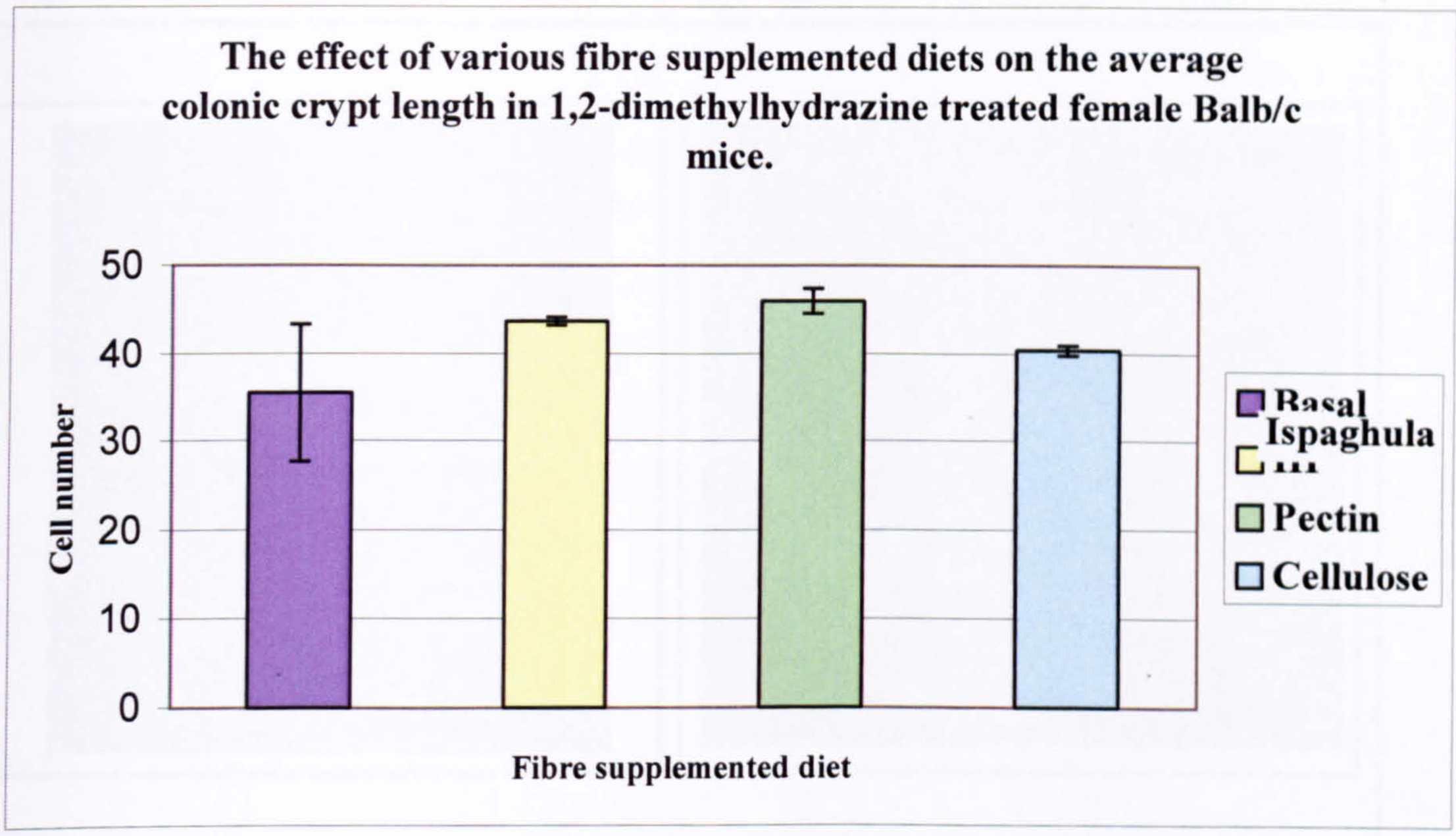


For each group N=2, n=50 each, except for one mouse within the basal where n=36

The average number of (A) BrdU positive cells and the (B) BrdU labelling index was greatest in the pectin supplemented basal diet, suggesting that pectin resulted in an increase in the number of cells in S phase, as determined by flash-labelling with bromodeoxyuridine. There was no statistically significant overall difference in BrdU-LI between the diets (*ANOVA p=0.0641) due to the variation between the two mice, particularly those fed the basal diet.

*Statistical analysis performed by J. Sykes, ReckittBenckiser Healthcare Ltd, Hull, UK

Figure 5.15a The effects of various fibre-supplemented diets on the average colonic crypt length in 1,2-dimethylhydrazine treated female Balb/c mice.

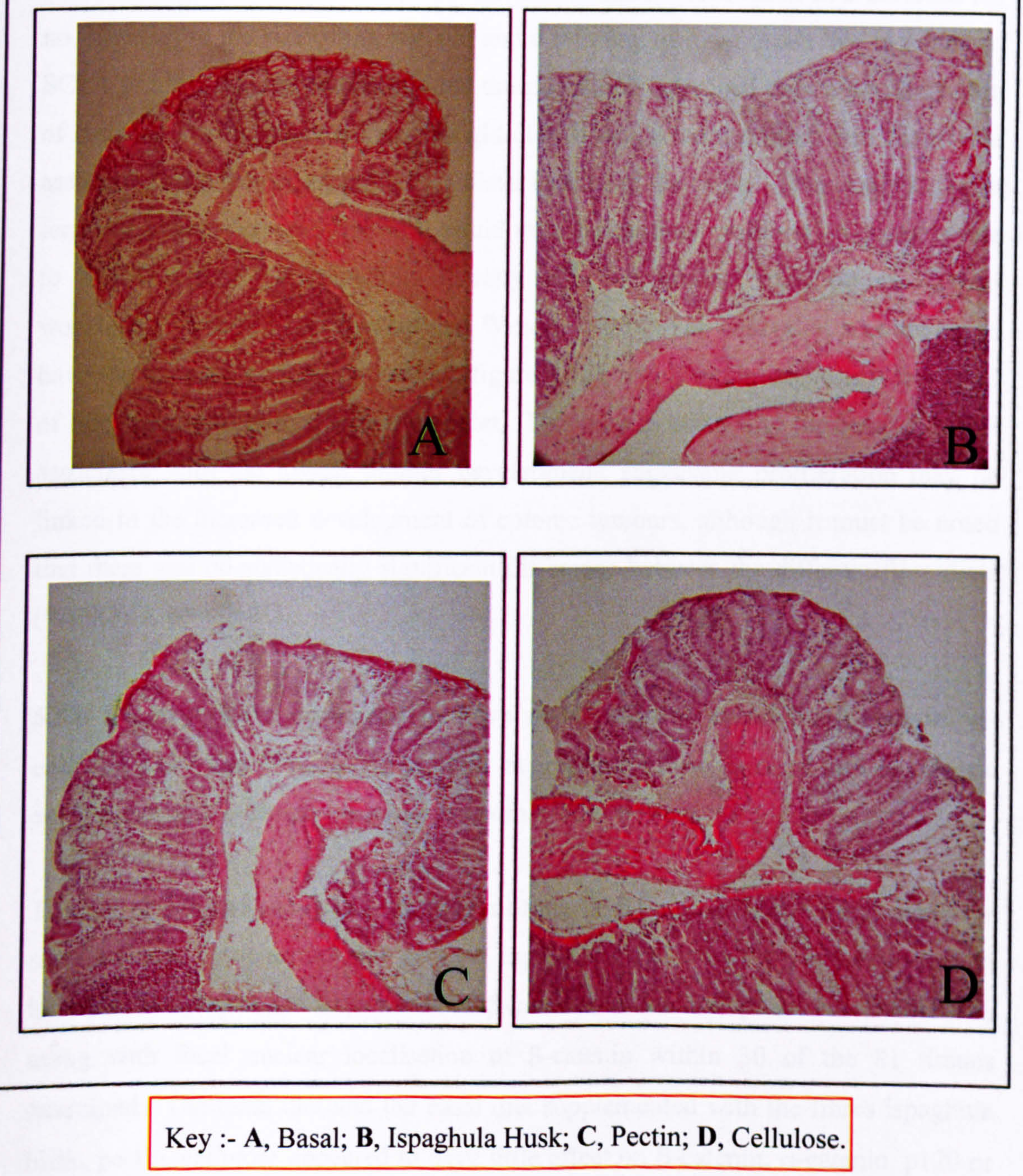


For each group N=2, n=50 each, except for one mouse within the basal where n=36

Comparison of the four fibre supplemented diets suggested that there was no difference between the groups in terms of crypt length due to the large standard deviations. The average crypt length appears to be greater within the pectin supplemented basal diet group and lowest in the basal diet group. There was no statistically significant overall difference in crypt length between all the fibre-supplemented diets (*ANOVA p=0.1835) this was because of the large mouse to mouse variation, particularly within the basal diet.

*Statistical analysis performed by J. Sykes, ReckittBenckiser Healthcare Ltd, Hull, UK

Figure 5.14b Representative light micrographs of colonic epithelium from DMH treated Balb/C mice treated with various fibre-supplemented diets.



Key :- **A**, Basal; **B**, Ispaghula Husk; **C**, Pectin; **D**, Cellulose.

Representative light micrograph pictures of colonic epithelium from DMH treated Balb/c mice fed on either a basal diet (**A**), ispaghula husk supplemented basal diet (**B**), pectin supplemented basal diet (**C**) or a cellulose supplemented basal diet (**D**), stained with haemotoxylin and eosin (Magnification X 20).

Resistant starch has been shown to reach the large intestine with the small bowel not being 100% efficient in humans (Reviewed by Hill, 1997c). The mice fed on the basal diet also exhibited smaller caecums and a thinner colonic mucosa (general observations). This could be explained by the fact that mice are caecal fermenters (Reviewed by Van Soest, 1995) thus the lack of fibre would result in a decrease (or no increase) in the size of the caecum and a thinning of the mucosa due to a lack of SCFA production, also indicating that the rice starch contained only a small fraction of resistant starch. Goodlad and Wright (1990) suggested that fibre free diets were associated with disuse atrophy of the distal ileum and the colon. They suggested that fermented fibre, but not inert bulk, could restore intestinal proliferation possibly due to SCFA release by microflora directly effecting luminal nutrition or luminal workload (Reviewed by Goodlad and Wright, 1990). The basal diet was shown to have the lowest proliferation indices (figure 5.15), which again suggests a low level of fibre (resistant starch) within this diet. This may indicate why the basal diet was associated with the lowest tumour development, suggesting proliferation may be linked to the increased development of colonic tumours, although it must be noted that there was no statistically significant difference between the different fibre diets (*ANOVA p=0.0641).

5.5.6 Dietary fibres appeared to have no significant effect on β-catenin, α-catenin, p120 and E-cadherin protein expression within normal and dysplastic epithelium of 1,2-dimethylhydrazine administered mice.

The non-dysplastic and dysplastic tissues of the DMH mouse model were scored for membranous, cytoplasmic and nuclear expression (refer to 5.4) and revealed an increase in cytoplasmic expression of β-catenin, α-catenin, p120 and E-cadherin, along with focal nuclear localisation of β-catenin within 30 of the 81 tissues examined. The basal diet and the basal diet supplemented with the fibres ispaghula husk, pectin, cellulose appeared to have little effect on β-catenin, α-catenin, p120 or E-cadherin cellular localisation within the non-dysplastic or the dysplastic tissues.

Figure 5.16 The effect of the dietary fibres Ispaghula husk, Pectin and Cellulose on (A) β-catenin, (B) E-cadherin, (C) α-catenin and (D) p120 expression in the 1,2-dimethylhydrazine mouse model of colorectal cancer.

A.

	Non-dysplastic tissue					Dysplastic tissue					
	Memb	SD	Cyto	SD	Nuc	Memb	SD	Cyto	SD	Nuc	N
Basal	2.75	0.44	1.5	0.52	0	3	0	2.95	0.22	8	20
Ispaghula	2.65	0.49	1.35	0.49	0	3	0	3	0	10	20
Pectin	2.71	0.56	1.71	0.56	0	2.95	0.22	2.95	0.22	6	21
Cellulose	2.75	0.44	1.5	0.52	0	2.9	0.31	2.9	0.45	6	20

B.

	Non-dysplastic tissue					Dysplastic tissue					
	Memb	SD	Cyto	SD	Nuc	Memb	SD	Cyto	SD	Nuc	N
Basal	2.74	0.45	1.63	0.6	0	2.84	0.37	2.79	0.54	0	19
Ispaghula	2.75	0.44	1.4	0.5	0	2.9	0.31	2.85	0.37	0	20
Pectin	2.86	0.36	1.57	0.6	0	2.76	0.44	2.52	0.51	0	21
Cellulose	2.45	0.51	1.55	0.6	0	2.55	0.69	2.45	0.83	0	20

C.

	Non-dysplastic tissue					Dysplastic tissue					
	Memb	SD	Cyto	SD	Nuc	Memb	SD	Cyto	SD	Nuc	N
Basal	3	0	2.06	0.56	0	3	0	2.89	0.33	0	17
Ispaghula	2.95	0.23	2.05	0.4	0	3	0	2.95	0.23	0	19
Pectin	2.9	0.3	2.05	0.38	0	2.9	0.44	2.62	0.5	0	21
Cellulose	2.85	0.37	1.95	0.51	0	2.9	0.45	2.9	0.31	0	20

D.

	Non-dysplastic tissue					Dysplastic tissue					
	Memb	SD	Cyto	SD	Nuc	Memb	SD	Cyto	SD	Nuc	N
Basal	2.68	0.48	1.68	0.58	0	2.89	0.32	2.74	0.45	0	19
Ispaghula	2.56	0.62	1.89	0.32	0	3	0	2.78	0.43	0	18
Pectin	2.81	0.4	1.71	0.64	0	2.81	0.51	2.76	0.54	0	21
Cellulose	2.61	0.61	1.67	0.59	0	2.89	0.32	2.67	0.49	0	18

The tables show that the different fibre supplemented diets, basal, ispaghula, pectin and cellulose resulted in no obvious difference in (**A**) β- catenin, (**B**) E-cadherin, (**C**) α-catenin and (**D**) p120 expression within the non-dysplastic and dysplastic colonic tissues from the DMH mouse model.

The dietary fibres also appeared to have little effect on the level of β- catenin nuclear localisation (figure 5.16). This may have been due to the fact that a small number of tissues (N=20) were examined within each fibre group.

5.6 Discussion.

Through the proposed multistep process of colorectal cancer, it is believed that by preventing the growth of adenomas in the colon and the rectum this will prevent the development of colorectal cancer (Reviewed by Byers, 2000). Thus the DMH model was used to examine whether the supplementation of a basal diet, primarily deficient in fibre, with dietary fibres resulted in a decreased incidence of tumour development. The study asked the question as to whether dietary fibre plays a role in the progression or prevention of tumour development after its initiation and promotion by the carcinogen 1,2-dimethylhydrazine.

5.6.1 The potential protective or causative mechanisms of dietary fibres on colon cancer development.

5.6.1.1 *Bulking effects of Fibres.*

This model involved the subcutaneous injection of a carcinogen, thus could not answer the question whether the bulking effect of fibre (by diluting down the carcinogen and decreasing transit time) played a role in preventing cancer development. If the carcinogen was secreted in the bile (Reviewed by Pozharisski *et al.*, 1979) then the fibres would affect the carcinogens actions, for example ispaghula husk, which is a bulking fibre would reduce the contact of the carcinogen with the colonic epithelium and result in its dilution, whereas pectin has little bulking effect thus could not elicit these actions. The basal diet results suggested that bulking is not the mechanism of action of the fibre in tumour development as the basal diet contained little fibre and thus would not elicit a bulking mechanism. The carcinogen is thought to act via the blood stream, rather than being extruded with the bile, with the active metabolites targeting the bottom of the crypt (Reviewed by Sunter, 1980) and this is thought to be the mechanisms of most environmental carcinogens (M.

Hill, London Reckitt Benckiser conference personal communication). Bulking effects, in terms of bowel transit have also been shown to alter stool SCFA concentrations including butyrate and distal colonic pH. Increasing the rate of intestinal transit was shown to increase butyrate concentrations in the distal colon (Lewis and Heaton, 1997). Butyrate production has been shown to protect against DMH induced tumourigenesis *in vivo* (McIntyre *et al.*, 1993; Avivi-Green *et al.*, 200a).

5.6.1.2 *Fermentation.*

The findings from this study posed the question as to why pectin enhanced tumour development. Pectin is fermented readily, if not completely, in the caecum producing high levels of intracolonic short chain fatty acids (SCFA) levels (Avivi-Green *et al.*, 2000b) and this could have many implications. Fermentation of pectin in the caecum (lowering in pH observed by Cameron *et al.*, 2000) could have potentially lowered the SCFA concentration within the distal colon, as the majority of the SCFA production would have been within the caecum or proximal colon. This would have influenced the availability of energy for colonocytes and the pH of the colonic lumen. Although Avivi-Green *et al.* (2000a) suggest that pectin produce high levels of intracolonic levels of butyrate and that pectin and butyrate infusions resulted in a decrease in the number of aberrant crypt foci (ACF) induced by DMH, which theoretically is in contrast to our findings, although ACF numbers were not determined in this study. SCFAs result in a lowering in pH within the colon, which has many implications on enzyme activity and bile acid solubility. If the SCFA levels decreased, the pH would increase, and would subsequently affect primary bile acid levels and their conversion to secondary bile acids by influencing the activity of the enzyme involved, 7-α-dehydroxylase (section 5.5.1.4) (Reviewed by Ink and Hurt, 1987). Lipids themselves have been shown to stimulate proliferation within the small intestine, although in the presence of bacteria had little effect on colonic proliferation (Pell *et al.*, 1995). pH has been shown to affect cell proliferation with an increase in the environments acidity being associated with an increase in proliferation. If pectin is fermented rapidly within the caecum an increase in pH within the distal colon is unlikely to be the cause of the increased proliferation within

the colonic epithelial cells. Another study has shown pectin to result in an increase in markers of apoptosis within the colon (Avivi-Green *et al.*, 2000a), which could elicit the increase in proliferation to help maintain a balance within the colon, colonic homeostasis. Our findings are similar to those of Jacobs and Lupton (1986) where they found that pectin resulted in 5 times the number of adenomas than the fibre free diet, showing that it was also the greatest stimulus for cell proliferation and that a lower luminal pH was associated with the greater tumour number (Jacobs and Lupton, 1986).

SCFAs, such as butyrate, have previously been shown by Medina *et al.* (1998) to inhibit 1,2-dimethylhydrazine induced cancer development in rats. Weaver *et al.* (1996) also showed that in rats treated with azoxymethane (AOM), butyrate was protective, whereas propionate was thought to enhance tumour development. Slow release pellets within F344 male mice administered with AOM have been shown to increase apoptosis without affecting ACF or proliferation within colonic tissues (Caderni *et al.*, 1998). The protective effects of butyrate have also been shown *in vitro* (section 1.3), thus the SCFA profile produced by these dietary fibres is important. In freshly isolated rat colonic cells both butyrate and acetate were shown to reduce the induction of DNA damage by H_2O_2 (oxidative stress), whereas propionate was ineffective (Abrahamse *et al.*, 1999). A number of studies have shown that when fermented different fibres produced different levels of the three main SCFAs, butyrate, acetate and propionate (section 3) (Thomsen *et al.*, 1984; Edwards and Eastwood, 1992; Marteau *et al.*, 1994; Reviewed by Edwards, 1995). Interestingly, Clausen *et al.* (1991) also showed that the ratio of butyrate production to total SCFA production from fibre was reduced in patients with colonic cancer and adenomas compared to healthy controls. Interestingly physiological mixtures of SCFAs representing that for pectin, acetate, propionate and butyrate ratio of 41:21:38 respectively, were shown not have significant antigenotoxic effects (oxidative stress) (Abrahamse *et al.*, 1999). A study examining the effects of resistant starch and cellulose on DMH induced colonic tumours within rats found that resistant starch resulted in an increase in buyrate concentration but did not inhibit colonic carcinogenesis (Sakamoto *et al.*, 1996).

5.6.1.3 *Microflora*

It has been proposed that different fibres select for different microflora within the hindgut, resulting in changes in microbial metabolism (Costa *et al.*, 1989). This alteration of microflora will in turn affect their ability to ferment other dietary fibres (Monsma and Marlett, 1996). Narushima *et al.* (1998) results indicated that intestinal bacteria could substantially modify colorectal tumourigenesis induced by DMH. Further studies also showed the role of microflora in DMH induced tumour development, germfree mice had a greater incidence of tumour development than conventionalised mice but they were smaller in size (Horie at al., 1999a). This could be suggestive that microflora helps to slow down the initiation but increases rate of progression (possibly due to SCFA induction increased proliferation *in vivo*, or any effect of chemical conversion i.e. maybe it slows down the decomposition rate of MAM to methydiazonium thus slowing down initiation). The effect of a number of individual intestinal bacteria on DMH induced colorectal adenoma formation has been examined by developing gnotobiotic mice. The incidence of adenoma formation in gnotobiotic mice with *Mitsuokella multiacida, Clostridium butyricum, Bifidobacterium longhum, Clostridium paraputrificum, Escherichia coli* and *Lactobacillus acidophilus* was 68%, 68%, 63%, 50%, 50% and 30% respectively (Horie *et al.*, 1999a), suggesting bacteria influenced tumour development. There are a number of proposed mechanisms as to how these bacteria influenced tumour incidence, for example the effects of *L.acidophilus* may be mediated by faecal pH and the effects of *Cl. paraputrificum* and *Cl. butyricum* may be through the deconjugation of bile acids (refer to section 5.5.1.4) (Horie *et al.*, 1999a). Horie *et al.* (1999b) developed gnotobiotic mice each with a single species of intestinal bacteria to look at the effects on DMH induced colorectal cancer. The incidence of adenoma formation in gnotobiotic mice with *Lactobacillus acidophilus*, gnotobiotic mice with *E.coli* and germ-free mice were 30, 50 and 74% respectively. Probiotics (viable microbial dietary supplement which benefits the host through its effects within the intestinal tract) have been shown to have an inhibitory effect on the development of aberrant crypts and tumours in animal models (Reviewed by Brady *et al.*, 2000) again suggesting that microflora influences tumour development. This clearly showed the influence that microflora had on DMH induced chemical

carcinogenesis, thus pectin could result in changes in the microflora of the gut that modify the colorectal cancer induced by DMH.

5.6.1.4 *Bile Acids.*

Unconjugated bile acids have been shown to be tumour promoters (Bayerdorffer *et al.*, 1995), and the presence of these bile acids in the intestinal tissues is regulated by dietary factors (Weisburger *et al.*, 1977; Mahmoud *et al.*, 1999). Influence of diet can be seen through their fermentation resulting in changes in pH within the colonic lumen. Increases in pH can theoretically result in the release of primary bile acids due to changes in their solubility, and loss of fibre as bulk could also result in the same end. This release of primary bile acids potentially results in their increased conversion to secondary bile acids, which have been shown to be involved in colorectal cancer development (Reviewed by Harris and Ferguson, 1993; Reviewed by Chaplin 1998). The increase in pH affects the enzyme activity of 7-α-dehydroxylase with a decrease in pH reducing the activity of this enzyme, an enzyme involved in the conversion of primary bile acids into secondary bile acids. Pectin is fermented rapidly in the caecum thus would result in a dramatic lowering of pH in the proximal colon inhibiting the enzyme 7-α-dehydroxylase (Reviewed by Ink and Hurt, 1987) but this would also result in the release of primary bile acids due to a loss of bulking. pH would increase as the bile acids move along the colon and the level of SCFAs would decrease, thus the activity of the enzyme would increase particularly in the distal colon. Whereas ispaghula husk has been shown to bind primary bile acids and as it is fermented relatively slowly would result in a steady release of SCFAs keeping the pH low and the concentration of primary bile acids low possibly resulting in a reduced production of secondary bile acids. Dietary fibres such as ispaghula husk have already been shown to affect bile acid concentrations by lowering faecal lithocholic and isolithocholic acids (Chaplin *et al.*, 2000). Interestingly, Furukawa *et al.* (1995) showed that the dietary fibre yugao-melon suppresses DMH induced colonic tumours in mice by lowering bile acid concentration and pH within the colon, thus bile acids could play a role in DMH induced tumour pathology. Kozoni *et al.* (2000) looked at the biological effects (proliferation and apoptosis) of lithocholic acid (LCA), a bile acid implicated in

human and experimental cancers, in DMH treated mice. They found that DMH on its own increased apoptosis and so did LCA but LCA and DMH together resulted in a decrease in apoptosis. They also showed that DMH and LCA on their own resulted in an increase in proliferation but together resulted in a shift in proliferation site. Thus this suggests that LCA, in the presence of a chemical carcinogen, alters the colonic homeostasis resulting in tumour formation (Kozoni *et al.*, 2000). Bile acids/salts have also been shown to induce apoptosis *in vitro* in colon cancer cell lines (Schlottman *et al.*, 2000).

5.6.1.5 *Colonic homeostasis*

Diet (fibre) and the products of its fermentation, the SCFAs, have already been shown to stimulate intestinal proliferation (Jacobs and Lupton, 1984; Reviewed by Jacobs, 1988; Goodlad *et al.*, 1989; Goodlad *et al.*, 1995; Reviewed by Goodlad and Wright, 1990; Reviewed by Wasan and Goodlad, 1996). Pectin (via non-specific irritation), the products of its fermentation (Ichikawa and Sakata, 1998; Reviewed by Wasan and Goodlad, 1996) could affect the natural homeostasis of the colon by altering the balance between cell proliferation, differentiation and apoptosis. DMH itself increases proliferation and alters the distribution of proliferating and apoptotic cells within colon crypts early in carcinogenesis (Richards, 1977; Baril *et al.*, 1990; Barnes *et al.*, 1999; Carter *et al.*, 1994), and studies using AOM have found that colonocyte energy metabolism (depressed butyrate metabolism) also differs with further changes during tumourigenesis (Zhang *et al.*, 1998). Edwards *et al.* (1992) showed that a long-term high fibre diet was associated with an increase in cellular proliferation in the proximal but not the distal colon of rats. Robblee *et al.* (1989) has previously shown an increased labelling index with pectin compared to cellulose thus suggesting an increase in cell proliferation. Ispaghula husk in the form of Metamucil has also been shown to increase proliferation when compared to cellulose in rats (Calvert *et al.*, 1985). Zhang and Lupton (1994) found that the production of SCFAs through the fermentation of fibre (cellulose, pectin or oat bran) stimulated caecal cell proliferation but not in colonic cells within the distal colon and proposed that other factors may influence proliferation within distal colonocytes. Changes in apoptosis have also been involved in tumour development by unbalancing colonic homeostasis. Avivi-Green *et al.* (2000a) has previously shown that pectin increased

caspase 1 activity and increased cleaved poly (ADP-ribose) polymerase (PARP). The caspases have been shown to play a fundamental role in apoptosis; caspase 1 (ICE) belongs to a subfamily of caspases who are predominantly involved in the control of inflammation (Reviewed by Earnshaw *et al.*, 1999) although it has been implicated in initiating the apoptotic pathway (Avivi-Green *et al.*, 2000a). Caspase 1 has also been shown to be involved in the cleavage of β-catenin (Fukuda, 1999) and caspase 3 cleaves E-cadherin during apoptosis (Steinhusen *et al.*, 2001). PARP is a DNA repair enzyme that is cleaved during the initial phases of apoptosis, thus an increase would suggest that there has been an increase in apoptosis (Avivi-Green *et al.*, 2000a). A pectin supplemented basal diet was shown to increase the proliferation (Figure 5.15) of colonic epithelial cells in 1,2-dimethylhydrazine treated female Balb/c mice. This increase was not accompanied by a significant increase in crypt length (figure 5.14) this could be due to an increase in apoptosis as shown previously (Avivi-Green *et al.*, 2000a). The increase in proliferation as a result of pectin supplementation could also elucidate the increase in tumour development in terms of initiation and progression. Although Chang *et al.* (1997) suggest that measurements of differentiation and apoptosis may have a greater prognostic value to detect dietary effects on tumour incidence than proliferation.

5.6.1.6 *Changes in gene and protein expression.*

Pectin could also result in changes in gene or protein expression. Other dietary factors, including the SCFAs, have been shown to affect a number of proteins in terms of their expression levels. Tappenden and McBurney (1998) showed that systemic SCFAs increase plasma *GLP-2* (Glucagon-like peptide-2) and *ileal proglucagon* mRNA, *GLUT 2* expression, c-myc, c-jun and c-fos expression *in vivo*. Although *in vitro* butyrate has been shown to downregulate c-myc protein levels (Basson *et al.*, 1998). Pectin could result in changes in enzyme activity such as 7-α-dehydroxylase, which converts primary bile acids into secondary bile acids. Other dietary factors such as morin (a flavonoid) have also been shown to affect enzyme activity such as Glutathione S-transferase and quinone reductase (Tanaka *et al.*, 1999). Mahmoud *et al.* (1999) found that the bile acid chenodeoxycholate resulted in increased β-catenin expression and increased PGE2 expression, in duodenal cells and

tissues respectively, in the multiple intestinal neoplasia (MIN) mice. Mei *et al.* (1999) also showed that cells containing a mutant APC express higher levels of *PGHS-Z* mRNA and protein thus increasing PGE2 production. This was found to correlate with the formation of β-catenin-Lef-1 complexes. Hormone levels may also play a role as suggested by the differences of tumour incidence between sexes (Reviewed by Pozharisski *et al.*, 1979). For example Toth *et al.* (1984) examined the effects of metamucil (ispaghula husk) on the development of tumours in DMH treated Swiss mice. They found that the administration of metamucil enhanced the appearance of colonic tumours in males only. Psyllium (ispaghula husk) was shown to protect against colorectal cancer when tumours were induced in rats by gastric intubation (Roberts-Andersen *et al.*, 1987) this suggests that ispaghula may protect against colorectal cancer by diluting down the carcinogen. Alabaster *et al.* (1993) showed that in F-344 rats psyllium (ispaghula husk) could protect against AOM induced colon cancer promoted by high fat diets, and this effect was enhanced when in combination with wheat bran. Fibres have also been shown to modulated bacterial β-glucuronidase activity, with guar gum resulting in lower levels of activity than pectin, although this was not thought to be related to tumour number (Bauer *et al.*, 1981). Psyllium (ispaghula husk) has also been shown to have lower levels of β-glucuronidase than cellulose (Costa *et al.*, 1989b) and wheat bran (Leng-Peschlow, 1991). Thus, pectin could result in changes in gene and protein expression either directly or indirectly by the production of SCFAs or changes in bile acid concentrations. In this study the different fibres, ispaghula husk, pectin, cellulose and the basal diet, had little effect on β-catenin, α-catenin, p120 or E-cadherin cellular localization.

5.6.1.7 *Immune responses*

Fibres may also affect the gut associated lymphoid tissue (GALT) of the colonic mucosa. Within this study lymphoid aggregates were generally observed associated with dysplastic tissue. Exposure of Balb/c mice to DMH has been shown to stimulate an immune response (Tendler *et al.*, 2000). Although Madar *et al.* (1998) found that in the ileum of rats exposed to the carcinogen DMH/AMO, the carcinogen even at low levels inhibited lymphoid system in the mucosa. Horie *et al.* (1999b)

showed that gnotobiotic mice with *L.acidopilus* resulted in a decrease in DMH induced tumours. The protective effects of *L.acidophilus* are thought to be due to activation of extrathymic T cells in the liver and granulocytes in the colonic mucosa. The SCFAs also affect the immune response (ref to section 1.5.5).

5.6.2 Other dietary intervention studies and the biological effects.

Other studies have looked at the biological effects of a number of dietary factors. Epidemiological studies have consistently indicated that the consumption of fruits and vegetables lower cancer risk in humans and suggest certain dietary constituents may be effective in preventing colorectal cancer (Mahmoud *et al.*, 2000). This has lead to a number of groups studying the effects of a number of different dietary factors in rodent models. Many of these studies have examined changes in the colonic homeostasis and the effects on a number of important genes and proteins, such as β-catenin, in the development of colorectal cancer. Taniyama *et al.* (2000) showed that 24R,25-dihydroxyvitamin D3 decreased the number of tumours present in rat colonic mucosa. The decrease in tumour number was accompanied by a decrease in the bromodeoxyuridine (BrdU) labelling index suggesting a decrease in proliferation. Tanaka *et al.* (1999) found that the flavonoid, morin, also resulted in a decrease in tumour incidence (AMO treated rats) with changes in the expression of a number of proteins. These changes included a decrease in proliferating cell nuclear antigen (PCNA) in aberrant crypt foci (ACF) and an increase in Glutathione S-transferase (involved in the production of leukotrienes by the immune system, part of the aracadonic acid metabolic pathway) and quinone reductase. Bartsch *et al.* (1999) review the findings that a high intake of certain dietary polyunsaturated fatty acids (PUFAs) promotes several stages of mammary and colon cancer from an increase in oxidative DNA damage and changes in cell proliferation and hormone levels and catabolism. In contrast the fish oil derived w-3 fatty acids seem to protect against cancer development by influencing the activity of enzymes and proteins involved in intracellular signalling and ultimately cell proliferation. In the MIN mouse, Mahmoud *et al.* (1999) examined the effects of chenodeoxycholate (unconjugated bile acid) on tumour incidence and found an increase in the number of duodenal tumours. This was associated with an increase in β-catenin expression in duodenal

cells and an increase in Prostaglandin E2 (PGE2,) a product of the arachidonic acid pathway involving cyclooxygenase (COX) in duodenal tissue, suggesting that bile acids contribute to tumour formation where there is an APC mutation. Rao *et al.* (2000) showed that 1,4-phenylene bis (methylene) selenocyanate (P-XSC) also has antitumour activity and appears to involve the modulation of β-catenin expression and COX-2 activity. NSAIDs modulate COX-2 activity and epidemiological evidence suggests that NSAIDs protect against colon cancer development (Reviewed by Choi and Mason, 1998). High doses of asprin (NSAID) have been shown to reduce tumour formation in MIN mice (Barnes and Lee, 1998). Moorghen *et al.* (1998) showed that the NSAID sulindac had a marked inhibitory effect on the development of colonic tumours in mice treated with the carcinogen DMH. Sulindac was shown to increase cell proliferation (BrdU index), which may be secondary to the loss of crypt epithelial cells by apoptosis induced by sulindac. Sulindac was also shown to reduce the number and volume of tumours with DMH treated rats (Sprague-Dawley), this was found to be independent of cyclooxygenase inhibition but possibly due to the increase in apoptosis observed, although in contrast to Moorghen *et al.* (1998) a reduction in proliferation (Brd-U) was also observed (Brown *et al.*, 2001). Interestingly Kishimoto *et al.* (2000) found that sulindac also resulted in an increase in *APC* mRNA in AOM treated rat colonic mucosa. The Cox-2 inhibitor celecoxib resulted in the suppression of occurrence and advancement of crypts overexpressing β-catenin, suggested to be premalignant lesions, induced by AOM within F344 rats (Yamada *et al.*, 2001b). The relationship between β-catenin and Bcl-2 expression in sulindac induced regression of intestinal tumours in MIN mice was examined by McEntee *et al.* (1999). They found that in this model there was a decrease in the expression of β-catenin with Bcl-2 conferring resistance to these effects. Ishizuka and Kasai (1997) found that rats treated with DMH and fed on a diet deficient of fibre resulted in a significant increase in the PCNA-LI (proliferating cell nuclear antigen-labelling index) of the distal colon and rectum. They found that the ingestion of wheat bran diminished this increase in PCNA-LI. Cameron *et al.* (1997) found that lignin supplementation has good bulking characteristics, and had a significant effect on several factors that have previously been linked to reduction of colon cancer risk, but the consumption of high levels of lignin did not decrease the risk for colon carcinogenesis. Within the colon of DMH

induced ACF in mice found a protective effect of carotenoids and curcumins (Kim *et al.*, 1998). Mahmoud *et al.* (2000) has also shown that the plant phenolics decrease intestinal tumours in an animal model of familial adenomatous polyposis coli (FAP). They examined the effects of caffeic acid phenethyl ester (CAPE), curcumin, quercetin and rutin. They found that only CAPE and curcumin resulted in a decrease in tumour development and this was associated with an increase in enterocyte apoptosis and proliferation and a decrease in β-catenin expression in enterocytes. Thus suggesting that CAPE and curcumin suppresses APC associated intestinal carcinogenesis. Mikhailowski *et al.* (1998) looked at TGF-β1, a cytokine known to play a key role in the control of cell growth inhibiting human and rodent epithelial cell proliferation. They found that *in vivo* TGF-β1 markedly inhibited colonic ACF formation and significantly reduced colonic tumour formation and size.

5.6.3 Animal studies

The findings from these dietary studies, especially those looking into fibre, reviewed by Jacobs (1990) and Klurfeld (1990), vary considerably. This could be attributed to a number of factors such as the use of different animal models with varying genetic background, the age, sex, strain of the animal, the dosage of dietary supplement, carcinogen administration site, all of which can effect the results (Reviewed by Bingham, 1990; Reviewed by Pozharisski *et al.*, 1979). Rats and mice are very popular experimental animals for studying human nutrition (Reviewed by Van Soest, 1995). However, these models have many disadvantages especially when it comes to studying dietary fibre. Firstly they are smaller, have inferior fermentation capacities with their main site of fermentation being in the caecum whereas in humans it is the colon. They are also coprophagous, which can confound results (Reviewed by Van Soest, 1995). Although tumours are produced mainly in the descending colon and rectum in rodents after administration of DMH, the mode of tumour formation appears to be species specific: the neoplasms isolated in the rat are border-based but widespread in the mouse (Chang, 1980). Thus the mouse appears to be the better experimental model with which to analyse the pathogenesis of multiple adenomas and the subsequent development of adenocarcinomas (Chang, 1980). All species

differ in some way or other; there is no species that behaves exactly like humans (Reviewed by Van Soest, 1995).

Further problems with the DMH model include the unknown genetic background with the possibility of a non-uniform genetic background as with all carcinogen-based models. It has previously been shown that the active metabolite of DMH and AOM is MAM, which, in the case of AOM, has been shown to cause mutation in the GSK-3β consensus motif of β-catenin (Takahashi *et al.*, 1998 and 2000). This was implied by the finding that within microadenomas there is an over expression and redistribution of β-catenin to the cytoplasm and nucleus, suggesting a disruption in the regulation of β-catenin expression. Although this model has many histological changes that are seen in humans the initial stages of DMH induced colorectal cancer in rodents involve hyperplasia, which is not seen in humans. The microflora in murine species is likely to be different to humans thus there are a number of problems with these models. Another problem with this model was the use of a carcinogen that is not usually found in the human diet (Reviewed by Harris and Ferguson, 1993) along with fibres that are not usually found in a murine diet. Although Pozharisski (1975) reports that 1,1-dimethylhydrazine occurs in tobacco and methylhydrazine is found in edible mushroom (*Gyromitra esculenta*), thus hydrazines may be a suspect in the aetiology of colon cancer.

Chapter 6

Examination of the effects of SCFAs and dietary fibres on colonic cell homeostasis and the expression and cellular localisation of β-catenin.

6.1 Introduction

The aim of this chapter was to elucidate the biological mechanisms behind the *in vivo* responses to luminal factors using *in vitro* techniques. Within the *in vivo* study, pectin was shown to increase tumour development in terms of tumour incidence, number and burden (size), and this was accompanied by an increase in the number of cells undergoing DNA replication suggesting an increase in proliferation within the colonic tissues. Using an *in vitro* system of colonic epithelial cells, of adenoma and carcinoma origin, the role of the dietary fibres pectin, ispaghula husk, cellulose and rice starch and their associated short chain fatty acids, butyrate, acetate and propionate on colonic cell homeostasis and protein expression was examined.

Colonic homeostasis is an important factor in the development of colorectal cancer. Colonic homeostasis is the result of a balance between cell growth and cell death with colorectal cancer occurring when one of these processes is disrupted. Escaping one of the controlling mechanisms of colonic homeostasis results in an increase in cell proliferation and/or a loss of cell apoptosis results in the clonal expansion of abnormal cells. Perturbation of the cadherin/catenin complex, important in the regulation of a number of cellular processes, results in changes in intercellular adhesion and cell transformation (Valizadeh *et al*, 1997; Guilford, 1998). APC has a diverse functionality within the cell and is particularly important in regulating levels of β-catenin within the cell (Reviewed by Moon and Miller, 1997; Reviewed by Morin *et al.*, 1997; Reviewed by Ben-Ze'ev and Geiger, 1998; Reviewed by Gordon, 1998; Reviewed by White, 1998). 'Gain of function' mutations in β-catenin or 'loss of function' mutations in APC have been shown to result in an overexpression and change of cellular localisation of β-catenin, from the membrane to the cytoplasm and nucleus, within colonic dysplastic tissues (Rubinfeld *et al.*, 1996; Takahashi *et al.*,

1998; Lamlum *et al.*, 2000; Reviewed by Fearnhead *et al.*, 2001). The nuclear translocation of β-catenin and the association with the transcription factors Tcf-4/Lef-1 have been shown to result in the expression of a number of genes important in colonic homeostasis and colorectal cancer development including c-myc and cyclin-D1 (He *et al.*, 1998; Shtutman *et al.*, 1999). Thus E-cadherin, β-catenin and APC all play a fundamental role in the regulation of the normal colon with disruptions playing a role in colorectal cancer development.

The effects of fibres *in vitro* have yet to be studied, but the effects of other luminal factors have been studied particularly the short chain fatty acids acetate, propionate and butyrate. These SCFA's profoundly alter the milieu of the colon and have a wide range of biological effects. The SCFA butyrate has been the most extensively studied *in vitro* and revealed a number of biological effects including those involved in colonic homeostasis, being powerful promoters of differentiation, apoptosis and cell migration (Sakata and Yajima, 1984; Hague *et al*, 1995; Wilson and Gibson, 1997; Basson and Sgambati, 1998; Wang and Friedman, 1998; Tappenden and McBurney, 1998; Bordonaro *et al*, 1999; Siavostian *et al*, 2000), inhibition of growth (Archer *et al*, 1998a), membrane synthesis and sodium absorption of colonocytes (Schwartz *et al.* 1998) and changes in gene and protein expression (Archer *et al.*, 1998a; Litvak *et al.*, 1998; Coradini *et al.*, 2000; Siavoshian *et al.*, 2000; Lührs *et al.*, 2002; Menzel *et al.*, 2002).

6.2 Aims

To identify the biological mechanisms responsible for the effects seen *in vivo,* using in vitro techniques.

To examine the effects of luminal factors (fibres and SCFAs) on a number of parameters of colonic homeostasis and cadherin/catenin protein expression in human and murine colonic epithelial cell lines.

6.3 Methodology

A panel of previously characterised human colon carcinoma cell lines HT29, HCT116, LS174T, and SW1222, the adenoma cell line RG/C2 and a murine rectal carcinoma cell line CMT-93 were used in these experiments. The cell lines were chosen due to their different genetic makeup with some containing APC mutations (HT29 and SW1222), others containing known β-catenin mutations (HCT116) and one with E-cadherin mutation (LS174T). The remaining cell lines, RG/C2 and CMT-93, remain to be characterised as they have unknown quantities in their genetic make up. Immunocytochemistry along with mutational analysis (PCR, SSCP and sequence analysis) were used to determine the β-catenin status of LS174T and RG/C2 cell lines. This work was carried out with the help of Nari Janghra who performed the SSCP and sequence analysis used within this thesis for the cell line LS174T. Western blotting, immunocytochemistry and immunofluoresence were used to examine protein expression. Proliferation was measured using cell counts in all cell lines and confirmed using the MIB-1 antibody, which determines Ki-67 expression, for the cell lines HT29 and SW1222. Other studies were originally undertaken to determine total cell numbers and possible cell adhesive properties after treatment with various dietary fibres and SCFAs using colorimetric assays using a microtitre plate and an ELISA plate reader. The colourimetric substrates used were trypan blue and the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrzoliumbromide). These studies were unsuccessful; firstly due to the false positive results obtained with trypan blue coupled with solubilisation of the cell membrane by SDS (Sodium dodecylsulfate), with ispaghula husk. Ispaghula husk appeared to bind trypan blue thus further methods were explored. MTT was then employed to determine cell numbers, by measuring mitochondrial activity, but problems arose with MTT crystal solubilisation. Different methods for solublising MTT tetrazolium salts were examined, the solvent Dimethyl Sulphoxide (DMSO) together with a glycine buffer and acidified (HCl) Triton-X, but neither of the methods were successful. In hindsight these techniques, if they had been successful, may have had confounding results as both could result in the measurement of apoptotic cells. Apoptosis was measured using annexin-V binding and floating cell numbers were also examined but due to the nature of the fibres these counts gave

inconsistent results. This was due to the difficulties in distinguishing between apoptotic cells and fibre residue along with the mucilaginous nature of ispaghula husk restricting the ability of suspending the cells.

Initial problems arose with the sterility of the fibres used in the *in vitro* experiments. The dietary fibres used within this study were not sterile, for example ispaghula husk was obtained after sterilisation at 10Kgreys of gamma-irradiation, this did not render the fibre sterile as they still resulted in microbial counts including fungal spores (M. Havler, personal communication). This posed the possibility of a lack of usability, along with a number of sterilisation procedures being ruled out as they changed the properties of fibres. An example of this was the finding that γ-irradiation of ispaghula husk resulted in changes in properties including waterholding capacity thus would not be representative of the ispaghula husk used in the study (M. Havler, ReckittBenckiser Healthcare Ltd, and S. Al Assaf, North East Wlaes Institute, Wrexam, personal communication,). To keep the problem of sterility to a minimum, antibiotics within the cell culture medium along with the use of minimal incubation times and a low percentage of fibres were adopted.

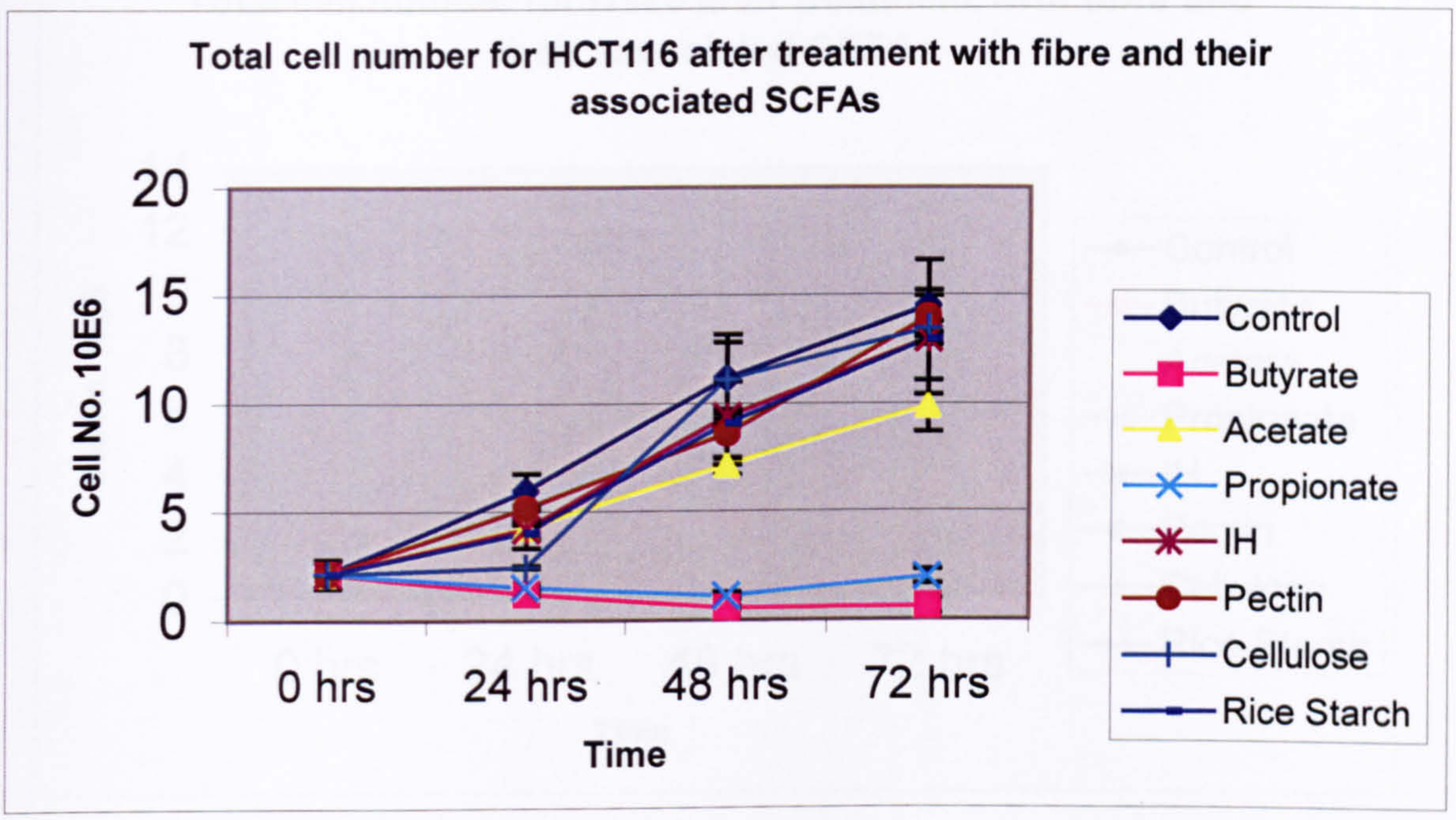
Original studies on protein expression using western blotting techniques used protein assays to determine equal loading. These studies revealed that after butyrate treatment equal loading could not be achieved using protein assays (BioRad Laboratories, Protein assay kit). This was most likely due to the induction of differentiation by butyrate, resulting in the production of glycoproteins. This resulted in the alteration of protocol to incorporate equal cell numbers rather than cellular protein levels.

6.4 Results

6.4.1 The effects of the SCFAs (butyrate, acetate, propionate) and various fibres (ispaghula, pectin, cellulose and rice starch) on the proliferation of a number of carcinoma and adenoma cell lines.

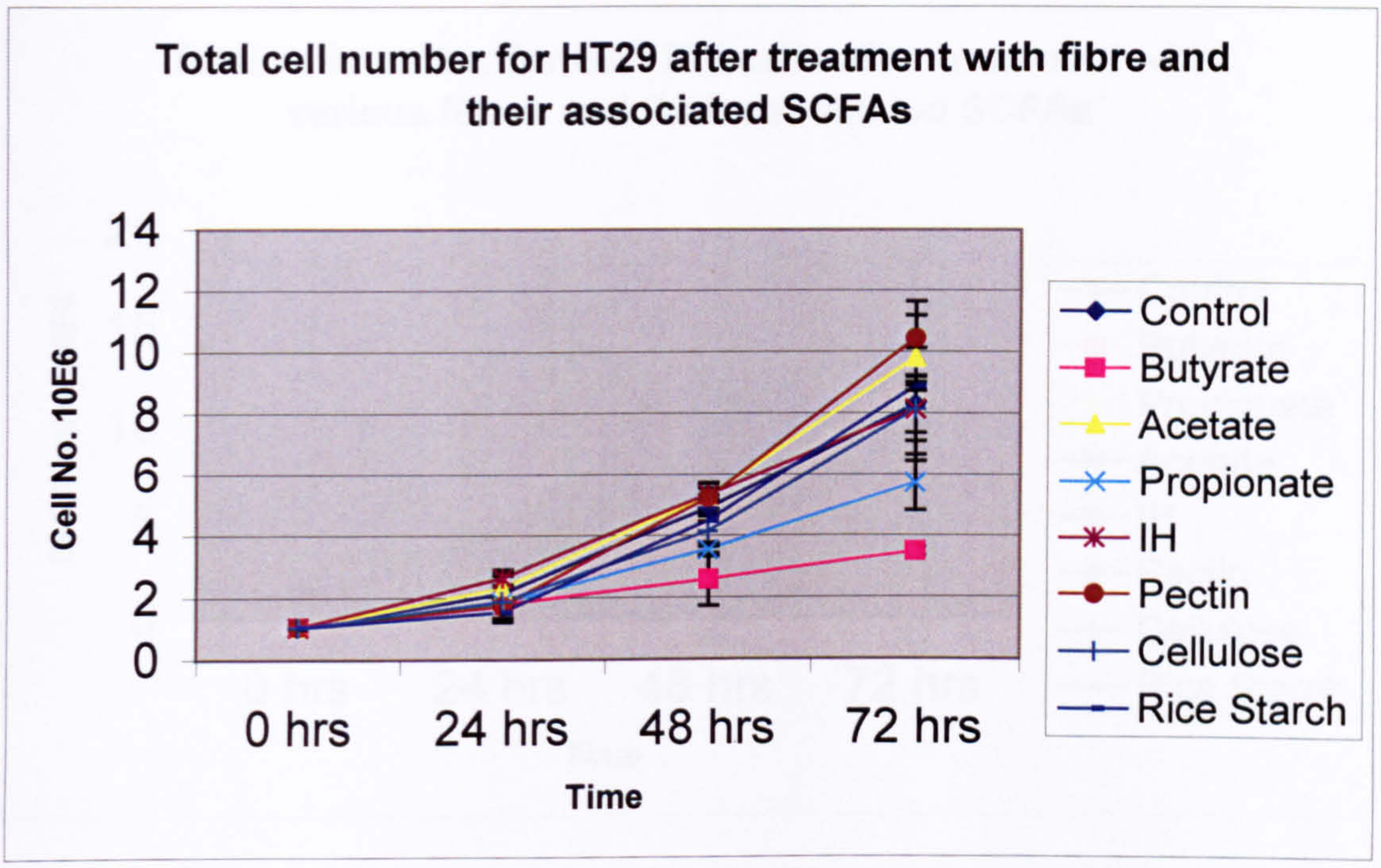
The results from this study revealed that in the carcinoma cell lines HCT116 (Figure 6.1), HT29 (Figure 6.2), SW1222 (Figure 6.3), LS174T (Figure 6.4), the adenoma cell line RG/C2 (figure 6.5) and the murine rectal carcinoma cell line CMT-93 (Figure 6.6) that 4mM butyrate and to a lesser extent 10mM propionate resulted in a reduction in total cell number over a 72 hour period. The difference in total cell numbers at 72 hours with butyrate and propionate treatment was only shown to be stastically significantly different to the control in the cell lines HCT116 (Student T-test $p=0.008$ and $p=0.009$ respectively) and LS174T (Student T-test $p=0.037$ and $p=0.031$ respectively). The reduction in total cell numbers suggested a decrease in proliferation and possibly the induction of growth arrest. The carcinoma cell line HT29 appeared to be less susceptible to the effects of butyrate and propionate (Figure 6.2) but this result is inconclusive. The inhibition of proliferation by the SCFAs butyrate and propionate in the carcinoma derived cell lines HT29 (figure 6.7) and SW1222 (figure 6.8) were confirmed using ki-67 nuclear expression. These results revealed that in the cell line SW1222 cell numbers after butyrate treatment was shown to be statistically significantly different to the control at concentrations of 1mM, 2mM and 4mM (Student T-test $p=0.0006$, $p=0.0001$ and $p=0.0004$ respectively). This was also shown for treatment with propionate at concentrations of 3mM, 6mM and 10mM there was a statistically significant difference in ki-67 expression when compared to the control (Student T-test $p=0.001$, $p=0.0007$ and $p=0.00008$ respectively). For the cell line HT29 a statistically significant difference in ki-67 expression from the control was only observed at a concentration of 4mM for butyrate and 10 mM for propionate (Student T-test $p=0.02$ and $p=0.04$ respectively). Thus these findings suggested that both butyrate and propionate treatment resulted in a reduction in cell proliferation. The fibres, ispaghula husk, pectin, cellulose and rice starch appeared to have little effect on total cell numbers at

Figure 6.1. The SCFAs butyrate and propionate reduced total cell number in the colonic epithelial cell line HCT116 (p23).



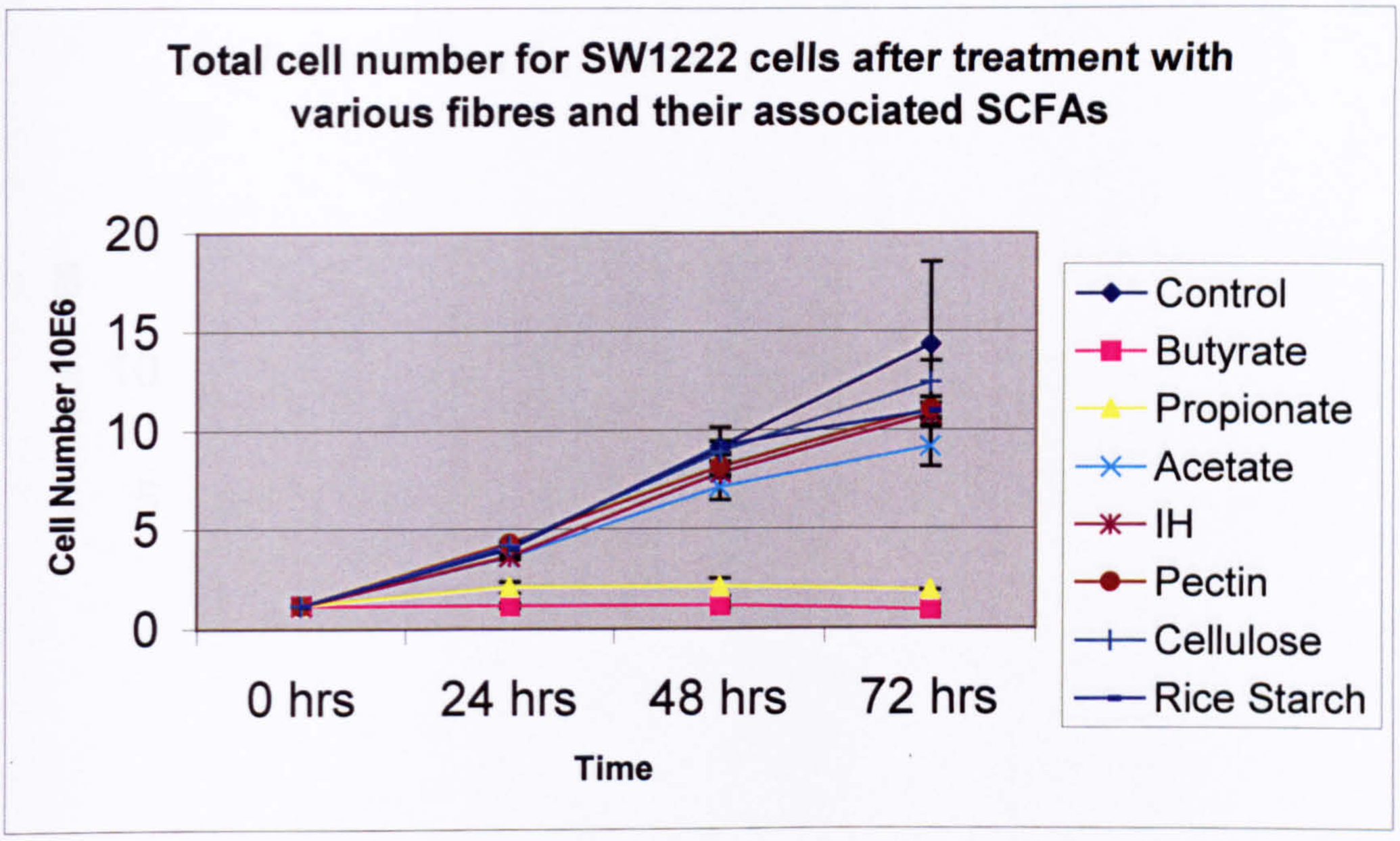
The graph revealed that the short chain fatty acids butyrate and propionate reduced total cell numbers by inhibited cell proliferation (reducing cell growth, causing growth arrest) or induced apoptosis when compared to the control. Both Butyrate and propionate were shown to be significantly different to the control at 72 hours (Student T-test $p=0.008$ and $p=0.009$ respectively). The remaining short chain fatty acid acetate along with the fibres: pectin, ispaghula, cellulose and rice/resistant starch have little effect on total cell numbers when compared to control (From one independent study $N=1$) (Student T-test $p>0.05$).

Figure 6.2. The SCFAs butyrate and propionate reduced total cell numbers in the colonic epithelial cell line HT29 (p186).



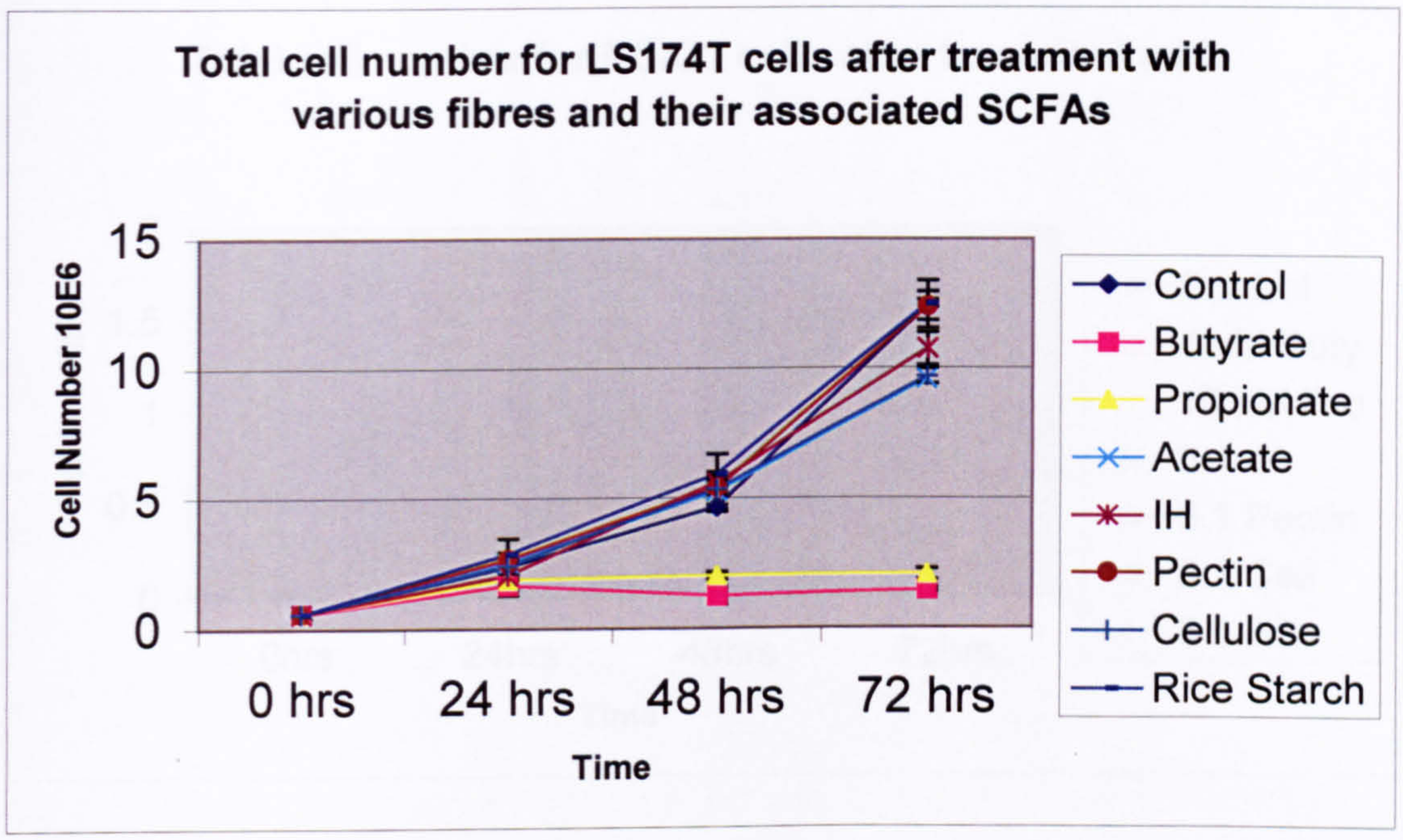
The graph revealed that the short chain fatty acids butyrate and propionate reduced total cell numbers by inhibited cell proliferation (reducing cell growth, causing growth arrest) or increased cell apoptosis compared to the control. This was found not to be significantly different to the control at 72 hours (Student T-test p=0.1.67 and p=0.462) for butyrate and propionate respectively. The remaining short chain fatty acid acetate along with the fibres: pectin, ispaghula, cellulose and rice/resistant starch had little effect on total cell numbers when compared to control (Results representative of findings from one independent study N=1) (Student T-test p>0.1).

Figure 6.3. The SCFAs butyrate and propionate reduced total cell *number in the colonic epithelial cell line SW1222 (p119).*



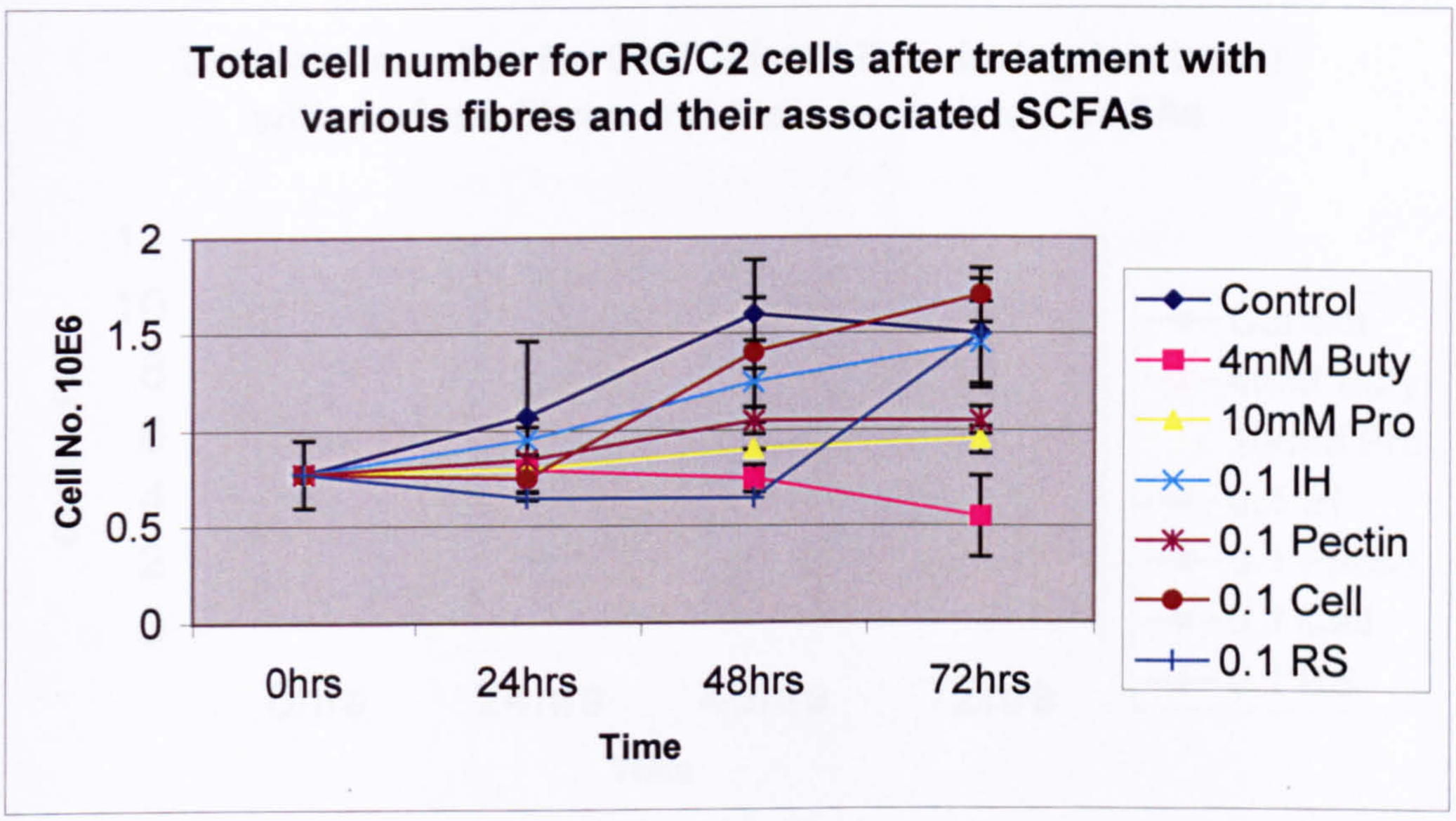
The graph revealed that the short chain fatty acids butyrate and propionate reduced total cell numbers by inhibited cell proliferation (reducing cell growth, causing growth arrest) or increased apoptosis compared to the control. This was found not to be significantly different to the control at 72 hours (Student T-test $p=0.137$ and $p=0.148$) for butyrate and propionate respectively. The remaining short chain fatty acid acetate along with the fibres: pectin, ispaghula, cellulose and rice/resistant starch had little effect on total cell numbers when compared to control (Representative results from two independent studies N=2) (Student T-test $p>0.3$).

Figure 6.4. The SCFAs butyrate and propionate reduced total cell numbers in the colonic epithelial cell line LS174T (p40).



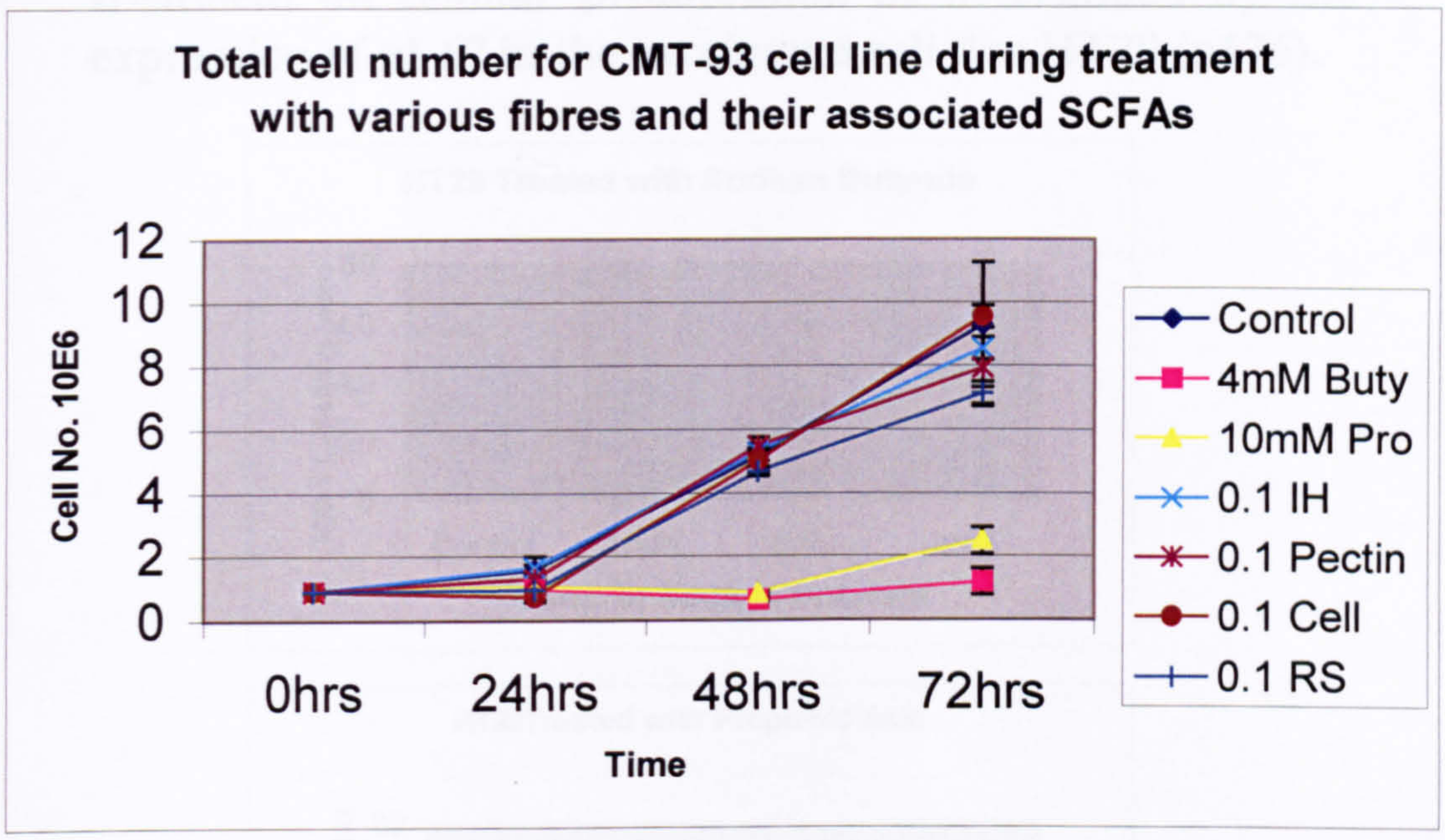
The graph revealed that the short chain fatty acids butyrate and propionate reduced total cell numbers by inhibited cell proliferation (reducing cell growth, causing growth arrest) or increased apoptosis compared to the control. Both Butyrate and propionate were shown to be significantly different to the control at 72 hours (Student T-test $p=0.037$ and $p=0.03$ respectively). The remaining short chain fatty acid acetate along with the fibres: pectin, ispaghula, cellulose and rice/resistant starch had little effect on cell proliferation when compared to control (N=2) (Student T-test $p>0.1$).

Figure 6.5. The SCFAs butyrate and propionate reduced total cell numbers in the colonic epithelial cell line RG/C2 (p42).



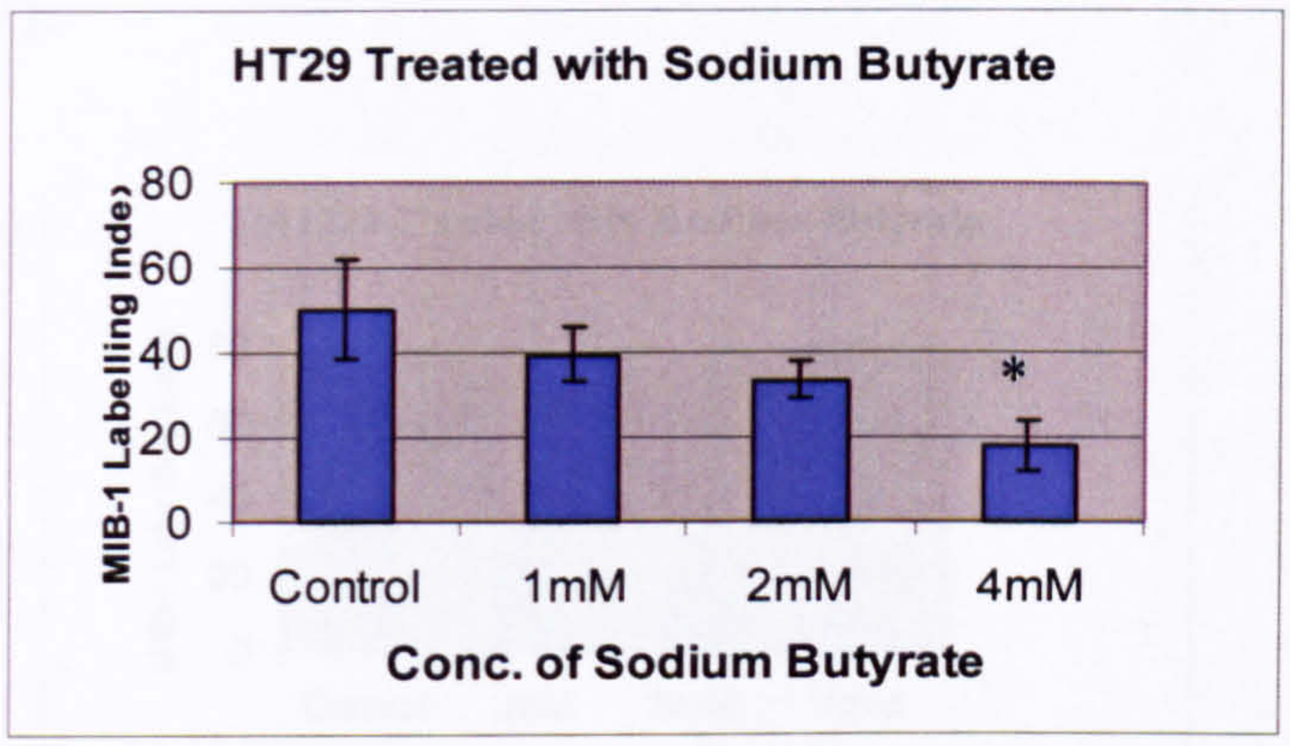
The graph revealed that the short chain fatty acids butyrate and propionate reduced total cell numbers by inhibited cell proliferation (reducing cell growth, causing growth arrest) or increased apoptosis compared to the control. This was found not to be significantly different to the control at 72 hours (Student T-test $p=0.07$ and $p=0.2$) for butyrate and propionate respectively. The remaining short chain fatty acid acetate along with the fibres: pectin, ispaghula, cellulose and rice/resistant starch had no obvious effect on total cell numbers when compared to control at 72 hours ($N=1$) (Student T-test $p>0.2$).

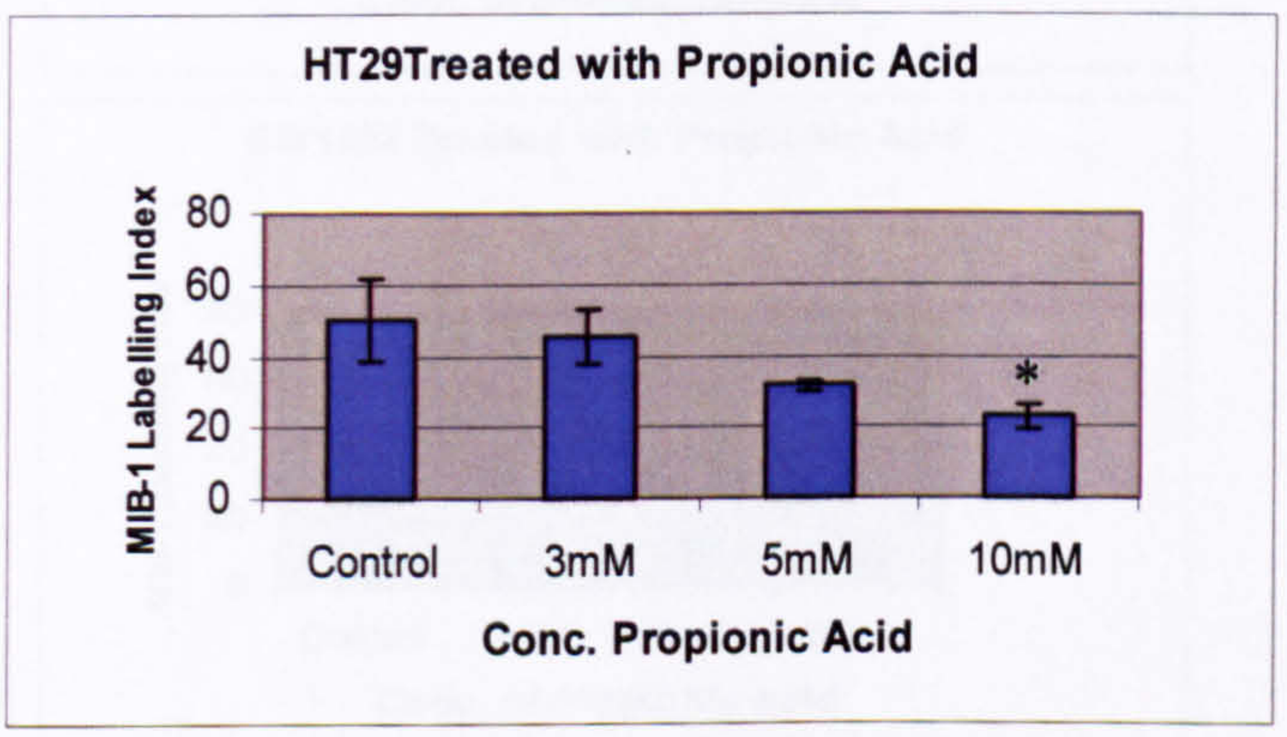
Figure 6.6. The SCFAs butyrate and propionate reduced total cell number in the colonic epithelial cell line CMT-93 (p5).

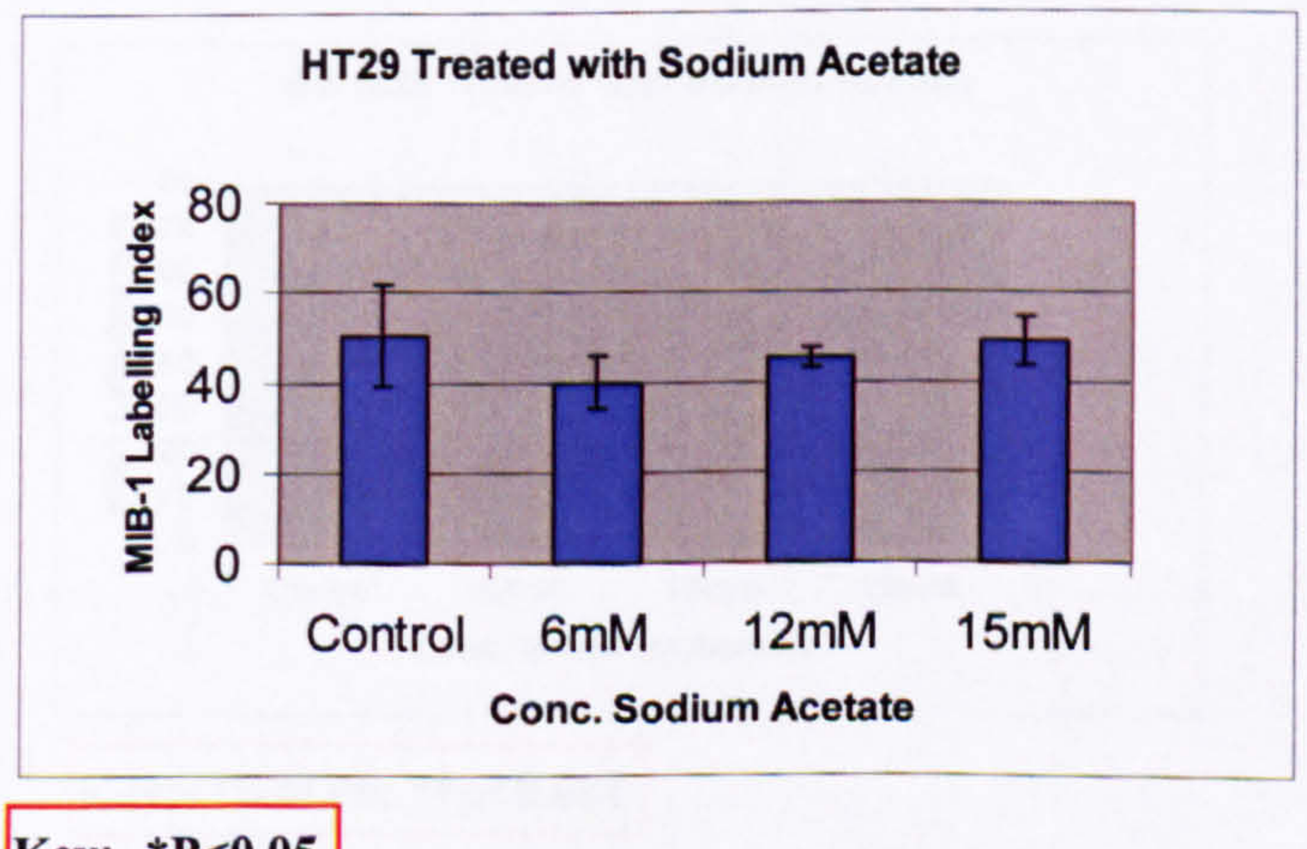


The graph revealed that the short chain fatty acids butyrate and propionate reduced total cell numbers by inhibited cell proliferation (reducing cell growth, causing growth arrest) or increased apoptosis compared to the control. This was found not to be significantly different to the control at 72 hours (Student T-test $p=0.097$ and $p=0.117$) for butyrate and propionate respectively, this could possibly due to the large variation within the control. The remaining short chain fatty acid acetate along with the fibres: pectin, ispaghula, cellulose and rice/resistant starch had little effect on total cell numbers when compared to control ($N=2$) and were shown to have no significant difference to the control at 72 hours (Student T-test $p>0.5$).

Figure 6.7 Histograms showing the effects of SCFA treatment on cellular proliferation as determined by the expression of κI-67 in the carcinoma cell line HT29 (p176).



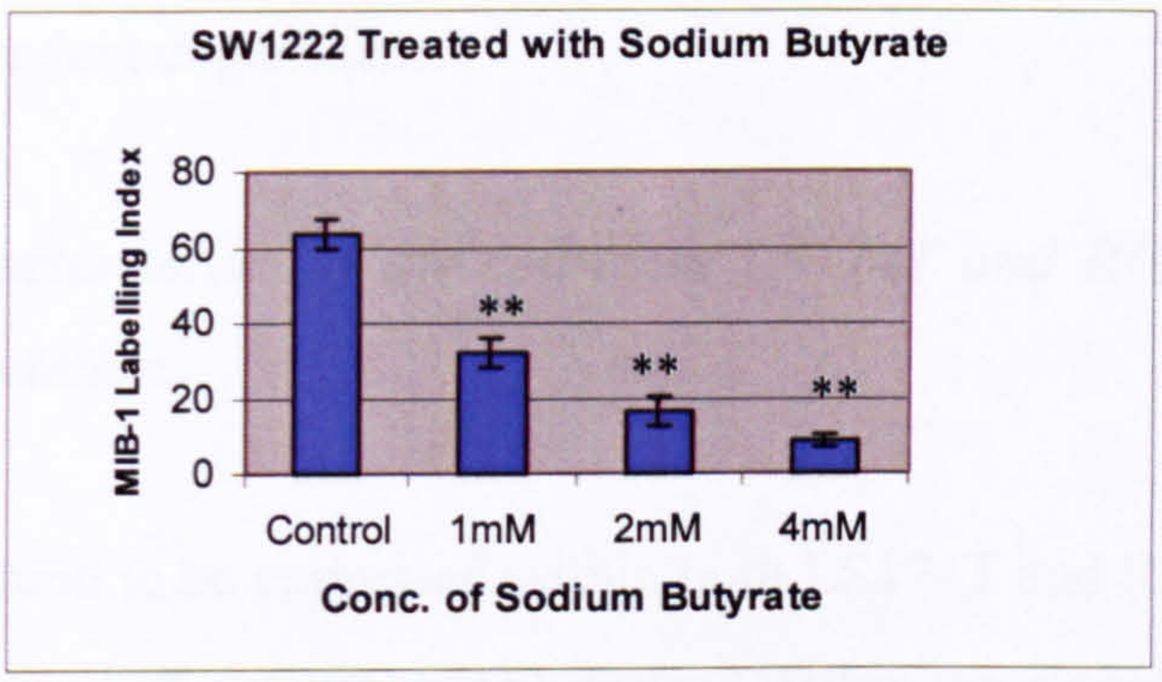


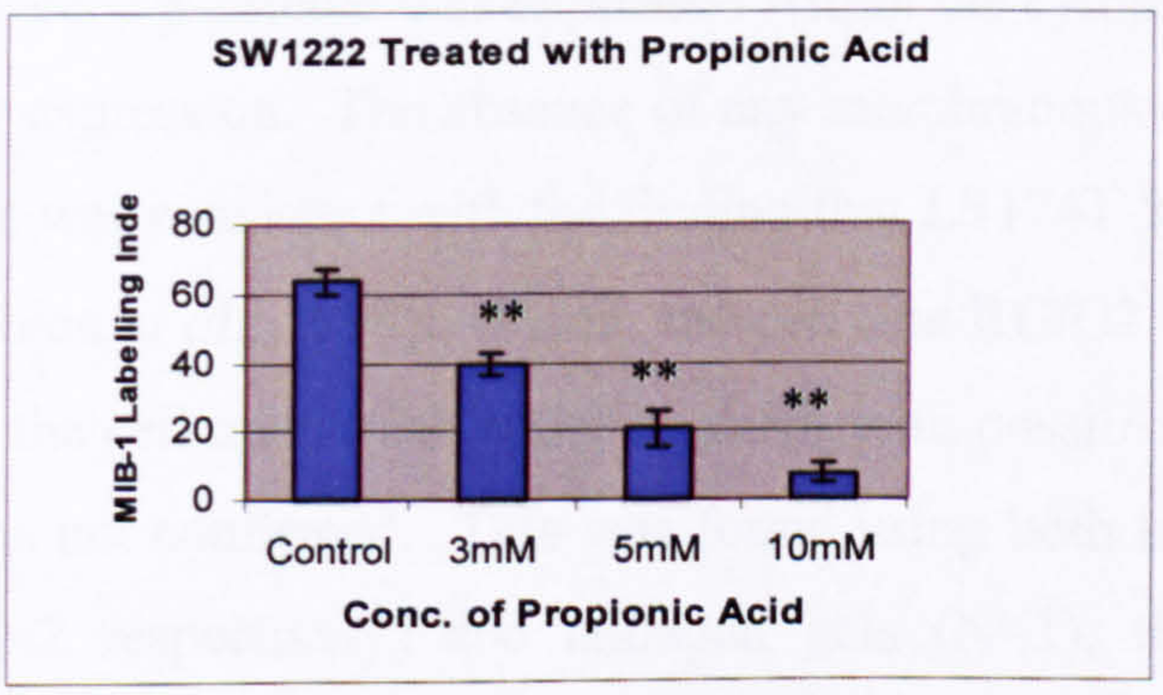


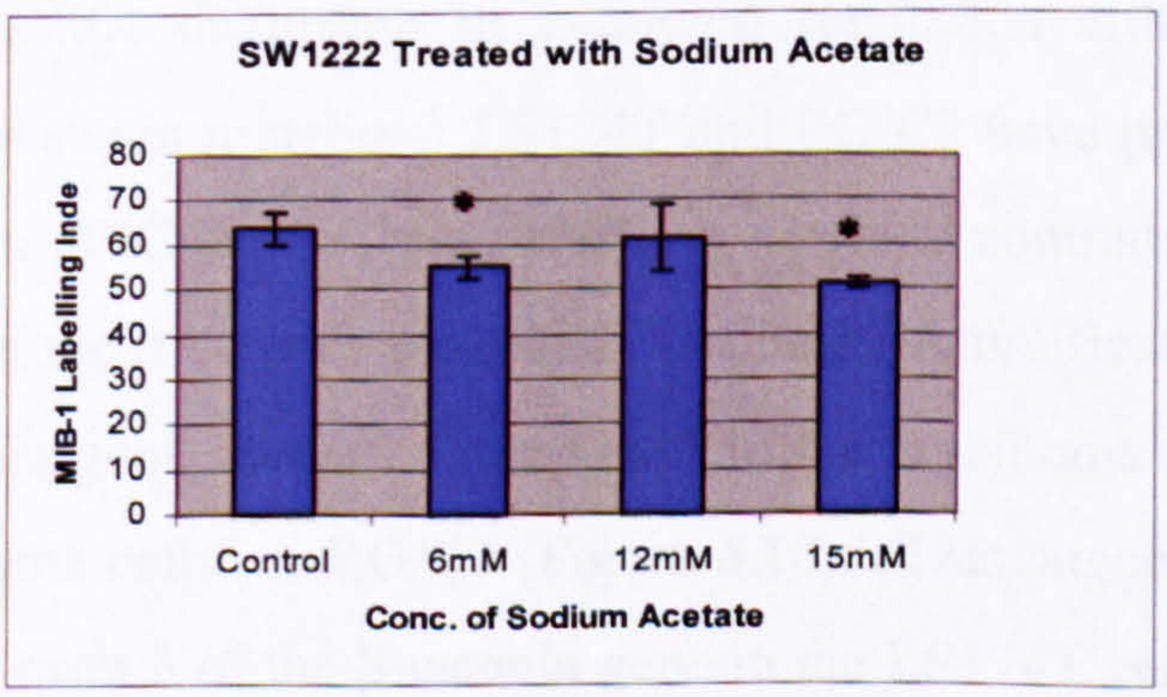
Key:- *P<0.05

The histograms revealed that both butyrate and propionate resulted in a reduction in κI-67 expression which suggested they resulted in a reduction in cell turnover. This was shown to be statistically significant different from the control at a concentration of 4mM for butyrate and 10 mM for propionate (Student T-test p=0.02 and p=0.04 respectively) only.

Figure 6.8 Histograms representing the effects of SCFA treatment on cellular proliferation as determined by the expression of κi-67 in the carcinoma cell line SW1222 (p107).







Key:- *p<0.05; **p<0.001

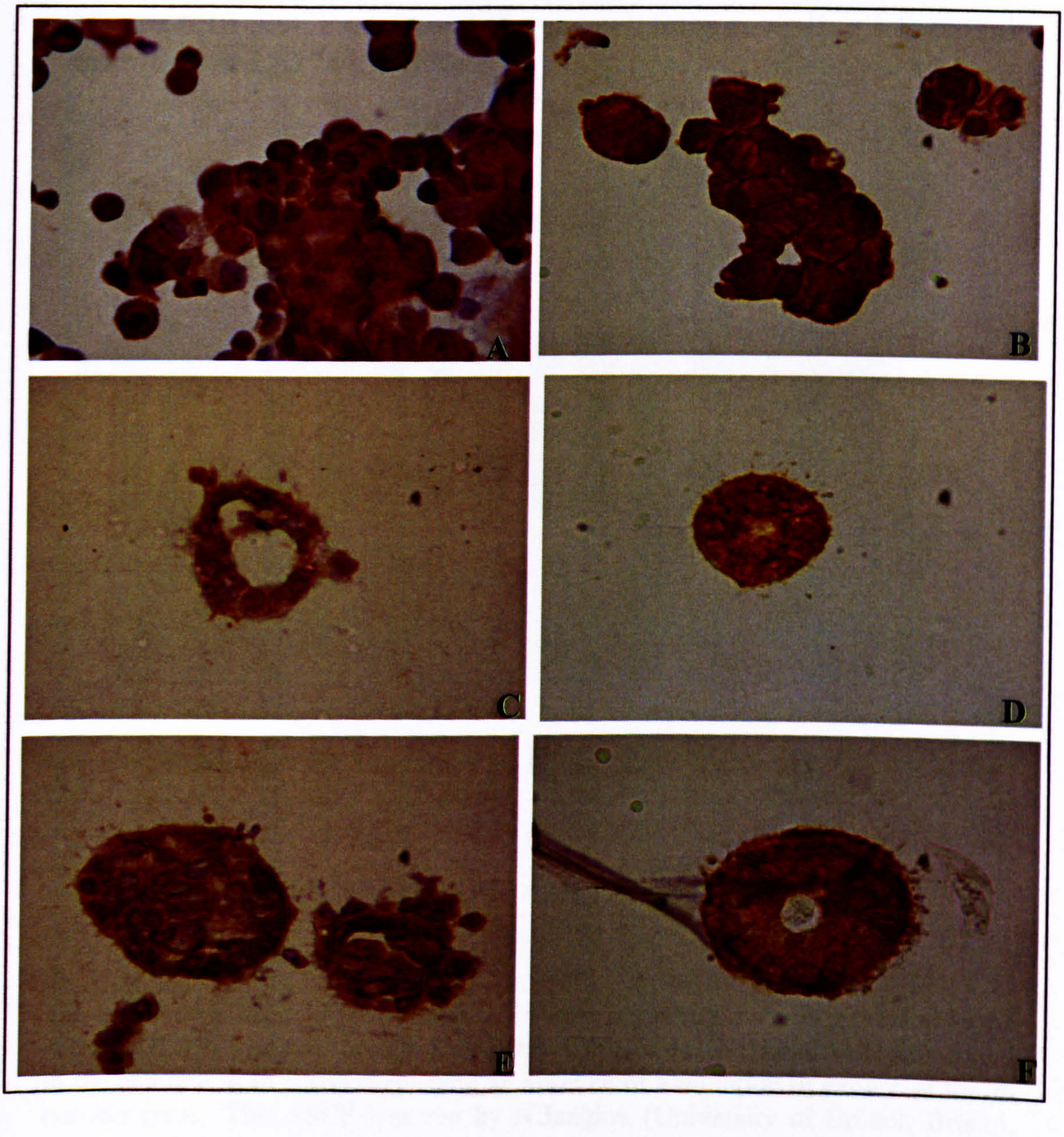
The histograms revealed that both butyrate and propionate resulted in a reduction in κI-67 expression which suggested they resulted in a reduction in cell turnover. This was shown to be statistically significant different from the control for all concentrations of butyrate and propionate (Student T-test $p<0.001$). There were also differences observed at 6mM and 15mM concentrations of acetate (Student T-test $p<0.05$).

a concentration of 0.1% in any of the carcinoma or adenoma derived cell lines (Student T-test $p>0.05$), although this does not concisely prove that there was no alteration in cell proliferation. Using ki-67 expression acetate was shown to case a significant difference in ki-67 expression compared to the control at concentration of 6mM and 15mM (Student T-test $p=0.037$ and $p=0.022$ respectively) within the colonic carcinoma cell line SW1222, although this result is inconclusive because it is from one independent experiment.

6.4.2 *The characterisation of the cell lines LS174T and RG/C2 for a exon 3 β-catenin gene mutation.*

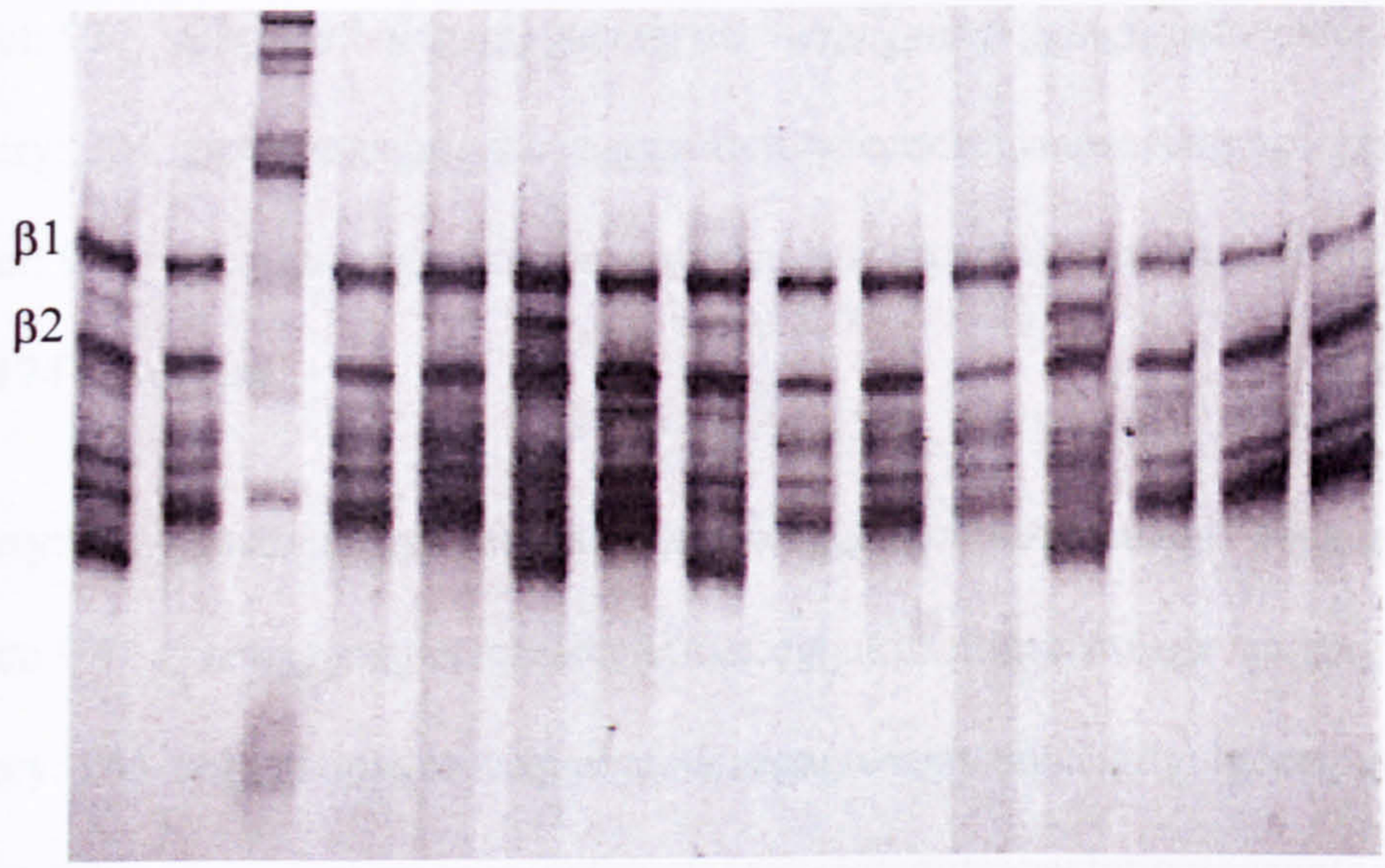
β-catenin was found to be expressed within both LS174T and RG/C2 cell line (figure 6.9). In LS174T cells β-catenin was expressed within the cytoplasm accompanied by possible nuclear expression. The absence of any membranous expression within the LS174T cell line was consistent with the finding that LS174T lacks expression of E-cadherin (Efstathiou *et al.*, 1999). Within the cell line RG/C2, β-catenin expression was localised at the cell membrane and cytoplasm with possible nuclear localisation, however this was not confirmed. This was found using both immunocytochemistry (ICC) (N=1, N=2 respectively) and collagen gels (N=1), these results together suggested a possible disruption in β-catenin regulation either through an APC mutation or a β-catenin mutation. LS174T and RG/C2 have previously been shown to have wild type APC (Ref; Chris Paraskeva personal communication respectively) thus the status of the β-catenin gene was examined. Amplification products of exon 3 of the β-catenin gene showed a band shift in the carcinoma cell line LS174T but not in the adenoma cell line RG/C2 (Figure 6.10). This suggested that there was a mutation within exon 3 of the β-catenin gene in the LS174T cell line, a result which was confirmed using sequence analysis of the gene product (Figure 6.11), revealing a C to T point mutation. The lack of a band shift for the cell line RG/C2 suggested no such mutation in exon 3, which was confirmed by sequence analysis (Figure 6.12). These results suggest that the cell line RG/C2 may not exhibit nuclear localisation, carry a mutation in the β-catenin gene or may affect another exon that has not been studied, or another possible mechanism that results in the deregulation of β-catenin. Further experiments are required to determine whether β-catenin is expressed within

Figure 6.9 Expression of β-catenin in LS174T (p18) and RG/C2 (p46) growing as a monolayer (1) and in a 3D collagen gel (2).



For LS174T ICC (**1**) revealed cytoplasmic and possible nuclear expression (**A**), the accompanying 3D collagen gels also revealed this pattern of expression at 5 days (**C**) and 1 week (**E**), although there appeared to be no nuclear localisation within the cells grown in collagen. For RG/C2 ICC (**2**) revealed membranous, cytoplasmic coupled with possible nuclear localisation and the collagen gels (**B**) after 1 week (**D**) and 2 weeks (**F**). (LS174T N=1; RG/C2 N=2 (ICC) and N=1) (x40 Objective plus telescopic zoom).

Figure 6.10 SSCP on four different cell lines revealing a β-catenin mutation in LS174T cell line.



Key:- A= LS174T, B= RG/C2, C= AA/C1, D= HT29, M=Marker

The SSCP revealed a possible mutation in exon 3 of the *β-catenin* gene within the cell line LS174T (**A**) as there is a clear band shift seen. No band shift was found in the cell line RG/C2 (**B**) suggesting an absence of a mutation in exon 3 of the *β-catenin* gene. This SSCP was run by N.Janghra (University of Bristol, Bristol, UK).

Figure 6.11 Sequence analysis of LS174T (p21) for exon 3 of the *β-catenin* gene.

LS174T Forward

Query: 84 gttagtcactggcagcaacagtcttacctggactctggaatccattctggtgccactacc 143

Sbjct: 867 gttagtcactggcagcaacagtcttacctggactctggaatccattctggtgccactacc 926

Query: 144 acagctcctttctgagtggtaaaggcaatcctgaggaagaggatgtggatacctcccaa 203

Sbjct: 927 acagctccttctctgagtggtaaaggcaatcctgaggaagaggatgtggatacctcccaa 986

Query: 204 gtcctgtatgagtgggaacagggattttctcagtccttcactcaagaacaagtagctggt 263

Sbjct: 987 gtcctgtatgagtgggaacagggattttctcagtccttcactcaagaacaagtagctggt 1046

LS174T Reverse

Query: 85 ggacttgggaggtatccacatcctcttcctcaggattgcctttaccactcagaaaaggag 144

Sbjct: 990 ggacttgggaggtatccacatcctcttcctcaggattgcctttaccactcagagaaggag 931

Query: 145 ctgtggtagtggcaccagaatggattccagagtccaggtaagactgttgctgccagtgac 204

Sbjct: 930 ctgtggtagtggcaccagaatggattccagagtccaggtaagactgttgctgccagtgac 871

The sequence analysis for LS174T revealed a C ---> T point mutation in exon 3 of the *β-catenin* gene.

Figure 6.12 Sequence analysis of RG/C2 (p46) PCR product of exon 3 of the *β-catenin* gene.

RG/C2 forward

Query: 5 gtttcgtatttatagctgatttgatggagttggacatggccatgggaaccaagacagaaa 64

Sbjct: 802 gtttcgtatttatagctgatttgatggagttggacatggccat-ggaacc-agacagaaa 859

Query: 65 agcggctgttagtcactgggcagcaacagtcttacctggactctggaatccattctggtg 124

Sbjct: 860 agcggctgttagtcact-ggcagcaacagtcttacctggactctggaatccattctggtg 918

Query: 125 ccactaccacagctccttctctgagtggtaaaggcaatcctgaggaagaggatgtggata 184

Sbjct: 919 ccactaccacagctccttctctgagtggtaaaggcaatcctgaggaagaggatgtggata 978

Query: 185 cctcccaagtcctgtatgagtgggaacagggattttctcaagtccttcactcaagaacaa 244

Sbjct: 979 cctcccaagtcctgtatgagtgggaacagggattttctc-agtccttcactcaagaacaa 1037

Query: 245 gtagctggtaagagtattatttttcattgccttactgaaagtcagaatgcag 296

Sbjct: 1038 gtagctggtaagagtattatttttcattgccttactgaaagtcagaatgcag 1089

RG/C2 Reverse

Query: 22 cagctacttgttcttgantgaaggactgagaaaa-ccctgttcccactcatacaggactt 80

Sbjct: 1044 cagctacttgttcttgagtgaaggactgagaaaatccctgttcccactcatacaggactt 985

Query: 81 gggaggtatccacatcctcttcctcaggattgcctttaccactcagaaaagganctgtgg 140

Sbjct: 984 gggaggtatccacatcctcttcctcaggattgcctttaccactcagagaaggagctgtgg 925

Query: 141 tantggcaccaagaatggnttccagagtccaggtaagactgttgctgccantgnctaaca 200

Sbjct: 924 tagtggcacca-gaatggattccagagtccaggtaagactgttgctgccagtgactaaca 866

Query: 201 gccgcttttctgtctggttccatggccatgtccaactccatcaaatcagctataaatacn 260

Sbjct: 865 gccgcttttctgtctggttccatggccatgtccaactccatcaaatcagctataaatacg 806

Query: 261 aaacagtattatcattagtagattggaaatg 291

Sbjct: 805 aaacagtattagcattagtagattggaaatg 775

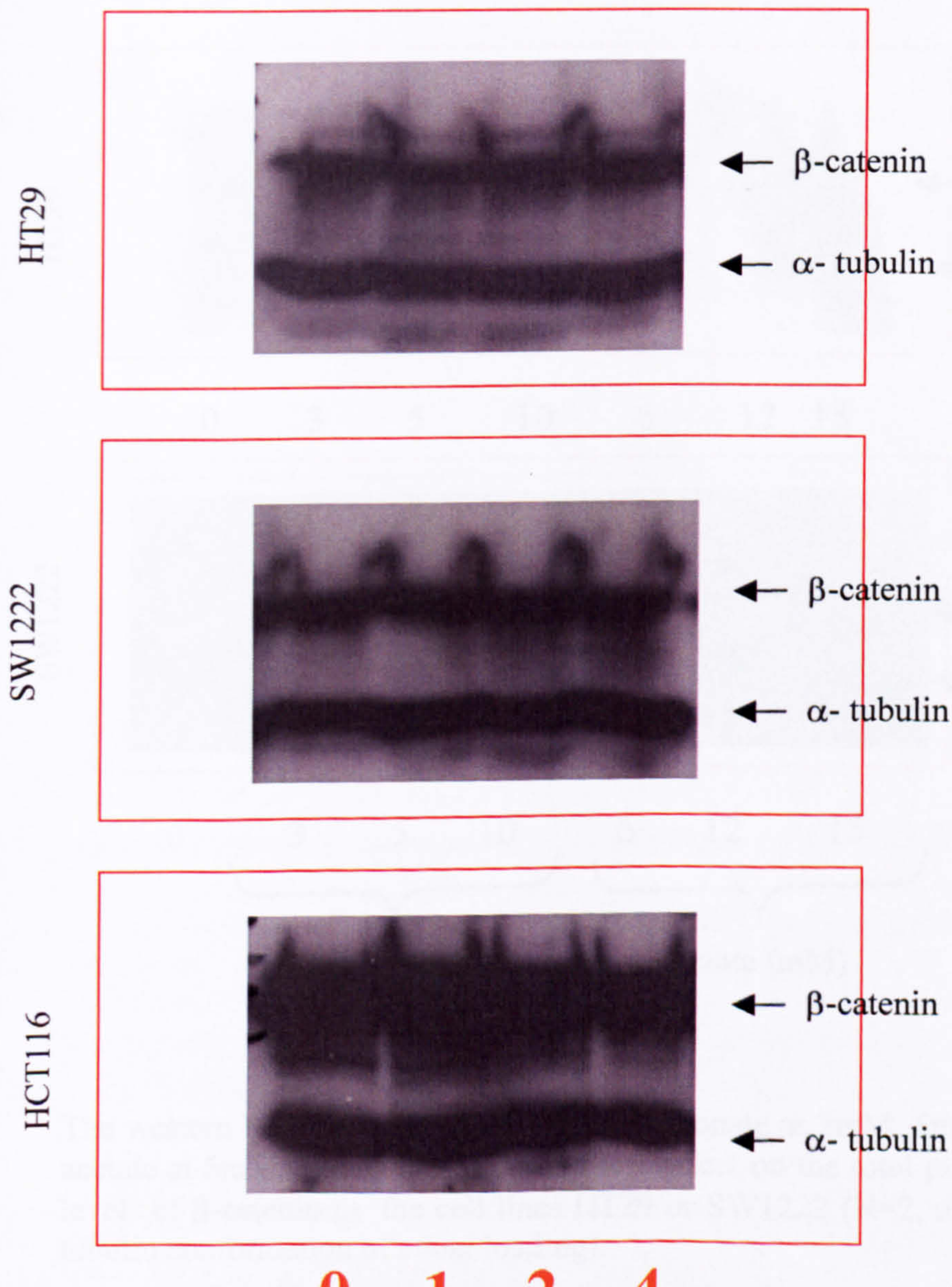
The sequencing analysis confirmed that there was no mutation in exon 3 of the *β-catenin* gene in the RG/C2 colonic adenoma cell line.

the cytoplasm and nucleus of the RG/C2 cell line and whether RG/C2 carries a β-catenin mutation.

6.4.3 *The effects of SCFAs acetate, propionate and butyrate on β-catenin protein expression.*

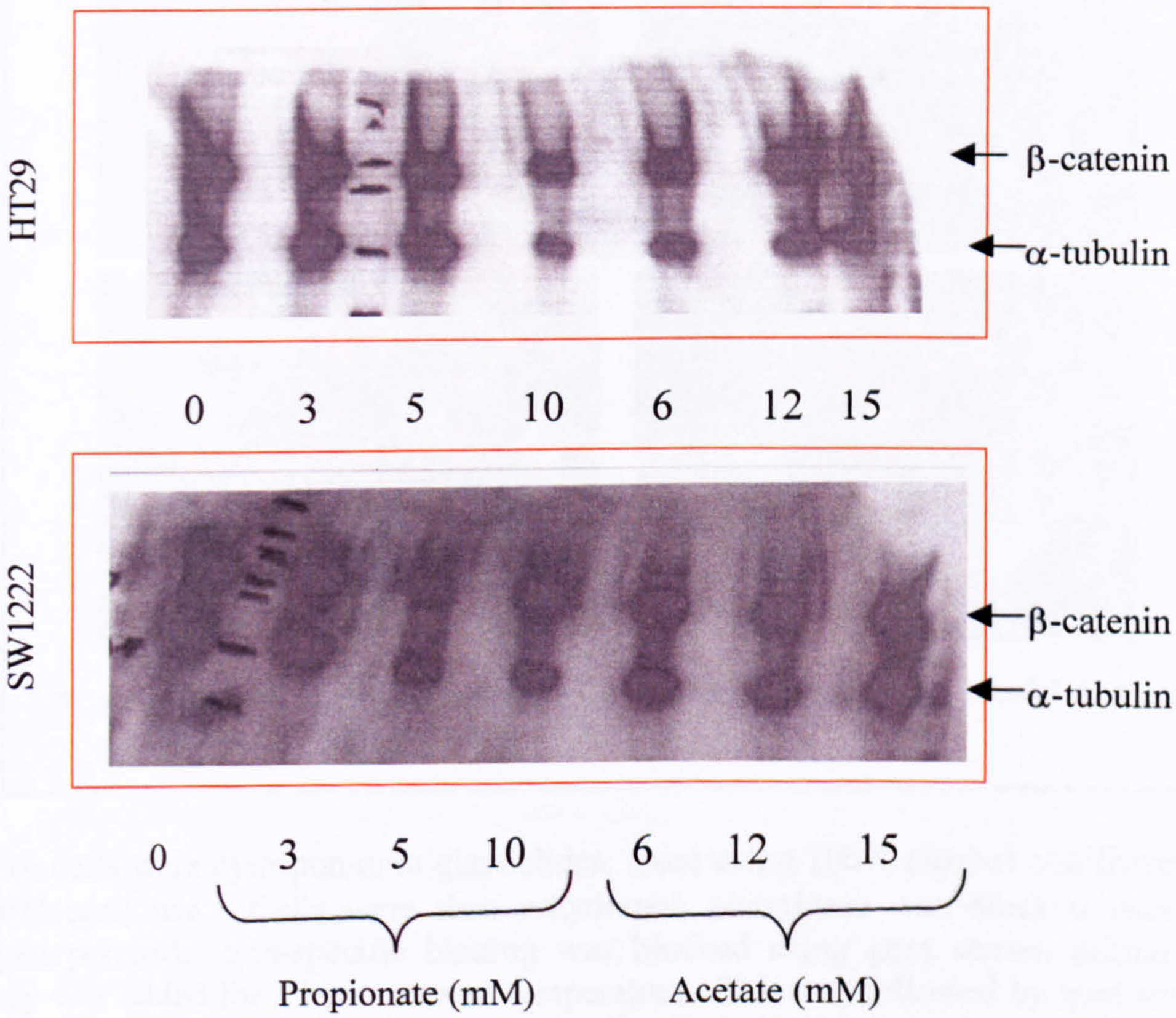
After 48 hours incubation of HT29, SW1222 and HCT116 with varying concentrations of the SCFAs butyrate, propionate and acetate there were no alterations in the total protein levels of β-catenin when examined using western blotting (figure 6.13 and 6.14) although for HCT116 this result was from one independent experiment thus was not conclusive. Using immunocytochemistry there were differential responses to butyrate in the three cell lines. Within the cell line HCT116 (figure 6.15), which has a β-catenin mutation, β-catenin localisation changes from membranous and cytoplasmic with an accompanied loss of membranous expression in the butyrate treated cells. The other SCFAs had little effect on cellular localisation of β-catenin in the HCT116 cell line, although this result was not conclusive as this result was from only one independent study. In the carcinoma cell lines HT29 (figure 6.16) and SW1222 (figure 6.17) there was little effect on β-catenin localisation after treatment with the SCFAs butyrate, propionate and acetate. Immunofluorescence using confocal microscopy confirmed the relocalisation of β-catenin into the cytoplasm within the HCT116 carcinoma derived cells when treated with 4mM butyrate for 48 hours (Figure 6.18). Immuoflouresence also confirmed that this did not occur in the carcinoma derived cell line HT29 when treated with 4mM butyrate for 48 hours (Figure 6.19). Using western blot analysis to examine the different fractions of the cell, soluble (cytoplasm and membrane) and the insoluble (actin cytoskeleton) fractions revealed that there was an increase in the soluble fraction in the HCT116 cells but not the HT29 cells upon treatment with 4mM butyrate for 48 hours (Figure 6.20). This suggested that butyrate treatment resulted in a reduction in the amount of β-catenin associated with the actin cytoskeleton, although these experiments did not elucidate whether this was due to an increase in membranous or cytoplasmic expression. The western blotting results were not conclusive as they were from one independent experiment and require repeating. Although when taken together with the immunocytochemistry and the

Figure 6.13 Effect of varying concentrations of butyrate on β-catenin total protein expression in HT29 (p185), SW1222 (p115) and HCT116 (p34) cell lines.



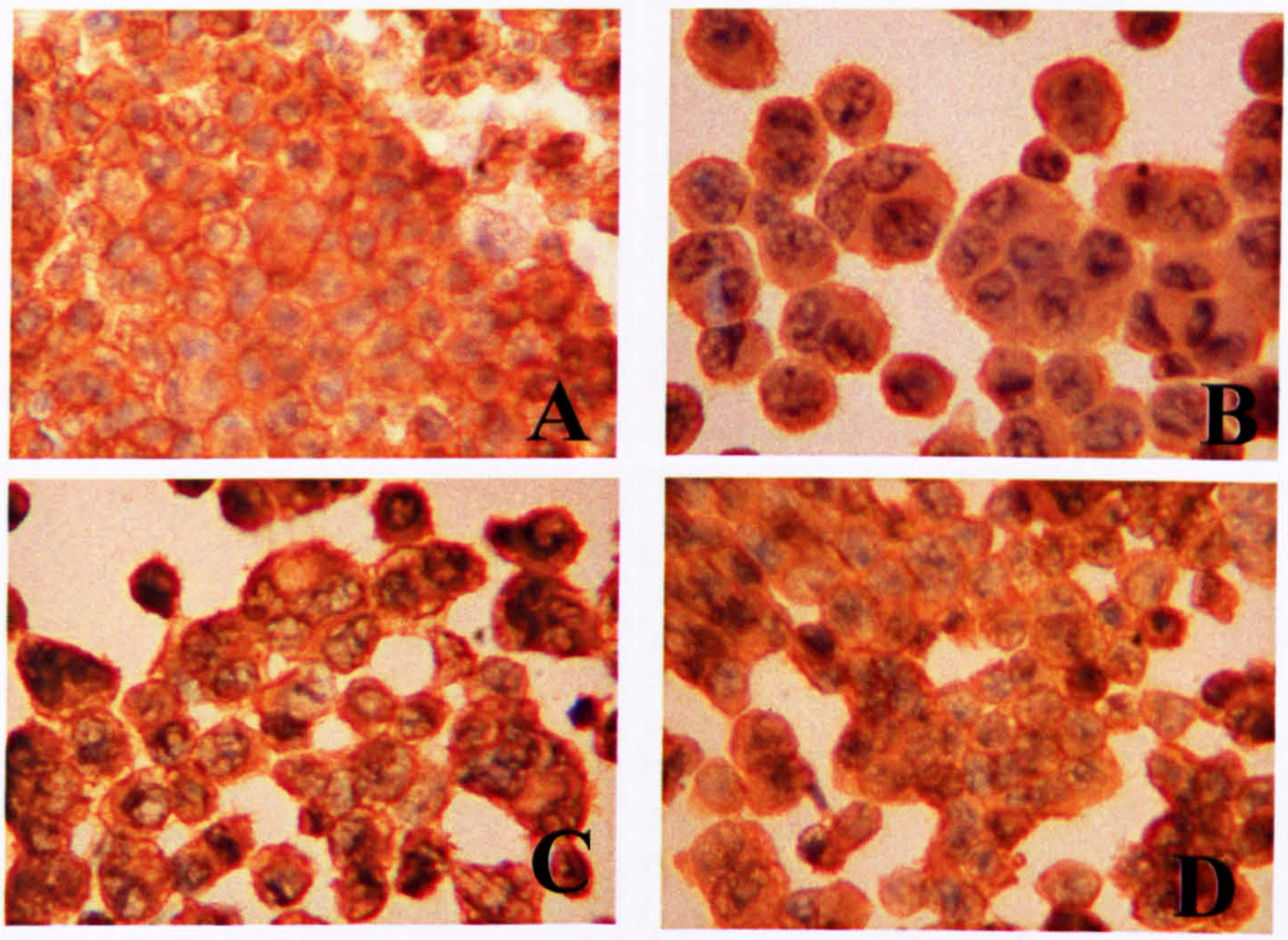
β-catenin protein expression was determined by western blotting (SDS-PAGE). The blot shown is a typical example of those obtained from at least 2 independent experiments, except for the cell line HCT116 (N=1). After 48 hour incubation of HT29, SW1222 and HCT116 with varying concentrations of butyrate (0, 1mM, 2mM and 4mM) there was no obvious effect on total β-catenin protein expression levels.

Figure 6.14 The effect of varying concentrations of the SCFAs propionate and acetate on β-catenin protein expression in the cell lines HT29 (p180) and SW1222 (p113).



The western blots revealed that neither propionate at 3mM, 5mM or 10mM or acetate at 6mM, 12mM or 15mM had any effect on the total protein expression levels of β-catenin in the cell lines HT29 or SW1222 (N=2, although N=1 for tubulin conformation of equal loading).

Figure 6.15 Butyrate treatment of colonic carcinoma derived HCT116 (p23) cell line results in the relocalisation of β-catenin from the cell membrane to the cytoplasm (x40).

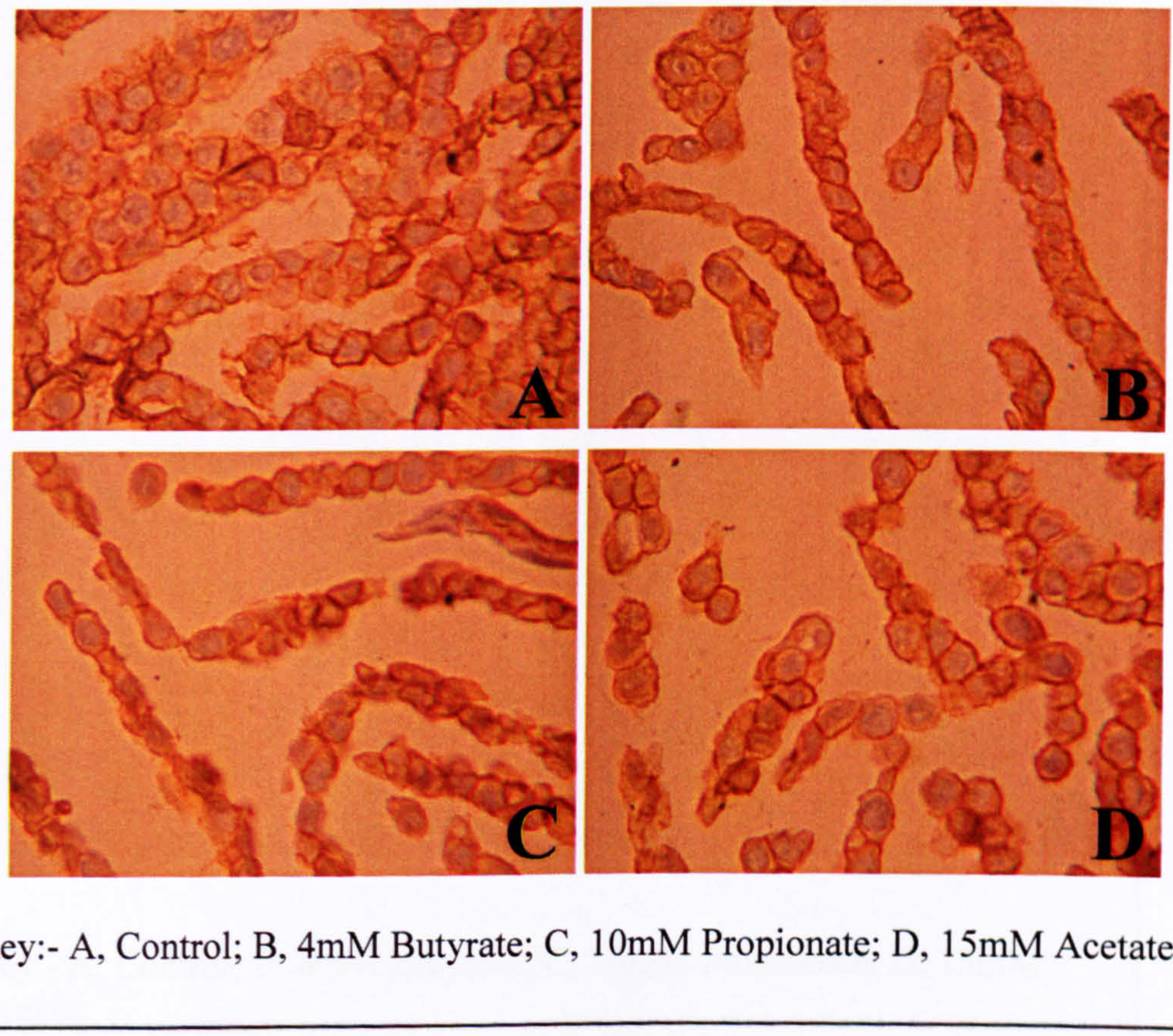


Key:- A, Control; B, 4mM Butyrate; C, 10mM Propionate; D, 15mM Acetate.

HCT116 cells were cytospun onto glass slides, fixed using 100% alcohol and frozen at -20°C until use. Cells were then rehydrated, peroxidase was blocked using hydrogen peroxide, non-specific binding was blocked using goat serum, primary antibody was added for 1 hour at room temperature. This was followed by goat anti mouse antibody (1:250, 30 minutes), streptavidine (1:250, 30 minutes) and DAB (10 minutes) dehydrated and mounted (N=1). (Magnification x40)

β-catenin localisation changed from membranous in control cells to cytoplasmic in 4mM butyrate (**B**) treated cells along with a number of morphological changes including an increase in size of the butyrate treated cells. The other SCFAs propionate (**C**) and acetate (**D**) had no obvious effect on the localisation of β-catenin when compared to control, with β-catenin being found at the cell membrane and cytoplasm with the nuclei remaining negative for expression.

Figure 6.16 The SCFAs, butyrate, propionate and acetate, had no obvious effect on the localisation of β-catenin in the cell line HT29 (p175) (x40).

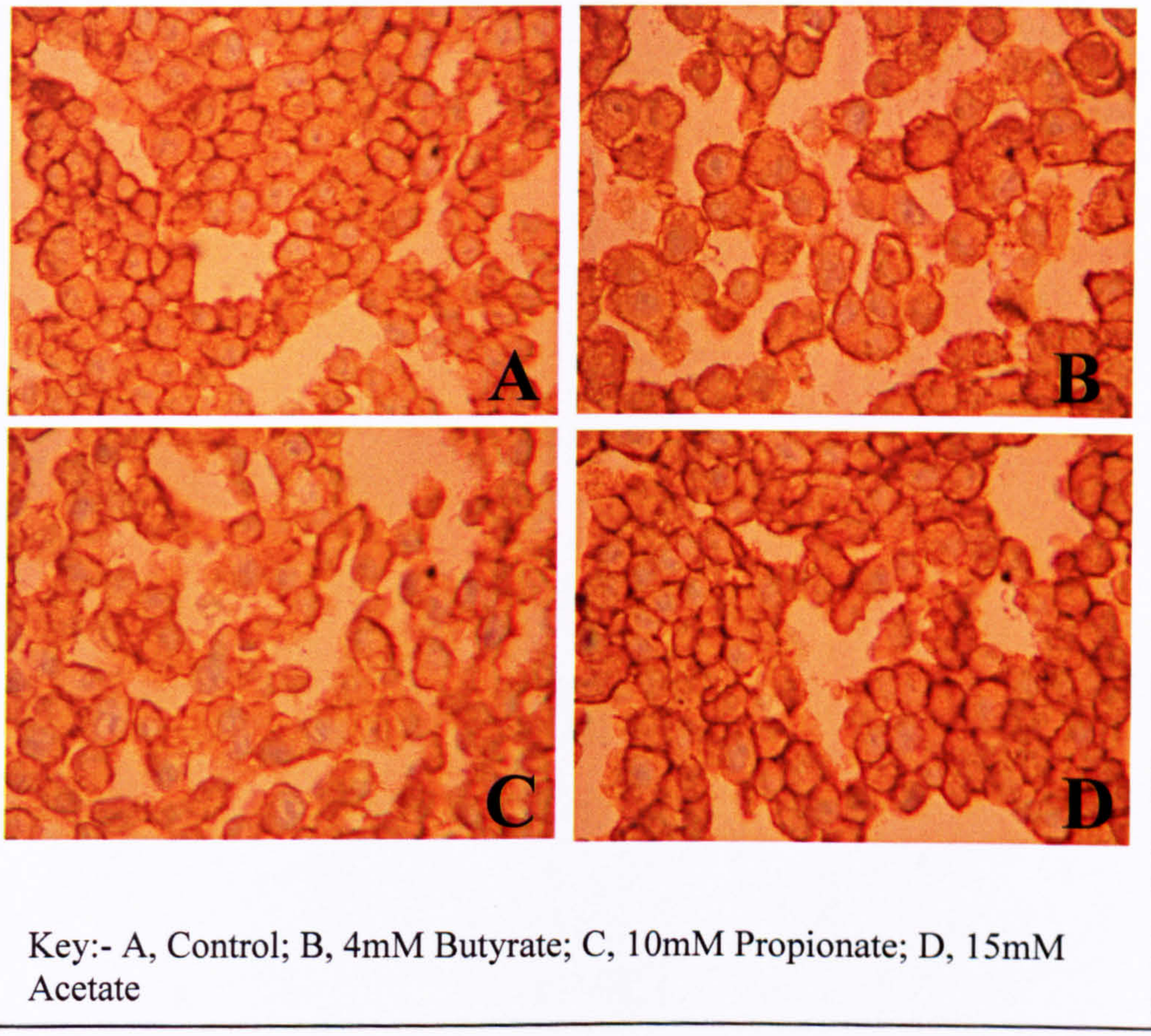


Key:- A, Control; B, 4mM Butyrate; C, 10mM Propionate; D, 15mM Acetate

HT29 cells were fixed using BNF, embedded in agar followed by paraffin and treated as tissues. Cells were rehydrated, peroxidase activity was blocked using hydrogen peroxide, non-specific binding was blocked using goat serum, primary antibody was added for 1 hour at room temperature. This was followed by goat anti mouse antibody (1:250, 30 minutes), streptavidine (1:250, 30 minutes) and DAB (10 minutes) dehydrated and mounted (N=3).

The luminal factors; butyrate (4mM) (**B**), propionate (10mM) (**C**), acetate (**B**) had no obvious effect on the localisation of β-catenin when compared to the control (**A**), with β-catenin being found at the cell membrane and cytoplasm with the nuclei remaining negative for expression.

Figure 6.17 The SCFAs, butyrate, propionate and acetate, had no obvious effect on the localisation of β-catenin localisation in the cell line SW1222 (p113) (x40).



Key:- A, Control; B, 4mM Butyrate; C, 10mM Propionate; D, 15mM Acetate

SW1222 cells were fixed using BNF, embedded in agar followed by paraffin and treated as tissues. Cells were rehydrated, peroxidase activity was blocked using hydrogen peroxide, non-specific binding was blocked using goat serum, primary antibody was added for 1 hour at room temperature. This was followed by goat anti mouse antibody (1:250, 30 minutes), streptavidine (1:250, 30 minutes) and DAB (10 minutes) dehydrated and mounted (N=3).

The luminal factors; butyrate (4mM) (**B**), propionate (10mM) (**C**), acetate (15mM) (**D**) had no obvious effect on the localisation of β-catenin when compared to the control (**A**), with β-catenin being found at the cell membrane and cytoplasm with the majority of the nuclei being negative for expression.

Figure 6.18 Butyrate treatment of colonic carcinoma-derived HCT116 cell line results in the relocalisation of β-catenin from the cell membrane to the cytoplasm.

HCT116 cells (p32) were grown on collagen coated glass slides, and were stained with mouse monoclonal anti-Beta-catenin primary antibody, followed by anti-mouse FITC secondary antibody. The slides were mounted using Vectorshield mounting medium and cells were viewed under the Leica TCS-NT confocal laser scanning microscope with leica TCS-NT software for 2D and 3D image analysis. Results are representative of triplicate experiments (N=3), each looking over 5 different fields (Magnification x63).

Visualisation of cells for FITC staining, Showing β-catenin localisation
Visualisation of cells for DAPI staining, showing Nuclei

A. Longitudinal sections.
B. Transverse sections.

β-catenin staining changes from predominantly membranous in control cells to an increased cytoplasmic expression in butyrate (4mM) treated cells.

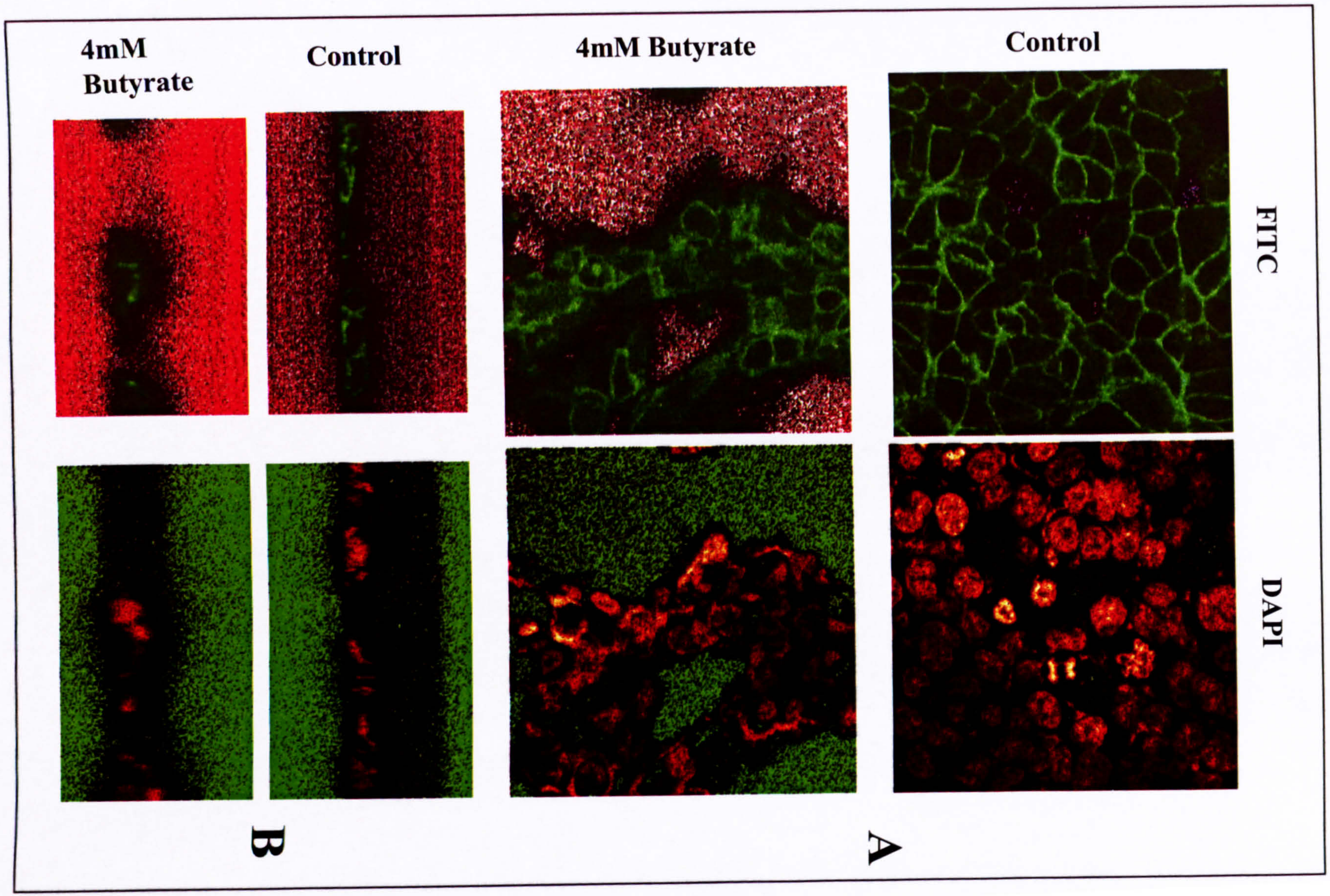

4mM Butyrate
Control
4mM Butyrate
Control
FITC
DAPI
A
B

Figure 6.19 Butyrate treatment of colonic carcinoma-derived HT29 cell line has no effect on the localisation of β-catenin.

HT29 cells (p185) were grown on collagen coated glass slides, and were stained with mouse monoclonal anti-Beta-catenin primary antibody, followed by anti-mouse FITC secondary antibody. The slides were mounted using Vectorshield mounting medium and cells were viewed under the Leica TCS-NT confocal laser scanning microscope with leica TCS-NT software for 2D and 3D image analysis. Results are representative of triplicate experiments, each looking over 5 different fields (Magnification x63).

Visualisation of cells for FITC staining, Showing β-catenin localisation
Visualisation of cells for DAPI staining, showing Nuclei

A Longitudinal sections.
B Transverse sections.

β-catenin staining remains unchanged in the butyrate (4mM) treated cells when compared to the control, with it being primarily found at the cell membranes.

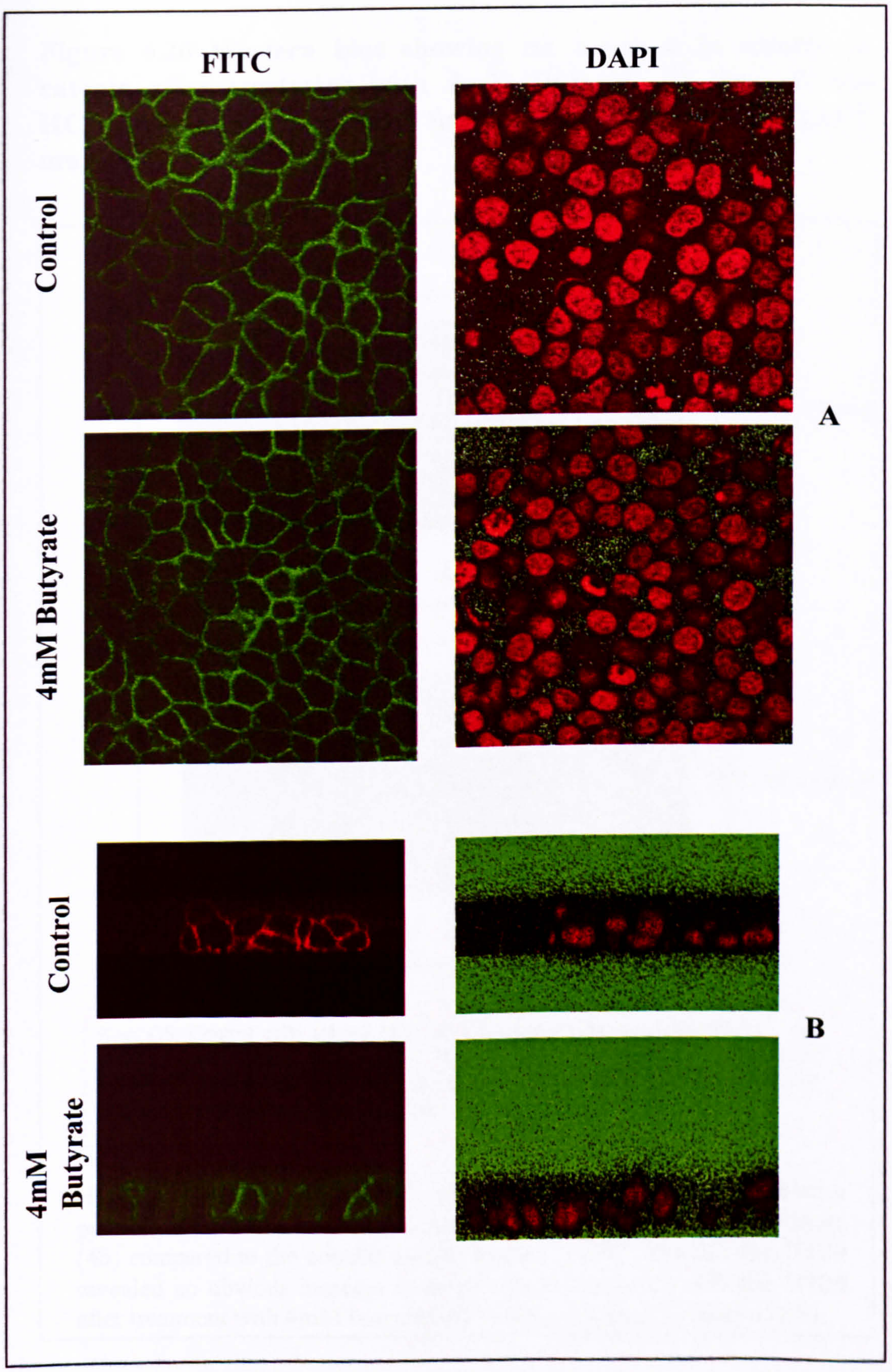
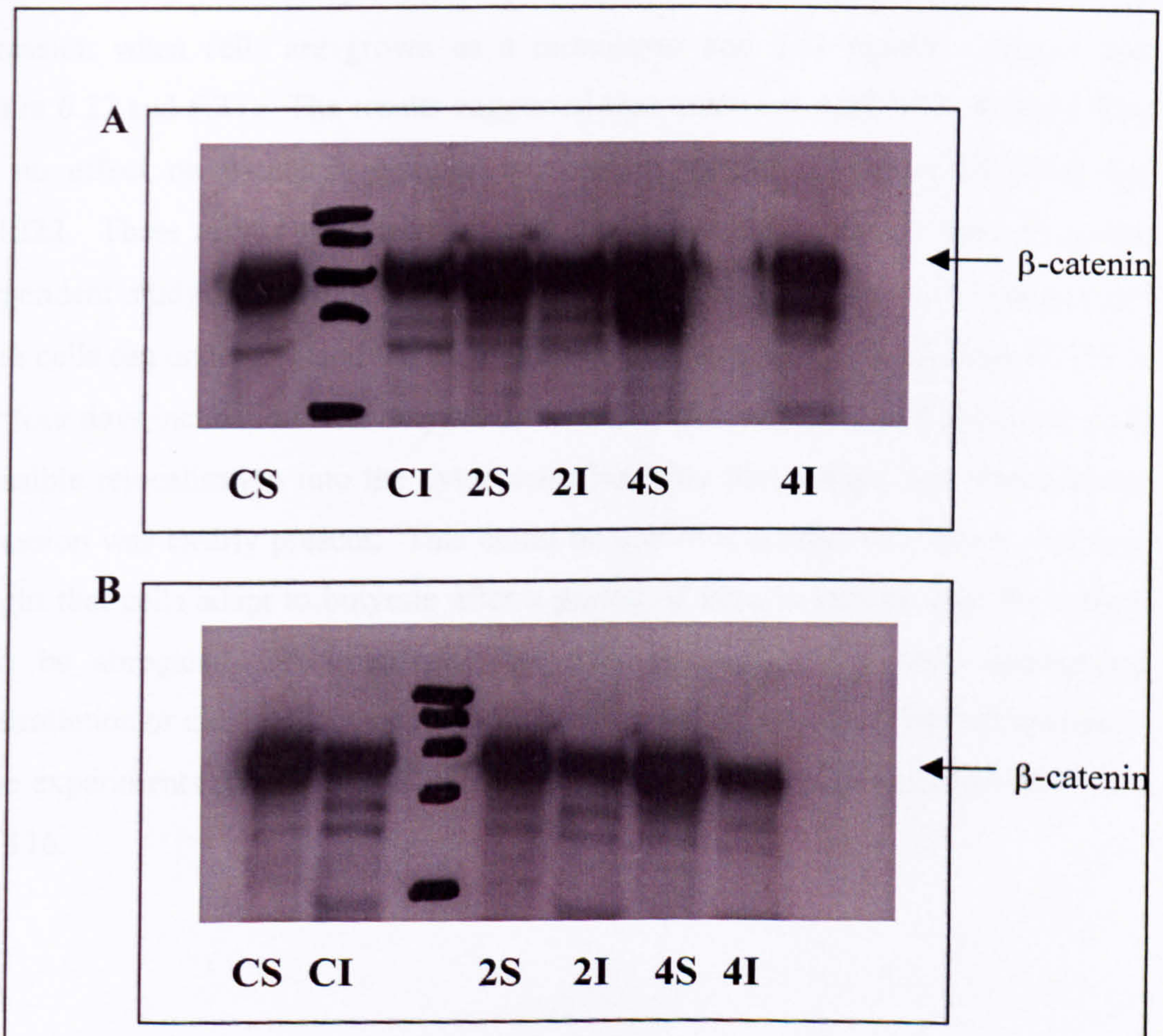

FITC
DAPI
Control
4mM Butyrate
A
Control
4mM Butyrate
B

Figure 6.20 Western blot showing an increase in soluble β-catenin after treatment with 4mM Butyrate in the cell line HCT116 (β-catenin mutation) but not in the cell line HT29 (APC mutation).



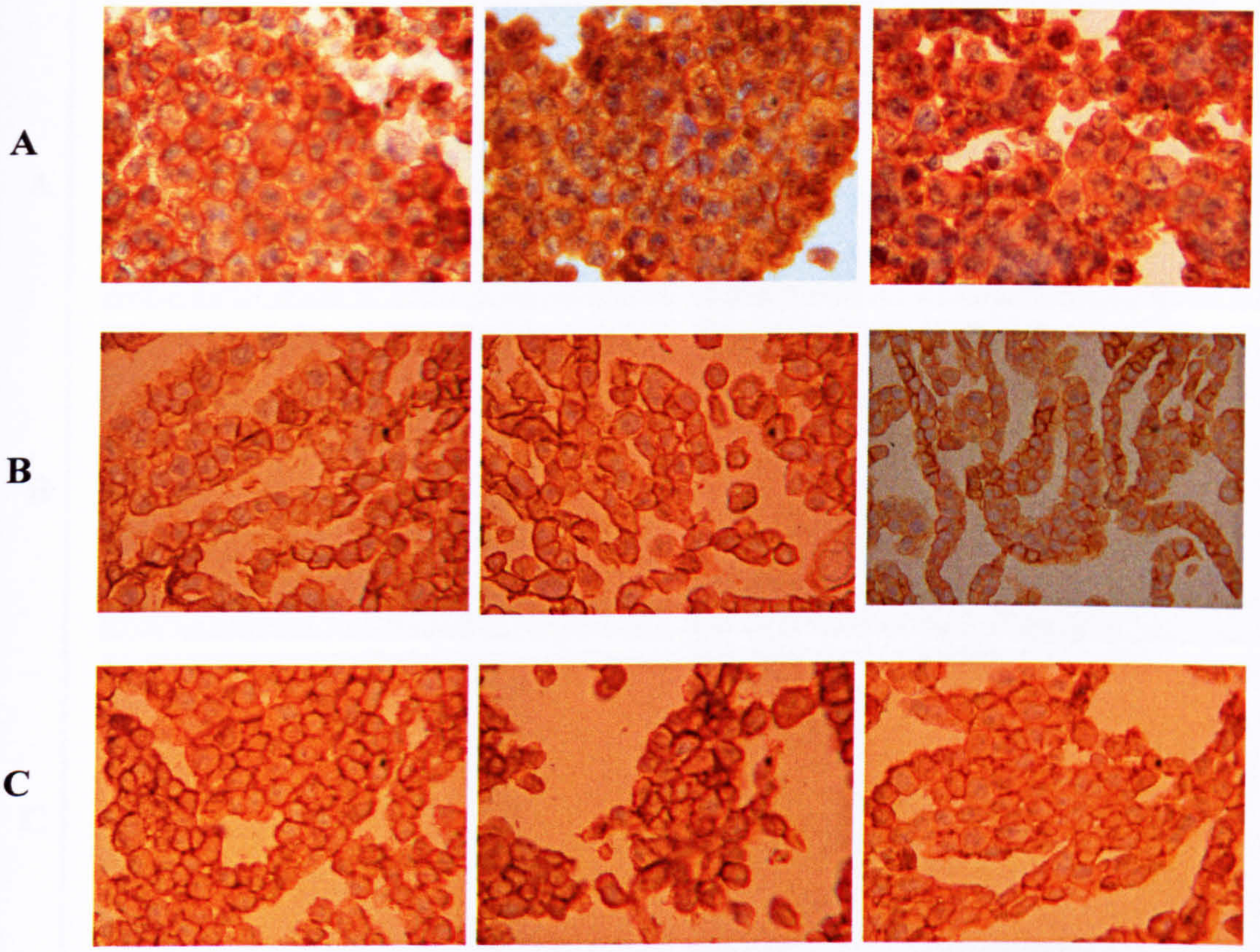
Key: **CS**, Control cells soluble fraction, **CI**, control cells insoluble fraction, **2S**, 2mM butyrate treated cells soluble fraction, **2I**, 2 mM butyrate treated cells insoluble fraction, **4S**, 4mM butyrate treated cells soluble fraction, **4I**, 4 mM butyrate treated cells insoluble fraction.

HCT116 (N=1) (**A**), HT29 (N=1) (**B**). The HCT116 cell line revealed a possible increase in soluble β-catenin when treated with 4mM butyrate (**4S**) compared to the control soluble fraction (**CS**). The cell line HT29 revealed no obvious increase in soluble β-catenin in the cell line HT29 after treatment with 4mM butyrate (**4S**) when compared to control (**CS**).

immunofluorescence (at least three independent experiments) there was an accumulation of β-catenin within the cytoplasm after treatment with 4mM butyrate for 48 hours within the cell line HCT116.

The effects of the fibres ispaghula husk and pectin on β-catenin expression were also examined using immunocytochemistry (Figure 6.21), which assessed protein expression when cells are grown as a monolayer and 3-D matrix collagen gels (Figure 6.22 and 6.23). The results suggested that treatment with 0.1% of each fibre had no effect on β-catenin cellular localisation in the cell lines HCT116 and SW1222. These results were inconclusive due to the fact that they were from one independent study. β-catenin expression was also examined within a collagen gel where cells can undergo glandular differentiation. Interestingly the cell line HCT116 after four days incubation with butyrate revealed similar expression of β-catenin with a possible relocalisation into the cytoplasm, but after seven days this membranous expression was clearly present. This could be due to a number of reasons, but it is thought that cells adapt to butyrate after a period of time in culture thus the effects could be abrogated. Alternatively this may be due to the cells undergoing differentiation or due to differences with growth in monolayers and 3D collagen gels. These experiments remain inconclusive as they are from one independent study for HCT116.

Figure 6.21 The luminal factors pectin and ispaghula husk (two dietary fibres) had no obvious effect on the localisation of β-catenin in the cell lines HCT116 (p23), HT29 (p175) or SW1222 (p113) (x40).

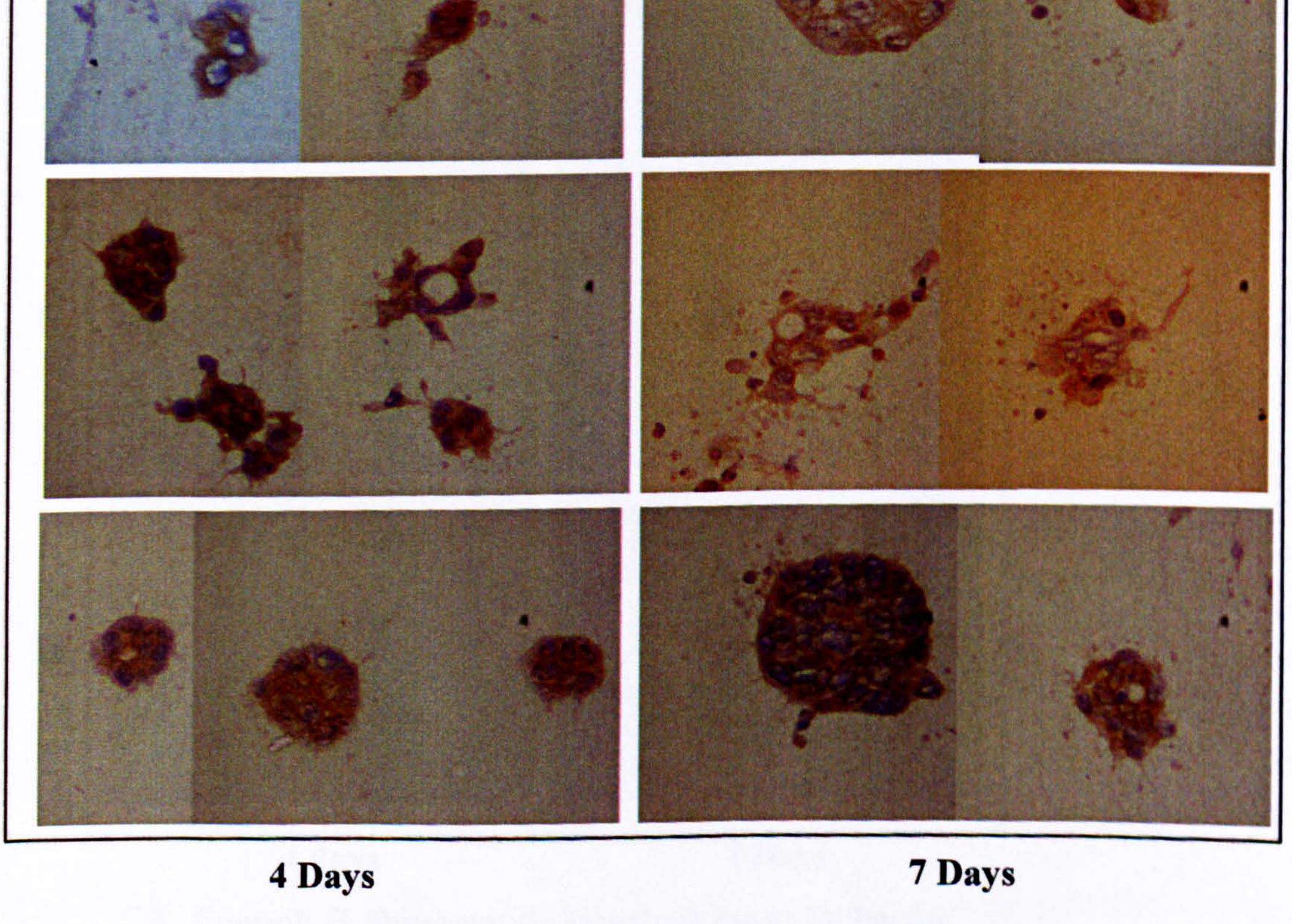


Key:- A, HCT116; B, HT29; C, SW1222.

The cells were cytospun or embedded in agar. Cells were then rehydrated , peroxidase were blocked using hydrogen peroxide, non-specific binding was blocked using goat serum, primary antibody was added for 1 hour at room temperature or over night at 4°C. This was followed by goat anti-mouse antibody (1:250, 30 minutes), streptavidine (1:250, 30 minutes) and DAB (10 minutes) dehydrated and mounted (N=1) (Magnifcication x40).

The luminal factors ispaghula husk (0.1%), pectin (0.1%) had no obvious effect on the localisation of β-catenin when compared to control with β-catenin being found at the cell membrane and cytoplasm with the nuclei remaining negative for expression.

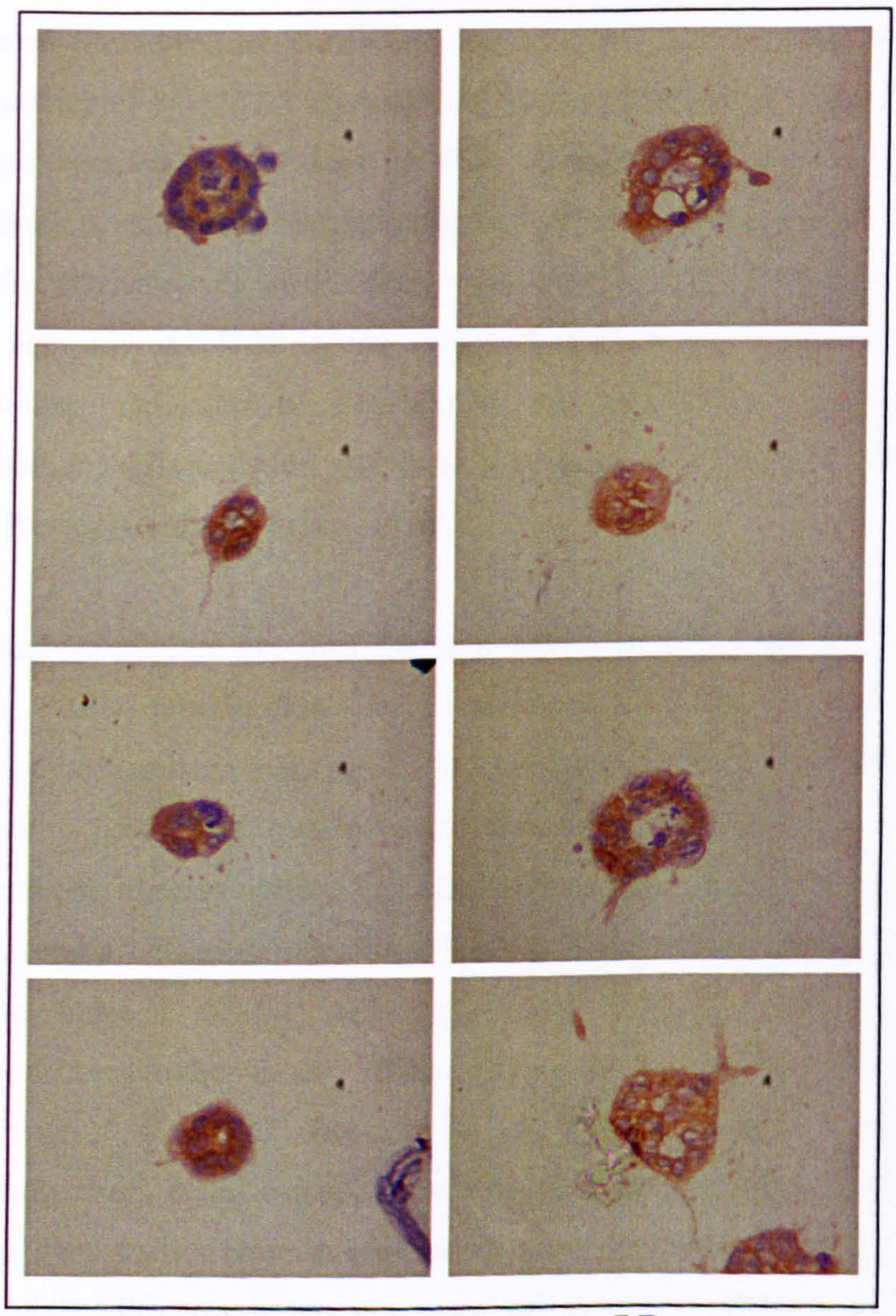
Figure 6.22 The effect of butyrate, ispaghula husk and pectin on the cellular localisation of β-catenin in the cells HCT116 during growth in collagen.



A, Control; B, Butyrate; C, Ispaghula Husk; D, Pectin.

The luminal factors appeared to have little effect on the cellular localisation of β-catenin on HCT116 in collagen, except butyrate at day 4 appears to result in a possible redistribution of β-catenin into the cytoplasm, this is not seen at day 7. The cells within the collagen gels treated with ispaghula husk appear to be poorly cohesive and disorganised (x40 Objective plus telescopic zoom).

Figure 6.23 Treatment of SW1222 collagen gels with butyrate, ispaghula husk and pectin.



A, Control; B, Butyrate; C, Ispaghula husk; D, Pectin

The luminal factors butyrate, ispaghula husk and pectin had very little effect on the cellular localisation of β-catenin in SW1222 cells, with it being found at the membrane and in the cytoplasm (x40 objective plus telescopic zoom).

6.5 Discussion

SCFAs are rapidly absorbed by colonocytes in the large intestine giving them the potential to exert a number of biological effects with possible beneficial roles within the colon (Reviewed by Burrin and Reeds, 1997; Review Cook and Sellin, 1998). Dzierzewicz *et al.* (2002) showed that treatment of transformed Caco-2 cells with butyrate resulted in the loss of their malignant phenotype including reduced proliferation, enhanced differentiation, and stimulation of apoptosis and interleukin-8 secretion (Dzierzewicz, *et al.* 2002). Investigations led to the discovery that SCFAs were not only involved in ion transport but also as nutrients for the colonic epithelium, modulators of intracellular pH, cell volume, regulators of cellular proliferation, differentiation and gene expression (Reviewed by Burrin and Reeds, 1997; Review Cook and Sellin, 1998).

This study confirmed previous findings that *in vitro* butyrate resulted in decreased colonic cell proliferation as determined by total cell numbers and immunocytochemistry using the MIB-1 antibody which measures the level of Ki-67 antibody. This study also revealed that propionate resulted in a decrease in proliferation, whereas acetate had little or no effect. Cyclin D3, p21 (mRNA and protein) and a reduction in cyclin D1 has previously been shown to be involved in butyrate induced G0-G1 cell cycle arrest affecting cell proliferation and differentiation (Archer *et al.*, 1998a; Archer *et al.*, 1998b; Coradini *et al.*, 2000; Siavashian *et al.*, 2000; Buecher *et al.*, 2001). Butyrate results in histone hyperacetylation, being a non-competitive inhibitor of histone deacetylases, which changes gene transcription thus effecting gene expression (Reviewed by Cress and Seto, 2000). Proteolysis has also been implicated as a means by which butyrate modulates the expression of key proteins in the control of cell cycle, apoptosis and differentiation (Tan *et al.*, 2002). Butyrate activates β-catenin-Tcf signalling, the mechanism of action and the consequences of this action remain unclear. Activation of Wnt has been associated with modulation of genes involved in cell proliferation, differentiation and tumour progression (Bordonaro *et al.*, 2002). The mechanism of action of propionate is unclear, although it could involve similar mechanisms of

inducing cell cycle arrest involving possible inhibition of histone deacetylases or possibly modulating induced β-catenin-Tcf-4 signalling pathways.

The activation of the Wnt signaling pathway through mutations in *APC*, *β-catenin* or activation of Wnt-1 modulates the transcription of genes linked to proliferation, differentiation and tumour progression (Bordonaro *et al.*, 2002). Mutations within the *adenomatous polyposis coli* (*APC*) gene have previously been shown to directly target the protooncogene *c-myc*, by elevating β-catenin-Tcf (T-cell factor) signalling (Wilson *et al.*, 2002). APC has recently been shown to consist of nuclear export signals which have been found to regulate β-catenin-Tcf signalling. Loss of function mutations within APC resulting in the loss of these export signals and APCs ability to modulate β-catenin-Tcf signalling as well as the ability to regulate β-catenin levels (Rosin-Arbesfeld *et al.*, 2000; Rosin-Arbesfeld *et al.*, 2003). Butyrate influences β-catenin-Tcf dependent wnt signaling stimulating β-catenin-Tcf complex formation and Tcf activity *in vitro* (Bordonaro *et al.*, 1999; Wilson *et al.*, 2002), in the presence of a membrane potential using SW620 colonic carcinoma cells (Bordonaro *et al.*, 1999). Butyrate induced β-catenin-Tcf complex formation and subsequent increase in Tcf activity was found to be independent of the down regulation caused by the expression of wild-type APC (Bordonaro *et al.*, 1999). The finding that butyrate increased β-catenin-Tcf-4 signalling in the presence of a membrane potential was interesting as butyrate is usually found in association with the dissipation of membrane potential (Bordonaro *et al.*, 1999), thus this increase in Tcf-4 signalling could be temporary within the first 24 hours. Tcf-4 target genes include *c-myc*, *cyclin D1* and *Tcf-1* (He *et al.*, 1998; Shtutman *et al.*, 1999; Reviewed Barker and Clevers, 2000) proteins involved in cell proliferation and apoptosis. Butyrate treatment resulted in decreased c-myc (Taylor *et al.*, 1992; Basson *et al.*, 1998) and cyclin-D1 (Coradini *et al.*, 2000) expression, thus suggesting butyrate may not only increase but also modulate β-catenin-Tcf-4 target gene transcription. The down-regulation of c-myc by butyrate has been attributed to a transcriptional pause mechanism in exon 2 of the *c-myc* gene, thus presumably overriding the effects of Tcf on initiation of transcription (Bordonoaro *et al.*, 1999b). These findings could explain why butyrate induced β-catenin-Tcf-4 transcription may not result in the transcription of all its target genes and may explain why butyrate did not increase

proliferation. The upregulation of β-catenin-Tcf activity by butyrate, in the absence of wild type APC (SW620 cells) has been associated with an increase in colonic epithelial cell apoptosis (Bordonaro *et al.*, 1999), rather than proliferation or differentiation. After examination of a number of cell lines it was found that there was no correlation between the sensitivities to proliferation inhibition and the sensitivities to apoptosis induction by butyrate (Galfi *et al.*, 2002) suggesting possible independent signaling pathways. This suggests that perhaps histone deacetylation was involved in the inhibition of proliferation (Galfi *et al.*, 2002) with β-catenin-Tcf-4 being involved in the induction of apoptosis (Bordonaro *et al.*, 1999). It has been suggested that APC maybe more closely linked to induction of differentiation (Bordonaro *et al.*, 1999). Further studies by Bordonaro *et al.* (2002) have found that butyrate effects on wnt-signalling are partly due to the inhibition of histone deacetylases resulting in changes in DNA structure.

Butyrate has been shown to effect the expression of a number of genes critical to colonic tumorigenesis (Wilson *et al.*, 2002) (section 1.5.5), including *p53*, *Bcl-2* and *c-myc*. Butyrate was shown to result in the upregulation of β-catenin in the colonic epithelial cell line MCE301 (Tabuchi *et al.*, 2002). In this study western blotting (SDS-PAGE) suggested that there was no obvious change in total β-catenin expression in the cell lines HCT116, HT29 or SW1222 following 48 hour butyrate treatment at 1mM, 2mM and 4mM concentrations. The results for HCT116 were inconclusive due to a lack of repetition. Although these results were in fact consistent with the findings of Bordonaro *et al.* (2002) which revealed that treatment of HCT116 and SW620 cell lines with 5mM butyrate for 24 hours had no effect on total levels of β-catenin. β-catenin expression levels appeared to be unaltered by the addition of the SCFAs propionate and acetate, even at concentrations of 10mM and 15mM respectively, within the cell lines HT29 and SW1222. Thus butyrate, propionate and acetate did not appear to affect total protein expression of β-catenin. The upregulation of β-catenin was not observed in this study or that of Bordonaro *et al.* (2002), this could be due to the use of different cell lines, different techniques (cDNA microarrays versus western blotting over different time periods, although 48 hours consistent in both) or could be the difference in examining genes verse protein expression possibly indicating a modification in translation.

This study revealed that butyrate at a concentration of 4mM resulted in the relocalisation of β-catenin into the cytoplasm in HCT116 cells, however this phenomenon was not observed in HT29 cells. β-catenin expression was determined by three techniques; immunocytochemistry, immunoflouresence confocal microscopy and western blotting using an antibody against the C-terminal region of the β-catenin protein. Different reasons could account for the cytoplasmic accumulation of β-catenin within HCT116 cell lines. The two cell lines differ in their genetic make up; HT29 has a wildtype *β-catenin* and mutant *APC* whereas HCT116 cell line has a wild type *APC* and a heterozygous mutation in the *β-catenin* gene (Ilyas *et al.*, 1997; Morin *et al.*, 1997). Difference in APC and β-catenin status between the two cell lines could affect β-catenin-Tcf-4 signaling and protein-protein interactions of β-catenin with the proteins E-cadherin, GSK-3β or APC.

The HCT116 cell line is replication error positive (RER+) (Efstathiou *et al.*, 1999) and has previously been shown to express full length APC (Su *et al.*, 1993; Ilyas *et al.*, 1997; Morin *et al.*, 1997). HCT116 was also shown to express intact β-catenin as determined by western blotting, however sequence analysis revealed a mutation within the *β-catenin* gene (*CTNNB1*) (Morin *et al.*, 1997). HCT116 had a heterozygous 3-bp deletion in codon 45, an inframe deletion within exon 3 within the *β-catenin* gene (*CTNNB1*) (Ilyas *et al.*, 1997; Morin *et al.*, 1997). This mutation resulted in the loss of the highly conserved serine residues (Ser^{45}) in a region of the protein that may be a target for GSK-3β (Ilyas *et al.*, 1997; Morin *et al.*, 1997). HCT116 has been shown to have constitutively active β-catenin-Tcf transcription (Morin *et al.*, 1997). Morin *et al.* (1997) demonstrated that the heterozygous β-catenin acts in a dominant negative fashion for mutant β-catenin that resulted in an increase in β-catenin-Tcf-4 mediated transcriptional activity (Ilyas *et al.*, 1997; Morin *et al.*, 1997) and was unaffected by the presence of wild type APC (Morin *et al.*, 1997). Mutagenesis experiments demonstrated that substitution of the serine residues in the glycogen synthase kinase 3β (GSK-3β) phosphorylation consensus motif of β-catenin inhibits the ubiquitination and results in the stabilisation of the protein (Aberle *et al.*, 1997).

Agents such as butyrate, ascribed as having chemopreventive activity for colon cancer and induce G0-G1 cell cycle arrest, in fact also stimulate β-catenin-Tcf activity *in vitro* (Bordonaro *et al.*, 1999; Bordonaro *et al.*, 2002; Wilson et al., 2002) although it has been suggested that this is not accompanied by an increase in target gene expression (Vincan *et al.*, 2000). Within the cell line HCT116, Bordonaro *et al.* (2002) found that butyrate resulted in a decrease in free Tcf within the cytoplasm and this was accompanied by an increase in β-catenin-Tcf binding to DNA, although this study also revealed that 5mM butyrate had little effect on the nuclear levels of β-catenin after 24 hours. This could partially explain the increase in cyotplasmic β-catenin expression found within the cell line HCT116.

It is also possible that the increase in cytoplasmic localisation of β-catenin within HCT116 cell line following butyrate treatment was due to the induction of apoptosis. This would result in the cleavage of β-catenin and as such an increase in cytoplasmic β-catenin although there were no other physical signs of apoptosis were observed. β-catenin-Tcf-4 signalling is thought to be more associated with apoptosis (Bordonaro *et al.*, 1999). The response to butyrate in terms of differentiation and apoptosis by HT29 is decreased during prolonged exposure (Kucerova *et al.*, 2002). This may explain why, after 7 days prolonged treatment of the HCT116 cells in collagen, there appears to be no effect on β-catenin localisation with butyrate treatment although this could be due to a lack of repetition, so no strong conclusions could be ascertained.

Aberle *et al.* (1997) revealed that the ubiquitin-dependent proteolysis system was involved in the regulation of β-catenin turnover. β-catenin, but not E-cadherin, p120 and α-catenin, becomes stabilised when proteosome-mediated proteolysis was inhibited leading to the accumulation of multi-ubiquitinated forms of β-catenin. Research showed that ubiquitination of β-catenin was greatly reduced in Wnt-expressing cells, providing the first evidence that the ubiquitin-proteosome degradation pathway may act down stream of GSK-3β in the regulation of β-catenin (Aberle *et al.*, 1997). Butyrate has been shown to have differing effects on proteosome activity depending on the protein and cell system being examined. Butyrate treatment resulted in alterations in the proteosome of HT-29 cells (Tan *et al.*, 2002) resulting in changes in the expression of various components of the

ubiquitin-proteosome system (Tan *et al.*, 2002). Butyrate did not appear to effect proteosome activity, although inhibition of proteosomes by other inhibitors did appear to increase cyclin D3 expression (Buecher *et al.*, 2001). Butyrate was shown to suppress the activation of NF-κB and cellular proteosome activity (Yin *et al.*, 2001), thus there are conflicting studies as to whether butyrate affects proteosome activity. A change in proteosome activity could profoundly affect the ubiquitination and degradation of β-catenin in HCT 116, again postulating another mechanism for relocalisation and accumulation of β-catenin in the cytoplasm. Modulation of proteosome activity was probably not the cause of cytoplasmic accumulation observed in this study, as there were no changes in overall β-catenin levels. Analysis of the glycogen synthase kinase-3β (GSK-3β) activity in several colon cancer cell lines suggested a correlation between comparatively low enzyme activity and moderate to high differentiation status. Butyrate has been shown to result in the phosphorylation of GSK-3β inhibiting its ability to phosphorylate target proteins (Tuhackova *et al.*, 1999). GSK-3β activity was shown to decrease with butyrate treatment, resulting in an increase in the stabilization of β-catenin (as in wnt) although there was no increase in β-catenin target gene expression (Vincan *et al.*, 2000). The modulation of GSK-3β activity is unlikely to affect β-catenin cytoplasmic accumulation due to HCT116 having mutant β-catenin, which is resistant to GSK-3β induced degradation.

Mutation of the second allele of HCT116 was shown to reduce β-catenin-TCF/LEF transcription and this in turn was shown not to affect growth rate *in vitro* but adhesive functions of β-catenin (Sekine *et al.*, 2002). Thus the relocalisation of β-catenin to the cytoplasm by butyrate could be due to changes in cellular adhesion. Wnt-1 expression has been shown to result in the accumulation of β-catenin, plakoglobin and stabilisation and increased expression of cadherin strengthening cell-cell adhesion (Bradley *et al.*, 1993; Hinck *et al.*, 1994a). *C-myc*, a target gene for β-catenin-Tcf-4 and its associated proteins (c-Myc-1, c-Myc-2) have been shown to result in the upregulation of E-cadherin when simultaneously expressed (Batsche and Cremisi, 1999). Butyrate has also been shown to result in the upregulation of E-cadherin (Kucerova *et al.*, 1999) suggesting that it is a possible target of β-catenin-

Tcf-4 (Reviewed by Ben-Ze'ev, 1998). Within HCT116 there is a heterozygous mutation within the *E-cadherin* gene with the E-cadherin protein being expressed by this cell line (Efstathiou *et al.*, 1999). The mutation within HCT116 was shown to be a deleted frameshift, a single base deletion in repeat regions of exon 3 (codon 120), causing a downstream premature stop codon (TGA) at codon 216 within HCT116 (Efstathiou *et al.*, 1999). The mutation within HCT116 results in a truncation within the N-terminal region, the extracellular domain of the protein, which may interfere with the normal function of full-length E-cadherin (Efstathiou *et al.*, 1999). The steady state levels of β-catenin are partly dependent upon cadherin expression (Munemitsu *et al.*, 1996). E-cadherin has been shown to be upregulated in some cell lines by butyrate treatment (Kucerova *et al.*, 1999), which could possibly be due to the induction of differentiation. Butyrate was also shown to result in an increase in membrane bound β-catenin, suggesting a possible mechanism as to how butyrate induces cell differentiation (Vincan *et al.*, 2000). In HCT116, β-catenin coprecipitated with APC, E-cadherin and α-catenin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993; Ilyas *et al.*, 1997). E-cadherin binds to all 12 Arm repeats within β-catenin (Huber and Weis, 2001). APC contains sequences homologous to the phosphorylated region of cadherin and is likely to bind similarly (Huber and Weis, 2001). APC association with the catenins is consistent with the previous observation that APC is localised in the detergent insoluble fraction (microtubule network) (Su *et al.*, 1993) although in HCT116 APC would be unable to elicit its degredation. The binding of the β-catenin to APC could account for the increase in cytoplasmic accumulation observed within this study although this would not account for the western blotting results, which suggests that APC-β-catenin interactions would be found within the soluble fraction. Cadherins appear to associate with similar amounts of α- and β-catenin whereas APC associates preferentially with β-catenin (Su *et al.*, 1993). Due to the heterozygous mutation within the cell line HCT116, it is unknown whether or not E-cadherin or α-catenin complexes contain mutant protein (Ilyas *et al.*, 1997). The β-catenin mutation in the cell line HCT116 is found within exon 3, which consists of the GSK-3β consensus sequence, that lies upstream from the E-cadherin binding site within the β-catenin *Arm* repeat region so it would be unlikely to affect these interactions (Nusse, 2002). In HCT116 the mutation was in a region outside the *Arm* repeats 5 prime to the α-catenin binding site (Ilyas *et al.*,

1997). β-catenin forms independent complexes with either APC or E-cadherin (Rubinfeld *et al.*, 1993), E-cadherin and APC directly compete for binding to the internal, armadillo-like repeats of β-catenin, the NH_2-terminal domain of β-catenin binding to α-catenin (Hülsken *et al.*, 1994; Reviewed Ilyas and Tomlinson., 1997). This leads to the conclusion that the mutation within β-catenin would not interfere with its interactions with E-cadherin, APC or α-catenin. Immediately after synthesis, E-cadherin, β-catenin and plakoglobin cosedimented as complexes. α-catenin was not associated with these complexes after synthesis, but a subpopulation of α-catenin joined the complex at a time coincident with the arrival of E-cadherin to the plasma membrane (Hinck *et al.*, 1994b). In addition to cadherin/catenin complexes, there were cadherin-independent pools of catenins present in both TX-100 soluble and insoluble fractions (Hinck *et al.*, 1994b). Cadherin-β-catenin complexes were present in the TX-100 soluble fraction (Hinck *et al.*, 1994b). Approximately 50% of complexes were titrated into the TX-100 insoluble fraction coincident with the arrival of the complexes at the plasma membrane and the assembly of α-catenin (Hinck *et al.*, 1994b). The increase in soluble pool could also be due to an increase in E-cadherin-β-catenin at the membrane but not linked to the actin cytoskeleton by α-catenin. E-cadherin expression appeared to be maintained at the cell membrane within the HCT116 (results not shown) but the increase in E-cadherin expression by butyrate could result in the increased transcription of the mutant E-cadherin, which could affect β-catenin interactions with the cell membrane, possibly leading to this cytoplasmic localisation.

Chapter 7

Concluding remarks.

7.1 Concluding remarks.

Colorectal cancer is the second biggest cause of cancer mortality in the western world. In advanced disease the prognosis is poor and available forms of treatment are largely ineffective, thus there is a need to develop preventive approaches. Moreover there is a national strategy towards health improvement and prevention of disease, which includes recommendations to increase the consumption of fruit and vegetables (five portions a day) along with the cessation of smoking. Dennis Burkitt was the first to propose that dietary fibre, contained within cereals, fruit and vegetables, may protect against a number of prevailing diseases in the western world including bowel cancer. Recent studies have produced conflicting data as to whether dietary fibre protects against colorectal cancer development. This study attempted to examine the effects of individual fibres on colonic tumourigenesis, particularly those used to supplement foods. Loss of function mutations within the tumour-suppressor gene *APC* occurs in 80-85% of sporadic colorectal cancers; loss of function of APC results in β-catenin over expression within the cytoplasm along with nuclear translocation. Thus this study has investigated the effects of dietary fibres on β-catenin expression and cellular localisation.

This study has confirmed that different fibres produce different levels of SCFAs and at differing rates along with revealing the SCFA production for the fibres used in this study; ispaghula husk, pectin and cellulose. Pectin was shown to be rapidly fermented, which is consistent with previous findings that it is completely fermented in the caecum of mice. This finding may partially explain the results that pectin increased the incidence and increased development of colonic tumours within the distal colon of mice treated with 1,2-dimethylhydrazine (DMH). Complete fermentation of pectin would result in the loss of bulk, which would result in the release of bile acids and as a consequence an increase of primary bile acids being converted into secondary bile acids promoting proliferation and other tumourigenic

processes. The release of SCFAs have previously been shown to result in an increase in proliferation *in vivo* (Goodlad *et al.*, 1989; Goodlad *et al.*, 1995), in the case of pectin the level of SCFAs reaching the distal colon would presumably be low. If this is not the case high levels of SCFAs could be another cause of an increase in cell proliferation. Pectin has also been shown to result in an increase in markers of apoptosis (Aviv-green *et al.*, 2000a), so this increase in apoptosis could explain the increase in proliferation due to a balance of colonic homeostasis. Other constituents of the diet also appeared to affect fermentation, although the constituents examined within this study would not reach the colon so the relevance of these results are difficult to determine. Future studies could involve a repetition of the *in vitro* fermentation process after mimicking digestion throughout the gastrointestinal tract. This would be achieved by subjecting the fibres to digestion conditions found within the gastrointestinal tract. Replicating the conditions found within the mouth could be achieved by using the enzyme amylase and mechanical force reflecting mastication and possibly exposure to a representation of the commensal microflora. The next stage would involve mimicking the stomachs digestion process, which involves mechanical, enzymatic digestion using pepsin and acid hydrolysis (Reviewed by Moore and Agur, 1995). Digested food enters the small intestine where nutrients are absorbed and bile secreted, a process that would be difficult to mimic. The remaining residue would possibly reflect more accurately that which enters the large intestine providing a more accurate reflection of SCFA production from each dietary fibre. This would be particularly true for rice starch where only the portion of resistant starch would actually reach the large intestine dramatically altering the levels of SCFAs achieved. It would also give a more accurate picture of the SCFA profiles for the fibre supplemented diets as there are components within these diets that have the potential to affect fermentation within the large intestine, such as protein. A further extension of the analysis of the properties of dietary fibres would be to repeat the *in vitro* experiments using faecal matter obtained from normal and DMH-treated mice. This would reflect more accurately the fermentation patterns within the mouse, as there are variations in the resident bacteria within the large intestines of mice and humans (Interpretation from Reviewed by Holdeman *et al.*, 1977). Although this study may be difficult due to the small quantities of murine faecal matter produced, this could be overcome by making an artificial faecal slurry which incorporates and reflects the microbial colonisation of these mice.

The 1,2-dimethylhydrazine (DMH) mouse model was used to examine the effects of the dietary fibres used as food supplements on tumourigenesis. This is an established animal model of colonic tumourigenesis, which is characterised by the development of multiple adenomas in the large intestine which eventually progress to adenocarcinomas. The aim of this study was to examine alterations in the expression of the catenins (α-, β-catenin and $p120^{(ctn)}$) and E-cadherin expression in colonic adenomas induced by DMH. β-catenin, α-catenin, $p120^{(ctn)}$ and E-cadherin expression were all altered within the dysplastic epithelium of the 1,2-dimethylhydrazine induced adenomas. Within the dysplastic tissues there was an increase in cytoplasmic catenin and cadherin localisation along with maintenance of membranous expression. This is different to human adenomas where relocalisation of β-catenin into the cytoplasm is usually associated with a loss of membranous expression (Hao *et al.*, 1997). Takahashi *et al.* (2000) also examined β-catenin staining and found an increase in cytoplasmic staining and nuclear localisation but a decrease in β-catenin membranous expression. This difference could be due to the differences between the two models, including the use of a metabolite of DMH, AOM, and the use of different immunohistochemical techniques. Increased cytoplasmic and nuclear localisation of β-catenin suggests either activation of WNT-1 or a mutation within β-catenin or APC. For β-catenin we failed to show a mutation within the β-catenin gene. Other studies have shown that AOM, a metabolite of DMH, results in a mutation of exon 3 of the β-catenin gene within a murine model (Takahashi *et al.*, 2000; Yamada *et al.*, 2003). Thus future work could include finding a more sensitive method to specifically amplify up the mutant β-catenin including real time PCR, high fidelity PCR, nesting primers or the use of more accurate polymerases. The PCR method used may not have been sensitive enough to detect single base-pair changes and any masked mutations. The techniques could also be improved through the use of microdissection to reduce the contamination of non-dysplastic colonic tissues.

The maintenance of E-cadherin expression and catenin expression at the cell membrane as well as cytoplasmic localisation is a significant finding. This could be due to a specific type of mutation, which does not interfere in cell adhesive

properties of the proteins. *E-cadherin* mutations within the extracellular domain, exon 8 or 9, maintained E-cadherin and catenins at the cell membrane, along with an increase in catenins within the perinuclear region within diffused gastric cancer cells (Luber *et al.*, 2000). Thus it would be interesting to examine *E-cadherin* for mutations within exon 8 and 9, as this may explain the unusual finding. Another interesting analysis would be the evaluation of dysplastic tissues for functional interactions within the cadherin/catenin complex using western blotting and immunoprecipitation.

Another possible explanation are changes in proteosome activity with a reduction in proteosome activity explaining the accumulation of the catenins and cadherin within the cytoplasm, accompanied by their maintenance at the cell membrane. Butyrate has been shown to alter proteosome activity and resulting in a reduction in the degradation of a number of proteins (Refer to section 6.5). It could also be the result of changes in tyrosine phosphorylation, DMH possibly inducing proteins that result in the tyrosine phosphorylation of the catenins, disrupting their cellular function (refer to 1.3.3). Examination of the tyrosine phosphorylation status of β-catenin could be achieved using western blotting and the antibody 4G10. There is also the possibility of changes in methylation with the carcinogen previously been shown to result in pro-mutagenic O^6-methylguanine lesions (Reviewed by Sunter, 1980).

The status of *APC* gene and the functionality of the protein could also be examined, as this is an important protein in the regulation of catenin levels and cellular localisation within the cell. APC in a complex with GSK-3β and axin have shown to result in the degradation of β-catenin. Loss of function mutations in APC results in the loss of its ability to down regulate β-catenin. APC has recently been found to have a highly conserved nuclear export signal, cells with mutant APC lacking these export signals results in β-catenin nuclear accumulation (Rosin-Arbesfeld *et al.*, 2000) suggesting that the ability of APC to exit from the nucleus appears to be critical for its tumour suppressor function (Rosin-Arbesfeld *et al.*, 2000). The export signals are crucial for APCs function by reducing the transcriptional activity of β-catenin (Rosin-Arbesfeld *et al.*, 2003). Thus a loss of APC function could reflect the increase in catenin expression within the cytoplasm, with the maintenance of

catenin expression at the cell membrane and the nuclear localisation of β-catenin. Within colonic tumours induced by either AOM or DMH within rats and mice APC expression has been shown to be negative, with reductions in mRNA expression and low levels of APC mutations (Maltzman *et al.*, 1997; Endo *et al.*, 2001; Kishimoto *et al.*, 2002). If the mutation within APC involves its export signals it could also account for the nuclear localisation of β-catenin. Mutations in APC and β-catenin are thought not to be the only reasons for loss of the regulation of β-catenin levels. A number of other complexes involved in β-catenin degradation, such as mutations in axin (Reviewed by Gordon, 1998; Ikeda *et al*, 1998) could also play a role in the fundamental disruption of this multiprotein complex. Mutations in GSK-3 could also be a significant factor with mutant GSK-3β, lacking the kinase domain, being unable to phosphorylate either APC or β-catenin.

The accumulation of β-catenin within the cytoplasm leads to translocation of β-catenin into the nucleus where it associates with the transcription factors LEF and TCF (Behrens *et al.*, 1996; Reviewed by Barth *et al*, 1997b; Reviewed by Jankowski *et al*, 1997; Korinek, 1997; Reviewed by Nusse, 1997; Morin *et al*, 1997a; Muller *et al*, 1998). β-catenin forms a complex with TCF/LEF transcription factors which binds to DNA and consequently activates gene transcription. The nuclear localisation of β-catenin could have a number of implications in terms of modulation of TCF signalling resulting in the transcription of a number of target genes such as c-myc, cyclin D1 and cox-2 (He *et al.*, 1998; Shtutman *et al.*, 1999; Howe *et al.*, 1999). Thus the modulation of gene expression of target genes of β-catenin/tcf-4 could be examined, along with an examination of β-catenin/TCF-4 interactions. The target genes c-myc (He *et al.*, 1998), cyclin D1 (Shtutman *et al.*, 1999) and Cox-2 (Howe *et al.*, 1999) expression have been examined within similar models, for example rats exposed to azoxymethane (AOM) for 4 weeks were shown to have elevated Cox-2 and c-myc mRNA expression (Kishimoto *et al.*, 2002).

The effect of the characterised fibres; ispaghula husk, pectin and cellulose along with a basal diet being relatively fibre free on tumour development were examined in a murine model using the complete carcinogen 1,2-dimethylhydrazine. The results revealed that pectin, the soluble fibre, resulted in an increase in all aspects of tumour

development and progression. This included the initiation, even though DMH initiates tumour development the fibre pectin also seemed to increase the rate of initiation in terms of microadenoma number (although not significant) and tumour incidence (significantly when compared to the basal diet), tumour numbers (although not significantly) and tumour size (not significantly). The reason these experiments failed to prove a significant difference between the fibre-supplement groups was attributed to the large variation between groups on the same diet. These results are in keeping with previous findings that soluble fibres tend to be less protective against tumour development than insoluble fibres (Harris and Ferguson, 1993). The proposed chemopreventive effects of fibres include their bulking abilities which help dilute carcinogens down and decrease transit time within the colonic lumen, changes in bile acids and short chain fatty acids (SCFAs) along with other systemic effects through reduction in appetite, reduction in insulin, a reduction in glucose absorption helping to control type II diabetes and lowering of the risk of coronary heart disease (CHD) could all have an indirect effect on colorectal cancer development. Pectin was shown to increase (although not significantly due to the large variations between the mice) in the number of cells replicating their DNA as indicated by an increase in bromodeoxyuridine-labelling index (BrdU-LI). This increase in proliferation indicates a possible disruption in colonic homeostasis, through either a direct increase in proliferation or an increase in apoptosis; suggested by the average crypt lengths within each fibre supplemented group not being significantly different. Interestingly the lowest number of tumours, with the lowest incidence and the lowest level of proliferation (cells undergoing DNA replication) was in those mice fed the basal diet, which was fibre free. This leads to a number of possibilities but the most significant being the increase in proliferation being associated with the increase in tumour development within this model. DMH itself has been shown to increase proliferation and the addition of fibre may accentuate this causing a greater imbalance in colonic homeostasis. The disruption of catenin expression within the dysplastic tissues and the nuclear localisation of β-catenin could also be a consequence or the cause of the increase in proliferation within the dysplastic. Furihata *et al.* (2002) found that an overexpression of β-catenin was not associated with an increase in proliferating cell nuclear antigen-labelling index (PCNA-LI), and our study revealed no significant pattern associated with catenin expression and the

fibre supplemented diets to support this hypothesis. The finding that there was no obvious difference in catenin expression between the mice fed on different fibre diets but this could have been because of the small sample number; alternatively this could simply be a reflection of the limitations of immunohistochemistry. Future studies could involve the repetition of these experiments using different colon cancer models such as the MIN mouse or APC knockout mice, or supplementation of a normal diet, although this may not be good use of resources. Further studies within the tissues already obtained could involve measuring the level of apoptosis (apoptotic body count or use of the TUNEL immunohistochemistry) within the colonic tissues, and determine whether there are any significant differences between the fibres. In terms of exploring the possible chemopreventive properties of fibre supplements an extension of the examination of different types of fibre supplement on the market would be important. For example in this study ispaghula husk was examined, this fibre is the main constituent of fybogel® a product used the treatment of constipation and diahhorea and regulation of bowel habit. This study could be extended to examine the different types of fybogel® as fybogel® lemon also contains curcumin, with curcumin previously being shown to have possible chemopreventive properties (Reviewed by Choi and Mason, 1998; Anto *et al.*, 2002). Thus it would be interesting to examine the effects of the two different supplement on colorectal cancer development and determine whether there is any synergy between the two. The use of an *ex vivo* model such as the growth of colonic biopsies in culture (organ culture) either human or murine in origin may provide a link between the *in vivo* and *in vitro* results and be a more precise representation of the *in vivo* system.

The possible biological effects of the dietary fibres and the SCFAs associated with the fermentation of these fibres were investigated *in vitro*. The SCFAs butyrate and propionate resulted in a decrease in proliferation and an increase in apoptosis (Reviewed Rombeau and Roth, 1995; Hague *et al.*, 1995; Reviewed by Cummings, 1997; Reviewed Lührs *et al.*, 2002). The fibres were found to have no effect on total cell number suggesting that proliferation is unaffected by the addition of fibres. To extend this further, cell turnover could be examined using tritiated thymidine, which would help confirm whether the total cell number obtained was due to no changes in proliferation or the result of an increase in apoptosis increasing cell turnover.

Examination of combinations of SCFAs would be relevant as this would more closely reflect the *in vivo* situation and would also allow for investigations into the interactions between different SCFAs.

Butyrate was shown to induce relocalisation of β-catenin from the cell membrane into the cytoplasm within the cell line HCT116 but not HT29. The changes in β-catenin expression within HCT116 but not HT29 using butyrate could be examined further to determine the mechanism behind this. HCT116 has a mutant β-catenin whereas HT29 has a wild type β-catenin. Thus it would be important to examine other cell lines with mutant β-catenin or without a mutant β-catenin for example LS174T and SW1222 respectively. Changes in proteosome activity within the two different cell lines after butyrate treatment could also be examined along with the ubiquitination of β-catenin protein. There are conflicting studies as to whether butyrate affects proteosome activity, butyrate did not appear to affect proteosome activity when examining the effects on cyclin D3 expression (Buecher *et al.*, 2001); whereas Butyrate was shown to suppress the activation of NF-κB and cellular proteosome activity (Yin *et al.*, 2001); this could be the result of examination of different cell systems and target genes. A change in proteosome activity could profoundly affect the ubiquitination and degradation of β-catenin in HCT 116, again postulating another mechanism for relocalisation and accumulation of β-catenin in the cytoplasm. Changes in β-catenin transcription could also be examined with butyrate being a known histone deacetylase inhibitor. The Wnt signaling pathway modulates the transcription of genes linked to proliferation, differentiation and tumour progression (Bordonaro *et al.*, 2002). Butyrate has also been shown to influence β-catenin-Tcf dependent Wnt signaling (Bordonaro *et al.*, 2002). Butyrate induced increase in Tcf activity, which is reflected in an increase in β-catenin-Tcf complex formation, is independent of the down regulation caused by expression of wild-type APC (Bordonaro *et al*, 1999). Thus, butyrate and wild-type APC have different and independent effects on β-catenin-Tcf signalling (Bordonaro *et al*, 1999). Examination of β-catenin/TCF-4 interactions could be examined. HCT116 has a wild type APC with a mutant β-catenin accompanied by low levels of β-catenin-Tcf complexes and higher levels of free Tcf (Bordonaro *et al.*, 2002). Butyrate in HCT116 cells results in the downregulation of free Tcf-4 which was

accompanied by an increase in β-catenin/Tcf-4 complex formation (Bordonaro *et al.*, 2002). Thus the relocalisation of β-catenin into the cytoplasm could be the result of an increase in β-catenin-TCF complex formation. Examination of any possible nuclear localisation of β-catenin or changes in target gene transcription, such as c-myc (He *et al.*, 1998), cyclin D1 (Shtutman *et al.*, 1999) or cox-2 (Howe *et al.*, 1999) between the 2 cell lines would be of interest with butyrate previously shown to down regulate c-myc protein expression within Caco-2 cells (Basson *et al.*, 1997). This could be extended to examine whether propionate has a similar effect although the initial immunocytochemistry results suggest this is not the case. The tyrosine phosphorylation status of β-catenin could also be examined using western blotting and the antibody 4G10. It would also be possible to examine the interaction of β-catenin with E-cadherin and extending the studies using the murine cell line CMT-93 as it may give further evidence to how the fibres affect tumour development in the murine model.

The increase in cell proliferation *in vivo* could be attributed to the production of SCFA from the fermentation of the dietary fibres. This is supported by the fact that pectin was shown to produce the highest level of SCFAs at the fastest rate (Refer to 3.5) and previous experiments revealing that SCFAs increase proliferation *in vivo* (Goodlad *et al.*, 1989; Goodlad *et al.*, 1995; Ichikawa and Sakata, 1998). SCFAs have also been shown to produce the biological effects *in vitro,* by decreasing proliferation, with the fibres having little effect (refer to 6.4). The reduction in proliferation *in vitro* versus the increase in proliferation *in vivo* brings into play the *in vivo* vs *in vitro* debate with the difference responses possibly relating to the availability of glucose and the ability of cells to metabolise butyrate (Singh *et al.*, 1997). The rapid fermentation of pectin within the caecum, resulting in a possible lowering SCFAs levels in the distal colon, suggests that other factors maybe eliciting the increase in proliferation observed including an increase in apoptosis, release of bile acids.

7.2 Conclusion

The results suggest that the DMH mouse model is a good model for studying the effects of chemopreventive agents on catenin expression and associated gene products in relation to colonic neoplasia. In this model the carcinogen acts systemically but the development human colorectal neoplasia could be related to either systemic or luminal carcinogens. Despite this difference, this model retains sufficient similarities to human colon cancer to allow for valid comparisons to be made. The original hypothesis to that dietary fibres protect against tumour development could not be proved. The studies revealed that in DMH-treated mice fibres did not protect against tumour development; the more soluble fibre pectin indeed resulted in a significant increase in tumour incidence with a possible increase in development when compared to a fibre-free diet (basal). The fibres used in this study: ispaghula husk, pectin and cellulose did not appear to affect β-catenin expression *in vivo*, but *in vitro* butyrate was shown to affect β-catenin expression in a cell line, which has a β-catenin mutation (HCT116) but not in a cell line with an APC mutation (HT29).

This study revealed that individual dietary fibres were not sufficient to prevent against colon cancer induced by systemic carcinogens. The fact that epidemiological studies have revealed that fruit and vegetables have a negative effect on colon cancer maybe attributed to other factors such as antioxidants (vitamins) within them. Dietary fibre may have other chemopreventative effects on colon cancer that may not have been revealed by this study. Dietary fibre has a bulking effect, which reduces the contact of luminal carcinogens with the colonic mucosa, the carcinogen used in this study was systemic. Epidemiological studies revealed that high fat and red meat intake has been positively associated with colon cancer; dietary fibres reduce protein digestion, which has been associated with amine production, which are possible tumour promoting compounds. Butyrate, a SCFA produced during fermentation, has also been proposed to be chemopreventative, the fibres used in this study did not appear to produce high levels of butyrate (resistant starch and rice starch excluded as levels of these would be minimal) apart from pectin, although the intercolonic levels of butyrate within distal colon would be predicted to be low where the majority of

tumours were observed. Butyrate was also found to have an unexplained effect on mutant β-catenin, but not wild type, which may be a further chemopreventative biological action. Dietary fibre may also protect against colon cancer indirectly, dietary fibres have previously been shown to help treat and prevent type II diabetes, which has been positively associated with an increased risk of colon cancer.

Thus this study revealed that the dietary fibre pectin enhanced tumour development in this model. This suggests the supplementation of foods with excessive pectin and the consumption of fruit jams may not be beneficial to the colon. The clinical implication of these studies highlights the need for a well balanced diet and the adoption of the '5 a day policy' with a consumption of both fruit and vegetables ensuring the consumption of all their constituents, not just an increase in the consumption of dietary fibre.

Chapter 8
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