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Protein Fermentation, Gut Microbiota and Colorectal Cancer

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Declaration of authorship

I confirm that this is my own work and the uses of all material from others sources has been properly fully acknowledge.

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Abstract

The purpose of this project was to determine the interaction between dietary protein and the gut microbiome in the production of genotoxic metabolites, with a particular focus on the poorly characterised metabolite 4-cresol.

The thesis describes, in the first instance, data from a large human observation study (n=205 healthy Omani adults). In which dietary records and urinary nitrogen excretion were used to estimate protein consumption in relation to urinary 4-cresol excretion. The study observed positive correlations between excreted 4 cresol and protein intake and then sought to explain the inter-individual variance in this by evaluating the influence of the colonic microbiota.

Then the study focused on predicting 4-cresol exposures in the colon using *in vitro* gut fermentation models. The microbiota composition and metabolic profiles from these models are evaluated against different substrates, including comparisons of animal and plant proteins. We show that the total production of 4-cresol is dependent both on the host microbiota and also upon the dietary nitrogen source. The metabolite profiles of these fermentations may be used to predict DNA damage, with 4-cresol emerging as the greatest correlate of fermentation supernatant mediated genotoxicity.

Finally, the study explored whether specific tumour isolates of *F. nucleatum* produce 4-cresol, or other genotoxins, that could drive intestinal carcinogenesis. At this stage the study is unable to conclude whether or not these isolates are passengers or drivers of intestinal disease. This work suggests the need for better models of the effects of the tumour environment on microbial growth.

The most significant aspect of this thesis is that it evidences both the potential genotoxic contribution of 4-cresol in the colonic milieu, but also that urinary 4-cresol sulfate may be used as a biomarker of genotoxic colonic fermentation and thus, may be of use as a cancer risk endpoint in future dietary intervention study.

List of abbreviations

ACF: Aberrant crypt foci

AICR: American Institute for Cancer Research

AOM: Azoxymethane

APC: Adenomatous polyposis coli

ANOVA: Analysis of variance

ASRs: Age-standardised rate

BMI: Body mass index

CA: Cancer

CACO-2: Cell Line human colon from human

CAPP1&2: The Colorectal Adenoma/Carcinoma Prevention Programme 1&2

CFU: Colony forming Unit

CHO: Carbohydrates

CI: Confidence interval

CIF: Cycle inhibiting factor

CIN: Chromosomal instability

CIMP: CpG island methylator phenotype

CRC: Colorectal cancer

COMET: Single-cell gel electrophoresis

DAPI: 4,6-diamidino-2-phenylindole dihydrochloride

DCC: Deleted in colorectal cancer

DMH: Dimethylhydrazine dihydrochloride

DMSO: Dimethyl sulfoxide

DNA: Deoxyribnucleic acid

E. coli: Escherichia coli

EDTA: Ethylenediaminetetraacetic acid

EPIC: European Prospective Investigation of Cancer

FAP: Familial adenomatous polyposis

FFQ: Semi-quantitative food frequency questionnaire

FBS: Fetal bovine serum

FFAR 2/3: Free fatty acid receptors 2 and 3

FISH: Fluorescent *in situ* hybridization

FOS: Fructooligosaccharides

G: Gram or gravity

GalOS/GOS: Galacto-Oligosaccharides

G1: Gap 1

G2: Gap 2

G phase: Gap phase

GC: Gas Chromatography

GC-MS: Gas chromatography–mass spectrometry

GI: Gastrointestinal

GDP: Guanosine diphosphates

GPAQ: Global Physical Activity Questionnaire

GTP: Guanosine triphosphate

GST: Glutathione-S transferases

HCA: Heterocyclic amines

H_2O_2: Hydrogen peroxide

HPLC: High performance liquid chromatography

HT-29: Human colon adenocarcinoma cells

IBD: Inflammatory bowel diseases

IBS: Irritable bowel syndrome

ISE: Ion selective electrodes

KRAS: Kirsten-RAS

LAB: Lactic acid bacteria

LMA: Low melting point agarose

M: Mitosis

MAPK: A mitogen-activated protein kinase

MDF: Mucin-depleted foci

METs: Metabolic Equivalents

Mm: millimolar

μM: micromolar

M phase: Mitosis phase

MS: Mass spectrometry

NMR: ^{1}H Nucleic magnetic renascence

NOCs: N-nitroso compounds

PA: Phenyl acetate

PBS: Phosphate buffered saline

PC: *p*-cresol

PDX; Polydextrose

PKS: Polyketide synthase

PI: Propidium iodide

qPCR: Quantitative polymerase chain reaction

ROS: Reactive oxygen species

16S rRNA: 16S ribosomal RNA

RR: Relative risk

RS: Resistant starch

S: synthesis

SCFA: Short chain fatty acid

SCG-MRC/FFQ: Scottish Collaborative Group-MRC Food Frequency Questionnaire

SD: Standard deviation

SEM: Standard error of the mean

SB: Strand break/ DNA strand break

Sg: Streptococcus gallolyticus

SPME: Solid-phase microextraction

TEM: Transmission electron microscopy

TP53: Tumour protein

UC: Ulcerative colitis

USA: United States of America

V: Vessel

WBE: Wheat bran extract

WHO: World Health Organization

WCRF: World Cancer Research Fund

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Chapter I

1. Gut bugs and colorectal carcinogenesis

1.1 Abstract

In this review we evaluate current thinking on the role of the gut microbiota in colorectal carcinogenesis. We argue that the microbiota plays an important role in colorectal carcinogenesis. Saccharolytic fermentation yields short chain fatty acids which may protect against adenocarcinoma formation, although its role in other tumour pathways is less certain. Some beneficial bacteria may also inhibit tumour formation through the suppression of inflammation and/or the enhancement of immune surveillance. In contrast, the collective proteolytic biota yield toxic metabolites including hydrogen sulphide, ammonia, cresol and phenol which enhance colonic genotoxicity and promote tumorigenesis. Further, we consider the tumour associated biota as a source of oncogenic peptides or inflammatory stimuli. The current literature supports interventions with pre or probiotics intended to optimise the balance of microbial activity in the colon, although efficacy in relation to carcinogenesis has not been demonstrated in man. Going forward, there is a need to integrate advances in microbiology with the different molecular pathways observed in colorectal carcinogenesis.

1.2 Introduction

The comparative incidence of colorectal to oesophageal and stomach cancer is approximately 30:1:2 [1, 2] making the colon the primary anatomical location of gastrointestinal tumours. A distinguishing characteristic of the colon is the relative abundance of a resident microbiota, with microbial populations present in much higher numbers than elsewhere in the GI tract.

In gnotobiotic (germ free) mice, the colon architecture is visibly aberrant, there is an under-developed immune system [3]. Furthermore, there is a reduction in epithelial cell differentiation , mediated potentially through impaired *wnt* signalling [4] leading to a functionally impaired epithelial barrier [5]. These models clearly demonstrate the importance of host-microbe interactions to normal host physiology. Further, the incidence of chemically induced tumours in mice models varies dependent upon the presence or absence of a functional microbiota [6] suggesting a role for the microbiota in neoplastic transformation. Experimental intervention studies in non-germ free animal models, with both probiotics and prebiotics have been shown to potentially suppress tumour development (reviewed previously) [7]. Epidemiological evidence indicates a protective role for dietary fibre in CRC in meta-analysis, which may be coupled to favourable microbial metabolism. Several meta-analyses show that consuming a high-fibre diet reduces CRC risk [8-10]. These strands of evidence point to roles for the microbiota in both tumour development and perhaps suppression. Understanding these host microbiome interactions in cancer is of continuing interest. The emergence of microbial culture independent technologies is beginning to bring some clarity to the complex interactions between the gut microbiota in neoplastic disease, the purpose of this review is to assess this current literature.

1.3 The healthy microbiota

In a healthy host, the colonic microbiome is typically dominated at the phyla level by Bacteroidetes and Firmicutes, with a smaller but sizable abundance of Actinobacteria and Verrucomicrobia [11, 12]. The proportions of these phyla are not fixed, and different phyla and/or species may compete to fulfil distinct ecological niches, thus there is considerable inter-individual variation between phenotypically similar and healthy individuals [13]. Furthermore, age, gender, genetics, diet, and disease may all influence the composition of the microbiome through the life-course, potentially to the benefit or detriment of the host.

1.4 Colorectal cancer

Genetic analysis of colorectal tumour samples reveals inter tumour pathogenic heterogeneity; at least 4 distinct common CRC molecular subtypes have now been established [14]. Broadly speaking, the descending colon and rectum demonstrate high levels of chromosomal instability (CIN) and a strong upregulation of *wnt* signaling [15], in contrast, the microsatellite instability (MSI) subtype shows a higher prevalence in the ascending colon as do cancers developing through the serrated sessile polyp (CIMP phenotype) pathway. Thus the favoured anatomical distribution of these tumour subtypes hint at distinct aetiologies [16]. From a developmental perspective, the right and left side of the colon have different embryological origins. Physiologically, these portions of the colon may be characterised as having distinct microbial activities, and distal and proximal colonocytes may be exposed to diverging metabolite exposures. Saccharolytic fermentation dominates in the ascending colon, where the high fluid volume make the luminal contents quite dilute [17]. Microbial metabolites, including short chain fatty acids (SCFA), may be absorbed, with water and electrolytes, *in situ* and through the transverse colon, such that the contents of the descending colon are more concentrated. In *in*

vitro models at least, microbial activity may be decreased in the latter portions of the bowel but proteolysis becomes favoured [18, 19]. Mechanisms have been proposed through which the gut microbiota may influence the cancer process including, via eliciting chronic inflammation in the host, potentially by disrupting the epithelial barrier [20], or through the production of genotoxic metabolites [21]. Similarly, mechanisms of protection mediated through a healthy microbiota are proposed [22], notably through the production of beneficial SCFA, and through the competitive inhibition of pro-carcinogenic genera. Mechanisms have similarly been proposed to explain the role of diet in CRC. However, to this point, these mechanisms and aetiologies have been poorly considered in relation to the diversity in tumour sub-type.

1.5 The saccharolytic microbiota in protection against cancer

Meta-analysis of prospective cohorts show dose-dependent protection against CRC with increasing dietary fibre intake, corresponding to a 10% decrease in risk per additional 10g consumed per day [9]. Suggested mechanisms underpinning this protection include the displacement of other foods from the diet, the co-linearity of fibre with phytochemical intakes, and decreasing colonic genotoxin exposure (via both hastening bowel transit times and the diluting of stool) [23, 24]. Of note, in the small intestine fibre chelates both iron and bile acids, and the delivery of these to the colon could feasibly increase genotoxicity in the gut.

An additional protective mechanism involving the colonic fermentation of fibre has been well characterised; SCFA are produced as a consequence of microbial fermentation with fibre as a substrate. Predominantly acetate, propionate and butyrate are produced in ratios which may vary according to diet and microbial composition, but are often approximately 3:1:1 [25]. The SCFAs are natural ligands for free fatty acid receptors 2 and 3 (FFAR 2/3), which activate MAPK; this pathway is suggested to exert direct downstream anti-inflammatory and gut barrier

function enhancing responses [26, 27]. Conversely an increase in the permeability of tight junctions is associated with translocation of bacteria and a subsequent pro-inflammatory response which may represent an independent risk pathway for CRC [28].

Of the colonic SCFAs, butyrate is the preferential energy source for normal colonocytes [29]. In addition, in healthy cells, butyrate may confer some protection against genotoxins via the induction of glutathione-S transferases (GST) [30]. Moreover, in colorectal cancer cell lines, butyrate has consistently been observed to induce cell cycle arrest, differentiation and apoptosis [31]. Tumour cells are inherently glycolytic; butyrate is therefore not oxidised and its accumulation inhibits the activity of histone deacetylases in favour of histone acetyltransferases, thus transcriptionally activating regions of the genome that were otherwise silent, and which favour apoptosis [32] **Figure 1.1**. *In vitro* these effects on tumour cells are concentration dependent; *in vivo*, there exists a concentration gradient through the bowel, with the highest concentrations likely to be present in the caecum where saccharolytic activity is at its greatest, and the lowest concentrations in the sigmoid colon/rectum where the upregulated *wnt*/canonical signalling subtype of cancer predominates [33, 34]. The potential relationship of butyrate in the caecum to the development of right side tumours is also not well explored; at high concentrations, butyrate provides the cell with an abundance of acetyl groups, which may, if not oxidised or shuttled into lipogenesis, potentially be utilised by histone acetyltransferases [35] in a region of the colon characterised by a susceptibility to epigenetically sensitive serrated polyps and tumours.

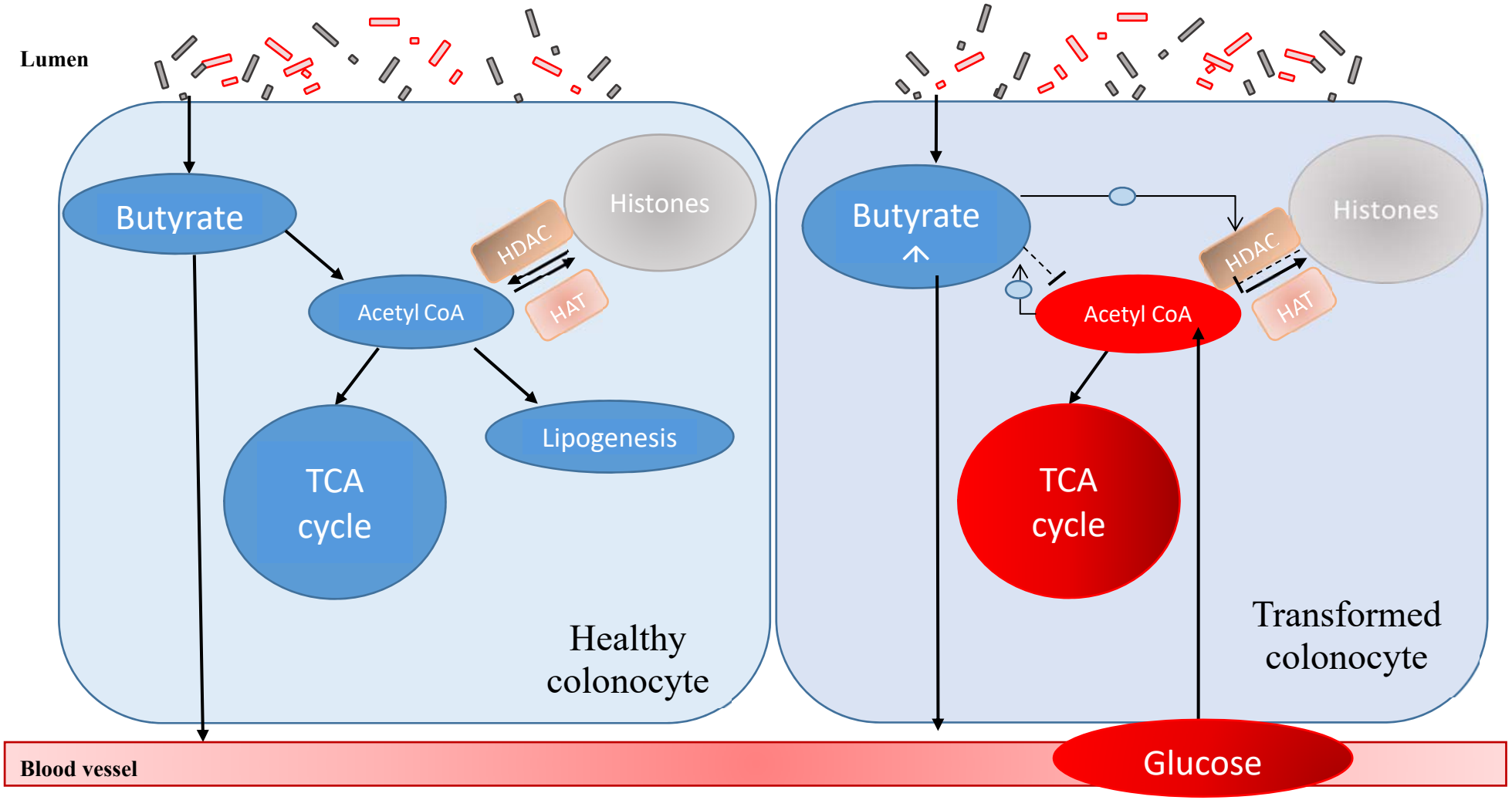

Figure 1.1: The effects of butyrate accumulation on histone deacetylases in glycolytic neoplastic cells. Here two models of butyrate-induced histone acetylation mechanisms. In addition to acting as an HDAC inhibitor (at the left side), butyrate can act as an acetyl-CoA donor and stimulate HAT activity (at the right side).

As a consequence of the observed biological responses to butyrate *in vitro*, dietary interventions have been explored utilising prebiotics to enhance the production of butyrate *in situ*. In *in vivo* models, with animals fed experimentally high doses of prebiotics, chemically induced tumour and development and pre-neoplasia can be suppressed **Table 1.1**. These interventions show greatest efficacy when the dietary prebiotic is given to the test animals prior to tumour induction. In addition to changing microbiota composition, post-tumour induction the prebiotics suppress inflammation which might help explain their anti-cancer action.

Table 1.1: Intervention studies in rodent models evaluating the anti-cancer effects of prebiotic supplements

Intervention	Rodents	Carcinogenesis protocol	Treatment	Outcome	Ref.
Resistant starch in a colitis-associated colorectal cancer model	4 week old Male sprague-Dawley rats (n=100, 25 per group)	2 weeks of tests diets, then 2 injections of Azoxymethane (AOM) (10mg/kg) 1 week apart, then 2% dextran sodium sulphate indrinking water for 7 days. Test diets maintained for a further 20 weeks	20 week intervention with diets containing 10% Resistant starch and/or 0.5% Green tea extract.	RS and RS + GTE but not GTE diets decreased tumour multiplicity and adenocarcinoma formation compared to control diet ($p<0.05$). RS changed microbial composition, increasing *Parabacteroides, Ruminococcus, Marvinbryantia* and *Bifidobacterium*. RS increased SCFAs and decreased inflammation markers	[36]
Galacto-Oligosaccharides and biomarkers of Colorectal Cancer in Wister Rats	Six-week old male Wister rats (n=90, 15 per group)	40 mg/kg body weight 1,2-dimethylhydrazine dihydrochloride (DMH) given twice a week for two weeks prior to intervention with (GalOS)	16 week intervention diets with GalOS at doses equivalent to 4, 6 or	GalOS reduced ACF formation, increased SCFA and decreased the activity of microbial β-glucuronidase, β-glucosidase, azoreductase and nitroreductase.	[37]

			8 g per day in man by weight		
Inulin type fructans and a colitis associated murine model	Five-week old male BALB/cAn Nhsd mice (n-105, 15 per group)	Azoxymethane (10 mg/kg i.p.) plus aqueous DSS 2% for 4 days given either before or after (or both) the dietary intervention	1 month inulin prior to induction then 8 months without inulin Or 1 month inulin prior to induction then 8 months with inulin Or induction then 9 months inulin	Inhibition of colitis, and a reduction in polyp number with Inulin type fructans. Reduction in inflammatory markers was greater in animals fed the inulin pre AOM:DSS exposure than post exposure.	[38]

High-amylose CS, high amylose-octenyl succinic anhydride-modified CS, and a novel RS, high amylose-stearic acid-complexed CS.	1/ 5-week-old male F344 rats (n=90) 2/ 5-week-old male A/J mice (n=120)	1/ Rats- Azoxymethane (15 mg/kg i.p) twice over two weeks 2/ Mice- Azoxymethane (7.5 mg/kg i.p.) weekly for 4 weeks	Control diet (corn starch) for two weeks, then induction with AOM, then transition to treatment diets for 10 weeks	Resistant starch did not inhibit ACF formation	[39]

Dietary intervention studies in high-risk cohorts with cancer endpoints in man using high fibre diets and/or prebiotics have shown less efficacy. The Polyp Prevention Trial was a polyp recurrence dietary intervention study initially involving 1905 participants, enrolled on a high fruit and vegetable/low fat arm (~18 g fibre 1000 $kcal^{-1}$) or a control diet. Based on intention to treat analyses, there were no observed differences in polyp recurrence at either 4, or 8 years [40]. Adherence to a long term high fibre intervention is difficult to achieve in a large cohort, in a post hoc analysis only 210 individuals from this study were identified as being 'super compliant', these individuals did show a 35% reduction in the risk of recurrence [41]. The production of butyrate was not quantified in this intervention study, and it is not possible to determine the contribution of microbial metabolism to this observation.

In mixed culture *in vitro* gut fermentation models, resistant starch stimulates the growth of *Ruminococcus bromii*, *Faecalibacterium prausnitzii, Eubacterium rectale* and *Eubacterium hallii* and induces a significant increase in the concentration of butyrate in supernatant [19, 42-45]. It was selected therefore as a potential butyrate delivery vehicle for use in the CAPP1 and 2 polyp prevention trials. The Colorectal Adenoma/Carcinoma Prevention Programme (CAPP 1) study involved a 30g day^{-1} dietary intervention with resistant starch (n=30), or with resistant starch plus aspirin (n=31) or with placebos. The study was conducted amongst very high risk adolescents with familial adenomatous polyposis (FAP) for period of 17 months. At the end of the study the resistant starch elicited a reduction in the length of the crypts of Lieberkühn relative to placebo but it did not suppress polyp formation (RR 1·05, 95% CI 0·73–1·49). In follow up, CAPP2 used the same intervention arms to evaluate chemoprevention in individuals with Lynch syndrome over almost 4 years. Lynch syndrome has a slightly lower absolute risk of CRC than FAP, and the resultant colonic changes are characterised by mismatch repair as opposed to APC mutation; this evaluation of different subtypes of tumour

is a strength of the collective CAPP programme. However, again they observed no anti-cancer effects of resistant starch consumption in this genetically high risk cohort [46].

A common critique of CAPP and indeed of all polyp recurrence studies is that subjects have already progressed quite far along the cancer former phenotype, and accordingly that chemoprevention needs to begin earlier.

Evidence from short term dietary interventions using high fibre diets, or prebiotics, in healthy volunteers with the earlier, and poorly validated against neoplasia, end-point that is faecal genotoxicity, suggest an alternative butyrate-independent mechanism through which these saccharolytic fermentation substrates might protect against cancer **Table 1.2**.

Table 1.2: Human prebiotic dietary intervention studies with faecal genotoxicity as an endpoint.

Intervention food	Subjects	Protocol	Faecal water genotoxicity	Reference
β-glucan in a bread product versus β-glucan free control bread	Polypectomised patients (n = 69) recruited in Greece. (Mean age 63)	Double blind randomised control trial in which participants consumed breads containing 3 g/day β-glucan or control over 3 months	Faecal water induced DNA damage assessed against Caco-2 cells via the comet assay decreased significantly in the treatment group	[47]
Wheat bran extract (AXOS) or oligofructose or placebo in a sucrose drink	Healthy Belgian adults (n=19)	Double-blind, randomized cross-over trial. 2 week intervention periods with 15g day in week 1 and 30 g day in week 2. 2 week washout period	Both WBE- and oligo-fructose tended to reduce faecal water induced DNA damage in Ht29 cells compared to placebo but the effect was not quite statistically significant (WBE: $p = 0.060$; oligofructose: $p = 0.057$) (Comet assay)	[48]

Polydextrose versus maltodextrin	Healthy British adults (n= 31)	Double blind placebo-controlled, crossover study polydextrose PDX; 8g/d and placebo 8g/d, or control for three weeks then crossover after a 3 week wash out	Faecal water induced DNA damage assessed against Ht29 cells (Comet assay) was significantly lower following consumption of PDX versus the maltodextrin	[49]
Galacto-oligosaccharide (GOS) in an orange juice vs. a juice control	Healthy older British volunteers aged 50+, (n=37) (Comet analysis n= 5)	Randomised, double-blind, placebo-controlled crossover trial with a juice containing 4 g GOS or a placebo. With a three week treatment and three week wash out.	DNA damage against Ht29 cells assessed pre and post treatment for five volunteers (Comet assay). No change in faecal water genotoxicity reported, although the study may be underpowered.	[50]

Saccharolytic 'probiotic' bacteria are also prepared as dietary supplements; and in rodent models of cancer, with animals fed experimentally high doses of the probiotic, they are shown to strongly inhibit tumorigenesis but with significant strain and species variability (**Table 1.3**). The probiotics most commonly demonstrating anti-cancer effects in experimental models are of the lactate producing lactobacillus genus; Lactobacilli do not directly produce large quantities of butyrate, they do temporarily shift the metabolic activity of the intestinal biota, through both competitive inhibition and the production of hydrogen peroxide and bacteriocins [51]. The activity of microbial enzymes implicated in the activation of intestinal carcinogens may therefore be attenuated; a double blind placebo control human feeding trial with combined *Lactobacillus rhamnosus LC705* and *Propionibacterium freudenreichii* ssp. shermanii JS led to a reduction in the activities of both beta-glucosidase and urease [52] in recovered stool. Whilst a separate intervention, this time in elderly volunteers, with the same probiotic combination reduced the activity of faecal azoreductase [53].

The lactobacilli may also inhibit DNA damage directly, by binding and potentially metabolising genotoxins [54], or alternatively through the induction of the DNA repair [55] apparatus of the colonocytes. This anti-genotoxicity is not just shown against experimental carcinogens; the genotoxicity of human faecal samples may be significantly reduced by post collection *in vitro* incubation with viable probiotic bacteria [56].

In randomised control trials supplemental lactobacilli and/or selected other probiotic strains reduce inflammatory markers in healthy volunteers [57-59] and may induce remission and maintenance of remission in ulcerative colitis [60, 61] though not in Crohn's. This suppression of inflammation may be mediated through interaction with toll like receptors, and their subsequent influence on the intestinal barrier [62]. Lactobacilli mediated suppression of the pro-carcinogenic enzyme COX-2, which activates pro-inflammatory prostaglandins has been shown in *in vivo* and *in vitro* models of carcinogenesis [63, 64]. Conversely, in interesting

tumour xenograft or implantation models *Lactobacillus casei* BL23, *Lactobacillus plantarum* are shown to strongly suppress the subsequent development of initiated non GI tumours [65, 66], presumably through activation of the immune response. Indeed enhancement of NK (Natural Killer) cell activity has been shown in dietary interventions in both animal models and in human volunteers [67-69]. Thus it seems that select probiotics downregulate systemic inflammation whilst potentiating immune surveillance through NK cell activity, thus targeting different phases of cancer development. We are unaware of studies demonstrating the anti-cancer benefits of chronic probiotic ingestion on these systems. The observed responses may well be transient as the host adapts to novel intestinal antigen, with adaptation these anti-cancer immune effects may or may not persist.

As with the prebiotics, human data proving efficacy in cancer prevention is lacking. Epidemiological studies have not assessed probiotic intake in any detail, and to our knowledge, aside from those interventions showing amelioriation of inflammation in UC, there are no experimental intervention data in healthy volunteers with probiotics using a well validated colorectal cancer endpoint such as polyp recurrence. As with prebiotic intervention trials researchers have had to use poorly validated endpoints in probiotic feeding studies; for example, a 4 week randomised crossover trial in 17 volunteers with *Bifidobacterium lactis* (and resistant starch) induced no significant changes in crypt cell kinetics or DNA methylation patterns [70] in colonic biopsy material. In contrast, a 12 week dietary intervention with a synbiotic (Inulin, *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12) decreased DNA damage in intestinal mucosa and reduced faecal water genotoxicity [71]. The animal experimental data are highly promising and well controlled dietary interventions in high cancer risk human cohorts with probiotics are now perhaps justified.

Table 1.3: Probiotic treatments in experimental models of tumorigenesis

Intervention	Rodents	Carcinogenesis protocol	Treatment	Outcome	Reference
Lactobacillus rhamnosus GG CGMCC 1.2134 (LGG)	64 four week old female Sprague Dawley rats (16 per treatment group)	1,2-Dimethyl hydrazine (40 mg/kg i.p) weekly for 10 weeks	1 x10^9 CFU *Lactobacillus rhamnosus* GG CGMCC 1.2134 (LGG) given daily from initiation of carcinogenesis through 25 weeks. Or DMH control, LGG control, or -/-control.	~40 % reduction in tumour incidence in *L*.GG/DMH group versus DMH group. And a relative suppression of TNFα, NF-kβ, iNOS and VEGF versus DMH group.	[72]
Activia(R) (*Bifidobacterium animalis lactis* DN-173 010/CNCM I-2494)	70 Swiss (Mus musculus) male, sexually mature mice, in 7 experimental groups (n = 10)	Four doses of 1,2-DMH (20 mg/kg b.w., ip), two doses per week. Given to relevant treatment arms during weeks 3	Activia® groups received the Activia® product by oral gavage (0.1 mL/10 g b.w., vo) either just prior to initiation with DMH, just post initiation, or simultaneously with DMH, or pre and post initiation every day until the 12th	The numbers of aberrant crypt foci were reduced by up to 79% in mice given the activia relative to DMH alone. The reduction in ACF was greatest in the mice pretreated with the probiotic, suggesting suppression of the initiating DNA damage.	[73]

		and 4 of the intervention	week, plus relevant controls. Sacrifice at week 12.		
Lactobacillus rhamnosus GG (ATCC 53013)	Seven week old Apc Min/+ mice (n=24)	Sporadic polyp development in this model, Ad libitum sulindac (180 ppm in drinking water) used as a protective control.	1×10^8 CFU LGG in dried form as part of the experimental diet every day for 8 weeks	LGG reduced the polyp burden in this genetically post initiation model, but not quite as well as sulindac.	[74]
Synbiotic preparation LBB (oligofructose-*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and	Three week old Sprague Dawley rats (n=40) ten per group	Ampicillin (75 mg/kg), daily for five days, Relevant treatment arms then received 1,2-DMH sub-cutaneously weekly for 10 weeks	LBB in chow at 0.9 g/ kg body weight daily equivalent to 6.4×10^{11} cfu *Lactobacillus acidophilus* and 1.9×10^{10} cfu *Bifidobacteria* spp. for 23 weeks alongside initiation	20% reduction in tumour burden with rats co-administered the probiotic versus the DMH alone. It also preserved epithelial integrity and suppressed β catenin.	[62]

Bifidobacterium infantus)					
Lactobacillus casei BL23 Or *L. lactis* MG1363	6 week old Balb-c mice (N=30-35 per group)	Sub cutaneous 1,2 DMH weekly for 10 weeks 9 (20 mg/kg bw)	(1×10^9 CFU) *Lactobacillus casei* BL23 or *L. lactis* MG1363 daily in chow beginning the day of first DMH injection.	~40 % reduction in multiple plaque lesions in mice fed *Lactobacillus casei* BL23 but not *L. lactis* MG1363 relative to DMH positive control.	[65]
Lactobacillus salivarius Ren	Five-week-old male F344 rats (n=24, 8 per treatment group)	Subcutaneous 1, 2-DMH weekly for 10 weeks	5×10^{10} CFU/kg bodyweight per day *Lactobacillus salivarius* Ren for 32 weeks beginning 2 weeks prior to DMH initiation	~62 % reduction in tumour burden amongst the probiotic treated animals relative to DMH alone.	[75]
Lactobacillus plantarum and *Lactobacillus rhamnosus*	6-8 week old female BALB/c mice (n=30, 10 per group)	Tumour induction via subcutaneous implantation of CT26 tumour cells	Probiotics administered via oral gavage at 1×10^8 CFU per day for two weeks prior to implantation and then weekly at 1×10^9 CFU for three weeks post implantation	The mean implanted tumour volume was significantly suppressed in the mice fed *L. plantarum,* relative to the no probiotic group. It also significantly increased survival, *L.*	[66]

				rhamnosus did not have any effect on survival	
Lactobacillus plantarum (AdF10) or *Lactobacillus rhamnosus GG* (LGG)	Female sprague dawley rats in 6 groups (n=6 per group)	Subcutaneous 1,2 DMH 30 mg/kg body weight twice a week for four weeks, then once a week for 16 12 weeks	Probiotics administered at 10^{10} CFU per day for 16 weeks by oral gavage beginning at the same time as the tumour initiation treatment.	50 % reduction in the tumour burden with the *Lactobacillus plantarum* relative to DMH control. 34% reduction in tumour burden for the *Lactobacillus* GG	[76]
Lactobacillus salivarius Ren	50 male F344 rats aged 5 weeks. (n=10 per group)	Subcutaneous 1,2-DMH (30 mg Kg BW) once a week for 10 weeks	Probiotic given orally at high ($1x10^{10}$ CFU per day) or low doses ($5x\ 10^{8}$ CFU per day) for two weeks prior to initiation and then continuously until week 15	~35% reduction in aberrant crypt foci numbers in rats given either the high or low dose probiotic at week 15 relative to DMH control	[77]
Lactobacillus delbrueckii UFV-	Eight week old male Swiss mice were distributed	1,2-DMH (25 mg/kg)	Each probiotic prepared at $3x10^{8}$ CFU mL^{-1}, and given ad libitum	Both the lactobacilli and the bifidobacterium reduced the numbers of ACF by ~50% relative to DMH	[78]

H2b20 or *Bifidobacterium animalis* var. *lactis* Bb12; or a combination of the two, or *Saccharomyces boulardii*	five treatment groups (n=10/group)	subcutaneously beginning 1 week after first treatment with probiotic and then once weekly for 6 weeks	in the drinking water from one week prior to initiation through 14 weeks	control. Interestingly in combination they were not effective.	
Lactobacillus rhamnosus GG MTCC #1408, or *Lactobacillus casei* MTCC#1423, *Lactobacillus plantarum* MTCC #1407, or	Sprague Dawley rats, (n=6 per group with 12 treatment groups)	1,2-DMH intra-peritoneal (20 mg/kg bw) weekly for 6 wk.	1×10^9 CFU daily for 1 week prior to initiation then daily for six weeks	Significant decrease in ACF formation with all probiotic regimens relative to DMH control. Notably L.GG induced a 99% reduction in ACF numbers	[79]

Lactobacillus acidophilus NCDC #15 or *Bifidobacterim bifidum* NCDC #234

1.6 The proteolytic gut microbiota in colorectal cancer causation

1.6.1 Proteolytic fermentation

The epidemiological evidence implicating total protein intake in colorectal cancer is weak [80], there are however suggestions of differential risk according to protein source; plant protein consumption, from sources such as soy, may be associated with a decrease in risk [81] and animal protein intake, particularly from red and processed meat, associated with an increase in risk [82]. It has variously been proposed that the increased risk associated with animal protein intake is due to higher fat and haem intake, or that contaminants from processing or cooking of meat are the cause, or that meat is simply a proxy for an otherwise energy rich diet. More recently it has been suggested that animal proteins are a stronger stimulator for mitogenic hormones such as IGF-1 [83]. These mechanistic hypotheses remain generally poorly evidenced and somewhat neglect the potential involvement of the resident microbiota.

With a western diet somewhere between 6 g and 18 g of protein per day is thought to reach the colon [84, 85]. Saccharolytic fermentation is favoured in the caecum, but as a substrate it can be quickly utilised. With decreasing availability of fermentable carbohydrate in the distal colon, there is a shift towards the production of proteolytic end products in this slightly more cancer prone location [86]. In *in vitro* mixed culture models of gut fermentation, the addition of protein to media increases the concentrations of an assortment of metabolites, including phenolic compounds, amines, ammonia and hydrogen sulfide, these metabolites can be leveraged as nitrogen sources for bacterial growth, or they may be taken up by colonocytes and transported into the bloodstream [87]; their accumulation in the colonic lumen is associated with increasing toxicity [21, 88].

The amino acid composition of the protein substrate influences the overall composition of this potentially genotoxic fermentation supernatant. For example, methionine and cysteine may be

used as substrate by the sulfate reducing bacteria (SRB) (most notably *Desulfovibrio*, *Desulfotomaculum, Desulfobacter, Desulfobulbus* [89]), leading to the generation of H_2S [89, 90] **Table 1.6**. Hydrogen sulphide inhibits butyric acid oxidation [91-93], it increases cell proliferation *in vitro* [94] and is shown to be genotoxic [95]. In *in vitro* batch-culture fermentation with faecal inoculate, the rate of H_2S production differs according to whether albumin or casein is used as a substrate [96]. In human observational studies the sulfate reducing bacteria may be associated with inflammatory bowel disease [97], and are putatively implicated in its pathogenesis through the ability of H_2S to compromise barrier function [90, 98]. In both animal and human dietary intervention, diets high in protein increase the recovery of sulfide in faeces [99].

Additionally, fermentation of the aromatic amino acids leads to the production of phenols, indoles and 4-cresol. These are not well recovered in stool, but rather enter the hepatic circulation to be detoxified in the liver and eventually excreted in urine [100]. Studies have shown that with high protein intake, metabolites of *4*-cresol and phenol appear in the urine [101]. Phenol and 4-cresol are toxins and may be associated with disease, however due to their low concentration in stool they have not been thoroughly investigated as contributors to the colonic genotoxic load.

In contrast to the epidemiological data, the carcinogenicity of higher protein diets is consistently demonstrated, particularly in relation to colonic inflammation, in experimental animal models [102]. Higher protein dietary interventions in human volunteers do lead to increased excretion, in urine, of markers of amino acid fermentation, but the appearance of these metabolites in urine does not necessarily correlate with increased faecal genotoxicity [103, 104]. Colonic fermentation and absorption is dynamic, and stool can vary considerably in water content, therefore faecal samples may be poorly representative of colonic exposures; better biomarkers of cancer risk for human dietary intervention study are certainly needed to

bridge the gap between the lack of associations between protein intake and cancer in human subjects versus the mechanistic and animal experimental evidence to the contrary.

1.7 Towards a colon cancer specific microbiota

Perhaps the first specific bacteria implicated in the pathogenesis of colon cancer was *Streptococcus gallolyticus* (Sg). Endocarditis and bacteraemia associated with *SG* infection is associated with increased risk of colorectal neoplasia in observational studies [105, 106]. Similarly, case-control studies show an increased risk of colorectal cancer associated with serological evidence of previous exposure to (Sg) antigen [107-109]. Faecal samples from volunteers with colorectal cancer were found to be more likely to score positively for Sg, and tumour tissues show higher Sg counts than adjacent normal mucosa [110].

There is also good experimental evidence demonstrating the carcinogenicity of Sg; pre exposure of cultured HCT116 cells to Sg resulted in greater tumour mass in a mouse xenograft model, whilst oral gavage with Sg increased the tumour burden in an AOM mouse model of tumourigenesis [111, 112], From a mechanistic perspective the exacerbation of tumour development in the presence of Sg may be mediated via inflammation, indeed Abdulamir et al., [113] observed a higher expression of Nf-KB and IL-8 mRNA in tumour tissues from individuals seropositive for Sg antibodies versus Sg negative patients, indicating increased inflammation in the tumour environment which may influence cell turnover. Alternatively Sg. might also influence cell behaviour independently of inflammatory pathways, Kumar et al., [114] observed increased cell proliferation in cultured colon cancer cell lines (HT29, HCT116 and LoVo) exposed to Sg and demonstrated that this was driven by an increase in nuclear β-catenin.

The emergence of *Helicobacter pylori* as a risk factor in gastric cancer in the 1980s meant that it too has been considered as a candidate in CRC causation. Routine clinical screening for *Helicobacter pylori* infection facilitates opportunistic observational study into its role in CRC; a 2013 meta-analysis of 28 of these studies suggested an approximate 40% increase in CRC risk with *H. pylori* infection [115]. Separately Wang et al. analysed 27 studies to conclude that *H. pylori* infection increases risk of colonic adenocarcinoma by ~24%, adenoma by ~87%, tubular adenoma by 3 fold and villous adenoma by 2 fold [116], thus implying a role for *H. pylori* early in the disease process. In the stomach, strains of *H. pylori* secrete CagA which can be absorbed by the mucosa locating itself inside the cell membrane and presenting as antigen thus initiating systemic inflammation. Independently the CagA protein also aberrantly activates SH2 and the Ras-Erk MAP kinase signalling pathway [117] thus driving gastric carcinogenesis via different mechanisms. The potential mechanisms of action of *H. pylori* in the colonic mucosa have not been well investigated, however. Curiously Sonnenberg et al. recently reported an inverse association between *H. pylori* infection and the incidence of serrated polyps in a large US cohort [118]. In a DSS-induced murine colitis model $^{CagA+}$*H. pylori* infection significantly increased the number of dysplastic lesions observed at 48 days relative to DSS alone, via a process seemingly mediated through deregulated *wnt* signalling [119]. Whilst further mechanistic work is needed, the findings to date associate *H. pylori* infection with the adenocarcinoma pathway of CRC. In northern European populations about a third of the population screen positive for gastrointestinal *H. pylori,* levels of infection may be much higher in the developing world; given its already well established role in gastritis and gastric neoplasia, it may be singled out as a desirable target for elimination from the intestinal tract.

Other bacteria capable of capable of secreting oncogenic peptides include *Escherichia coli* and *Bacteroides fragilis*. Raisch et al. studied the abundance of *E. coli* by phylogenetic subgroup in mucosal biopsies from colorectal cancer versus mucosal samples from patients with

diverticular disease as a control. They report a much higher abundance of *E. coli* from the phylogenetic subgroup B2 in the cancer specimens (positively identified in 73.7 % of cancer specimens versus 41.9 % of controls) [120]. The phylogenetic B2 sub group is home to enteropathogenic *E. coli* strains; it may also be characterised by the presence of genes encoding cyclomodulins and genotoxins such as colibactin. Cycle inhibiting factor (CIF) is a cyclomodulin capable of blocking mitosis independently of DNA damage, at least *in vitro* [121]. Colibactin is a poorly characterised genotoxic polyketide-peptide synthesised in the gut by polyketide synthase (PKS) positive *Escherichia coli* [122]. These PKS positive bacteria have been identified in up to 20 % of healthy volunteers. In animal models of carcinogenesis, exposure to PKS may induce DNA strand breaks [123] and tumour formation [124, 125]. Transient infection of cultured epithelial cells with PKS positive *E.coli* induces chromosomal aberrations and increases the mutation frequency rate [123], in addition to influencing cell cycle behaviour [126]. Further, repeat infection of cultured intestinal cells with non-pathogenic *E. coli* abundant in the intestinal lumen are also shown to influence cell behaviour, enhancing cell survival and upregulating the B catenin apparatus consistent with a carcinogenic phenotype.

Strains of *Bacteroides fragilis* produce a metalloprotease toxin (BFT) which has been associated with inflammatory bowel disease [127] and which may be more abundant in samples from cancer patents than in controls [128] . Importantly an increased presence of BFT producing *Bacteroides fragilis* has been associated with pre-cancerous lesions suggesting its involvement early in the cancer pathway [129] . Chung et al. inoculated APC^{Min} mice with *B. fragilis* to promote tumorigenesis via an inflammatory cascade involving Stat3 and Nf-kB [130], which might explain the ability of BFT^{+} *B. fragilis* to induce colitis in gnotobiotic mice [129]. This pro inflammatory effect suggest a tumour promoting role for BFT.

Recent advances in culture independent technologies for characterising microbial communities have facilitated a shotgun approach towards the identification of tumour specific microbes through comparisons of tumour tissue and adjacent healthy tissues in the same individual (**Table 1.4**), or by comparing healthy mucosa or faecal samples between cases and controls, (**Table 1.5 and 1.6**) respectively. These approaches identify microbial communities which may be preferentially successful at utilising the tumour environment; it does not, of itself, well evidence causality. The tumour environment may be characterised by a disruption to the colonic stream, aberrant mucin production coupled to a depleted mucosal barrier, inflammation, potentially blood, and host derived lactate as a glycolytic metabolic by-product. Specialists within the biota could well thrive in this niche, however the potential of a tumour associated microbiome to influence the carcinogenic process is worthy of investigation. That said there is little consensus across studies, bacteria within the genera *Prevotella, Bacteroides, Roseburia* and *Faecalibacterium* are variously reported as being enriched in tumour tissue relative to normal mucosa, whereas between individuals *Escherichia coli, Actinomyces odontolyticus, Bacteroides fragilis, Clostridium nexile* are reported as being more abundant in cases but not consistently so across studies. Few of these studies to date have taken into account or had the power to well consider the microbiota by CRC subtype or location, and given the common methodologies there may now be scope for meta-analysis, the one stand out candidate tumour associated microbe emerging from these analyses appears to be *Fusobacterium nucleatum.*

Fusobacterium nucleatum are most commonly associated with the oral mucosa but they have been identified in, and cultured from, intestinal tumours [131, 132] **Table 1.4.** Evidence is emerging to suggest that this species may contribute to the tumour process. Yu et al. report an increased likelihood of tumour recurrence post treatment with a positive score for *F. nucleatum* [133]. Several groups report attenuation of the host immune response with the presence of *F.*

nucleatum [134-136], others suggest that these bacteria may increase cell proliferation and enhance *wnt* signalling through diverse cell mediated interactions [137-139]. Conversely, in recent work Amitay et al. were unable to identify *F. nucleatum* in pre-neoplastic adenomas, suggesting that this strain is more a passenger in advanced disease than a driver of early disease [140]. However others suggest *F. nucleatum* may be more strongly associated with serrated adenomas [141, 142] which are often characterised by aberrant CPG island methylation and microsatellite instability, and analysis of colonic tissue from patients with both ulcerative colitis and colorectal cancer shows hyper-methylation associated with the presence of fusobacteria [135].

Experimental proof of the potential role of *F. nucleatum* in human carcinogenesis is lacking, and a satisfactory mechanistic explanation is still needed [139]. In one experimental study $APC^{min/+}$ mice gavaged with *F. nucleatum*, developed significantly more tumours than non-*F. nucleatum* treated controls, and in the same study, colorectal cancers were more likely to form tumorous grafts after injection into nude mice when the cells were pre-treated with *F. nucleatum* [139]. In contrast, Tomkovich et al. noted no increase in either inflammation or cancer risk in tumour susceptible $APC^{Min/+;Il10-/-}$ and $APC^{Min/+}$ germ free animals colonised with *F. nucleatum* [124, 143] suggesting involvement in neither inflammation nor *Wnt* stimulating pathways of tumour promotion.

Table 1.4: A comparison of the adherent mucosa in colorectal cancer tissue versus adjacent normal mucosa

Study population	Characterisation Method	Findings	Reference
46 CRC patients	Pyrosequencing V1-V3 region of bacterial 16 S rDNA	Lower microbial diversity in tumour versus non tumour tissues from the same patient. The cancerous tissue had higher numbers of *Bacilli* and *Ochrobactrum* and lower numbers of *Phascolarctobacterium, Ruminococcaceae and Feacalibacterium* than the adjacent normal tissue.	[138]
6 CRC patients	454 pyrosequencing V1-V3 region of bacterial 16 S rDNA	Significant inter-individual differences in the normal mucosal microbiome and between normal and tumour tissues. *Fusobacterium, Roseburia* and *Feacalibacterium* were enriched in tumour specimens whilst the *Enterobacteriaceae*, *Citrobacter*, *Shigella*, *Cronobacter*, *Kluyvera*, *Serratia* and *Salmonella* spp. were decreased in the CRC samples relative to the normal tissue	[144]
65 colorectal cancer patients	V4 16 S RNA pyrosequencing	At the phylum level, the relative abundances of Bacteroidetes. Proteobacteria and Fusobacteria were	[145]

		more abundant in the tumour than the adjacent mucosa. At the genus *level Fusobacterium, Prevotella, Alloprevotella, Porphyromonas, Peptostreptococcus* and *Parvimonas* were enriched in the tumor tissue. In contrast, the relative abundances of the *Bacillus*, *Lactococcus*, *Acinetobacter*, *Pseudomonas* and *Parabacteroides* genera were significantly lower in tumour than in the adjacent non-tumour tissue	
52 CRC patients without symptoms of bacteraemia	qPCR targeted at *Streptococcus gallolyticus* -specific primers	33% of tumours versus 23% of matched normal colon tissues were Sg-positive	[146]
NGS 9 colorectal cancer patients. qPCR in samples from 95 patients	454 sequencing of the 16S gene, qPCR pyrosequencing and quantitative PCR specific to pan-*Fusobacterium* and Fluorescent in situ hybridisation with the *Fusobacterium*	*Fusobacterium* was the most differentially abundant taxon in colon tumour versus normal specimens via NGS. *Fusobacterium nucleatum* identified as the sub species most commonly enriched via qPCR.	[136]

	targeted probe (pB-00782)		
148 tumours and 128 adjacent matched normal tissues from patients without bactereamia	qPCR targeted using a *Streptococcus gallolyticus* -specific primer	4% of tumour tissues versus 47% of the normal tissues were positive for Sg	[77]

Table 1.5: Comparisons of the adherent mucosa microbiota in normal tissue from cancer patients versus mucosa from healthy controls

Study population	Characterisation Method	Findings	Reference
46 CRC patients 56 controls	Pyrosequencing V1-V3 region of bacterial 16 S rDNA	In swabs taken from the normal mucosa *Porphyromonas*), *Fusobacterium*, *Peptostreptococcus,* and *Mogibacterium* were more in CRC patients, whereas in the control patients *Faecalibacterium*, *Blautia*, and *Bifidobacterium* were more abundant.	[138]
6 CRC patients 6 serrated adenoma patients 6 advanced colorectal neoplasia patients 6 controls	16S RNA 454 pyrosequencing	The normal mucosa from the control group was characterised by a predominance of Proteobacteria with significant numbers of Firmicutes and smaller numbers of Bacteriodetes and Actinobacteria. The normal mucosa of the CRC group was less diverse with a very dominant Proteobacteria The ACN group had a much higher abundance of fusobacteria than controls or CRC. The serrated adenoma group showed a slightly higher abundance of Bacteriodetes and Firmucutes. At the genus level the proportion of *Eschericia coli* was markedly increased in the mucosa of CRC patients.	[142]

Table 1.6: Comparisons of the microbiota in stool samples from healthy volunteers versus cancer patients

Study population	Characterisation Method	Findings	Reference
46 CRC patients 56 controls	Pyrosequencing V1-V3 region of bacterial 16 S rDNA	The abundance of *Erysipelotrichaceae, Prevotellaceae, Coriobacteriaceae* (*Collinsella*), *Peptostreptococcus*, and *Anaerotruncus* (Clostridiales) was higher in cases than in the controls.	[138]
46 CRC patients 56 controls	454 pyrosequencing of the V3 region of the 16S ribosomal RNA gene	At the phylum level there was a slightly higher proportion of Firmicutes and a lower proportion of Bacteriodetes in the CRC patient stool samples relative to control. At the genus level *Enterococcus, Streptococcus Escherichia/Shigella* were enriched in the samples from the CRC patients	[147]
144 carcinoma patients 73 serrated polyp patients 323 polyp-free controls	16S rRNA sequencing	Lower diversity microbial diversity in CRC with reductions in the proportions of *Ruminococcaceae, Clostridiaceae*, and *Lachnospiraceae* and increases in the numbers of *Actinomyces* and *Streptococcus*	[148]

28 patients with malignant gastrointestinal disease 27 with non- malignant disease 50 controls	Identification of *S. bovis* (sic Galloctycus) by traditional culture methods (poorly defined)	The prevalence of *S. bovis* in stool samples from patients with malignant disease 36% In non-malignant gastrointestinal disease 18%, and in healthy controls 0%	[149]
50 colorectal adenoma patients 9 carcinoma patients 49 healthy controls	T-RFLP of all samples for genus level characterisation. NGS using V3–V4 region of 16S rDNA for species level characterisation in six CRC cases versus six controls	At the genus level cancer patients had higher numbers of actinomyces, fusobacteria, haemophilus and lower numbers of slackia than controls. At the species level, cancer patients had higher numbers of *Actinomyces odontolyticus, Bacteroides fragilis, Clostridium nexile, Fusobacterium varium, Heamophilus parainfluenzae, Prevotella stercorea, Streptococcus gordonii,* and *Veillonella dispar*	[150]
47 CRC case subjects and 94 control subjects	16S rRNA genes, sequenced by 454 FLX technology	CRC case subjects had decreased overall microbial community diversity, a lower relative abundance of Clostridia (68.6% vs 77.8%) and an increased abundance of *Fusobacterium*	[151]

1.8 Conclusions

Colorectal cancer constitutes at least four distinct molecular subtypes of disease, the precise roles of bad diet, unfortunate genotype, chance, an unfavourable microbial metabolome, and the presence of specific detrimental microorganisms, in the aetiology of these diseases is far from resolved. Here we have considered current evidence regarding the involvement of the intestinal microbiota. We have presented evidence to show that constituents of the microbiome may be oncogenic and/or protective. There are several candidate 'oncogenic' bacteria which, if appropriate technologies were available, might be selectively targeted for elimination from the gut due to their roles in inflammatory disease as well as cancer. Interventions with probiotics show promise as a cancer prevention approach, at least in experimental models, although human trials are needed. Pressingly there is a need to better understand the unique drivers of carcinogenesis in different colonic compartments; microbial activities that are potentially protective in recto-sigmoid disease may not be beneficial in the caecum and vice versa. A more precise grasp of these aetiologies would better enable dietary or chemoprevention strategies to optimally balance the microbiota through the gut. Better experimental models of the now well-defined colorectal tumour sub-types would help. Other important research questions remain; driver-passenger relationships are not well determined for the tumour associated microbiome. The particular carcinogenicity of red and processed meat is still inadequately explained from a mechanistic perspective given the similarity in composition with white meats and fish. And, despite the emerging field of nutrigenetics, there is a relative absence of studies integrating the microbiota, genetics and diet.

1.9 General hypothesis

4-cresol, derived from the gut microbial breakdown of meat is a carcinogen. Variation in the functionality of the microbiome is modifiable by dietary substrates and can modulate host exposure to this carcinogen and therefore influence the risk of CRC.

Objectives**:**

1. To identify healthy free living upper and lower quartile *4-cresol* excretors
2. To characterise and determine differences in their dietary exposures and gut microbiome composition
3. To compare microbial fermentation profiles from high and low excretors in simulated human gut models in order to:

    a/ assess the likely exposures to *4-cresol* for the gut epithelia.

    b/ study the potential effects of nutrients and prebiotics on the microbiota and the production of *4-cresol in vitro*.

    c/ assess the effects of faecal slurries generated from *in vitro* models on the cancer process

1.10 Rationale

Diet influences the metabolic activity of the gut microbiota with the potential to affect colorectal cancer. High levels of protein reaching the gut may increase the genotoxic load of the colonic milieu. Specific fermentation metabolites may be utilised as biomarkers of genotoxicity.

1.11 Thesis structure

Firstly, the study has considered the current literature on the role of the gut microbiota in colorectal cancer.

This thesis then describes the relationships between diet and microbial metabolism in an Omani cohort. The aim of the Omani study was to identify the dietary intake of and physical activity levels of in Omani population and consider associations with CRC risk.

Methods: A cross sectional study was carried out on 205 healthy study subjects (91 males and 114 females). Participants were aged between 19-60 years. Validated semi-quantitative food frequency questionnaires were collected. Anthropometric measurements and physical activity were assessed. Blood pressure was recorded and serum analysed for biochemical analysis. 24 hours urine samples were used to measure 4-cresol levels using nuclear magnetic resonance (NMR) analysis. Within faeces the microbial population of selected volunteers was ascertained using fluorescent *in situ* hybridisation attached to flow cytometry (flow FISH).

Afterwards, the study report on the in vitro fermentation of different protein sources and subsequent production of metabolic end products which were subsequently tested for genotoxicity in HT29 and Caco-2 cell lines models. We hypothesised that microbial 4-cresol is a colonic carcinogen and set out to model potential exposures in the colon and the effects of these exposures on colonic cells.

Methods: Batch culture fermentations with faecal inoculate were used to determine the synthesis of 4-cresol and other metabolites in response to various substrates. The microbiota was monitored and fermentation supernatants were evaluated for genotoxicity and the independent effects of 4-cresol on colonic cells were studied *in vitro*.

Finally the study explore whether specific tumour isolates of *F. nucleatum* produce 4-cresol and or other genotoxins that could drive intestinal carcinogenesis. Here, the aim was to assess the genotoxicity of culture supernatants from *F. nucleatum* samples isolated from CRC tissue and further, we assess the influence of these supernatants on the cell cycle activity of the intestinal HT29 cell line.

Methods: 18 *F. nucleatum* strains were isolated from tumour tissue and anaerobically cultured in a modified tryptic soy broth for 24 hrs. For the purity of these strains, these were isolated on selective agars, and the type strain were identified. The isolated fermentation supernatants were analysed for metabolite composition, and then used to treat HT29 cells, with assessment for DNA damage via comet assay, cells proliferation via DAPI, and cell cycle kinetics via propidium iodide staining with flow cytometry.

1.12 Impact

This work will establish gastrointestinal concentrations of a meat related carcinogen; it will shed new light on mechanisms linking diet and the gut microbiota to CRC. It may justify experimental and later, public health, interventions designed to beneficially modify the composition of the microbiota and/or sources of dietary protein for CRC prevention.

1.13 References

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Chapter II

2. General Methodology

This chapter provides an overview of all the methods used to investigate the research aims/ objectives. The merits of specific procedures or techniques used have been considered. This will aid in critically evaluating the overall validity and reliability of the data generated.

2.1 Food frequency Questionnaire (FFQ)

The semi-quantitative food frequency questionnaire (FFQ) consists of lists of foods and beverages with response categories to indicate usual frequency of consumption over the time period queried (e.g. times per day: daily; weekly and monthly). It is also used to obtain information on portion size; as standardised portions or as a choice of listed sizes [1]. Subsequent estimates for nutrient intake can be calculated via computerised software programs that multiply the reported frequency of each food by the nutrient quantities in the serving. FFQ are also used to describe a population's intake in cross-sectional studies of large numbers of individuals (e.g. 100 or more). The questionnaire can be self-administered, face-to-face interviewer administered or administered within a telephone interview; the questions are based around a given time period (e.g. in the past 6 months or 1 year) with the aim of capturing habitual intake [2].

Food frequency questionnaires are one of the most commonly used tools in epidemiologic studies to assess long-term nutritional exposure, they are less expensive than food records or diet recalls; can be easy for literate subjects to complete in a self-administered form and are suitable for very large studies and to examine the associations between diet and health or other variables. However, there are some disadvantages in the FFQs which are: the method relies upon the volunteer's memory and is less sensitive to measures of absolute intake for specific nutrients [3]. However, it is worth noting that several studies have used FFQs, such as European

Prospective Investigation of Cancer (EPIC) [4], Scottish Collaborative Group-MRC Food Frequency Questionnaire (SCG-MRC/FFQ). There are several methods that can be used to determine the habitual intake such as diet history, diet recall typically 24-hours, 4-day food record and weighed food records. However, we did not use these methods because they are costly to administer, analyse and require a trained interviewer to ask the volunteer to remember in detail all the food and drink they consumed. They are, however, accurate and feasible method to measure food intake for small group of people [5].

2.2 Cobas C111 analyser

The cobasc111 instrument is a continuous random-access analyser intended for determination of clinical chemical and electrolyte parameters in serum, plasma, urine or whole blood [6, 7]. It is optimised for small throughput workloads of approximately 50 samples per day, utilising photometric analysis with an optional unit for analysis with ion selective electrodes (ISE). This machine has been use for the blood biochemical analysis in the study; the advantages of this machine are as listed below:

1. Flexible sampling and high analytical performance
2. Efficient operation and the data management is good
3. Disposable cuvette which allows for easy loading and removal
4. High reliability, low maintenance and has very high safety standards
5. The touch screen process-driven software with reagent and sample barcode entry adapts to users of different skills and access levels
6. It can accommodate about 50 samples at one run which enabling continuous sample placing and removal during operation.

2.3 Gas chromatography–mass spectrometry (GC-MS)

Gas chromatography - mass spectrometry (GC/MS) is a core analytical method for metabolomics and has been used as a platform in non-targeted analysis, especially for hydrophilic metabolites **Figure 2.1**. The method is based on quantitative analysis of the concentration profile of free, low molecular mass metabolites, which can provide data relevant to metabolic disorders and colorectal cancer [8-10]. Volatile analysis was carried out by automated headspace solid-phase microextraction (SPME) (75 µm divinylbenzene/Carboxen on polydimethylsiloxane) followed by gas chromatography-mass spectrometry (GC-MS), using an Agilent 110 PAL injection system and Agilent 7890 gas chromatograph with 59705C mass spectrometer (Agilent, Santa Clara, CA). A 30m fused silica capillary column was used with helium as the carrier gas. The mass spectrometer operated in electron impact mode with an electron energy of 70 eV, scanning from m/z 20 to m/z 280 at 1.9 scans/s.

Figure 2.1: Typical gas chromatography-mass spectrometry.

GC/MS provides a highly efficient, highly sensitivity method with good peak resolution. The method is reproducible and there are extensive databases for identification of metabolites [11]. As such, this method was used within the thesis.

2.4 Gas chromatography (GC)

Gas chromatography is a common method used in analytical chemistry for separation and analysis of compounds that can be vaporised without decomposition **Figure 2.2**. The principle of it, is to run volatile samples with gaseous mobile phase and a stationary phase [12]. Velocity of the compound through the column depends upon affinity for the stationary phase, and the interaction of partitioned components with the gas phase. These differences lead to different retention times between compounds. The advantages of this method are: high resolution power compared to the other methods High performance liquid chromatography (HPLC). The method is very sensitive and highly accurate allowing detection and quantification of hundreds to thousands of molecules in a single measurement [11]. The analysis of the samples is quick and only a small volume of sample is required.

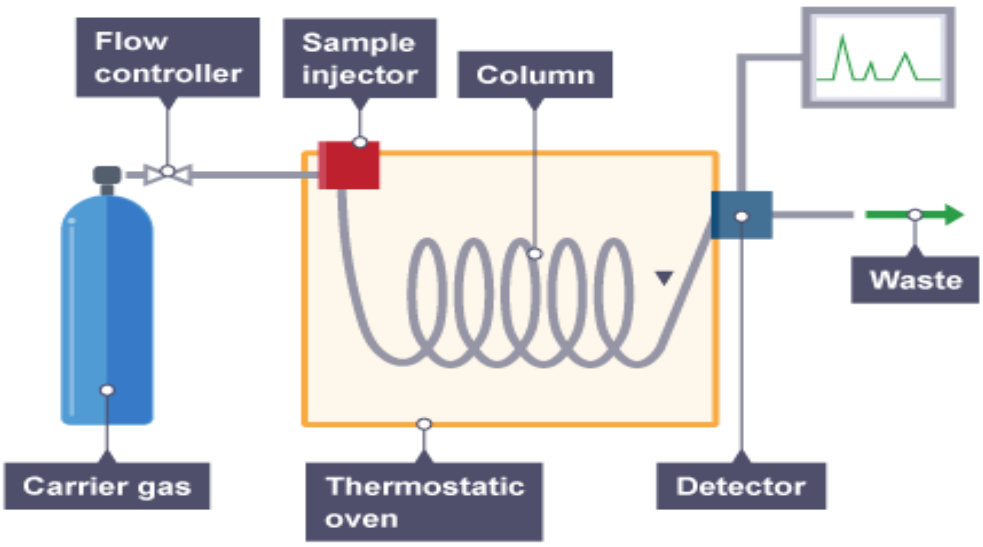


Figure 2.2: Fundamental of gas chromatography (GC). Figure adapted from [13]

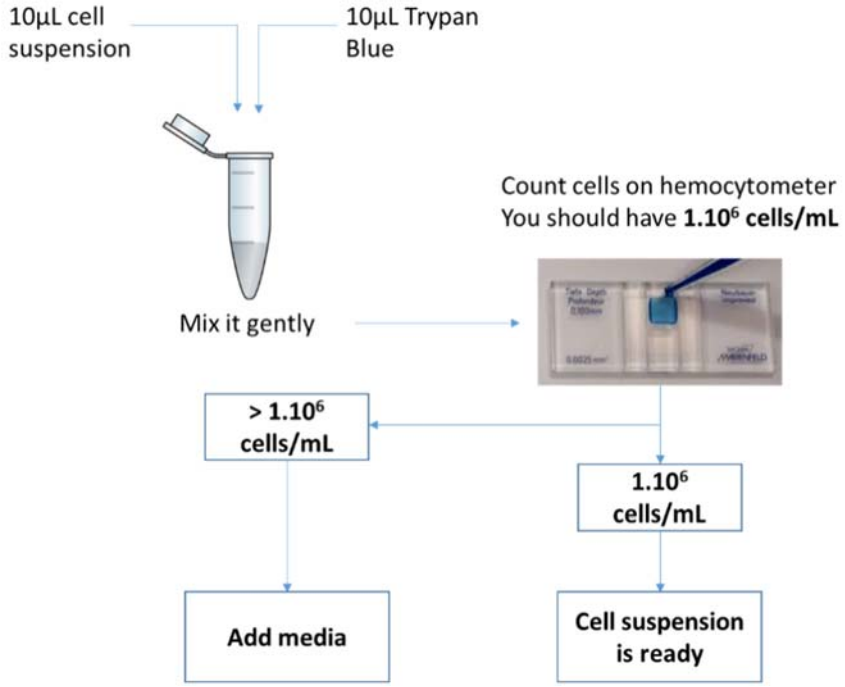
2.5 The single cell gel electrophoresis (comet assay)

The comet assay (single-cell gel electrophoresis) is a simple and sensitive method for measuring deoxyribonucleic acid (DNA) strand breaks and repair at individual cell level in eukaryotic and prokaryotic cells in low-melting point agarose [14].This assay has the potential to play an important role not only in the understanding of some of the fundamental aspects of human biology but also can be helpful in many practical ways **Figure 2.3**.

It was first described by Singh et al. in 1988 [15]. The idea was to combine DNA gel electrophoresis with fluorescence microscopy to visualise migration of DNA strands from individual agarose-embedded cells. Then the embedded cells on a microscope slide are lysed with electrophoresis buffer to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. The DNA is allowed to unwind under alkaline conditions. Following the unwinding, the DNA undergoes electrophoresis, allowing the broken DNA to migrate away from the nucleus. After staining with a DNA-specific fluorescent ethidium bromide dye, the gel is read for amount of fluorescence in head and tail and length of tail. Negatively charged DNA breaks migrate toward the anode during electrophoresis; whilst undamaged DNA remains in a tightly wound ball, thus there would be no migration. The broken ends are called the comet tail, which is relative to the head reflects the extent of DNA that has migrated from the central cell mass. DNA repair can be monitored by incubating cells after treatment with damaging agents and measuring the damage remaining at intervals. This is one of the techniques used in the area of cancer research for the evaluation of genotoxicity [16].

Several exogenous and endogenous metabolites can induce DNA damage. Therefore, the investigator needs to consider the dose and the time of the exposure to samples in relation to which genotoxic aspect that he or she would like to study.

In this thesis, comet assay was used to measure the level of DNA damage in HT29 cell line, this type of cell is a commonly used colon cancer cell line. The cells were exposed to different concentrations of 4-cresol for 24 hours to give an idea of how much the different doses will affect the DNA damage.



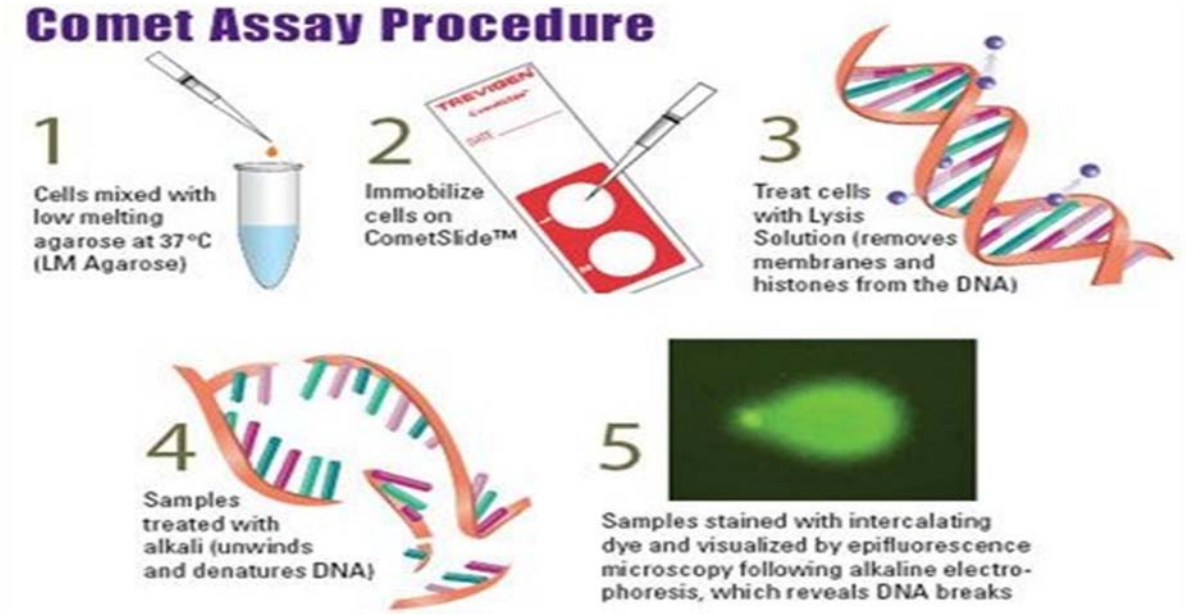


Figure 2.3: The process of the gel electrophoresis comet assay, to check DNA damage [17].

2.6 pH controlled, *in vitro*, faecal fermentation (Batch culture)

This is a closed bacterial batch culture system operated under anaerobic conditions with a specific temperature and pH. Nutrients to support microbial growth are supplied once before starting the fermentation process **Figure 2.4**. Although the batch model does lack biological brush boarder enzymes and intestinal epithelial cells, the models it does offer an environment where bacteria can grow under controlled conditions. This method can be used to assess how, within a mixed culture, microbes may behave with a specific treatment (e.g. substrate, pharmaceutic or a probiotic). The advantages of this method are that it is inexpensive and rapid. Finally it also aids assessment of the potential end products of the fermentation.

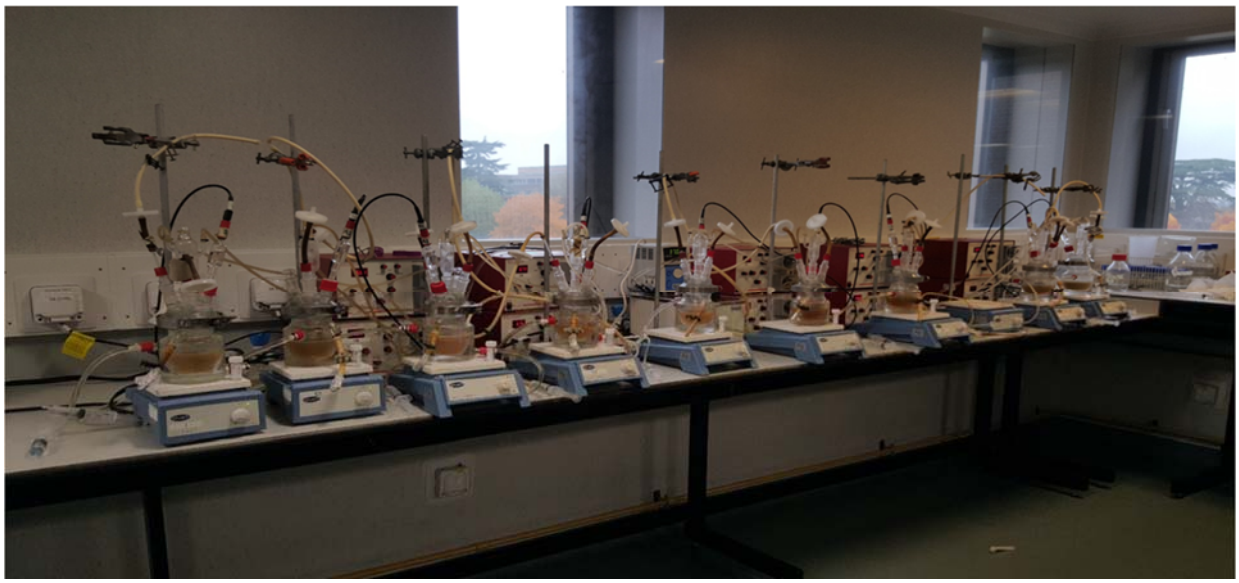


Figure 2.4: Batch culture fermentation setup.

2.7 Fluorescence *in situ* hybridisation (FISH)

The human gut microbiota has become the subject of extensive research in recent years. The gut microbiota plays an important role in immunological, metabolic, and neurological diseases [18]. The most prominent in mammalian gut inhabitants are within the phyla Bacteroidetes and Firmicutes. Individual bacterial species present unique pathological effects and, similarly, shifts in gut bacterial colonies can also prompt specific disease-inducing activity dysbiosis or disease-protective activity probiosis [19].

The methods of bacterial quantification have changed dramatically in the past fifteen to twenty years. This is because not all bacteria can be cultured. It is estimated that up to 70% bacteria are unculturable in the colon. Therefore, microbiological culture techniques do not give a clear picture of the changes in microbial numbers [20]. Gut microbiota can be determined and quantified by different techniques that bypass the limitation of culturing of a complex anaerobic community. One of the technique is FLOW FISH, which involves hybridisation of a specific fluorescent probe to 16S r RNA with maintaining the structure of the cell. FISH has become one of the most powerful tools developed in microbiology field for direct detection of target microorganisms in their natural environments. This can then identified using fluorescent microscopy or flow cytometry [21] **Figure 2.5**. FISH used to quantify the differences in bacterial populations with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA, can give an idea of broad phylogenetic changes in the gut microbiome.

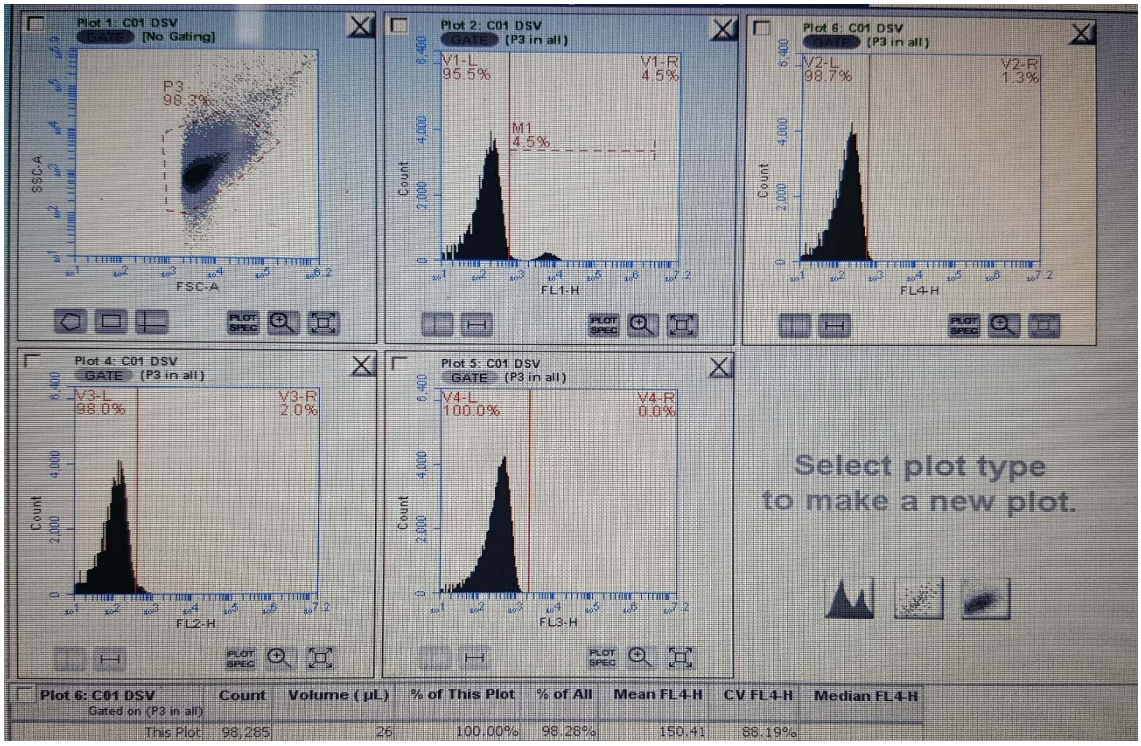


Figure 2.5: Peaks that generate from flow cytometry machine during FLOW-FISH analysis

In the first picture Flow-cytometry uses light scattering to assess and provide quantitative data for the cell size or granularity of cells bound to the probes of interest, as such can provide a measure of the number of cells per mL of sample. The detection limit for FLOW FISH is about 10^4 microbes/ ml. The second and third pictures show how differences in specific microbial groups, as compared to total bacteria. The final two images eanable background fluorescence to be measured.

Classical microbiological techniques are still important for identifying organism characteristics, but FISH overcomes issues of cultivation. Other modern molecular techniques could include the use of 16S characterisation using sequencing facilities (miseq); these techniques can record the abundance of DNA present belonging to different microbial groups; however, the method cannot provide full quantification; issues in initial polymerase chain reaction stages and DNA extraction can result in bias in the end results. Sequencing can give a

broader idea of bacteria that are present, or changing due to a treatment; however for full quantification of bacterial groups of interest FISH provides an appropriate tool; moreover, when coupled with flow cytometry, some of the bias associated with counting cells through the microscope, are reduced. As such, FLOW-FISH was used within this thesis.

2.8 Cell cycle

The cell cycle is the process by which eukaryotic cells duplicate and divide. It consists of two specific and different phases (Figure 6). The first one is interphase which consists of G1 (Gap 1), S (synthesis), and G2 (Gap 2). The second one is the mitotic phase which consists of M (mitosis). At each interphase, for instance, in G1, the cell grows, in S accumulates the energy necessary for duplication, finally in G2 prepares cell for division [22] **Figure 2.6**. There are several methods to assess the cell cycle, however, it is important to choose an appropriate method. The most common method for assessing the cell cycle is to use flow cytometry to measure cellular DNA content [23]. In this process, a fluorescent dye that binds to DNA is incubated with a single cell suspension of permeabilised or fixed cells. Then, the amount of fluorescence signal is directly proportional to the amount of DNA. There are several alterations that occur during the cell cycle, therefore, DNA content allows discrimination between G1, S, G2 and M phases. The value of the flow cytometry technique lies in the ability to take measurements of large numbers of single cells within a short period of time (tens of seconds to minutes). The heterogeneity of populations can be revealed and different subsets of cells identified and quantified.

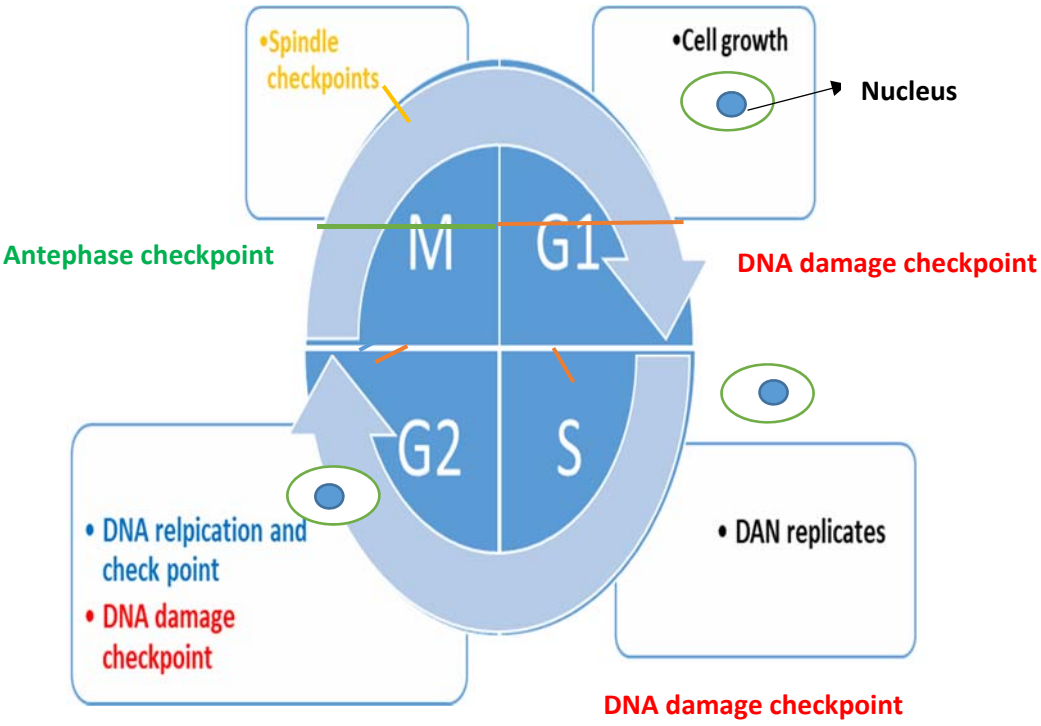


Figure 2.6: Cell cycle pathways adapted from [24]

The steps that the cells go through to be for cell cycle dynamics to be assessed by flow cytometry:

Centrifuge the sample

↓

Cells fixed with 70% ethanol

↓

Permeabilise

↓

Treated with RNase

↓

Allow the PI to enter the cell

↓

Staining the DNA

↓

DNA is measured by fluorescence

↓

Cell count vs propidium iodide

↓

Ensure only single cells are measured.

↓

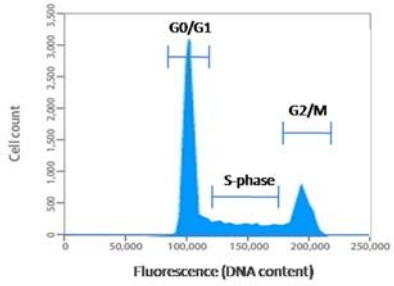


Figure 2.7: Peaks generated by flow cytometry indicating the cell cycle dynamics, adapted from [24]

3.9 Cell viability and proliferation

Cells can be distinct and characterised either by cell viability or/and proliferation. When measuring the number of living cells in a population, it is called cell viability [25]. Whereas, when measuring the cell division, then this is called cell proliferation. It should be noted that not all viable cells divide. Although proliferation can readily be interpreted as viability, absence of proliferation should not automatically be taken as a sign of cell death [26]. In this analysis several stains can be used such as 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), and others. PI and DAPI bind both RNA and DNA, and these can be assessed using a fluorescence microscope, flow cytometer or microplate reader. In this study, DAPI assay was used; with a microplate reader to analyse the data [27]. The advantages of cell viability and cell proliferation assays are easy, inexpensive, quick, accurate and good for initial screening. These assays are quick, allowing greater numbers of experiments to be performed within short timeframes [28]. However, the disadvantages are they all rely on the assumption that cell numbers remain in proportion to signal strength after treatments, an assumption that cannot be met.

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Chapter III

3. The effect of macronutrient intake on the microbial metabolite 4-cresol in an Omani population

3.1 Abstract

Background: It has been proposed that dietary factors are responsible for 70-90% of colorectal cancer (CRC) cases. Evidence has indicated that metabolites from colonic microbial protein metabolism, such as 4-cresol, contribute to increased genotoxicity when combined with high meat intake. Arab and Gulf countries are becoming increasingly afflicted by these conditions. Several studies have shown that shifting to a Western diet and changing to a sedentary lifestyle has a negative impact on human health. The aim of this study was to identify the dietary intake of and physical activity levels of in Omani population and consider associations with CRC risk.

Methods: A cross sectional study was carried out on 205 healthy study subjects (91 males and 114 females). Participants were aged between 19-60 years. Validated semi-quantitative food frequency questionnaires were collected. Anthropometric measurements and physical activity were assessed. Blood pressure was recorded and serum analysed for biochemical analysis. 24 hours urine samples were used to measure 4-cresol levels using nuclear magnetic resonance (NMR) analysis. Within faeces the microbial population of selected volunteers was ascertained using fluorescent *in situ* hybridisation attached to flow cytometry (flow FISH). Statistical analysis was performed using Statistical Analysis Software (SPSS) version 22.

Results: Results of this study revealed the Omani population have high protein intake, more than the recommended dietary intake and similar to that of a Western diet. The protein intake and high BMI had a higher significant affected on the biochemical analysis creatinine, urea, total protein and cholesterol compared to the CHO intake (p=0.028, 0.008, 0.046 and 0.013 respectively). Furthermore, there was a strong correlation in urine analysis, showing high protein consumers to have increased urinary 4-cresol (p=0.000). Moreover, the bacteriology

results indicated that diet impacted on the microbiota, with high protein and high carbohydrate consumers therefore, the results shows a significant effect with high protein intake in total bacteria, *Lactobacillus*, *Atopobium* cluster, Clostridial cluster IX, *Desulfovibrio* and *Clostridium histolyticum* group p = 0.000, 0.019, 0.014, 0.015, 0.017 and 0.031 respectively.

Conclusion: The Omani dietary intake and results, which correlated with high amounts of creatinine, urea and 4-cresol, which may affect health negatively. Nutrition awareness and health education should emphasis the importance of healthy balanced diets and active lifestyles**.**

Key words: dietary habits, lifestyle, colorectal cancer, dietary pattern, factor analysis, food frequency questionnaire, fluorescence *in situ* hybridisation and NMR.

3.2 Introduction

Adequate and balanced macronutrient intake is necessary to maintain human health. There are few dietary population based studies conducted in the Middle East; these have reported high consumption of red meat, junk food, fat and a decreased consumption of fibre, vegetables and fruits [1, 2]. It is likely that dietary changes have an impact on health status of the population. There is growing evidence, that nutrition and activity levels are major factors affecting a range of chronic diseases such as CRC, obesity, cardiovascular diseases and others.

The high incidence of sporadic CRC worldwide implies a role of environmental factors, specifically diet where 80% of colorectal cancer cases have been attributed to dietary factors, mainly animal protein consumption [3]. The highest rates of colorectal cancer are in some of the economically growing developed areas like United States, Europe and Australia; which are consuming a diet rich in animal products and fat content [4]. In addition, statistics have shown a 60% increase in cases in Africa, Asia and Central and South America [4]. Whilst in the Arab countries statistics have traditionally shown low incidence of colon cancer, however, more recently the incidence of CRC in people under 40 years of age has been found to be relatively high in the Gulf countries, such as Oman, Qatar, and Saudi Arabia [5].

The Gulf Centre for Cancer Registration (GCCR) reported that in the GCC states, CRC was the second most common cancer with overall age-standardised rate (ASRs) of 8·5 per 100,000 for men and 7·2 per 100 000 for women. In Omani men the ASR ranged between 6·6 per 100,000 men and 5·3 per 100,000 in women. The statistic showed increased incidence in both sexes between 1998 and 2009, with the total number of newly diagnosed colorectal cancer cases increasing by 3·4-times in men and 2·1-times in women [6]. Whereas in UK the age-standardised rates of cancer registrations per 100,000 people were initially at much higher rates 84.6 per 100,000 in males and 56.8 per 100,000 females and decreasing by less than 1% in 2015[7].

Indeed, the recent Oman World Health Survey reported a high prevalence of CRC with increased caloric, protein, fat and salt intake, tobacco use, combined with physical inactivity [7]. Such lifestyle changes are of importance, as eating less than five servings of fruits and vegetables and being physically inactive can increase the risk of obesity, CRC and cardiovascular diseases [8].

The World Cancer Research Fund and the American Institute for Cancer Research concluded that there was convincing evidence that red meat and processed meat intake increases CRC risk [8]. Arab and Gulf countries have been undergoing a rapid change in lifestyle, with alterations to food consumption patterns and socioeconomic status during the last few decades [9, 10]. Several migrant studies of dietary intake support the concept that a shift to a Western diet results in increases in CRC incidence [6, 11]. Furthermore, other evidence suggests that long-term consumption of red meat or processed meats may increase CRC risk. Therefore, a link has been hypothesed that high dietary intake of red and processed meats can influence all stages of carcinogenesis, starting from cell proliferation until differentiation and transformation to cancer [12-16].

Dietary impact on the colonic microbiota can mediate both changes in bacterial population and bacterial metabolic activity. Moreover, several studies have shown a link between diet, lifestyle and the composition and metabolic activity of the human gut microbiota [17-19]. In fact, there is growing evidence that imbalances in gut microbial populations can be associated with several diseases including colon cancer. This is because microbial pathways in the colon can lead to production of carcinogenic and genotoxic compounds [20]. Not all colonic microbiota members result in negative effects, there are some bacteria within this consortium that have a positive impact on colonocytes; thus diet can influence these factors [21].

The main fermentable substrates in the large intestine are carbohydrates and protein [22]. The main products of CHO fermentation include short-chain fatty acids (SCFA) which, as well as having positive systemic effects, lower the colonic pH, making the environment of the colon less favorable to pathogenic bacteria [23].

On the contrary, protein metabolism (as in a high meat diet) can lead to detrimental metabolic end products [24-26] e.g. ammonia, amines, 4-cresol, phenol, indole, hydrogen sulphide [27]. Some of these end products are retained within the gut and absorbed into the circulation and eventually secreted in the urine; these compounds are well known as potential uremic toxins [28]. Furthermore, 4-cresol is produced from tyrosine or phenylalanine fermentation by intestinal bacteria, this metabolite has been found to be elevated in the urine of CRC patients [29]. Several studies have shown that, 4-cresol increases cell proliferation and production of reactive oxygen species production in endothelial and mononuclear cells; such transformations may aid initiation and progression of CRC [30].

In contrast, several studies have shown that *Bifidobacterium* could inhibit ammonia production and enzymes such as beta-glucuronidase and tryptophanase that have been observed to be carcinogenic in rodents [31, 32]. Furthermore *Lactobacillus*, have also been observed to reduce the action of these enzyme in the colon. These genera have also been reported to possess anticancer potential by binding to amine, preventing its absorption from the colon [33]. As such, modulation of the microbiota to support the growth of lactobacilli and bifidobacteria could be of benefit to the host.

To date there are no studies in Gulf countries in general and in Oman specifically considering how current dietary intake influences metabolites, such as 4-cresol, and how these changes may impact on health. As such, this is the first study to address this issue. This will allow us to examine the potential metabolite production such as 4-cresol as a potential biomarker of CRC with respect to Omani diet.

3.3 Materials and Methods

A cross sectional study was carried out on 205 healthy study subjects (91 males and 114 females). Participants were aged between 19-60 years, from different regions of the Sultanate of Oman. The inclusion criteria were healthy subjects free of any metabolic disorders or chronic diseases and had not consumed antibiotic medication in the last 6 months. Written consent form was obtained from all study subjects before their interview. The study protocol was approved by the Research and Ethics Committee of Ministry of Health, Sultanate of Oman.

3.3.1 Semi-Quantitative Food Frequency Questionnaire

Adapted modified semi-quantitative food frequency questionnaires were developed and validated by the nutrition section, Sultan Qaboos University-Oman. Qualified dietitians asked the study subjects questions in a face-to-face interview and filled out the questionnaires. The food listed in the FFQ consisted of 128 food items categorised into nine major groups: 20 of vegetables; 36 of meat (Traditional Omani food) such as red meat (lamb and beef), chicken, fish, cold meat, and others; 14 of fruits and juices; nine of milk and dairy products; 11 of breads; four of nuts; six of beverages; ten of fast food; 18 of snacks and sweets. The questionnaire used measures to determine portion size, e.g. ½ cup and 1 cup standard serving and also considered frequency of food consumption (never, a few times/year, 1–2/month, 1–2/week, 3–4/week, 5–

6/week, 1/day, 1–3/day, 4–5/day, or 6/day). Dietary intake was analysed using NutriBase11 software (NutriBase 11 version: 11.5, Phoenix Arizona, USA).

2.3.2 Assessment Questionnaires

Qualified trained nurses collected assessment questionnaires from the volunteers in Oman. These questionnaires were used to obtain information from the study subjects about socio-demographic characteristics; including personal details, marital status and level of education, monthly income, permanent residential zone (urban, rural areas). The second section was about anthropometric measurements, third about blood pressure and fourth about lifestyle factors including physical activity, smoking and drinking habits.

The anthropometric measurements including body weight, height, BMI and waist/hip ratio (W/H) were taken by qualified trained nurses. Body weight was measure to the nearest 0.1kg with minimal clothing, using Tanita digital scale. Height was measured to the nearest 0.5 cm with the subject in the full standing position without shoes and using a calibrated portable measuring rod. BMI is defined as the individual's body mass divided by the square of their height (Kg/m^2). We used the cut-off points for adults (normal and overweight/obese) based on 18–24.9, ≥25 Kg/m^2, respectively). W/H was calculated as waist measurement divided by hip measurement using a non-stretchable measuring tape. The blood pressure was measured in the morning from the right arm of subjects while they were seat and at rest for at least 5 minutes, using an automatic blood pressure machine standardised daily against a mercury sphygmomanometer.

3.3.3 Global Physical Activity Questionnaire

A WHO developed Global Physical Activity Questionnaire (GPAQ) was filled out by trained nurses and was used to assess the physical activity levels of the study subjects [34]. The questionnaire focused on intensity, duration, and type of activity. Then the number of hours spent in different activity levels was obtained and converted into Metabolic Equivalents (METs). Average METs for walking 60-90 minutes = 3.3 METs, for moderate activity = 4.0 METs, for vigorous activity = 8.0 METs. Finally, the score expressed as MET-min/week; calculated as (MET level × minutes of activity / day × days per week). Total physical activity MET-min/week was obtained by METs summation and categorised as inactive (below 600 MET-min/week), and above 600MET-min/week) as active.

3.3.4 Urine and Faecal Collection

Urine and faecal samples were obtained from healthy volunteers age ranging from (19-60) years old by trained nurses. Volunteers had no history of gastrointestinal diseases and had not consumed antibiotics within the previous 6 months. Urine was collected over the course of 24 hours (all voids were within the collection container). Within the container, ascorbic acid was used as a preservative: 0.042 g. The urine container was stored within a cool bag. Following collection, 12.5 mL of urine specimen was taken into 25 mL falcon tube, this sample was stored at -80ºC for further analysis.

Faecal samples were provided on site. About 20 g of fresh faeces was transferred into mayo stool containers with glycerol preservative (20 mL glycerol/tube). Samples were stored at -80ºC for further analysis.

3.3.5 NMR spectroscopy

Urine is a biofluid and has been considered to be highly appropriate amongst metabolomics researchers [46]. Urine is an easily accessible biological fluid which can be obtained in large volumes, largely free from interfering proteins or lipids and other complex chemical [46]. Furthermore, it is known that urine contains significantly more metabolic end products and exhibits significantly more chemical diversity than other biofluids. More than 484 compounds have been identified in urine that were not previously reported to be in blood either experimentally or via literature review [48]. Therefore, in this study urine samples have been used. Therefore, 205 urine samples were analysed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Jonathon Swann from Imperial College London carried out this analysis. The frozen samples were prepared by adding 400 µl of urine with 200 µl of phosphate buffer (pH 7.4; 100% D2O) containing 1 mM of the internal standard, 3-trimethylsilyl-1-[2, 2, 3, 3-2H4] propionate (TSP). Samples were mixed by vortex, before centrifuging (10,000 x *g*) for 10 minutes before transfer to a 5 mm NMR tube. Spectroscopic analysis was carry out on a 600 MHz Bruker NMR spectrometer. Standard one-dimensional ^{1}H NMR spectra of the urine samples were acquired with water peak suppression using a standard pulse sequence. For each sample, 8 dummy scans were followed by 32 scans and collected in 64K data points. A recycle delay of 2 s, a mixing time of 10 µs and an acquisition time of 3.8 s was used. The spectral width was set at 20 ppm. Spectra were automatically phased and corrected for baseline distortions and the chemical shifts in the spectra were referenced to the TSP singlet at δ 0.0.

^{1}H NMR spectra (δ 0.2-10.0) were digitised into consecutive integrated spectral regions (~20,000) of equal width (0.00055 ppm). The regions between δ 4.50 - 5.00 were removed to minimise the effect of baseline caused by imperfect water suppression. Each spectrum was then normalised using a probabilistic quotient-based approach. Here, ^{1}H NMR spectroscopic

profiles were used as the descriptor matrix and class membership (e.g. control and infected) was used as the response variable. Correlation coefficient plots were generated by back-scaling transformation to display the contribution of each metabolite to sample classification. The colour scale represented the significance of correlation for each metabolite to class membership, with red indicating strong significance and blue indicating weak significance. The predictive performance (Q2Y) of the model was calculated using a 7-fold cross validation approach and model validity was established by permutation testing (1000 permutations).

3.3.6 Faecal samples - Bacterial enumeration

Faecal samples were obtained from 8 volunteers who consumed high protein, low CHO and another 8 volunteers who consumed high CHO, low protein. Bacteriological analyses performed in triplicate using standard published procedures. Differences in bacterial populations were assessed by fluorescence *in situ* hybridisation (FISH) coupled with flow cytometry, with oligonucleotide probes designed to target specific diagnostic regions of 16S rRNA. Probes for FISH were commercially synthesised and labelled at the 5′ end with the fluorescent dye Cy3 (Sigma Aldrich Ltd, Poole, Dorset, UK). The probes in **Table 3.1** used were EUB 338 mixture as a positive control consisting of EUB338, EUB338II and EUB338III for total bacteria count and Non EUB as a negative control. Other probes used such as : Ato 291 for *Atopobium* cluster (ATO), Lab 158 for *Lactobacillus/Enterococcus* (LAB), Bif 164 for *Bifidobacterium* (BIF), Erec 482 for *Eubacterium rectale–Clostridium coccoides* group (EREC) [47], Chis 150 for the *Clostridium histolyticum* group (CHIS), Bac 303 for *Bacteroides—Prevotella* spp. (BAC) and Fuso 664 for *Fusobacterium nucleatum.*

Seven hundred and fifty microliters of the samples was centrifuged at 12,400 × *g* for 5 min. Afterwards, pellets were resuspended in 375 µL of filtered PBS (using a 0.22 µm PVDF filter) and fixed in 1125 µL of 4% (v/v) paraformaldehyde. After 4 h of incubation at 4°C,

samples were washed twice using 1 mL of PBS, resuspended in 500 µL (1:1, v/v) PBS- 99% ethanol and stored at –20◦C. To hybridise the samples 75 µL sample was added to 500 µL PBS and centrifuged at 12,400 × *g* for 3 min. Pellets were resuspended using 100 µL of filtered TE-FISH buffer (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8, distilled H_2O, 0.22 µm filters). Then lysozyme was added (50 000 U) and incubated in the room temperature for 10 min. The samples were then vortexed and centrifuged at 12,400 × *g* for 3 min. Pellets washed with 500 µL PBS and centrifuged (12,400 × *g*, 3 min). Afterwards, the hybridisation process was performed by resuspending the pellets in 150 µL of hybridisation buffer (5 M NaCl, 1 M Tris/HCl pH 8, 30% formamide, ddH_2O, 10% SDS), vortexing and centrifuging (12,400 × *g*, 3 min). Pellets were resuspended in 1 mL of hybridisation buffer and 50ng/ µL aliquoted into each Eppendorf tube containing the relevant probe **(Table 3.1).**

The samples were read by flow cytometer, in a BD Accuri C6 flow cytometer. Scattered light and fluorescence of different wavelengths are then recorded to give number of cells per µL sample.

Table 3.1: Oligonucleotide probes used in this study for Flow- FISH analysis of bacterial populations.

Probes name	Sequences 5' To 3'	Target genus	Reference
Non Eub (Negative control)	ACTCCTACGGGAGGCAGC	Control	[35]
Eub338 I + (Positive control)	GCT GCC TCC CGT AGG AGT	Most bacteria	[36]
Eub338 II +	GCA GCC ACC CGT AGG TGT	Planctomycetales	[36]
Eub338 III +	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	[36]
Bif164	CATCCGGCATTACCACCC	Most *Bifidobacterium* spp.	[37]
Lab158	GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus, Leuconostoc* and *Weissella* spp.	[38]
Bac 303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	[39]
Erec 482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group	[40]
Rrec 584	TCAGACTTGCCGYACCGC	*Roseburia* subcluster	[41]
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group *(Clostridium* clusters I and II)	[40]
Prop 853	ATTGCGTTAACTCCGGCAC	Clostridial cluster IX	[41]
Ato 291	GGTCGGTCTCTCAACCC	*Atopobium, Colinsella, Olsenella* and *Eggerthella* spp.	[42]
Fprau 647	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and related sequences	[43]
DSV 687	TACGGATTTCACTCC T	Most *Desulfovibrionales* (excluding *Lawsonia*) and many *Desulfuromonales*	[44]
Fuso 664	CTTGTAGTTCCGCYTACCTC	*Fusobacterium* spp.	[45]

3.3.7 Assay of serum lipids profile

Fifteen mL of blood samples were collected from the subjects in the morning before breakfast after at least 12 hours of overnight fasting after dinner on the previous day of measurement. Fifteen mL of venous blood was collected into red cap plain vacutainer tube using vacuum blood collecting tube. Blood samples were centrifuged at 1500 g at 4^{o}C for 10 minutes and stored at -80^{o}C for subsequent analyses. The serum samples were thawed at room temperature (25 to 29°C) at Laboratories of food and nutrition department, Sultan Qaboos University, Oman, and using a COBAS C 111 analyser (Roche, Basel, Switzerland) analysed for levels of blood glucose, magnesium, creatinine, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and cholesterol using specific kits for each of these analysis. The COBAS C111 analyser uses a single point calibration for each analysis in which a calibration curve was created based on internal standard for the sample analysis. The internal quality control for the automatic analyser was performed using two levels of control materials purchased from manufacturer to calculate mean and standard deviation (SD). The results of lipid profile were expressed in mean ± SD.

3.4 Statistical analysis

Statistical Package for Social Sciences (SPSS Version 22, IBM, Chicago, Illinois, USA) was used for statistical analysis. The data are presented as mean ±SEM. Categorical variables were tested by Chi-square test; continuous variables were tested by one-way ANOVA. For bacteriology, comparisons between high protein consumers (≥60 g) and high CHO consumer (≥300 g) were made by analysis of variance. The correlation between protein consumption and 4-cresol production and the correlation between the biochemical analysis against 4-cresol production was assessed using a Pearson test. P values <0.05 were considered to be statistically significant.

3.5 Results

A total of 91 males and 114 females, with a mean of age 30.8±8.32 and 29.5±8.93 years respectively participated in this study. The general characteristics of the subjects are given in **Table 3.2. Figure 3.1** shows the distribution of the study subject according to their weight within the category. There was no significant difference in the age or BMI between the study subjects. However, a significant difference in physical activity levels was observed, males tended to be more active than females, with 72% of active males whereas just 35.6% of females were active p=000. BMI was categorised into 2 groups (Normal=18-24.9 and overweight and obese =≥25). The average BMI for males was 25.76± 5.69 whereas, the average BMI for females was 25.88±6.55.

Table 3.2: General Characteristics of the study subject's values are expressed by mean±SD

Gender	**Males**	**Females**
	N=(91)	**N=(114)**
AGE (years)	30.71 ± 8.32	29.57 ± 8.93
BMI (kg/m^2)	25.767 ± 5.69	25.881 ± 6.55
Waist/hip ratio (WHR)	1.6 ± 7.1	0.8 ± 0.1
Physical activity		
1. Active	68 (72.3%)	42 (36.8%)
2. Not active	26 (27.2%)	72 (63.2%)

Characteristics of study volunteers P-vales for categorical variables have been assessed by Chi-square test, where the continuous variables have been tested by one-way ANOVA. BMI = body mass index; WHR = waist-to-hip ratio. *Data collected using the Global Physical Activity Questionnaire (GPAQ).

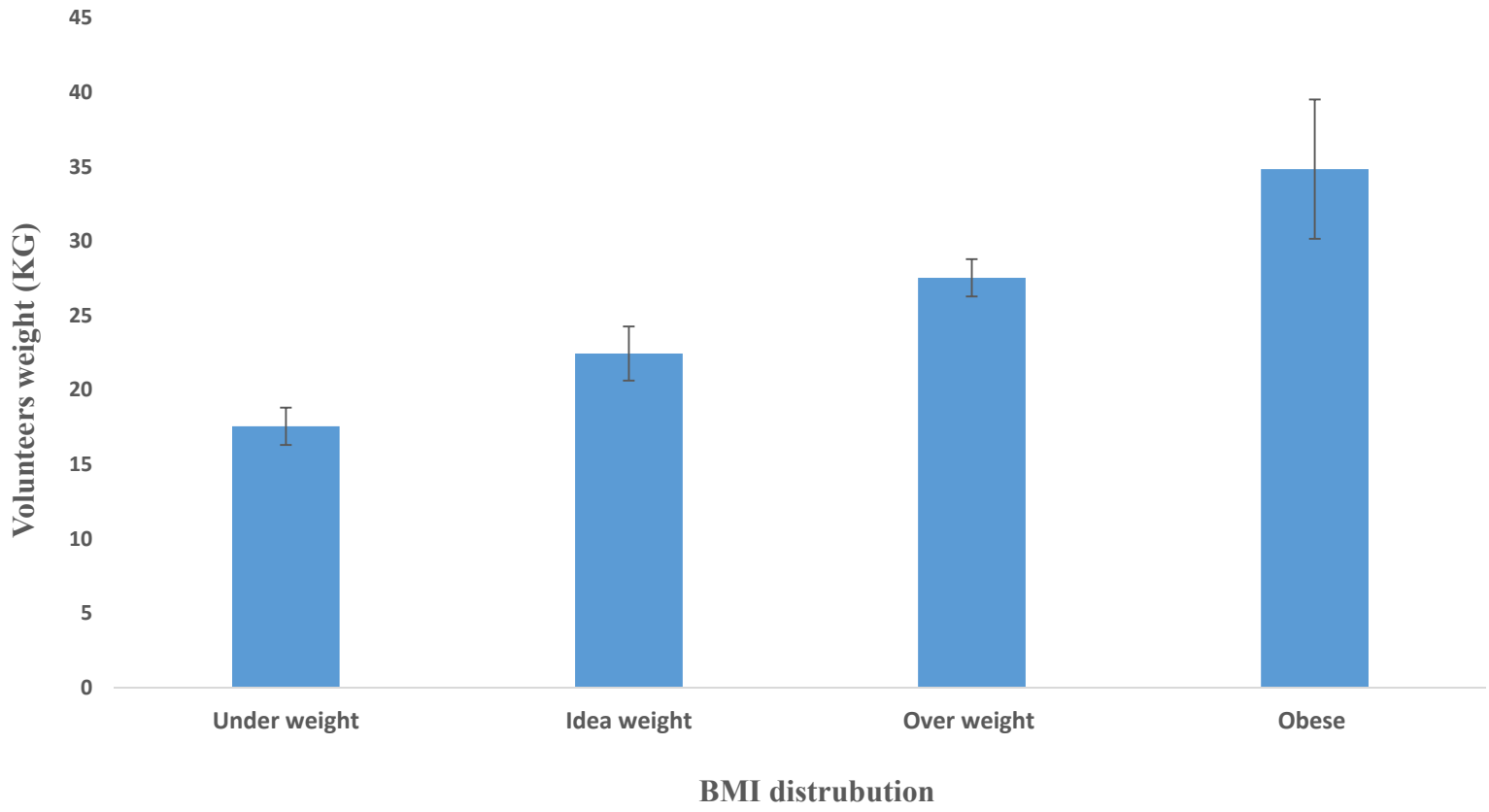

Figure 3.1: Distribution the number of the study subjects total of 205-study subject included: 94 males and 114 females weight within categories, values are expressed by mean ± SD.

Table 3.3 indicates the impact of BMI on systolic (SBP) and diastolic (DBP) blood pressure. For normal BMI study subject the mean of systolic blood pressure was 121.05±15.151mmHg, whereas for the overweight/obese BMI study subjects it was 127.24±14.790kg/m^2, which is increased significantly between the two groups (p= 0.003). Furthermore, the diastolic was also significantly higher in overweight/obese study subjects compared to the normal (72.73±10.539 mmHg and 76.79±12.875mmHg respectively, p= 0.012).

Table 3.3: Effect of BMI on the study subjects blood pressure, values are expressed by mean±SD

Variables	Normal BMI	Overweight/Obese	P-value
Systolic SBP	121.05 ± 15.151	127.24 ± 14.790	0.003**
Diastolic DBP	72.73 ± 10.539	76.79 ± 12.875	0.012*

Values listed are mean ± SD: P-vale obtained by one-way ANOVA. Total of 205-study subject included: 94 males and 114 females

Table 3.4 shows the macronutrient consumption for the study subjects by gender, along with the recommended intake. It can be seen from **Table 3.4** that total energy and fat intake of the study subjects was within the recommended values; however, both protein and carbohydrate intake were above the daily recommendations.

Table 3.4: Macronutrient consumption by the study subjects values are expressed as mean±SD. In this, study 8 volunteers who consumed high CHO diet and anther 8 volunteers who consumed high protein diet were analysed to check how much 4-cresol was produce from each diet.

Variables	Males N=94	Females N=114	Male & female, who consumed high CHO (n=8)	Male & female, who consumed high protein (n=8)	RDI Males	RDI Females
Total energy (Calories)	2265.5 ± 546.8	1958.1 ± 527.5	2134.862 ± 431.4	2019.8 ± 542.8	2500	2000
Carbohydrate (g)	349.9 ± 158.3	348.3 ± 261.6	349±157.1	215.7± 90.3	300	230
Protein (g)	91.5 ± 30.2	77.2 ± 27.8	48±10.5	84.3±30.9	55	45
Fat (g)	75.0 ± 27.8	61.3 ± 20.9	66.9 ± 30.6	62.9 ± 29.8	95	70

When blood serum was tested and analysed against the biochemical analysis it was observed that creatinine (CRE), Total protein and cholesterol (CHOL) were significantly in the study subjects who consumed more protein than CHO (p=0.028, 0.046 and 0.013) respectively **Table 3.5.**

Table 3.5: Biochemical analysis of the study subjects who consumed high protein diet using blood serum, values are expressed by mean±SD

Types	Participants n=18		P-value
	↑protein intake	↑CHO intake	
Magnesium (mmol/L)	0.89±0.192	1.78±0.991	0.61
Glucose (mmol/L)	5.06±1.990	3.02±1.621	0.16
Calcium (mmol/L)	2.40±0.348	4.59±1.248	0.55
Phosphorous (mmol/L)	1.29±0.171	2.10±0.634	0.77
Creatinine (mmol/L)	63.67±14.261	6.67±0.546	0.02*
Bile (mmol/L)	5.92±1.397	3.16±0.125	0.75
Urea (mmol/L)	4.16±1.325	12.60±4.676	0.00*
Total protein (mmol/L)	78.16±5.358	5.86±0.860	0.04*
Albumin (mmol/L)	52.30±20.206	6.40±1.348	0.06
Triglyceride (mmol/L)	1.15±0.731	1.34±0.651	0.91
High density lipoprotein (mmol/L)	1.51±0.403	2.43±0.563	0.98
Low density lipoprotein (mmol/L)	3.10±0.844	1.10±0.967	0.46
Cholesterol (mmol/L)	6.59±2.248	4.34±0.974	0.01*

Values listed are mean ± SD; P-values for the continuous variables were obtain by correlation.* indicate significant correlation with 4-cresol production. This table shows the results of biochemical analysis between the volunteers who consumed high protein diet (≥60 g) and high carbohydrate diet (≥300) and their effect in the serum.

Urine and Faecal samples

Urine results

Urine samples of the study subjects were analysed by NMR to examine 4-cresol produced when levels, which was compared to protein intake.

In **Figure 3.2 A** and **B** (top), the results of the full cohort did not show any significant correlation *p*= 0.250 as a full cohort. However, when the data for CHO adjustment was carried out using the residual method **Figure 3.1 B**. Pearson correlation coefficients were calculated using CHO adjusted data for CHO intake it showed a strong correlation, highly significant *p* =0.000 between the high protein intake and the production of 4-cresol within the study subjects.

Analysis was conducted considering the volunteers who were selected for faecal analysis; as such from all the study subjects 8 were selected who consumed high protein (≥60g and low CHO ≤300g per day) and another 8 from the study subjects who consumed high (CHO ≥300g per day) and low protein (≤ 60g per day). The results showed significant correlation between high protein intake who consumed 60 g/d or above and production of 4-cresol (r=0.571 and p= 0.030) **(Figure 3.2 C)**. However, for those study subjects who consumed high carbohydrate intake **Figure 3.2 D**, the result shows no significant correlation between the CHO intake and 4-cresol levels (r=0.078 and p = 0.520).

Moreover, high protein intake influences serum levels by generation of various metabolites such as urea and creatinine. The result also showed a strong correlation between urea and creatinine production and against 4-cresol (r= 0.718, p=0.000 and r =0.277, p =0.04 respectively), whilst there was no correlation with the other biomarkers of protein.

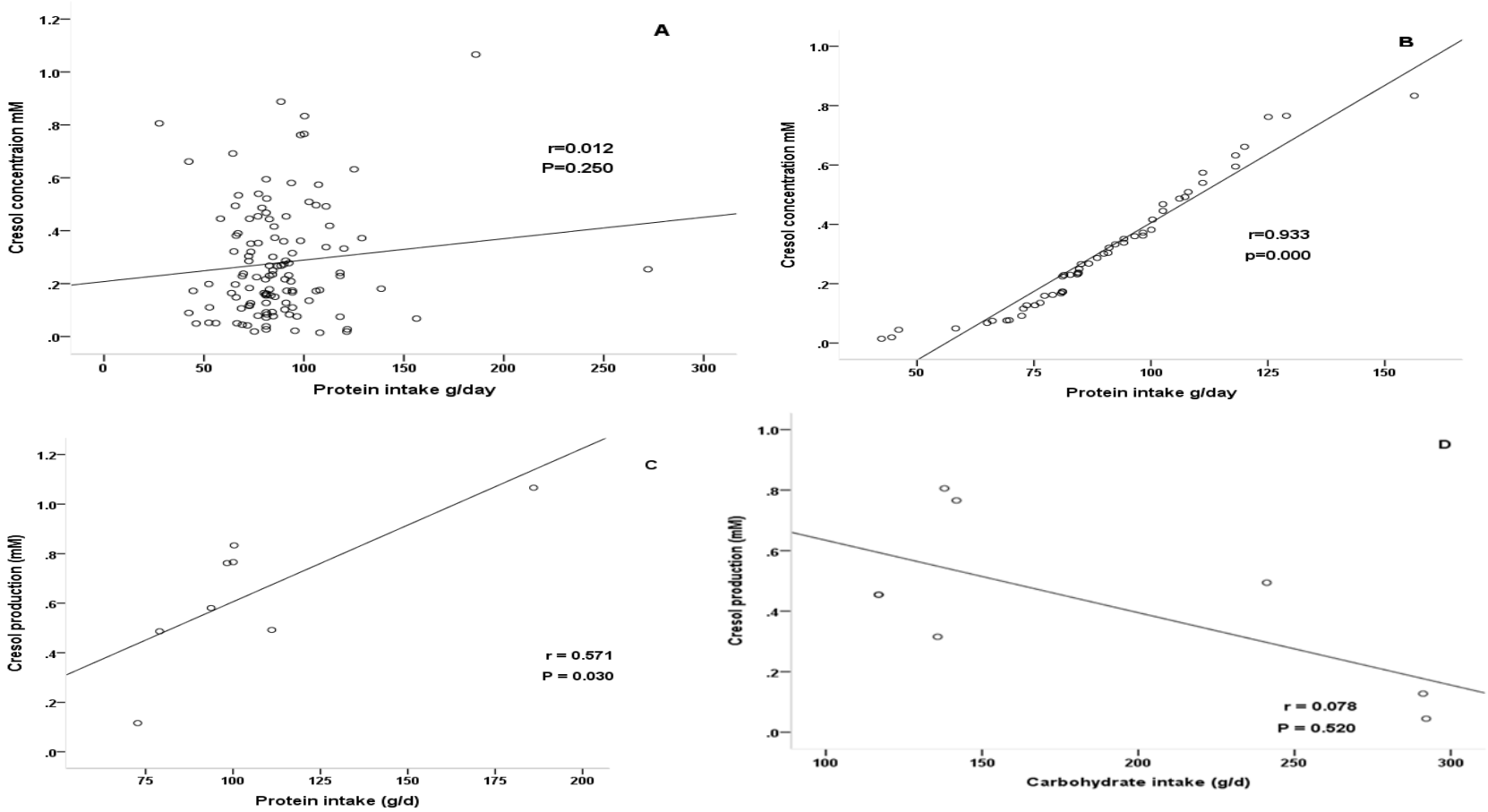

Figure 3.2: **A and B: A and B: A- the correlation of protein intake g and 4-cresol by the study subjects (n= 205). B- The correlation of protein intake in grams and 4-cresol Adjusted for CHO by the study subjects (n= 205) according to mean and SD. C and D: C- (8 study subjects) the correlation of high protein intake (g/d) and 4-cresol (mM) by the study subjects. D- (8 study subjects) the correlation of low protein intake g and 4-cresol by the study subjects according to mean and SD.**

Figure 3.2: A and B: A and B: A- the correlation of protein intake g and 4-cresol by the study subjects (n= 205). B- The correlation of protein intake in grams and 4-cresol Adjusted for CHO by the study subjects (n= 205) according to mean and SD. **C and D**: C- (8 study subjects) the correlation of high protein intake (g/d) and 4-cresol (mM) by the study subjects. D- (8 study subjects) the correlation of low protein intake g and 4-cresol by the study subjects according tomean and SD.

Faecal microbiota

The faecal samples were analysed by fluorescence *in situ* hybridisation to determine number of key bacterial groups. From all the study subjects 8 were selected from the study subjects who consumed high protein (≥60g and low CHO ≤300g per day) and another 8 from the study subjects who consumed high CHO ≥300g per day and low protein diet ≤60g per day. The study subjects who consumed a high protein diet had significantly more total bacteria, *Bacteriodes, Clostridium histolyticum* group, *Desulfovibrionales,* and *Fusobacterium* p= 0.024, 0.042, 0.007,0.022, 0.015 respectively when compared to high carbohydrate consumers **Table 3.6.**

Table 3.6: Microbial populations when comparing volunteers with protein and carbohydrate intake

Diet	Total bacteria	BIF	LAB	BAC	EREC	RREC	ATO	PRO	F-PRAU	DSV	CHIS	FUSO
High protein	7.93±0.44*	6.96±00.49	6.63±0.40	6.41±0.41*	7.32±0.47	6.72±0.64	6.69±0.62*	6.84±0.56	6.90±0.52	6.21±0.41*	6.40±0.36*	6.79±0.45*
High Carbohydrate	7.25±0.53	6.45±0.43	5.77±0.71	5.75±0.65	6.61±0.63	6.00±0.53	5.89±0.48	5.82±0.64	6.28±0.74	5.51±0.58	5.59±0.67	6.16±0.49

Bacterial numbers (log_{10}/g faeces determined by flow cytometry: fluorescence in situ hybridisation) from volunteers consuming a diet of over 60 g of protein, with less than 300g carbohydrate and volunteers consuming over 300g carbohydrate and less than 60 g protein. Values listed are mean ± SD; P-values for the continuous variables were obtained by two-way ANOVA comparing the two dietary habits * Indicate significantly different P>0·05. n=8 for high protein and n=8 for high carbohydrate. Eub= Total bacteria, BIF= *Bifidobacterium* spp. LAB= *Lactobacillus* spp. BAC= *Bacteriodes* spp. EREC= *Eubacterium rectal,* RREC= *Roseburia ,* ATO= *Atopobium,* PRO= *Clostridial*, FPRAU= *Faecalibacterium prausnitzii,* DSV= *Desulfovibrio,* CHIS= *Clostridium histolyticum.*

3.6 Discussion

Epidemiology studies have shown that a balanced diet and active lifestyle may play a crucial role in the prevention of non-communicable diseases. As such, changes in dietary patterns and lifestyle can lead to changes in health status [6, 49]. This study is a cross-sectional assessment of dietary regimes in the Omani population, with consideration of subsequent disease risk, and the microbial metabolite 4-cresol, associated with CRC.

Decades ago, people in Gulf countries were known to consume more traditional food than now. Such diets included dates, rice, high fibre breads and fish; which have now been replaced with red meat and others. This has been reflected negatively in the nutrition status and health of the Gulf countries [50, 51]. Indeed, in the current study the average intake of CHO and protein was 349.1g and 84.35g respectively, which is higher than the recommended dietary intakes (RDI) of 265g and 50g respectively [52]. This could highlight a dietary shift of Arab and Gulf countries to a more Western diet; containing higher levels of fast food, animal protein and sugary beverages as also reported by (Abdulrahman, et al, 1993) [53, 54].

The implications of these dietary patterns were explored, and it was observed that, study subjects who consumed combined high protein and high fat diets had higher serum creatinine, urea, total protein and cholesterol levels **(Table 3.5)**. This result was expected because protein catabolism is known to lead to increased serum creatinine and therefore, the urea level will also increase. Furthermore, high creatinine and urea levels may affect renal function by decreasing in glomerular filtration rate (GFR); which could be a factor in promoting renal disease.

It is well known that diet is a major factor driving the composition and metabolism of the colonic microbiota [55]. The amount, and type of macronutrients such as: carbohydrates, proteins and fats have a great impact on the large intestinal community [56]. Therefore, the dietary changes that have occurred within the Omani population are likely to impact on the gut

microbiota [57]. Meat provides a rich source of protein and high consumption of red and processed meats have been linked to microbial 4-cresol production and therefore, linked to colorectal cancer later in life [58]. Indeed, Tayyem et al, 2015 [59] observed an association between total energy intake, protein and fat intake and developing CRC. Whereas consumption of high amounts of vegetables, and fruit was seen to be protective against CRC development, this was attributed to their dietary fibre and antioxidant content [5]. The intake of animal source foods is indeed growing steadily; it replaced many typical diets in the region [8].The study subjects indicated they had a diet with a high frequency consumption of red meat and processed meat $p = 0.000$ and 0.000 respectively and a moderate consumption of fruits and vegetables. In the current study, it observed that those consuming higher protein levels, along with low CHO had significant differences in a range of microbial groups e.g. *Bacteriodes, Clostridium histolyticum* group, *Desulfovibrionales*, and *Fusobacterium*. These groups are known to contain some proteolytic bacteria. As such, diet can shift the gut microbiota community; therefore, it is possible that a high protein diet is leads to a shift in the microbiota that could be more favourable to production of genotoxic products.

Microbial protein fermentation metabolites such as 4-cresol are potentially harmful, when occurring at high concentrations, e.g. above 3 mM in endothelial lines [60]. The results in the current study show that high protein consumption was correlated with 4-cresol levels in the study subjects urine $p = 0.030$ **(Figure 3.1 A).** This results is in line with Windey, et al., [61] who found that 2 weeks of high protein intake by healthy volunteers led to increases in 4-cresol. Moreover, a study by Geypens, et al. [38] found that increased supplementary protein in healthy volunteers, resulted in a significant increase in urinary p-cresol ($p = 0.04$). Thus, a high protein diet, as was seen within the Omani population, may have a negative impact on gut health, resulting in increased 4-cresol. O'Keefe and coworkers found that when the gut bacteria composition shifted due to animal protein, there was fewer bacteria that produce the short chain

fatty acid butyrate, and more potentially harmful bacteria such as *Fusobacterium nucleatum, Rhodopseudomonas faecalis, Bacteroides vulgatus and Enterococcus faecalis,* which produce 4-cresol and that may damage epithelial DNA [62]. A study by Brinkworth et al., in obese individuals, found that urinary 4-cresol and phenol levels at baseline were considerably higher than those reported in normal weight adults (94·9 mg/d and 15·0 g/d for p-cresol and phenol, respectively) and decreased upon weight loss [63]. Within the gastrointestinal tract (GI), about 60% of the bacteria belong to the Bacteroidetes or Firmicutes phyla. Gut microbes are able to produce a wide range of products, the generation of which can be dependent on several factors such as nutrient availability and the luminal environment, particularly pH [64]. Therefore, competition between bacteria for substrates has a significant influence on which products are generated. Therefore, high protein intake will increase putrefactive bacteria and their fermentation end products which may lead to increased CRC risk [65]

On the other hand a high CHO diet, rich in fibre such as cereals, nuts, fruits and vegetables has been observed to have a positive effect, with consumption related to decreased incidence of several diseases, in some cases this may be mediated by production of short chain fatty acids (SCFAs) by the gut microbiota [66]. Indeed, in the current study, correlations of 4-cresol levels and protein intake were only apparent when high carbohydrate consumers were not considered in the analysis. As such, fibre intake may offer a protective effect against a high protein diet.

Asides from this a Western lifestyle involving decreased physical activity and overconsumption of energy-dense food leads to increased BMI. Indeed, several studies, along with our own, have found a positive association of BMI and blood pressure has been reported among different populations [67, 68]. Furthermore, the average waist/ hip ratio of the study subjects was 1.2, which is more than is recommended, [69]. This data is of interest as evidence indicates an association between elevated body weight, waist and hip ratio to cancer risk [70,

71]. All these factors are linked as several studies support the idea of an increased risk of adenomas and CRC in patients with obesity and metabolic syndrome [72, 73].

Furthermore, the, present study showed that only about half of subjects reached the recommended levels of physical activity of 60-90 minutes per day. This could be partly due to the environmental culture of people living in Oman. These results are in line with the results from Hashem Kilani, et al. [5] and Al-Hazzaa, et al. [6] study who found a high prevalence of sedentary behaviours and low levels of physical activity in Omani and Saudi populations respectively. However, this is of concern as a low level of physical activity can affect the general health of study subjects. Several studies have indicated that physical activity for 60-90 minutes practiced every day results in reduced incidence rates of cancer, cardiovascular disease, type 2 diabetes, and obesity [74]. As such, increased exercise could provide many benefits to the population.

This study, in line with several studies found a positive association of BMI and blood pressure [67, 68]. Hypertension can lead to other complications and diseases including heart disease, eye damage and stroke [75]. Furthermore, several epidemiological studies have revealed that excess body weight is associated with increased risk of colorectal cancer especially in men [76]. It has been calculated that compared to people with a BMI <23.0, the risk of CRC is increased by 14% for individuals with a BMI of 23.0–24.9; 19% for a BMI of 25.0–27.4; 24% for BMI of 27.5–29.9; and 41% for BMI of ≥30.0 [77]. A study by Koolhaas, found that, the volunteers who are overweight and obese with low physical activity had a higher CVD risk than normal weight participants with high physical activity [78]. Furthermore, it has been observed that leptin in obese people is directly related to CRC risk. Whilst the risk of developing tumours in patients with K-RAS mutation and p53 has been seen to be lower with

increase of the physical activity level [79]. Therefore, those in the overweight and obese category of the current cohort considered to be at higher risk of CRC development and other chronic diseases.

3.7 Conclusions

The present study indicated that the Omani population were consuming diets with high protein levels, combined with inactive lifestyles. High protein intakes were associated with elevated urinary 4-cresol, which could be increasing risk factors associated with non-communicable diseases such as CRC. Furthermore, high levels of physical activity have identified as being associated with a reduced risk of colon cancer. Therefore, health promotion policy should focus and set general strategies for encouraging increased physical activity and protein restriction to the recommended level and maintenance of a healthy diet. More research in this field is needed to explore the impact of different protein sources on the microbiota and the metabolites produced and whether fibre can mediate these effects.

3.8 Acknowledgements

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Chapter IV

4. Modelling the potential role of microbial 4-cresol in colorectal carcinogenesis

4.1 Abstract

Background: A greater understanding of mechanisms explaining the interactions between diet and the gut microbiota in colorectal cancer is desirable. Genotoxic microbial metabolites present in the colon may be implicated in carcinogenesis and potentially ameliorated by diet.

Aims: We hypothesised that microbial 4-cresol is a colonic carcinogen and set out to model potential exposures in the colon and the effects of these exposures on colonic cells.

Methods: Batch culture fermentations with faecal inoculate were used to determine the synthesis of 4-cresol and other metabolites in response to various substrates. The microbiota was monitored and fermentation supernatants were evaluated for genotoxicity and the independent effects of 4-cresol on colonic cells were studied *in vitro*.

Results: In batch culture fermentation supplementary protein increased the synthesis of phenols, indoles and 4-cresol, whereas supplementary fructooligosaccharide (FOS) increased the synthesis of short chain fatty acids. The 4-cresol was the greatest predictor of genotoxicity against colonocytes in the fermentation supernatants. Spiking fermentation supernatants with exogenous 4-cresol further increased DNA damage, and independently 4-cresol induced DNA damage in a dose dependent manner against HT29 and Caco-2 cells and influenced cell cycle kinetics.

Conclusions: High concentrations of microbial 4-cresol may contribute to carcinogenicity in the human colon.

Key wards: 4-cresol, tyrosine, colonic metabolites, microbiota, comet, cell proliferation, cell cycle, HT29, Caco-2, FOS, DAPI and PI.

4.2 Introduction

The colon is a pro-carcinogenic environment [1] with microbial activity being implicated in increased susceptibility to neoplastic transformation [2]. Environmental factors, particularly diet, modulate composition and metabolic activity of the colonic microbiota with implications for cancer risk [3, 4]. Current mechanistic models implicating diet in CRC risk propose that dietary fibre favourably improves the balance of the microbiome, increasing the abundance of saccharolytic species relative to proteolytic microbes. The latter are associated with increased production of an assortment of genotoxic metabolites from meat based or endogenous substrates [4-6]. Epidemiological studies implicate red and processed meat in particular as being pro CRC formation. Genotoxicity associated with haem, *N*-nitroso compounds, and heterocyclic amines is the proffered mechanism underpinning this association [7]. However, these compounds are also present in white meats and fish; foods which are not implicated in CRC risk. A deeper understanding of the relationship of diet to the production of genotoxic and tumour promoting microbial by products in the colon is therefore desirable.

Amongst proteolytic metabolites present in the colon, 4-cresol is a relatively under studied potential contributor to the genotoxic load [8]. 4-cresol is a methyl phenol produced via microbial degradation of tyrosine and phenylalanine [9, 10]. *In situ*, it is absorbed and metabolised in the liver, producing 4-cresol sulphate, which is excreted in the urine. Elevated urinary 4-cresol sulphate has been observed in patients with colorectal cancer [10], it may be associated with ageing [11] and more recently it has been suggested as a biomarker of protein intake [12].

Due to the efficient intestinal uptake of 4-cresol, and other luminal genotoxins, genotoxicity and chemical composition of faecal samples may be poorly representative of colonic exposures; this has, in part, limited the use of faecal water sample genotoxicity as a diet-related

biomarker of colorectal cancer risk [13]. The presence of 4-cresol sulphate in urine and its association with both diet and the microbiome may make it a useful, modifiable diet-sensitive, biomarker of colorectal genotoxicity and therefore CRC risk which could be applied in human intervention studies, assuming it is causally linked to carcinogenesis [12].

Here the objectives of this study were three fold, a) to consider the microbial fermentation of dietary protein sources. b) to establish the potential luminal exposure to 4-cresol using a simulated gut fermentation system. c) to determine the genotoxicity, and potential carcinogenicity, of 4-cresol, as part of the colonic metabolome, at levels of exposure consistent with those achievable *in vivo*, using two separate cell based models of the colonic epithelium.

4.3 Materials and methods

4.3.1 Chemicals

4-cresol, agarose, EDTA, Trizma base, Triton x100, hydrogen peroxide (H_2O_2), Hepes, BSA, ethidium bromide, propidium iodide (PI) and RNase A were purchased from Sigma-Aldrich Ltd. (Dorset UK). Sodium chloride (NaCl) and potassium chloride (KCl) were supplied by Fisher Scientific **(Loughborough, UK)**.

Tyrosine, Fructooligosaccharide, Albumin, soybean protein peptone meat extracts were all purchased from Sigma-Aldrich Ltd. (Dorset UK). Bacteriological growth medium supplements were obtained from Oxoid Ltd. **(Basingstoke, Hants, U.K.)**.

Probes for fluorescence *in situ* hybridisation were commercially synthesised and labelled at the 5′ end with the fluorescent dye Cy3 (Sigma Aldrich Ltd, Poole, Dorset, UK). The probes used were: EUB 338 mixture as a positive control consisting of EUB338, EUB338II and EUB338III for total bacteria count and Non EUB as a negative control. Other probes used such as : Ato 291 for *Atopobium* cluster (ATO), Lab 158 for lactobacilli/enterococci (LAB), Bif 164 for bifidobacterium (BIF), Erec 482 for *Eubacterium rectale–Clostridium coccoides* group

(EREC) [47], Chis 150 for the *Clostridium histolyticum* group (CHIS), Bac 303 for *Bacteroides—Prevotella* spp. (BAC) and Fuso 664 for *Fusobacterium* genus **(Table 4.1).** The HT29 and Caco-2 human colorectal cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC) (Salisbury, UK) and used between passages 45 and 70. Essential Medium (MEM), McCoy's 5A with L-gulatumate, Penicillin-Streptomycin and Fetal Bovine Serum (South America) were purchased from Biosera Ltd. (East Sussex, UK). Non-essential Amino Acid (NEAA) (Lonza group Ltd. Basel, Switzerland). Phosphate Buffered Saline (PBS), Trypsin-Versene and Ethylenediaminetetraacetic acid (EDTA) were purchased from Lonza group Ltd. (Basel, Switzerland).

4.3.2 Faecal inoculate for batch culture fermentation

Faecal samples were collected from three individuals (over 60 years of age). This is conducted to a certain if the microbiota of different volunteers behave in the same way, increasing the number of volunteers under same conditions would increase the statistical power of any data generated. A pooled sample however would mask differences observed due to different functional groups within the volunteers. All volunteers self-reported as being healthy, antibiotic free for at least 6 months prior to sampling and free from gastrointestinal issues. Samples were collected on the day of the experiment and were used immediately. Upon collection, they were diluted 1:10 (w/v) with anaerobic phosphate buffered saline (PBS; 0.1 M; pH 7.4) and homogenised in a stomacher for 2 min (460-paddle beats/min). (15 ml) of the resulting faecal slurries from each individual were used to inoculate batch culture vessels in triplicate.

4.3.3 Batch culture fermentation

Batch culture fermentation vessels were autoclaved and filled with 135 ml of basal nutrient medium (peptone water (2 g/L), yeast extract (2 g/ L), NaCl (0.1 g/ L), K_2HPO_4 (0.04 g/ L), KH_2PO_4 (0.04 g/ L), $NaHCO_3$ (2 g/ L), $MgSO_47H_2O$ (0.01 g/ L), $CaCl_{26}H_2O$ (0.01 g/ L), tween 80 (2 ml/ L), hemin (50 mg/ L), vitamin K_1 (10 ml/ L), L-cysteine (0.5 g/l), bile salts (0.5 g/ L), resazurin (1 mg/ L) and distilled water (Sigma, Aldrich, UK). The vessels were gassed overnight with O_2-free N_2 (15 ml/min).

Supplementary substrates: Alternative additional substrates were included to consider the influence of dietary substrate on cresol fermentation. These were prepared as a high tyrosine (supplementary tyrosine at 0.3:100 w/w), a low tyrosine treatment (supplementary tyrosine 0.003:100 w/w), a high tyrosine with fructoligosaccharide (FOS) (0.3:100 w/w Tyr + FOS 1.5:100 w/w), a low tyrosine with FOS (0.003:100 w/w Tyr + 1.5:100 w/w FOS), an albumin treatment (0.3:100 w/w), a soybean protein treatment (0.3:100 w/w) and a peptone meat extract treatment (0.3:100 w/w).

15 ml of faecal inoculate was added to the cultures and the subsequent fermentation carried out under anaerobic conditions. The temperature was maintained at 37°C by use of a circulating water bath and pH was maintained at 6.8 using a pH controller (Electro lab, UK). At six time points (0, 4, 8, 24, 30 and 48 hours), 10 ml of fermentation supernatant was collected for analysis. Fermentation supernatants were filter sterilised through a 0.2 mm PVDF single use filter (Sartorius Ltd. Surrey UK) for use as microbe free treatments in cell culture experiments. All fermentation conditions were conducted in triplicate, each time with a different donor- then to prove reproducibility one donor experiment was repeated three times.

4.3.4 Bacterial enumeration

4.3.4.1 Fluorescence *in situ* hybridisation

Bacterial populations from the batch culture samples were enumerated using fluorescence *in situ* hybridisation and flow cytometry (Flow-FISH), with oligonucleotide probes targeting specific regions of 16S rRNA. Probes were commercially synthesised and coated with the fluorescent dye, Cy3. Seven hundred and fifty µL of batch culture samples were centrifuged at 12,400 × *g* for 5 min. The resultant pellets were re-suspended in 375 µL of filtered PBS (using a 0.22 µm filters) and fixed in 1125 µL of 4% (v/v) paraformaldehyde. After 4 h of incubation at 4˚C, samples were washed twice using 1 mL of PBS, re-suspended in 500 µL PBS- 99% ethanol (1:1, v/v) and stored at –20˚C. 75 µL of the sample was added to 500 µL PBS and centrifuged at 12,400 × *g* for 3 min for premeabilisation. Pellets were re-suspended using 100 µL of filtered TE-FISH buffer (Tris/HCl 1 M pH 8, EDTA 0.5 M pH 8, distilled H_2O, 0.22 µm filters). Then lysozyme added (50 000 U) and the mixture incubated at room temperature for 10 min. The samples were then vortexed and centrifuged at 12,400 × *g* for 3 min. Pellets were washed with 500 µL PBS and centrifuged (12,400 × *g*, 3 min). The hybridisations process was completed by re-suspending the pellets in 150 µL of hybridisation buffer (5 M NaCl, 1 M Tris/HCl pH 8, 30% formamide, ddH_2O, 10% SDS), the mixture was vortexed then centrifuged (12,400 × *g*, 3 min). Pellets were then resuspended in 1 mL of hybridisation buffer and 50ng/ µL aliquoted into Eppendorf tubes for analysis.

Table 4.1: Oligonucleotide probes used in this study for Flow- FISH analysis of bacterial populations. concentration is 50 ng/µL

Probes name	Sequences 5' To 3'	Target genus	Reference
Non Eub (Negative control)	ACTCCTACGGGAGGCAGC	Control	[14]
Eub338 I + (Positive control)	GCT GCC TCC CGT AGG AGT	Most bacteria	[15]
Eub338 II +	GCA GCC ACC CGT AGG TGT	Planctomycetales	[15]
Eub338 III +	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	[15]
Bif164	CATCCGGCATTACCACCC	Most *Bifidobacterium* spp.	[16]
Lab158	GGTATTAGCAYCTGTTTCCA	Most *Lactobacillus, Leuconostoc* and *Weissella* spp.	[17]
Bac 303	CCAATGTGGGGGACCTT	Most Bacteroidaceae and Prevotellaceae, some Porphyromonadaceae	[18]
Erec 482	GCTTCTTAGTCARGTACCG	Most of the *Clostridium coccoides-Eubacterium rectale* group	[19]
Rrec 584	TCAGACTTGCCGYACCGC	*Roseburia* subcluster	[20]
Chis150	TTATGCGGTATTAATCTYCCTTT	Most of the *Clostridium histolyticum* group *(Clostridium* clusters I and II)	[19]
Prop 853	ATTGCGTTAACTCCGGCAC	*Clostridial* cluster IX	[20]
Ato 291	GGTCGGTCTCTCAACCC	*Atopobium, Colinsella, Olsenella* and *Eggerthella* spp.	[21]
Fprau 647	CGCCTACCTCTGCACTAC	*Faecalibacterium prausnitzii* and related sequences	[22]
DSV 687	TACGGATTTCACTCC T	Most *Desulfovibrionales* (excluding *Lawsonia)* and many *Desulfuromonales*	[23]

4.3.5 Metabolite characterisation

Standards of 4-cresol, phenol and indole were prepared in distilled water at concentrations from (0.1-1600 mM) and standard curves plotted following quantification via solid-phase microextraction (SPME) gas chromatography mass spectrometry (GC-MS) using an Agilent 110 PAL injection system and Agilent 7890 gas chromatograph with 5975C mass spectrometer (Agilent, Santa Clara, CA). The SPME fiber stationary phase was composed of 75 µm divinylbenzene/Carboxen™ on polydimethylsiloxane; Supelco, Bellefonte, PA). Sample (0.1 mL) was placed in a 20-mL headspace vial with magnetic screw cap and PTFE/silicone septum (Supelco). The samples were allowed to equilibrate for 10 minutes at 35 °C before being extracted for 30 min. Sample was agitated at 500 rpm (5 seconds on, 2 seconds off) during equilibration and extraction. After extraction, the contents of the fibre were desorbed onto the front of a Stabilwax-DA fused silica capillary column (30 min 0.25 mm i.e., 0.50 mm film thickness; Restek, Bellefonte PA). The GC temperature program and the fiber desorption step commenced at the same time. During the desorption period (45 s), the oven was held at 40 °C. After desorption, the oven was held at 40 °C for a further 255 s before incremental heating at 4 °C/min to 260 °C, where the temperature was maintained for 5 min. Helium was used as the carrier gas at a constant flow rate of 0.9 mL/min. The mass spectrometer was operated in electron impact mode with an electron energy of 70 eV, scanning from m/z 20 to m/z 280 at 1.9 scans/s.

4.3.6 Organic acid analysis

Samples from batch culture fermentation were screened for the organic acid short-chain fatty acid (SCFA) concentration which were determined using an acidification method adapted from Zhao, G et al [24]. Briefly fermentation samples were defrosted, vortexed and centrifuged for 5 minutes at 13,400 x g. The samples were filtered using a 0.22 µm filter and sulfuric acid was added to bring the pH down to 2. 200 µl of the resulting sample solution and 50 µl of internal standard was added to the vial. The internal standard used in this experiment was 2-ethylbutyric acid (Aldrich) made to concentration of 100 mM in HPLC grade water. The GC apparatus was calibrated for detection of acetate, propionate, iso-butyrate, butyrate, iso-valeric, valeric and caproic acid using standards of a range of concentrations (5mM – 50mM).

Analysis was conducted using a HP 5890 series II GC system (Hewlett Packard, Palo Alto, Calif) with an FFAP, capillary fused silica packed column 25 m by 0.32 mm; filter thickness, 0.25µm (Macherey-Nagel, Düren, Gemany). Afterwards, the sample was injected into the column, which was maintained at 140°C for 5 minutes. Then the column temperature was increased over 5 minutes to 240°C. The calibrated organic acids were detected in the samples and the concentrations calculated. An external standard with known concentrations of SCFAs were injected after every 10 samples to maintain appropriate calibration. Finally, peaks were analysed and integrated using HP GC ChemStation Software, Hewlett Packard.

4.3.7 Tissue culture

The HT29 and Caco-2 human cell lines are derived from colonic epithelial adenocarcinoma cells and are widely used in the study of tumourgenicity. The cells were obtained from the European Collection of Animal Cell Cultures (ECACC) (Salisbury, UK) and used between passages 45 and 70. HT29 cells were cultured routinely in McCoy's 5A with L-gulatumate supplemented with Penicillin-Streptomycin and 10% Fetal Bovine Serum (South America)

Biosera Ltd. (East Sussex, UK). Caco-2 cells were cultured in Minimal Essential Medium (MEM) supplemented Fetal Bovine Serum (Biosera Ltd. East Sussex, UK). Non-essential Amino Acid (NEAA) (Lonza group Ltd. Basel, Switzerland). Phosphate Buffered Saline (PBS), Trypsin-Versene and Ethylenediaminetetraacetic acid (EDTA) were purchased from Lonza group Ltd. (Basel, Switzerland). Routine culture was carried out at 37°C with 5% CO_2 and 95% humidity, the cell medium was changed every 2 days with trypsin mediated passage at 80-90% confluence.

4.3.8 Cell viability

The nuclear stain 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) was used to assess the cytotoxic effects of 4-cresol on HT29 and Caco-2 cells. HT29 and Caco-2 cells were seeded in each 96 well plate at 1×10^6 cells/ml concentration and incubated at 37ºC with 5% CO_2 and 95% humidity for 24 hours. 4-cresol was dissolved in PBS, then HT29 and Caco-2 cells were treated with 50 µl of McCoy's media and 50 µl of MEM media respectively containing serial dilutions of the test compounds. Concentrations used were 0-3 mM. The plates were then incubated at 37°C for 24 hours before removing the treatment solutions via aspiration. Cells were permeabilised with 100 µl of ice-cold methanol and left for 5 minutes to incubate at room temperature. Methanol was removed by pipette and plates were allowed to dry in a hood for 15 minutes, followed by addition of 100 µl of DAPI in PBS (70 µl of DAPI staining stock solution (3 mM) plus 10.43 ml of PBS per plate) and incubated in the dark for at least 30 minutes at 37°C prior to measurement. Absorption was measured using a GENios microplate reader (TECAN Group Ltd., Männedorf, Switzerland) with absorbance and emission at 340 nm and 465 nm, respectively.

4.3.9 DNA damage

DNA damage was assessed using the single strand comet assay which is widely used to detect single stranded breaks in cellular DNA. HT29 and Caco-2 cell lines were seeded into separate T75 flasks at a concentration of $1x10^6$ and maintained at 37ºC in an atmosphere of 5% CO_2 and 95% filtered air. Cells were treated in the tissue culture flasks at 80 % confluency; 4-cresol was made up in serial dilutions at 0, 0.5, 1.5 and 3.0 mM concentrations of growth media and applied directly to the cells for 24 hours. At 24 hours' cells were washed and detached with trypsin, following centrifugation 13000 x *g* for 3 minutes and the supernatant was removed via aspiration, followed by washing with PBS for 1 minute. A positive control was prepared with untreated cells exposed to 7.5 mM hydrogen peroxide for 15 minutes prior to lysis. Cells were counted with trypan blue and adjusted to give a working concentration of $3x10^6$ cells/m l20 µl of cells suspension re-suspended with 200µl of melted agarose were bedded on microscope slides, then left at 4°C for 15 minutes. The slides were placed into lysis buffer (2.5M NaCl, 0.1 EDTA, 0.01 M Tris and added 1% (v/v) Triton x100 prior to use) for 1 hour at 4°C, and then washed 3 times with neutralising buffer (96.9 g Trizma base, 1 L water and adjusted PH 7.5 with (6M HCl)) for 5 minutes before transfer into electrophoresis buffer (0.3M NaOH and 1mM EDTA). After 20 min at 4ºC the slides were placed horizontally in an electrophoresis tank containing electrophoresis alkaline buffer to allow the DNA to unwind. Electrophoresis was run at 26V, 300mA for 40 minutes in at 4°C in the dark. The slides were then washed with neutralising buffer (0.4 M Trizma base, pH 7.5) three times for 5 minutes each and then left for 5 minutes in 99% ethanol for 5 minutes, then left to dry overnight. Cells were stained with ethidium bromide (20µl/ml) and kept for 15minutes in the dark. Images of DNA integrity were captured by fluorescence microscopy using the Kinetic image software, Komet 4.0 UK. One hundred randomly selected cells from each replicate slides were evaluated for DNA tail damage by an analyst blinded to the treatment.

Fermentation supernatants: HT29 cells were prepared as described above. Filter sterilised fermentation supernatants were prepared at 10% (v/v) in McCoy's carrier culture medium with inactivated at 56°C Fetal Bovine Serum (FBS) and antibiotics at 37°C with 5% CO_2 and used to treat the cells for 24 hours, prior to the Comet assay as described.

Spiked cell line: to check the sensitivity of the HT29 cell line 4-cresol was added in a growth culture media alone with different concentrations (0, 0.2, 0.5,1, 1.5 and 3) mM in HT29 cell line without any fermentation supernatants.

4.3.10 Cell cycle assay

Cell cycle progression was assessed considering the percentage of cells in phases Gap0/1 (G0/1), Synthesis (S), Gap2/mitosis (G2/M) and apoptotic cells (sub G0/1) according to the fluorescence intensity of a PI nuclear stain, and based on the concentration of DNA within the cell. HT29 and Caco-2 cell cultures were treated at $2x10^5$ cells/well into 6 well plates at 80% confluence. The cells were exposed to 4-cresol at 0.2, 0.5, 1.0, 1.5, 3.0 mM for 24 hours. After removing treatments, the cells were washed with ice cold PBS and collected following trypsin harvest of the monolayer and pelleting by centrifugation at 300 x g for 3 minutes. The supernatants were discarded and then the cell tissues were resuspended in 200 µl ice cold PBS and fixed with 2 ml of fresh ice cold 70% ethanol. The cell pellets were stored in freezer at -20°C until analysis.

After chilling, the samples were centrifuged at 300 x g for 5 minutes and the supernatants discarded. The pellets were resuspended with 200 µl PBS before adding 25 µl of 1 mg/ml RNAse and the suspensions were then incubated at 37°C for 30 minutes. 2.5 µl of 400 µg/ml of PI were added to bind DNA and were left to incubate for 30 min at room temperature in dark condition. Cells suspensions were adjusted to a final volume of 600 µl with PBS. The DNA

content of 15,000 cells were then measured immediately via flow cytometry (BD Accuri C6 flow cytometer, Germany). Analysis was performed using the Flow Jo software (Tree star Inc, Oregon, USA).

4.4 Statistical Analysis

All statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) version 22. All data have been carried in three biological replicates for each analysis. The data are presented as mean ±SEM. Fermentation sample metabolites as predictors of genotoxicity were evaluated. Cell viability and cell cycle data were analysed using linear regression models. For bacteriology, comparisons between each volunteers made by analysis of variance. Similarly ANOVA was used to compare the effects of substrates on fermentation sample genotoxicity and cell cycle kinetics where appropriate comparison of individual treatments with negative control were performed using the Dunnett Post-hoc test.

P values <0.05 were considered to be statistically significant between the treatments.

4.5 Results

4.5.1 Characterisation of fermentation microbiota

Three faecal donors supplied specimens for the inter-individual biological replication of batch culture fermentations. An analysis of the microbial composition of the fermentation inoculate, was performed using 16S rRNA adherent molecular probes. There were statistically significant differences in the starting microbial composition of the batch culture fermentation which fed through to inter-individual differences in metabolite production. Volunteer 1 had a greater relative abundance of *Bifidobacterium* (BIF164) $P < 0.000$, *Atopobium* cluster (ATO 291) $P < 0.000$ and *Desulfovibrio* (DSV 687) $P < 0.000$ than volunteers 2 and 3. In contrast, volunteer 1 had a lower relative abundance of *Faecalibacterium* (FPRAU 655) $P < 0.000$, *Propionibacterium* (Prop 853) $P= 0.002$, and *Lactobacillus* (Lab 158) $P < 0.000$ **(Figure 4.1)**

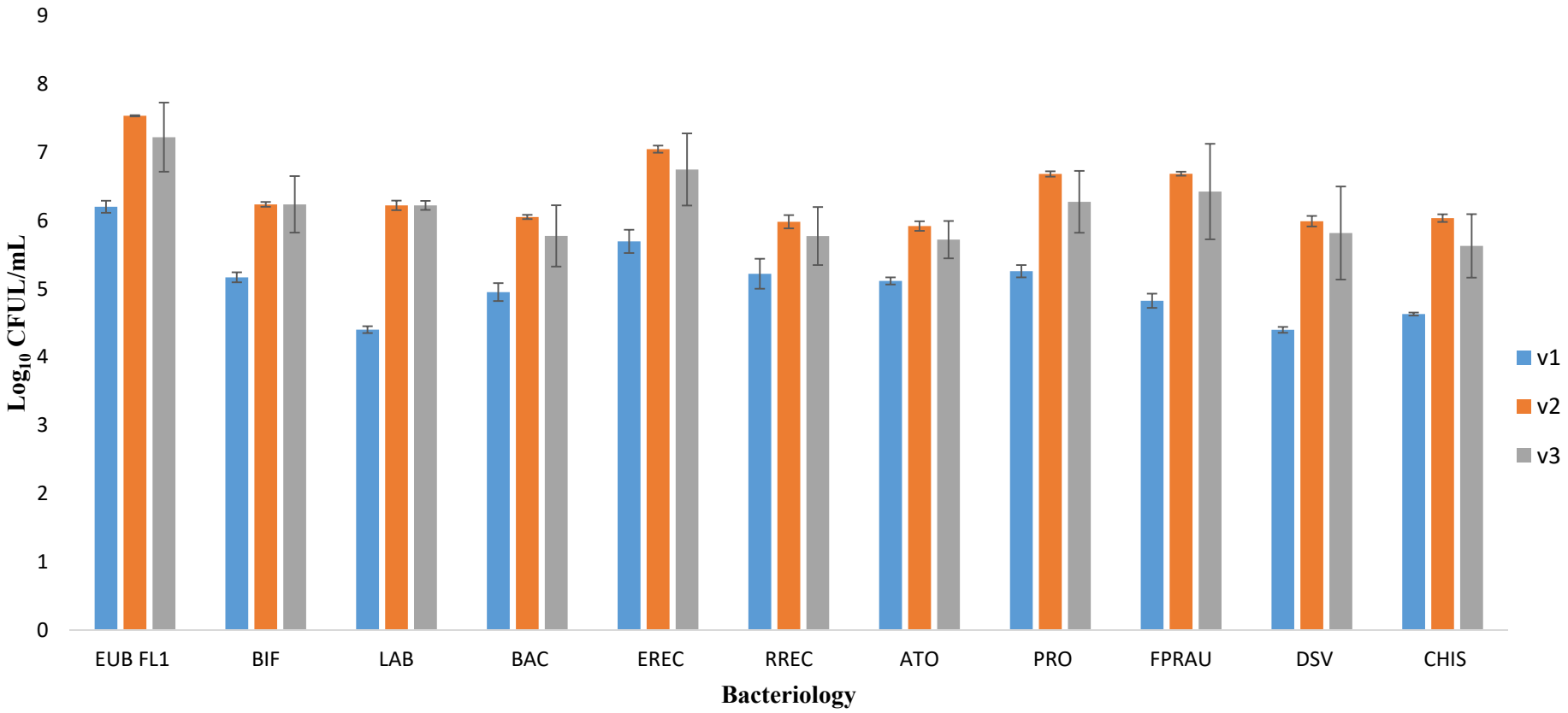

Figure 4.1: Differences in starting bacterial communities used within *in vitro* batch culture fermentation for all the three volunteers 1, 2 and 3 at t=0. Values are presented of the bacterial numbers (log_{10}/g faeces determined by flow cytometry: fluorescence in situ hybridisation) Values listed are mean ± SD. Eub= Total bacteria, BIF= *Bifidobacterium* spp. LAB= *Lactobacillus, Leuconostoc and Weissella spp*. BAC= *Bacteriodes* spp. EREC= *Clostridium coccoides-Eubacterium rectale* group, RREC= *Roseburia* subcluster, ATO= *Atopobium, Colinsella, Olsenella* and *Eggerthella* spp, PRO= clostridial cluster IX, FPRAU= *Faecalibacterium prausnitzii* and related sequences, DSV= *Desulfovibrionales* (excluding *Lawsonia)* and many *Desulfuromonales,* CHIS= *(Clostridium* clusters I and II).

Table 4.2: Microbial changes following fermentation of substrates in pH controlled batch cultures. Log_{10} number of bacterial cells/ml batch supernatant, +/- stdev. * indicated significantly different to blank, [a] indicates significantly different to t0.

TYR (L)	t0		t4		t8		t24		t30		t48		
EUB FL2	**6.71**	±0.74	**7.03**	±0.75	**6.92**	±0.61	**6.96**	±0.74	**6.92**	±0.18	**6.74**	±0.59	
BIF	**5.68**	±0.50	**5.91**	±0.74	**5.79**	±0.74	**5.93**	±0.70	**5.76**	±0.38	**5.63**	±0.65	
LAB	**5.15**	±0.98	**5.69**	±0.73	**5.33**	±0.67	**5.64**	±0.71	**5.67**	±0.36	**5.60**	±0.79	
BAC	**5.30**	±0.71	**5.69**	±0.72	**5.66**	±0.57	**5.79**	±0.72	**5.78**	±0.23	**5.78**	±0.65	
EREC	**6.19**	±0.73	**6.51**	±0.77	**6.32**	±0.65	**6.15**	±0.84	**5.88**	±0.43	**5.81**	±0.55	
RREC	**5.36**	±0.72	**5.29**	±0.95	**5.55**	±0.46	**5.44**	±0.83	**5.62**	±0.22	**5.55**	±0.51	
ATO	**5.41**	±0.43	**5.69**	±0.37	**5.53**	±0.39	**5.88**	±0.53	**5.72**	±0.40	**5.70**	±0.46	
PRO	**5.82**	±0.83	**5.88**	±0.92	**6.05**	±0.68	**6.03**	±0.94	**6.17**	±0.31	**5.88**	±0.67	
FPRAU	**5.69**	±0.88	**6.06**	±1.14	**5.89**	±0.91	**5.95**	±0.78	**5.79**	±0.41	**5.69**	±0.54	
DSV	**5.11**	±0.90	**5.48**	±0.82	**5.39**	±0.55	**5.55**	±0.62	**5.54**	±0.38	**5.26**	±0.68	
CHIS	**5.27**	±0.77	**5.55**	±0.56	**5.29**	±0.65	**5.64**	±0.48	**5.61**	±0.33	**5.50**	±0.61	

TYR (H)	t0		t4		t8		t24		t30		t48		
EUB FL1	**6.99**	±0.70	**7.19**	±0.69	a	**7.13**	±0.46	**7.07**	±0.81	**7.16**	±0.20	**6.85**	±1.00
BIF	**5.87**	±0.61	**5.85**	±0.79		**5.94**	±0.47	**6.04**	±0.71	**5.82**	±0.34	**5.78**	±0.90
LAB	**5.46**	±0.95	**6.01**	±1.10		**5.96**	±0.88	**5.79**	±1.22	**6.10**	±0.62	**5.61**	±1.22
BAC	**5.59**	±0.57	**5.87**	±0.70		**5.76**	±0.34	**5.86**	±0.67	**5.87**	±0.22	**5.74**	±0.85
EREC	**6.50**	±0.71	**6.64**	±0.62		**6.46**	±0.53	**6.31**	±1.01	**6.11**	±0.14	**5.89**	±0.95
RREC	**5.66**	±0.39	**5.52**	±0.36		**5.67**	±0.34	**5.69**	±0.74	**5.75**	±0.14	**5.48**	±1.08
ATO	**5.59**	±0.42	**5.69**	±0.58		**5.69**	±0.16	**5.96**	±0.51	**6.27**	±0.41	**5.80**	±0.71
PRO	**6.07**	±0.73	**6.06**	±0.95		**6.31**	±0.58	**6.16**	±1.03	**6.18**	±0.63	**6.11**	±1.23
FPRAU	**5.98**	±1.01	**6.13**	±0.96		**6.10**	±0.67	**5.94**	±0.77	**5.89**	±0.02	**5.63**	±0.94
DSV	**5.40**	±0.87	**5.51**	±0.58		**5.50**	±0.40	**5.56**	±0.78	**5.67**	±0.22	**5.31**	±0.99
CHIS	**5.43**	±0.72	**5.58**	±0.43		**5.55**	±0.41	**5.68**	±0.69	**5.76**	±0.31	**5.42**	±0.95

TYR (L)+FOS	t0		t4		t8		t24		t30		t48		
EUB FL4	**7.00**	±0.81	**7.33**	±0.78	**7.44**	±0.86	a	**6.59**	±1.37	**7.17**	±1.47	**7.57**	±0.13
BIF	**5.78**	±0.96	**6.28**	±0.94	a	**6.52**	±1.22	**5.75**	±1.62	**6.26**	±1.51	**6.45**	±0.83
LAB	**5.53**	±0.91	**6.22**	±0.89		**6.22**	±0.92	**4.99**	±1.18	**5.71**	±1.53	**6.31**	±0.43
BAC	**5.33**	±1.21	**5.93**	±0.86		**6.20**	±1.15	**5.62**	±1.45	**6.26**	±1.64	**6.52**	±0.22 *
EREC	**6.38**	±0.98	**6.62**	±0.82		**6.48**	±0.77	**5.55**	±1.38	**6.00**	±1.22	**6.21**	±0.17
RREC	**5.34**	±1.10	**5.75**	±0.46		**5.85**	±0.56	**5.17**	±1.11	**5.69**	±1.20	**5.98**	±0.19
ATO	**5.55**	±0.64	**5.82**	±0.16		**6.12**	±0.65	**5.56**	±1.67	**5.98**	±1.76	**6.38**	±0.44
PRO	**6.28**	±0.44	**6.17**	±1.04		**6.15**	±1.09	**5.52**	±1.45	**6.05**	±2.03	**6.80**	±0.57
FPRAU	**5.99**	±1.31	**6.34**	±0.91		**6.32**	±0.93	**5.47**	±0.98	**6.03**	±1.16	**6.18**	±0.12
DSV	**5.29**	±1.18	**5.69**	±0.88		**5.59**	±0.94	**4.97**	±1.21	**5.57**	±1.75	**5.91**	±0.18
CHIS	**5.31**	±1.16	**5.89**	±1.00		**6.16**	±1.05	a	**5.14**	±1.02	**5.83**	±1.14	**6.15**	±0.16

TYR (H)+FOS	t0		t4		t8		t24		t30		t48	
EUB FL3	**6.98**	±0.83	**7.42**	±0.98	**7.33**	±1.01	**7.31**	±0.54	**7.93**	±0.32 *	**7.59**	±0.30
BIF	**5.89**	±0.68	**6.45**	±1.06	**6.42**	±1.26	**6.53**	±0.84	**6.84**	±0.77	**6.58**	±0.75
LAB	**5.45**	±0.97	**6.30**	±1.31	**5.97**	±1.06 a	**5.93**	±0.85	**6.62**	±0.79	**6.30**	±1.04
BAC	**5.54**	±0.73	**6.07**	±1.04	**6.16**	±0.89	**5.64**	±0.18	**6.58**	±0.73	**6.24**	±0.21
EREC	**6.41**	±0.90	**6.68**	±0.89 a	**6.55**	±1.14	**6.28**	±0.81	**6.70**	±0.04	**6.30**	±0.53
RREC	**5.65**	±0.63	**5.74**	±0.60	**5.44**	±0.66	**5.41**	±0.57 a	**6.08**	±0.49	**5.88**	±0.37
ATO	**5.66**	±0.48	**5.97**	±0.74	**5.69**	±0.77	**5.96**	±0.72	**6.60**	±0.45 *	**6.34**	±0.43
PRO	**6.05**	±0.87	**6.17**	±1.12	**6.42**	±0.75	**6.16**	±0.63	**6.73**	±1.09	**6.61**	±0.87
FPRAU	**6.09**	±1.05	**6.37**	±1.04	**6.28**	±1.24	**6.18**	±0.50	**6.60**	±0.18 *	**6.11**	±0.29
DSV	**5.56**	±0.93	**5.84**	±0.87	**5.56**	±0.78	**5.35**	±0.32	**6.14**	±0.44	**5.71**	±0.56
CHIS	**5.56**	±0.79	**5.84**	±0.91	**5.83**	±1.08	**5.49**	±0.23	**6.21**	±0.62	**5.92**	±0.50

ALBUMIN	t0		t4		t8		t24		t30		t48	
EUB FL5	**7.07**	±0.44	**7.00**	±0.47	**7.13**	±0.52	**7.02**	±0.36	**7.09**	±0.05	**6.73**	±0.28
BIF	**5.90**	±0.47	**5.79**	±0.59	**5.94**	±0.73	**6.02**	±0.35	**5.90**	±0.37	**5.68**	±0.47
LAB	**5.57**	±0.73	**5.71**	±0.13	**5.49**	±0.58	**5.69**	±0.43	**5.85**	±0.05 *	**5.59**	±0.23
BAC	**5.75**	±0.36	**5.70**	±0.38	**6.03**	±0.30 a	**5.75**	±0.09	**5.84**	±0.26	**5.70**	±0.28
EREC	**6.56**	±0.39	**6.44**	±0.53	**6.46**	±0.67	**6.02**	±0.25	**6.11**	±0.15	**5.62**	±0.48
RREC	**5.63**	±0.47	**5.36**	±0.54	**5.68**	±0.50	**5.64**	±0.29	**5.51**	±0.33	**5.56**	±0.23
ATO	**5.72**	±0.26	**5.63**	±0.71	**5.67**	±0.37	**6.04**	±0.60	**6.09**	±0.23 a	**5.60**	±0.41
PRO	**6.13**	±0.59	**5.99**	±0.61 a	**6.31**	±0.52	**6.03**	±0.64	**6.28**	±0.46	**5.87**	±0.43
FPRAU	**6.12**	±0.69	**6.03**	±0.78	**6.12**	±0.89	**6.03**	±0.51	**5.97**	±0.20	**5.62**	±0.46
DSV	**5.54**	±0.65	**5.33**	±0.52	**5.40**	±0.63	**5.60**	±0.17	**5.66**	±0.37	**5.20**	±0.29 *
CHIS	**5.60**	±0.52	**5.22**	±0.61	**5.53**	±0.59	**5.56**	±0.27	**5.57**	±0.10	**5.49**	±0.21

SOYBEAN	t0		t4		t8		t24		t30		t48	
EUB FL6	**7.10**	±0.60	**7.12**	±0.41	**7.13**	±0.66	**6.88**	±0.87	**6.84**	±0.80	**6.97**	±0.67
BIF	**5.94**	±0.62	**5.99**	±0.63	**6.00**	±0.55	**5.94**	±0.63	**5.84**	±0.68	**5.96**	±0.73
LAB	**5.68**	±0.49	**6.18**	±0.44	**5.96**	±1.29	**5.76**	±1.34	**5.90**	±1.16	**5.96**	±0.97
BAC	**5.58**	±0.53	**5.87**	±0.36	**5.83**	±0.69	**5.64**	±0.94	**5.58**	±0.89	**5.84**	±0.88
EREC	**6.52**	±0.71	**6.52**	±0.44	**6.31**	±0.73	**5.83**	±0.97	**5.78**	±0.95	**5.97**	±0.66
RREC	**5.64**	±0.31	**5.63**	±0.57	**5.70**	±0.32	**5.58**	±0.86	**5.59**	±0.72	**5.78**	±0.61
ATO	**5.59**	±0.29	**5.59**	±0.25	**5.59**	±0.19	**5.65**	±0.69	**5.61**	±0.80	**5.67**	±0.77
PRO	**6.29**	±0.73	**5.97**	±0.52	**6.21**	±0.88	**5.99**	±1.12	**5.58**	±0.79	**6.04**	±0.50
FPRAU	**6.19**	±0.83	**6.08**	±0.66	**5.98**	±0.74	**5.56**	±0.84	**5.57**	±0.93	**5.65**	±0.72
DSV	**5.59**	±0.58	**5.46**	±0.46	**5.59**	±0.44	**5.37**	±0.98	**5.41**	±0.86	**5.49**	±1.05
CHIS	**5.53**	±0.58	**5.46**	±0.63	**5.57**	±0.44	**5.44**	±0.97	**5.68**	±0.75	**5.65**	±0.71

FOS	t0		t4			t8			t24			t30			t48		
EUB FL6	**6.81**	±0.57	**7.39**	±0.60		**7.82**	±0.15	*	**7.36**	±0.80		**7.71**	±0.16	*	**7.83**	±0.19	*a
BIF	**5.62**	±0.60	**6.21**	±1.03		**6.61**	±1.12		**6.42**	±1.07		**7.37**	±0.17	*a	**6.73**	±0.79	
LAB	**5.41**	±0.69	**6.00**	±1.02		**6.49**	±0.88		**6.05**	±1.42		**6.68**	±0.93		**6.61**	±0.98	
BAC	**5.43**	±0.57	**6.21**	±0.56	a	**6.36**	±0.38		**5.86**	±0.86		**6.32**	±0.38	a	**6.56**	±0.70	
EREC	**6.32**	±0.56	**6.29**	±0.40		**6.49**	±0.05		**6.34**	±0.33		**6.36**	±0.15		**6.30**	±0.40	
RREC	**5.44**	±0.62	**5.59**	±0.36		**5.76**	±0.48		**5.68**	±0.57		**5.97**	±0.31		**6.03**	±0.73	
ATO	**5.42**	±0.45	**5.92**	±0.78		**6.02**	±1.02		**6.28**	±0.87		**6.22**	±0.50		**6.54**	±0.26	*
PRO	**5.79**	±0.71	**6.40**	±0.78		**6.83**	±0.84		**6.05**	±0.81		**6.17**	±0.30		**6.72**	±1.14	
FPRAU	**5.89**	±0.65	**5.93**	±0.62		**6.18**	±0.18		**5.91**	±0.60		**6.20**	±0.21		**6.11**	±0.49	
DSV	**5.40**	±0.68	**5.38**	±0.32		**5.61**	±0.59		**5.60**	±0.69		**5.83**	±0.15		**5.82**	±0.80	
CHIS	**5.34**	±0.60	**5.99**	±1.13		**6.31**	±0.23	*	**5.74**	±0.81		**5.91**	±0.26		**5.94**	±0.71	

PEPTONE MEAT	t0		t4			t8			t24			t30			t48		
EUB FL6	**6.71**	±0.66	**6.89**	±0.43		**7.20**	±0.47		**7.01**	±0.55	a	**7.08**	±0.13		**7.00**	±0.53	
BIF	**5.45**	±0.70	**5.74**	±0.50		**5.99**	±0.54		**5.97**	±0.40		**5.97**	±0.32		**5.91**	±0.45	
LAB	**5.37**	±0.66	**5.24**	±0.26		**5.59**	±0.82		**5.76**	±0.98		**5.58**	±0.26		**5.76**	±0.57	
BAC	**5.36**	±0.60	**5.63**	±0.39		**5.74**	±0.97		**5.82**	±0.26		**5.95**	±0.34		**6.11**	±0.52	
EREC	**6.13**	±0.64	**6.34**	±0.51		**6.52**	±0.48	a	**6.34**	±0.46		**6.09**	±0.15		**6.10**	±0.50	
RREC	**5.38**	±0.53	**5.34**	±0.33		**5.83**	±0.44		**5.58**	±0.52	a	**5.76**	±0.29		**5.72**	±0.52	
ATO	**5.43**	±0.38	**5.47**	±0.54		**5.58**	±0.38		**5.77**	±0.55		**5.90**	±0.08		**5.80**	±0.41	
PRO	**5.91**	±0.70	**6.00**	±0.59		**6.30**	±1.12		**5.99**	±0.69		**6.18**	±0.58		**6.16**	±0.64	
FPRAU	**5.63**	±0.89	**5.97**	±0.61		**6.02**	±0.71		**5.93**	±0.66		**6.15**	±0.41		**5.91**	±0.61	
DSV	**5.25**	±0.84	**5.28**	±0.24		**5.56**	±0.62		**5.47**	±0.60		**5.49**	±0.29		**5.54**	±0.64	
CHIS	**5.25**	±0.68	**5.25**	±0.33		**5.51**	±0.57		**5.55**	±0.60		**5.53**	±0.19		**5.65**	±0.58	

Blank	t0		t4			t8			t24			t30			t48		
EUB FL6	**6.73**	±0.65	**6.95**	±0.65		**6.92**	±0.52		**6.78**	±0.53		**6.83**	±0.16		**7.03**	±0.29	
BIF	**5.42**	±0.68	**5.85**	±0.48		**5.75**	±0.27		**5.63**	±0.28		**5.84**	±0.22		**5.94**	±0.17	
LAB	**5.23**	±0.80	**5.41**	±0.52		**5.53**	±0.41		**5.96**	±0.97		**5.50**	±0.16		**5.81**	±0.19	
BAC	**5.33**	±0.71	**5.73**	±0.70		**5.73**	±0.55		**5.62**	±0.59		**5.67**	±0.24		**6.00**	±0.24	
EREC	**6.25**	±0.66	**6.21**	±0.86		**6.12**	±0.65		**5.80**	±0.53		**6.04**	±0.44		**6.01**	±0.34	
RREC	**5.40**	±0.48	**5.67**	±0.24		**5.66**	±0.27		**5.46**	±0.19		**5.63**	±0.09		**5.92**	±0.19	
ATO	**5.28**	±0.47	**5.56**	±0.38		**5.50**	±0.29		**5.67**	±0.33		**5.71**	±0.14		**5.90**	±0.23	
PRO	**5.74**	±0.69	**6.07**	±0.99		**6.18**	±0.92		**5.72**	±0.71		**5.88**	±0.32		**6.13**	±0.53	
FPRAU	**5.83**	±0.86	**5.93**	±0.71		**5.81**	±0.54		**5.69**	±0.51		**5.71**	±0.26		**5.95**	±0.28	
DSV	**5.28**	±0.78	**5.60**	±0.65		**5.55**	±0.36		**5.38**	±0.36		**5.47**	±0.05		**5.84**	±0.24	
CHIS	**5.24**	±0.66	**5.48**	±0.45		**5.52**	±0.22		**5.53**	±0.55		**5.54**	±0.19		**5.83**	±0.23	

Fermentation of the substrates resulted in changes in bacterial groups as observed using FLOW-FISH. Low tyrosine lead to no significant bacteriological changes whilst high tyrosine lead to an increase in total bacteria after 4h fermentation compared to baseline. The addition of FOS to low tyrosine resulted in significant increases in *Bifidobacterium* at t4 and of total bacteria and bacteria within the *C. histolyticum* group following 8 h fermentation when compared to t0. At time 48 h in the low tyrosine with FOS treatment there were more *Bacteroides*, compared to the blank vessel.

In the presence of FOS, the fermentation of high tyrosine resulted in significant increases in bacteria within the *E. rectale* group at t4; lactobacilli at t8 and *Roseburia* at t 24 as compared to t0. When compared to the blank vessel fermentation of high tyrosine with FOS resulted in more total bacteria, bacteria in the *Atopobium* group and members of the *Faecalibacterium prausnitzii* group.

Albumin treatment resulted in reduced clostridial cluster IX at t4, increased *Bacteroides* at t8 and increased *Atopobium* at t30 as compared to t0. Compared to the blank vessel, albumin lead to enhanced lactobacilli at t30 and reduced DSV following 48 hours fermentation. Fermentation of soybean protein resulted in no significant changes of the groups monitored.

Fermentation of FOS resulted in increases in *Bacteroides* at t4, *Bifidobacterium* and *Bacteroides* at time 30 h and total bacteria at time 48 h as compared to t0. When compared to the blank vessel FOS resulted in significant increases in *Bifidobacterium* after 30 hours, enhanced total bacteria after 8 hours and enhance *Atopobium* after 48 hours fermentation. Fermentation of peptone resulted in increases in *E. rectale* group after 8 hours fermentation and total bacteria and *Roseburia* after 24 hours.

4.5.2 Indole and Phenols

There was considerable inter-individual variation in the synthesis of organic metabolites in the batch culture fermentation supernatants according to the donor inoculum used; for the purposes of clarity, **Figure 4.2** shows metabolites produced with inoculae from volunteer 1 only, and shows variance in the production of proteolytic metabolites in fermentation supernatants, there was higher concentration of these metabolites at 30 hours, hence this data has been used. Data obtained using different fermentation substrates (three replicates within the same faecal donor) is in supplementary **Figure 4.1** for a comparison of inter-individual metabolite production. 4-cresol was produced in the highest concentrations using a basal media supplemented with a mixture of FOS (1.5:100 w/w) and tyrosine (0.3:100 w/w), reaching a concentration of 17.2 mM. Modest concentrations of 4-cresol were also observed in batch cultures supplemented with tyrosine at high concentration (0.3:100 w/w) reaching a concentration of 12 mM or low doses (0.003:100 w/w). Lower concentrations of 4-cresol were produced in fermentations where the media was supplemented with meat peptone (0.3:100 w/w), soy protein (0.3:100 w/w) or albumin (0.3:100 w/w). Using supplementary FOS alone in the media did not lead to appreciable production of 4-cresol (0.2 mM).

The highest concentrations of phenol (9.3 mM) were observed in the fermentation supernatant where the culture media was supplemented both tyrosine and FOS (0.3:100 w/w and 1.5:100 w/w). With all other substrates phenol concentrations were much lower. Indole production was also greatest (0.8 mM) in the fermentation supplemented with the combination of high tyrosine and fructo-oligosaccharide. It was produced in the lowest concentrations in fermentations supplemented with FOS alone (1.5:100 w/w) (0.06 mM), but was relatively abundant in fermentations with increasing concentrations of high tyrosine (0.3:100 w/w) tyrosine and FOS (0.3:100 w/w 1.5:100 w/w) and peptone meat (0.3:100 w/w)

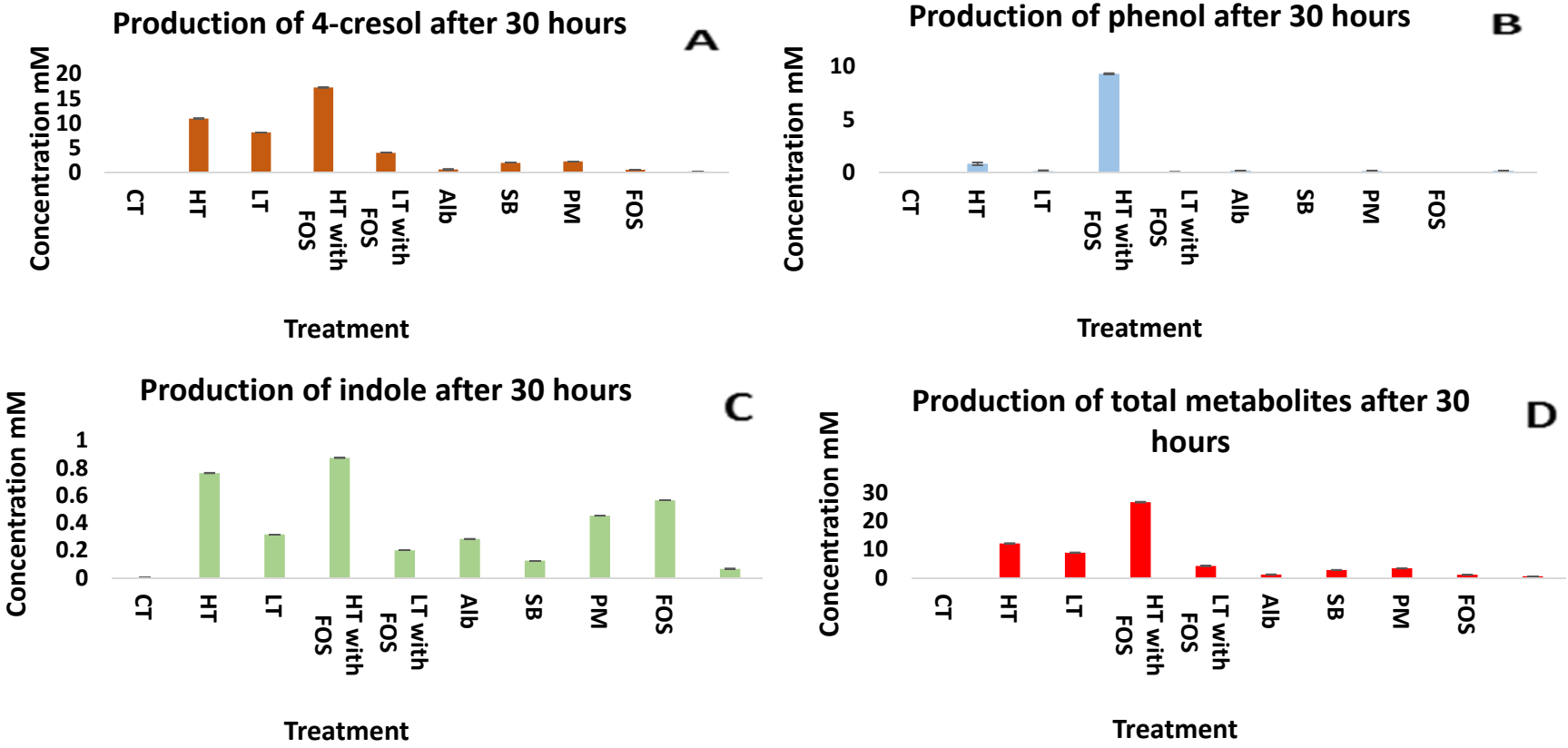

Figure 4.2: Concentrations of 4-cresol (A), phenol (B), indole (C) and total organic acid (D) using from mixed culture fermentation supernatants at 30 hours using faecal innoculate from volunteer 1; High tyrosine (HT) (0.3:100 w/w), Low tyrosine (LT) (0.003:100 w/w), High tyrosine with FOS (HT with FOS) (0.3:100 w/w and 1.5:100 w/w), Low tyrosine with FOS (LT with FOS) (0.003:100 w/w and 1.5:100 w/w), Soybean (SB) (0.3:100 w/w),, Peptone meat extract (PM) (0.3:100 w/w), and fructo-oligosaccharide (FOS) (1.5:100 w/w) after 30hrs incubation. The data presented as mean (±SEM) comparable to the control (n=3).

4.5.3 Short Chain Fatty Acids

Inter-individual variation in the synthesis of SCFA in batch culture fermentation was observed according to the inoculum used; again for the purposes of clarity, data are presented here for volunteer 1 only (see supplementary **Figure 2** for comparisons with volunteers 2 and 3). **Figure 4.3** shows the SCFA concentrations of fermentation supernatants at 30 hours with different supplementary substrates (three replicates within the same faecal inoculum). The highest production of SCFA was observed using a basal media supplemented with a mixture of tyrosine (0.3:100 w/w) and FOS (1.5:100 w/w), reaching concentrations of, 33 mM of acetate, 9 mM propionate and 6 mM butyrate. As anticipated, the presence of supplemental FOS led to higher SCFA concentrations with or without sources of supplemental nitrogen. The lowest concentrations of SCFA were produced in the negative control, indicating the baseline potential of the microbiota to produce SCFAs without the additional of substrates to the media.

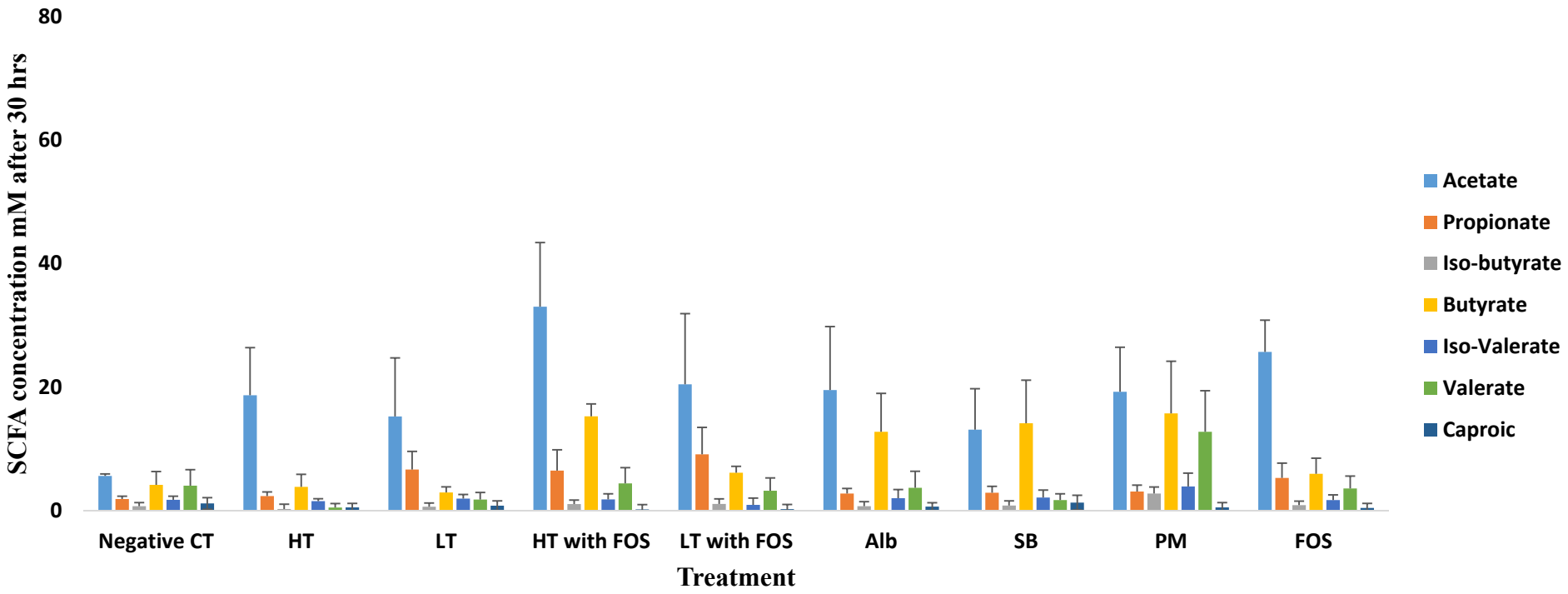

Figure 4.3: Concentration of SCFA from mixed culture fermentation supernatants at 30 hours using faecal inoculate from volunteer 1; High tyrosine (HT) (0.3:100 w/w), Low tyrosine (LT) (0.003:100 w/w), High tyrosine with FOS (HT with FOS) (0.3:100 w/w and 1.5:100 w/w), Low tyrosine with FOS (LT with FOS) (0.003:100 w/w and 1.5:100 w/w), Soybean (SB), Peptone meat extract (PM) and fructo-oligosaccharide (FOS) (1.5:100 w/w) after 30 hrs incubation. The data presented as mean (±SEM) comparable to the control (n=3).

4.5.4 Genotoxicity of fermentation supernatants

For clarity, in **Figure 4.4** data presented show the induction of DNA damage in HT29 cells by fermentation supernatants by supplementary regime for volunteer 1. DNA damage was assessed via the COMET assay following a 24 hour exposure to the filter sterilised supernatant at 10% of the carrier media. The highest observed levels of DNA damage were reported for the fermentation supernatant with the high tyrosine supplementation (with and without FOS), they were lowest in the fermentations supplemented with FOS alone. The low tyrosine, low tyrosine with FOS, albumin, soybean and peptone meat fermentations all produced moderately genotoxic fermentation samples.

Using the genotoxicity and metabolite data from all three volunteers the study were able to regress the measured metabolites for individual fermentation supernatants against the reported genotoxicity. The best predictors of genotoxicity were 4-cresol, acetate and iso-valerate. Of note however, acetate and iso-valerate are independently strong correlates of 4-cresol ($p= 0.001$ and < 0.000 respectively).

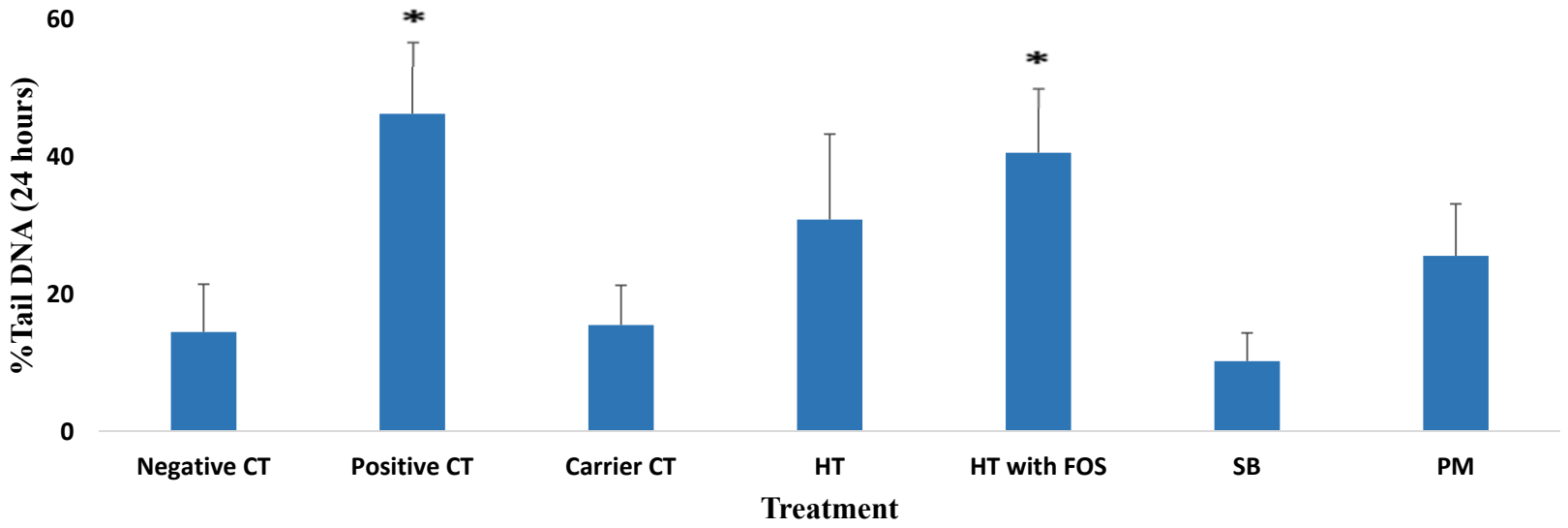

Figure 4.4: Effect of different fermentation supernatants (High tyrosine (HT), High tyrosine with FOS (HT with FOS), Soybean (SB) and Peptone meat (PM) on DNA damage for 24 hrs exposure on HT29 cell line. The data presented as mean (±SEM) percentage of DNA damage comparable to the control (*n*=3). * indicate a significant difference compared to the untreated control (Dunnett test; $^{*}p<0.05$).

Table 4.3: **Regression liner model test shows the effect of the metabolites from fermentation supernatants; 4-cresol, phenol, indole and SCFA and BSCFA in genotoxicity. Values are log_{10} biological replicates.**

	Metabolite predictor	**r**	***P value***
Model 1	4-cresol	0.775	0.002
	Phenol		0.317
	indole		0.708
Model 2	4-cresol	0.681	0.005
Model 3	SCFA		
	Acetate	0.759	0.001
	Iso-valerate	0.995	0.000
	Propionate	0.477	0.407
	Butyrate		0.820
	Iso-butyrate		0.199
	Valerate		0.249
	Caproic		0.241

4.5.5 4-cresol genotoxicity and cytotoxicity

The independent genotoxic effects of increasing concentrations of 4-cresol against both HT29 and Caco-2 cells, following a 24 hr treatment in carrier cell culture media, are shown in **Figure 4.5**. In the top pane of the figure cell viability established via DAPI assay with equivalent exposures is displayed. Cell viability was maintained above 85 % at each of the doses used although there was a trend towards increasing cytotoxicity at 3 mM (**Figure 4.5)**.

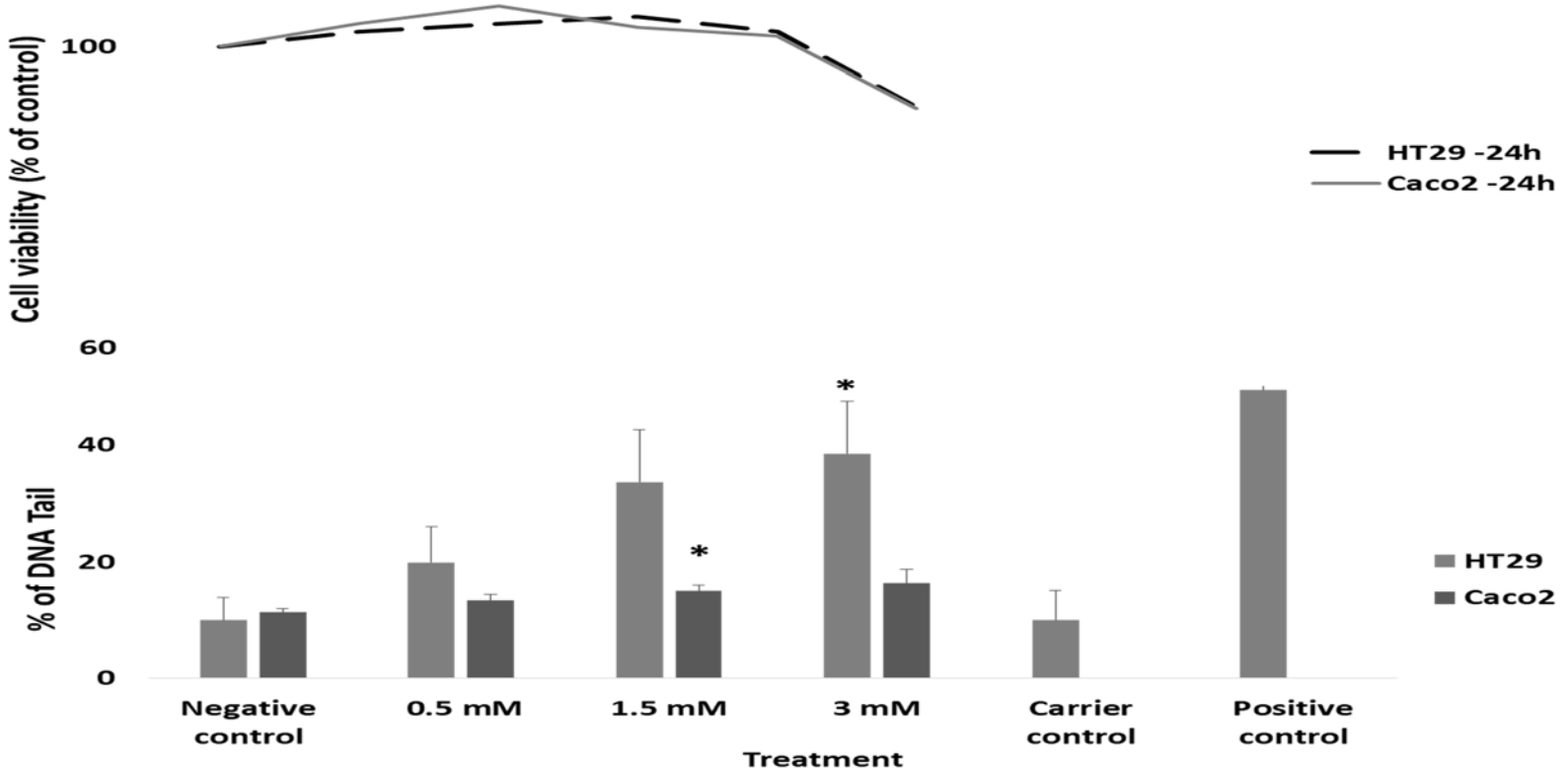

Figure 4.5: The top figure shows the cytotoxicity of 4-cresol (cell viability) present as mean (±SEM) percentage (n=4-6). Cells were incubated with different concentration of 4-cresol at 0, 0.2, 0.5, 1.0, 1.5 and 3 mM for 24 hours on HT29 and Caco-2. The data presented as mean (±SEM) percentage of viable cells comparable to the control (n=4-6).* indicate a significant difference compared to the untreated control (Dunnett test; *p<0.05). The lower figure shows the DNA strand breaks on HT29 and Caco-2 cells with different 4-cresol concentration 0,0.5,1.5 and 3.0mM for 24 hours. Values are means ±SEM biological replicates. * indicate a significant difference compared to the untreated control (correlation coefficient).

There was a dose dependent increase in DNA damage with increasing concentrations of 4 cresol reaching statistical significance at concentrations of 3 mM for both cells lines ($p = <0.05$) the observed DNA damage was slightly higher in the HT29 cells.

Internally spiked fermentation supernatants: To consider the effects of increasing 4-cresol concentrations within the context of the gut microbial environment the study spiked selected fermentation supernatants, post-fermentation with either low (0.2mM) or high (3mM) doses of exogenous 4-cresol and assessed genotoxicity against the HT29 cells **Figure 4.6** with the higher 4-cresol spike significant increase in DNA strand breaks was observed.

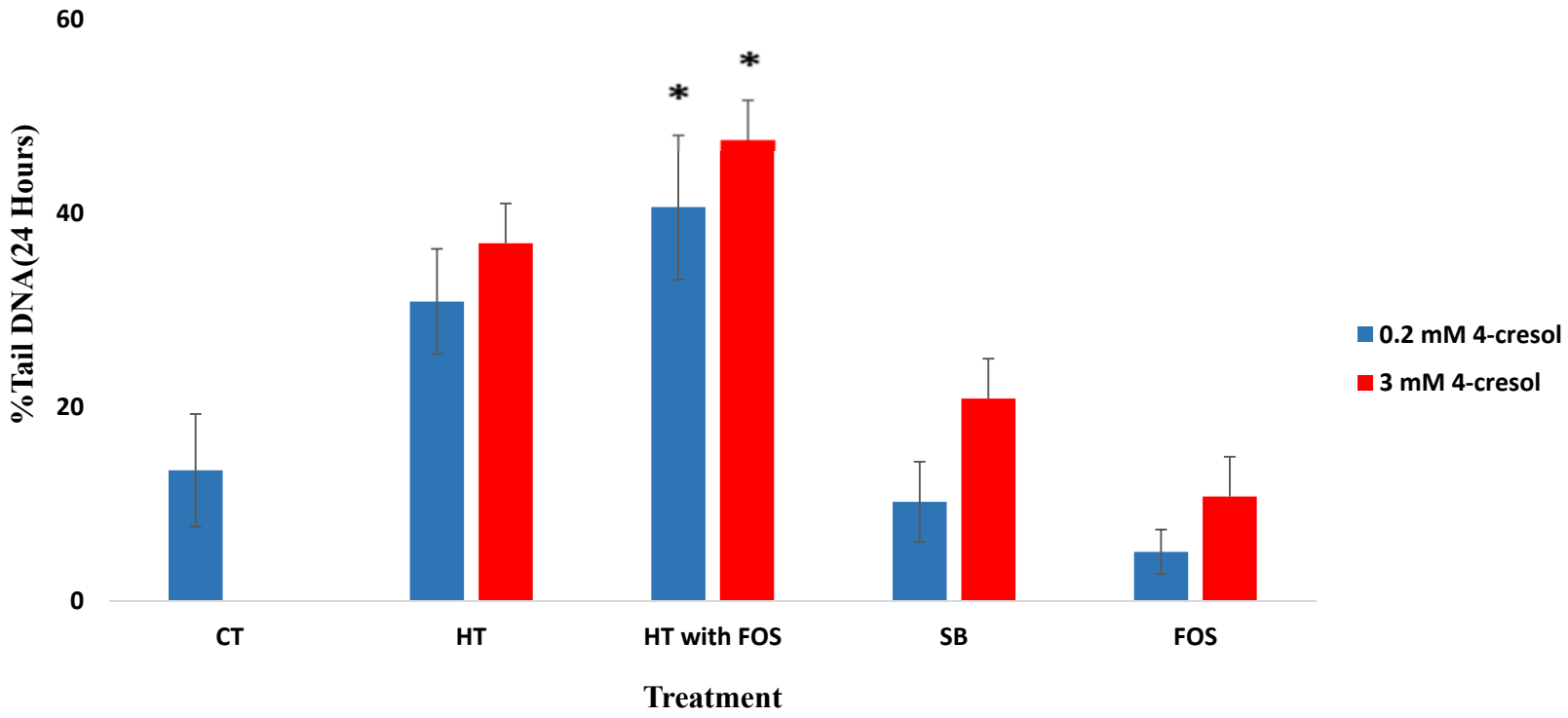

Figure 4.6: Spiked cell with low dose 0.2mM and high dose 3mM of 4-cresol in high production of 4-cresol from fermentation supernatants High tyrosine (HT), High tyrosine with FOS (HT with FOS), and low production of 4-cresol from Soybean (SB) and FOS alone on DNA damage for 24 hrs on HT29 cell line. The data presented as mean (±SEM) percentage of DNA damage comparable to the control (*n*=3).

4.5.6 4-cresol and cell cycle kinetics

Both the HT29 (A) and Caco-2 (B) cells treated with 4 cresol for 24 hours, before observing disruptions to cell cycle behaviour **Figure 4.7**. There was a non-linear dose response to 4-cresol, with the changes observed perhaps relating to levels of DNA damage. At lower exposures of up to 0.5 mM 4-cresol, the study observed decreases in the abundance of cells in G_0/G_1 with a compensatory increase in the proportion of cells in S phase, however at higher concentrations the proportion of cells in G_0/G_1 increased significantly relative to the proportion of cells in S phase in both cell lines, suggesting a slight growth promoting effect at lower doses and G_0/G_1 growth arrest in response to genotoxic insult at higher doses.

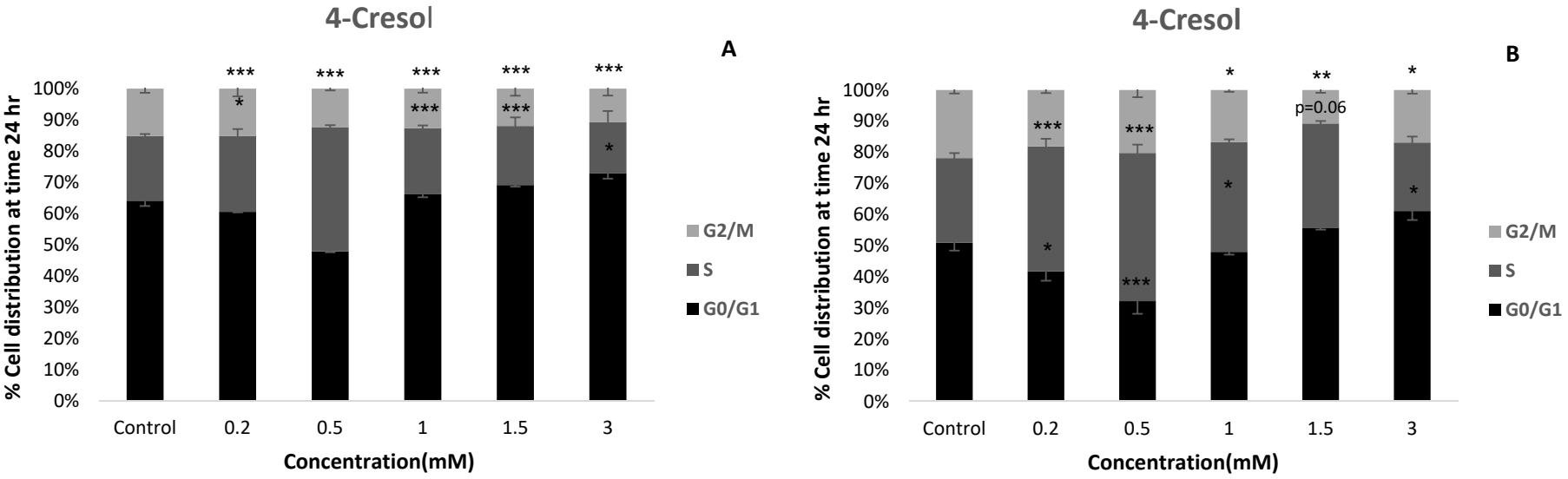

Figure 4.7: Cell cycle progression of HT29 (A) and Caco-2 (B) cell line treated with 0.2, 0.5, 1.0, 1.5 and 3mM of 4-cresol for 24hours. The percentage of cells in each phase of the cell cycle was determined using flow cytometry to quantify DNA content (n= 4). Asterisks indicate a significant difference compare to the untreated control (Dunnett test; * p<0.05, ** p <0.01, ***p<0.001)

4.6 Discussion and conclusions

Here, the synthesis of 4-cresol in a batch culture model of the human colon and assessed its effects on cultured human colonocytes has been evaluated. In this model, it was first observed that the resident microbiota influences the subsequent metabolic profile of gut fermentation supernatants given the, same dietary substrate. Then demonstrated that the genotoxicity of gut fermentation supernatants may be influenced by dietary fermentation substrates, and that this genotoxicity may, in part, be predicted by the concentrations of the metabolite 4-cresol. This study observed that 4-cresol induces genotoxic insult in colorectal cell lines, both independently, and as a supplementary component of faecal fermentation supernatants. Finally, the study show that 4-cresol interferes with normal cell cycle kinetics in two separate human colorectal cancer cell lines, stimulating DNA synthesis at low doses, and cell cycle arrest at genotoxic concentrations. The fluctuation of 4-cresol toxicity suggests a slight growth promoting effect at lower doses and G0/G1 growth arrest in response to genotoxic insult at higher doses.Changes in cell kinetics are a hallmark of carcinogenesis, and may be considered tumour promoting. These results support our original hypothesis that 4-cresol may contribute to a pro-carcinogenic colonic environment.

At baseline, the faecal inoculate from volunteer 1 was characterised by a higher relative proportion of *Bifidobacterium* (BIF164), *Atopobium* cluster (ATO 291) and *Desulfovibrio* (DSV 687) than volunteers 2 and 3; and a lower relative abundance of bacteria staining positive for *Faecalibacterium* (FPRAU 655) and *Propionibacterium* (Prop 853) and *Lactobacillus* (Lab 158) . The microbial composition of the faecal inoculate from volunteers 2 and 3 were more closely aligned and were associated with a lower production of 4-cresol and other metabolites. Importantly, the study also show that fermentation substrates can influence both composition of the microbiota and the subsequent production of beneficial and detrimental metabolites.

The total bacteria count was increased in particular following fermentation with broths supplemented with high levels of tyrosine, high levels tyrosine with FOS or with peptone meat

extract. Furthermore, the high tyrosine broth and the high tyrosine with FOS broths induced increases in the proportion of the proteolytic *Clostridium histolyticum* group (Chis 150) following fermentation. However, soybean and FOS supplemented broths favoured the growth of the saccharrolytic genera with observed increases in the proportions of bacteria staining for BIF, LAB and BAC.

According to the fermentation results in this study, microbial changes observed following fermentation indicated limited impact of tyrosine on the microbiota, however, enhanced activities were observed with the addition of FOS **(Table 4.2).** Such changes impacted on a range of groups, including *Roseburia*, a key butyrate producing group; but also *Atopobium* and *Faecalibacterium prausnitzii*. Whether these changes are of benefit are difficult to determine based on the bacterial changes, although alteration in a wide range of microbial groups points to reduced selectivity - this conclusion is further supported by the increased genotoxicity observed. A large increase in total bacteria was observed at 30 hours, this change implies a microbial group that was not monitored also increased at this time point and may account for some of the potentially negative changes seen. This result was not expected as a positive impact of the prebiotic was expected; however, in an *in vitro* environment with limited substrate availability the bacteria are competing in a different way as to how they would *in vivo* [25-27]. These results were in line with a study results by Vipperla O'Keefe who found that when the gut bacteria composition was shifted by animal protein diet and contained fewer bacteria that produce the short chain fatty acid butyrate, and more potentially harmful bacteria such as *Fusobacterium nucleatum, Rhodopseudomonas faecalis, Bacteroides vulgatus* and *Enterococcus faecalis,* the latter being a superoxide producer that may damage epithelial DNA [28]. Furthermore, a results from Hildebrandt et al, study found that Sulfate reducing bacteria is considered harmful for the gut epithelium and can damage the DNA through production of free radicals [29]. In the present study it was seen that several

bacterial groups, that include proteolytic genera, such as *Bacteroides, Clostridium* and *Fusobacterium* increased, along with genotoxicity after 24 hours. These observation are associated with high proteolytic activities and thus DNA damage [30].

In this study, it was selectively measured the concentration of several known faecal metabolites in the fermentation supernatants. The short chain fatty acids are generally seen as beneficial products of both the proteolytic and saccharolytic fermentation; whereas indoles, phenols and cresol might be viewed as potential toxins produced during proteolytic fermentation. As expected, supplementing fermentation broth with the prebiotic FOS led to enhanced synthesis of the saccharolytic metabolites (SCFA) with little effect on the production of cresol, indole or phenol, whereas supplementing broths with tyrosine or sources of protein induced increases in the production of the proteolytic metabolites but also increased the production of acetate and butyrate. Furthermore, combining prebiotic FOS with supplemental tyrosine in the fermentation broth led to the highest concentrations of the measured fermentation supernatants. This was a surprising result, as a rescuing effect of FOS may have been expected in terms of protein fermentation end-products. However, enhanced SCFA produced when FOS is additionally present may offer benefits to the host [31]. 4-cresol can be found in human faeces at concentrations of up to 0.5 mM; *in vivo* 4-cresol is largely absorbed and metabolised to 4-cresol sulphate appearing in urine at concentrations of up to 0.3 mM [32]. Therefore faecal 4-cresol poorly reflects colonic concentrations, and further the normal range of concentrations through the colon remains uncertain. In this study, *in vitro* batch culture fermentation system were flawed, in that there is no absorptive, or flow through, clearance of 4-cresol from the system and no replenishment of substrate, as would occur *in vivo*; nevertheless, by experiencing this build up 4-cresol production can be observed; and as the study observed 4-cresol at concentrations of up to 17 mM, suggesting that the 0.5 mM concentrations reported in faeces may significantly under represent potential intestinal epithelial exposures.

The study assessed the genotoxicity of our fermentation supernatants against HT29 cells using the Comet assay. The post fermentation supernatant resulting from broth supplemented with high tyrosine plus FOS was observed to be the most genotoxic. Regressing genotoxicity against the metabolites measured in the fermentation samples from all three volunteers, it was found that the best independent predictor of genotoxicity was the concentration of 4-cresol **(Table 4.3).** The study observed considerable co-linearity in the concentrations of the metabolites in our fermentation samples, and further, the study was uncertain of the potential for collinearity between 4-cresol and other un-characterised potential genotoxins in our fermentation supernatants. Therefore in a follow up experiment the study spiked a selection of fermentation supernatants with 0.2 or 3 mM of 4-cresol and observed a consistent dose-dependent increase in supernatant genotoxicity.

Having established that 4-cresol contributes to the observed genotoxicity in the fermentation samples from this gut model, the next phase was to establish the independent effects of 4-cresol against two separate colonic cell lines. 4-cresol was observed to be cytotoxic at doses 3 mM and up for both HT29 and Caco-2 cells; HT29 cells appeared more sensitive to 4-cresol mediated genotoxicity than the Caco-2 cells, however in both cell lines this study observed a linear dose dependent increase in DNA damage up to 3 mM. The study observations are consistent with previous work by Andriamihaja et al, who used the γH2AX assay and observed genotoxicity against both HT29 $Glc^{-/+}$ and LS-174T human colonic cell lines at concentrations of >1.5 mM [33].

Then proceeded to study the effects of exposure to this genotoxin on cell cycle activity. At lower concentrations the abundance of cells in S phase was increased in both cell lines with a subsequent decrease in the abundance of cells in G_0/G_1. This observation of a mitogenic response to low dose 4 cresol might explain the tumour promotion demonstrated by Boutwell

and Bosch [34] for 4-cresol in a classical murine papilloma study following tumour initiation with 9,10-dimethyl-l-benzanthracene.

At higher doses the study observed a reduction in the proportion of cells in S phase and an increase in proportion of cells in G_0/G_1 and in G2/M, perhaps indicating cell cycle arrest in response to DNA damage. The observed genotoxicity and mitogenicity therefore represent two separate, and complimentary, potential pro-carcinogenic properties of colonic 4-cresol. The models have employed in this study are widely used and accepted in mechanistic studies of dietary exposures related to colorectal cancer; they represent different aspects of the carcinogenic process, and so it is interesting that 4-cresol exerts effects on each of these models. Having said that, these are *in vitro* systems, the anti-cancer defence mechanisms of the colonic epithelium are potentially very different *in vivo*, and the complexity of the microbiota and the environment of the gut lumen confounds the ability to draw firm conclusions regarding the potential carcinogenic effects of 4-cresol *in vivo*. One human randomised crossover trial with high and low protein diets reported a weak correlation between urinary 4-cresol excretion and FW genotoxicity [35].

Carcinogenicity in humans has not yet been proven for 4-cresol; based on a very small (n=6 cases) case control study by Bone and Tamm [36] argued that comparable urinary concentrations of 4-cresol in from volunteers with bowel cancer to that from the urine of healthy controls was evidence that this metabolite is not affecting CRC risk; ours and other emerging data would challenge this. Evaluation of 4-cresol in stored urinary samples from existing prospective cohort studies may help establish the strength of any relationship with cancer risk and further validate the use of urinary 4-cresol as a biomarker of risk for intervention studies.

4.7 Acknowledgment

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Chapter V

5. Genotoxicity of culture supernatants derived from intestinal tumour associated fusobacteria.

5.1 Abstract

Background: *Fusobacterium nucleatum* are typically present in oral mucosa but not in healthy gastrointestinal mucosa; several groups now report the adherence of fusobacteria to inflammatory bowel specimens as well as to colorectal adenoma and tumour tissues. It remains unclear as to whether fusobacteria act as a driver or a passenger in colorectal cancer.

Aim: Here, we assess the genotoxicity of culture supernatants from *F. nucleatum* samples isolated from CRC tissue and further, we assess the influence of these supernatants on the cell cycle activity of the intestinal HT29 cell line.

Methods: 18 *F. nucleatum* strains were isolated from tumour tissue and anaerobically cultured in a modified tryptic soy broth for 24 hrs. For the purity of these strains, these were isolated on selective agars, and strain type identified. The isolated fermentation supernatants were analysed for metabolite composition, and then used to treat HT29 cells, with assessment for DNA damage via comet assay, cells proliferation via DAPI, and cell cycle kinetics via propidium iodide staining with flow cytometry.

Results: All 18 *F. nucleatum* specimens produced supernatants which induced DNA damage at levels above those observed for the carrier control. The range in associated DNA damage correlated most positively with the concentration of 4-cresol in the culture supernatant. The *F. nucleatum* supernatants increased the rate of cell proliferation after a 24-hour incubation in some strains, however, with strain specific effects observed on cell cycle kinetics.

Conclusions: We demonstrate that supernatants from the fermentation of *F. nucleatum* contain metabolites which may be both genotoxic and growth promoting to intestinal tumour cells, this work potentially implicates tumour associated *F. nucleatum* in carcinogenic process, although further studies are needed.

Key words: *F. nucleatum*, IBD, colorectal cancer, HT29 cell line, DNA damage, cell proliferation and cell cycle.

5.2 Introduction

Understanding passenger-driver relationships in relation to the colonic tumor-adherent microbiome is of interest. The emergence of culture independent techniques in microbiology has revealed an enriched presence of adherent *F. nucleatum* in colorectal carcinoma tissues [1, 2]. Normally identified as an oral-commensal bacteria, the presence of *F. nucleatum* in tumour tissue raises questions about disease causality. The tumour microenvironment is characterised by a dysplastic mucosa, inflammation, occult blood and disruption to the colonic flow, it may therefore provide an ecological niche for adaptable bacteria [3, 4]. Alternatively, commensals from within the intestinal microbiota are implicated in inflammation, the production of genotoxins, and of metabolites which influence host cell behaviour through epigenetic mechanisms [5]. The role of individual members of the gut microbial community remains poorly defined, whilst developing an understanding of optimal microbiota composition for colon cancer prevention might allow for nutritional or pharmaceutical strategies which beneficially attenuate the microbiome [6]. Here, we focus on the specific role of *F. nucleatum* in colorectal cancer. Several studies have shown that the adherence of *F. nucleatum* in tumour samples may be specifically associated with a high level of microsatellite instability and the CPG island methylator phenotypes (CIMP) [9]. This association with a molecular sub type of intestinal tumour favours the causation/driver hypothesis, although a mechanistic explanation is needed.

In the present study, we have obtained *F. nucleatum* strains previously isolated from tumour mucosa and kindly donated by Dr Alasdair Scott of Imperial College London to generate fermentation supernatants which were characterised for the presence of genotoxic and other metabolites, we then took these supernatants and applied them to the HT29 cell line to evaluate their influence on cell behaviour. Colonic genotoxicity is implicated as an exposure which may initiate the tumour process through the induction of loss or gain of function in tumour

suppressor or oncogenes respectively [7]. On the other hand, mitogenic exposures which lead to enhanced cell cycle kinetics may be considered tumour promoting [8, 9]. Thus our models were selected to represent different phases of the cancer process.

5.3 Materials and methods

28 *F. nucleatum* strains isolated from human tumour mucosa were gifted by Dr Alasdair Scott St Mary's Hospital, London, UK. Since *F. nucleatum* are often present in mixed cultures with other obligate anaerobes and with facultative species, the use of a selective medium is often necessary for their isolation. 18 *F. nucleatum* strains were isolated from tumour tissue and anaerobically cultured in a modified tryptic soy broth for 24 hrs. For purity of these strains, they were isolated on selective agars, and strain the type strain were identified through amplicon sequencing. The strains in this study were transferred into modified tryptic soy broth (tryptic soy broth with hemin (5mg/L), menadione (0.5mg/L) and, L-cysteine HCl 0.25mg). 18 of the received strains proved culturable. These were transferred from broth into modified tryptic soya agar and incubated for several days anaerobically. Plates were inspected every day for growth. From the agar plates individual colonies were aseptically transferred into hungate tubes (modified tryptic soya media) and incubated for 24 hours at 37 ºC. After that, in a new fresh hungate tubes containing 10 mL of basal media (chapter 5-has the recipe of basal media) with peptone meat added as a protein source, the strain was transferred and incubated again for 24 hrs at 37°C. After 24 hours' incubation, 1 mL of fermentation supernatant was transferred into an Eppendorf and stored in -20°C for further analysis. The remaining 9 mL transferred into a falcon tube, centrifuged (13,000 x *g*) for 10 minutes; the resulting supernatant was filter sterilised (0.22 mm filter Milipore) and transferred into 1 mL Eppendorf tubes. This experiment was conducted in triplicate. The fermentation supernatants of the strains were analysed by GC

MS to determine the concentrations of 4-cresol, phenol and indole and the short chain fatty acids (SCFAs), acetate, propionate, butyrate, isobutyrate, valerate, isovalerate and caproic acid.

5.3.1 Chemicals

4-cresol ($CH_3C_6H_4OH$), Phenol (C_6H_6OH), indole (C_8H_7N), McCoy's 5 A with L-glutamate, Trizma base, agarose, EDTA, Triton x100, hydrogen peroxide (H_2O_2), Fructooligosaccharides (FOS), ethidium bromide, propidium iodide (PI) and RNase A were purchased from Sigma-Aldrich Ltd. (Dorset UK). Sodium chloride (NaCl) and potassium chloride (KCl) were supplied by Fisher Scientific (Loughborough, UK). The HT29 colorectal adenocarcinoma cells line was obtained from the European Collection of Animal Cell Cultures (ECACC) (Salisbury, UK) and used between passages 70 and 85. Essential Medium (MEM), McCoy's 5A with L-gulatumate, penicillin-streptomycin and fetal Bovine Serum (South America) were purchased from Biosera Ltd. (East Sussex, UK). Non-essential Amino Acid (NEAA) (Lonza group Ltd. Basel, Switzerland). Phosphate Buffered Saline (PBS), Trypsin-Versene and Ethylenediaminetetraacetic acid (EDTA) were purchased from Lonza group Ltd. (Basel, Switzerland). Non-essential Amino Acid (NEAA). Bacteriological growth medium supplements were obtained from Oxoid Ltd. (Basingstoke, Hants, U.K.).

5.3.2 GC MS Analysis

4-Cresol, phenol and indole analyses were carried out by automated headspace solid-phase microextraction (SPME). Then followed by gas chromatography-mass spectrometry (GC-MS), using an Agilent 110 PAL injection system and Agilent 7890 gas chromatograph with 59705C mass spectrometer (Agilent, Santa Clara, CA). The SPME fibre stationary phase was composed of 75 µm divinylbenzene/Carboxen™ on polydimethylsiloxane; Supelco, Bellefonte, PA).

Sample (0.1 mL) was placed in a 20-mL headspace vial with magnetic screw cap and PTFE/silicone septum (Supelco). The samples were then equilibrated for 10 minutes at 35 °C before being extracted for 30 min. Sample was agitated at 500 rpm (5 s on, 2 s off) during equilibration and extraction. After extraction, the contents of the fibre were desorbed onto the front of a Stabilwax-DA fused silica capillary column (30 m ′ 0.25 mm i.d, 0.50 mm film thickness; Restek, Bellefonte PA). The GC temperature program and the fibre desorption step commenced at the same time. During the desorption period of 45 s, the oven was held at 40 °C. After desorption, the oven was held at 40 °C for a further 255 s before heating at 4°C/min to 260°C, where the temperature was maintained for 5 min. Helium was used as the carrier gas at a constant flow rate of 0.9 mL/min. The mass spectrometer operated in electron impact mode with an electron energy of 70 eV, scanning from m/z 20 to m/z 280 at 1.9 scans/s.

5.3.3 Organic acid analysis (SCFAs)

The main organic acid production was determined by Gas Chromatography (GC) (Hewlett Packard, UK). Samples from *F.nucleatum* fermentation were screened for the short-chain fatty acid (SCFA) concentrations using an acidification method adapted from Zhao, G et al [10]. Briefly fermentation samples were defrosted, vortexed and centrifuged for 5 minutes at 13,400 x *g*. The samples were filtered using a 0.22 µm filter and sulfuric acid was added to bring the pH down to 2. 200 µl of the resulting sample solution and 50 µl of internal standard was added to the vial. The internal standard used in this experiment was 2-ethylbutyric acid (Aldrich) made to concentration of 100 mM in HPLC grade water. The GC apparatus was calibrated for detection of acetate, propionate, iso-butyrate, butyrate, iso-valeric, valeric and caproic acid using standards of a range of concentrations (5mM – 50mM).

Analysis was conducted using a HP 5890 series II GC system (Hewlett Packard, Palo Alto, Calif) with an FFAP, capillary fused silica packed column 25 m by 0.32 mm; filter thickness,

0.25µm (Macherey-Nagel, Düren, Gemany). Afterwards, the sample was injected into the column, which was maintained at 140°C for 5 minutes. Then the column temperature was increased over 5 minutes to 240°C. The calibrated organic acids were detected in the samples and the concentrations calculated. An external standard with known concentrations of SCFAs were injected after every 10 samples to maintain appropriate calibration. Finally, peaks were analysed and integrated using HP GC ChemStation Software, Hewlett Packard.

5.3.4 Tissue culture

The HT29 colorectal adenocarcinoma cell line was obtained from the European Collection of Animal Cell Cultures (ECACC) (Salisbury, UK). Cells were cultured in McCoy's 5A media with L- gulatumate, 10% FBS 1% Pen Strep (Biosera Ltd. East Sussex, UK) and 1% NEAA (Lonza Ltd, Basel) The HT29 colorectal adenocarcinoma cell line was used to model the intestinal epithelium. Assays were performed between passages 45-55 with routine culture at 37°C with 5% CO2 and 95% humidity and passage via trypsin-versene and PBS supplied by Lonza (Basel) McCoy's 5A with L-gulatumate was used for HT29 and. The rest of the chemical has been used for HT29 cells line such as: Penicillin-Streptomycin and Fetal Bovine Serum (South America) (FBS) were purchased from Biosera Ltd. (East Sussex, UK). Phosphate Buffered Saline (PBS), Non-essential Amino Acid (NEAA), Trypsin-Versene and Ethylenediaminetetraacetic acid (EDTA) were purchased from Lonza group Ltd. (Basel, Switzerland). HT29 cells was cultured into tissue culture flasks as monolayers in the growth medium (containing 10% FBS, 1% Penicillin-Streptomycin and 1% NEAA).

5.3.5 Comet Assay

DNA damage was assessed using the single strand comet assay which is widely used to detect (SB) in single cells. HT29 cells line were seeded into separate T75 flasks at a concentration of $1x10^6$and maintained at 37ºC in an atmosphere of 5% CO2 and 95% filtered air. Cells were treated, in tissue culture flasks at 80 % confluency, with filter sterilised supernatants from the *F. nucleatum* fermentations at 10% (v/v) in McCoy's carrier culture medium with inactivated at 56°C Fetal Bovine Serum (FBS) and antibiotics at 37°C and 5% CO_2 and applied directly to the cells for 24 hours. The negative control was composed of 10% v/v unfermented modified tryptic soy broth in carrier media. The carrier control was McCoy's carrier culture medium (as described above). The positive control was additional H_2O_2 (75mM) applied in carrier media 5 minutes before cell harvest. At 24 hours' cells were washed and detached with trypsin, following centrifugation 300 x *g* for 3 minutes the supernatant was removed via aspiration, followed by washing with PBS for 1 minute. A positive control was prepared with untreated cells exposed to 7.5 mM hydrogen peroxide for 15 minutes prior to lysis. Cells were counted with trypan blue and adjusted to give a working concentration of $3x10^6$ cells/ml, 20 µl of the cell suspension was re-suspended in 200µl of melted agarose and coated on to microscope slides, then left at 4°C for 15 minutes. The slides were placed into lysis buffer (2.5M NaCl, 0.1 EDTA, 0.01 M Tris and 1% (v/v) Triton x100) for 1 hour at 4°C, and then washed 3 times with neutralising buffer (96.9 g Trizma base, 1 L water, adjusted to PH 7.5 with 6M HCl) for 5 minutes before transfer to electrophoresis buffer (0.3M NaOH and 1mM EDTA). After 20 min at 4ºC the slides were placed horizontally in an electrophoresis tank containing electrophoresis alkaline buffer to allow the DNA to unwind. Electrophoresis was run at 26V, 300mA for 40 minutes in at 4°C in the dark. The slides were then washed with neutralizing buffer (0.4 M Trizma base, pH 7.5) three times for 5 minutes each and then left for 5 minutes in 99% ethanol for 5 minutes, then left to dry overnight. Cells were stained with ethidium bromide (20ul/ml)

and kept for 15minutes in the dark. Images of DNA integrity were captured by fluorescence microscopy using the Kinetic image software, Komet 4.0 UK. One hundred randomly selected cells from each replicate slides were evaluated for DNA tail damage by an analyst blinded to the treatment.

5.3.6 Cell Proliferation Assays

HT29 cells were seeded into 96-well microplates (Thermo Fisher Scientific Inc., Roskilde, Denmark) at 1.5×10^4 cells/well and incubated at 37ºC with 5% CO_2 and 95% humidity for 24-hours. Then the different *F. nucleatum* strain supernatants were removed via aspiration. Cells were permeabilised with 100 µl of ice-cold methanol and left to incubate at room temperature for 5 minutes. Methanol was removed carefully by pipette and plates were allowed to dry in a hood for 15 minutes. Afterwards, 100 µl of DAPI in PBS (70 µl of DAPI staining stock solution (3 mM) plus 10.43 ml of PBS) was added. Finally, cells were incubated in the dark for 30 minutes at 37°C prior to measurement using a GENios microplate reader (TECAN Group Ltd., Männedorf, Switzerland) with absorbance and emission at 340 nm and 465 nm, respectively.

5.3.7 Cell Cycle Assays

Cell cycle progression was assessed considering the percentage of cells in phases Gap0/1 (G0/1), Synthesis (S), Gap2/mitosis (G2/M) and apoptotic cells (sub G0/1) according to the fluorescent intensity of a PI nuclear stain, and based on the concentration of DNA within the cell [25]. HT29 cell cultures were treated at 2 $x10^5$ cells/well in two different 6 well plates at 80% confluence. Each cell suspension was exposed to different *F. nucleatum* supernatant 10% v/v for 24 hours where the negative control was only cell suspension and the carrier control was added McCoy's carrier culture medium. After removing treatments, the cells were washed with ice cold PBS and collected following the trypsin harvest of the monolayer and pelleting

by centrifugation at 377 x *g* for 3 minutes. The supernatants were discarded and then the cell tissues were resuspended in 200 µl ice cold PBS and fixed with 2 mL of fresh ice cold 70% ethanol. The cell pellets were stored in freezer at -20°C until analysis.

After chilling, the samples were centrifuged at 277 x *g* for 5 minutes and the supernatants discarded. The pellets were resuspended with 200 µl PBS before adding 25 µl of 1 mg/ml RNAse and the suspensions were then incubated at 37°C for 30 minutes. 2.5 µl of 400 µg/ml of PI were added to bind DNA and were left to incubate for 30 min at room temperature in dark condition. Cells suspensions were adjusted to a final volume of 600 µl with PBS. The DNA content of 15,000 cells were then measured immediately via flow cytometry (BD Accuri C6 flow cytometer, Germany). Analysis was performed using the Flow Jo software (Tree star Inc, Oregon, USA).

5.4 Statistical Analysis

All statistical analyses were carried out using the Statistical Package for Social Sciences (SPSS) version 22. All data have been carried in three biological replicates for each analysis. The data are presented as mean ±SEM. Gc Ms analysis for 4-cresol, phenol and indole and GC analysis for SCFA and BSCFA were analysed by LSD one-way ANOVA. Correlation coefficient runs for total SCFAs versus total metabolites of 4-cresol, phenol and indole. *F. nucleatum* stains supernatants as predictors of genotoxicity, cell proliferation and cell cycle were evaluated using linear regression models. P values <0.05 were considered to be statistically significant between the treatments.

5.5 Results

5.5.1 Metabolite production: The production of proteolytic metabolites varied between the *Fusobacterium* isolates, although generally it remained low, with phenol reaching maximal observed concentrations of ~0.05 mM for one strain. This same strain produced the highest concentrations of 4-cresol and indole, each also in the 0.02-0.05 mM range. Most other fermentation supernatants were characterised by very low concentrations of these metabolites >0.01 mM **(Figure 5.1).**

There was similar variability in the production of SCFAs with a predominance of acetate observed in most but not all the fermentations. This was produced at concentrations between 0 and 7 mM **(Figure 5.2).** There was no apparent correlation between the production of proteolytic and saccharolytic metabolites $r = -0.25$ and $p = 0.35$.

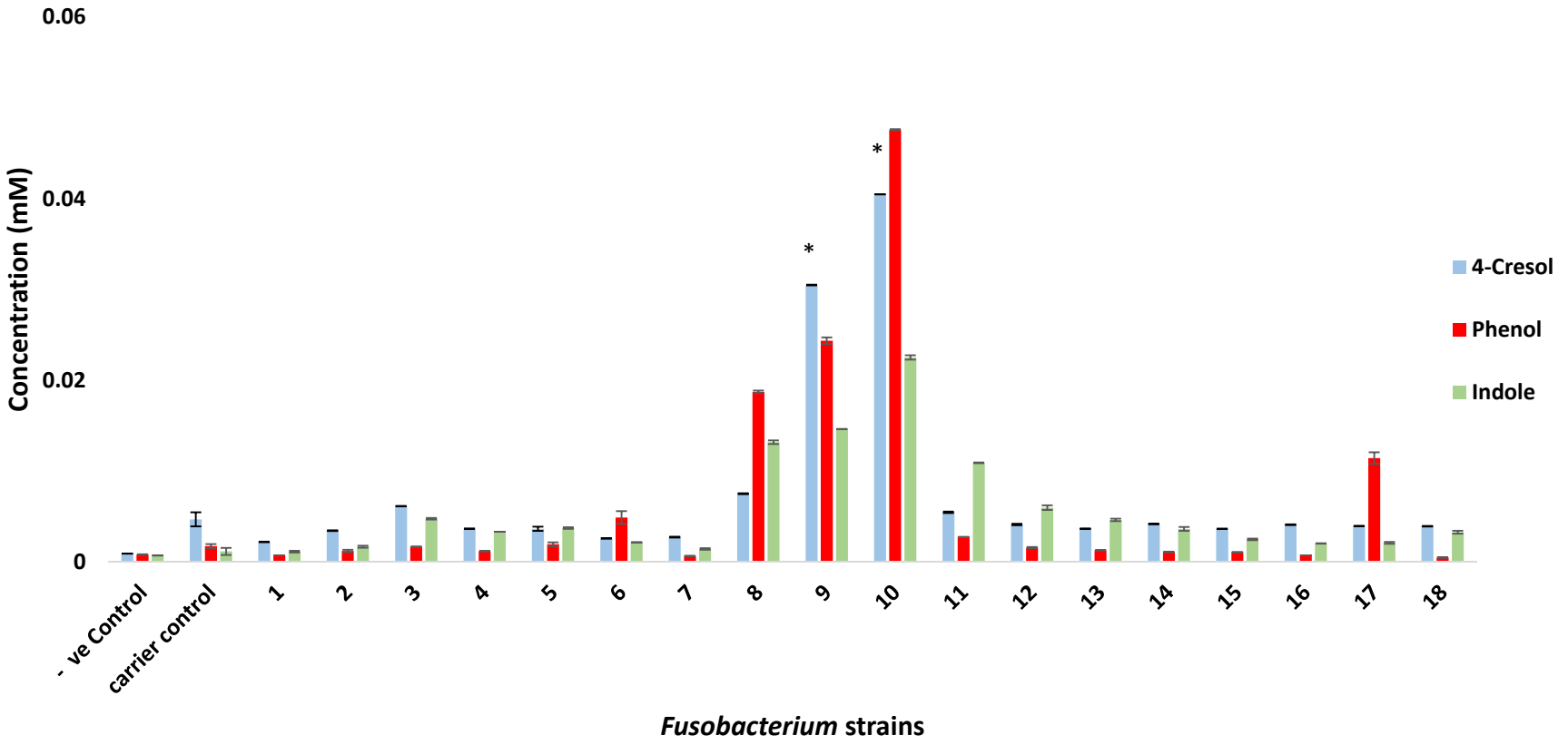

Figure 5.1: Proteolytic metabolite production, 4-cresol, phenol and indole (mM) from *F. nucleatum* fermentation supernatants. Values are means ±SEM with three replicates per sample. P values are calculated using one-way ANOVA, * indicate significant difference compare to carrier control with P<0.05

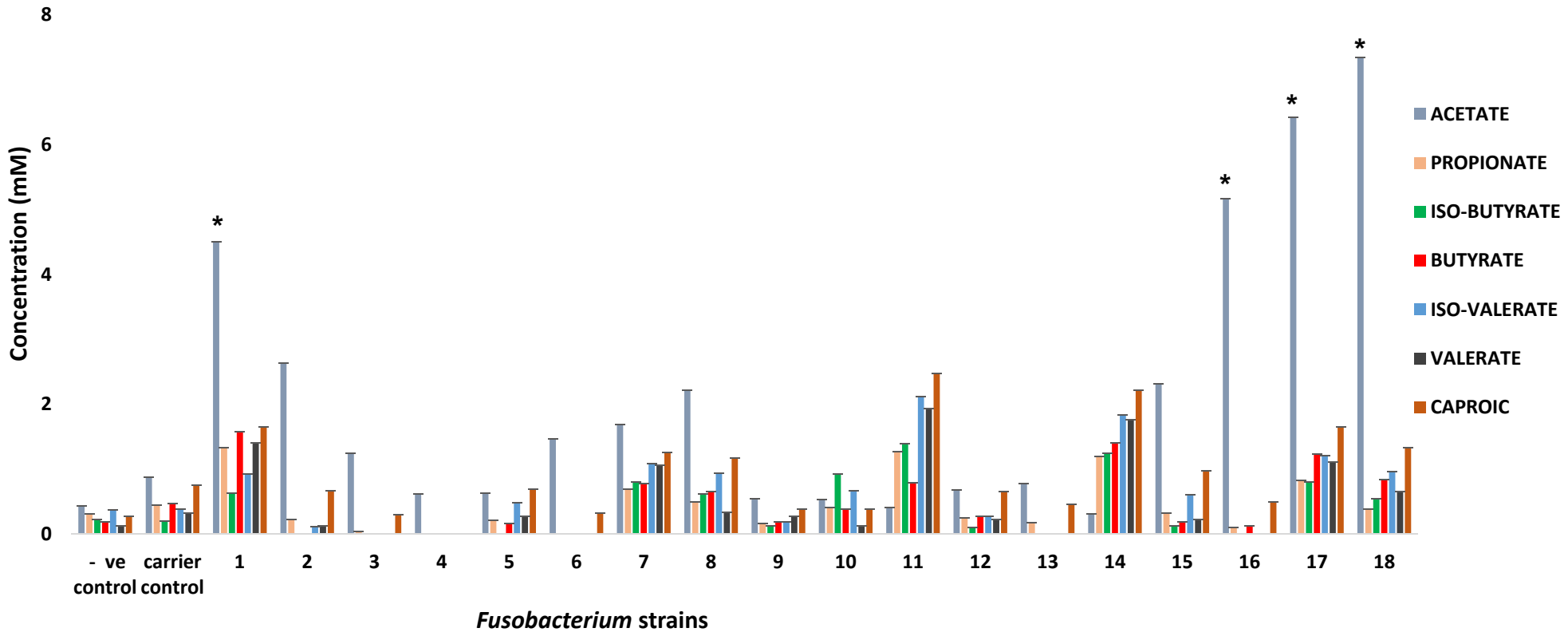

Figure 5.2: The production of SCFA and BSCFA (mM) by different *F. nucleatum* strains supernatants detected by gas chromatography (GC). Values are means ±SEM with three replicates per samples. P values are calculated using one-way ANOVA, * indicate significant difference compare to carrier control with P<0.05

5.5.2 Genotoxicity

HT29 cell viability was maintained above 85 % after 24 hour exposure to fermentation supernatants at a 10% v/v concentration as assessed via DAPI staining. DNA damage was then assessed in HT29 cells via the COMET assay following a 24 hour exposure to the filter sterilised fermentation supernatant at 10% of the carrier media. The highest observed levels of DNA damage were reported for *F. nucleatum* isolates 7, 8, 9 and 10) **Figure 5.4**. However, fermentation supernatants from other *F. nucleatum* isolates were either only moderately or not at all more genotoxic than the negative control. The strongest metabolic correlate of fermentation genotoxicity was the 4-cresol concentration of the supernatant ($r = 0.54$, $p = 0.001$), whereas phenol and indole concentrations did not significantly correlate with genotoxicity ($r= 0.19$, $p = 0.074$ and $r = 0.117$, $p = 0.165$ respectively).

5.5.3 Cell proliferation and cell cycle kinetics

HT29 cells were incubated with filter sterilised supernatants from the fermentations of the *F. nucleatum* isolates at 10% v/v of carrier medium for 24 hours, with cell cycle kinetics assessed via PI staining. Some of the fermentations, notably isolates # 4 and 5 that at lower exposures of up to 0.5 mM 4-cresol induced decreases in the abundance of cells in G_0/G_1 with a relative increase in the proportion of cells in S phase. In contrast the fermentation supernatants from isolates # 2, 6 and 14 induced increases in the proportion of cells in G_0/G_1 relative to the proportion of cells in S phase in cell line. This suggests a slight growth promoting effect at lower doses and G_0/G_1 growth arrest in response to genotoxic insult at higher doses.

Changes in cell kinetics are a hallmark of carcinogenesis and may be considered tumour promoting.

Taken together we therefore report both the induction DNA damage and the encouragement of a more carcinogenic phenotype in cells cultured in vitro when exposed to 4-cresol. These observations were not deemed significant after adjustment for multiple testing $p > 0.05$.

Figure 5.5. The strongest metabolite predictors of the abundance of cells in S phase were total metabolites (4-cresol, phenol and indole) $r=0.162$, $p=0.243$

Neither the abundance of cells in G_0/G_1 or the abundance of cells in the S phase were correlated with genotoxicity $r=0.083$, $p=0.763$

There were significant differences in the growth curves of the HT29 cells post exposure to the fermentation supernatants. Isolates # 9, 10, 12, 15 and 16 induced increases in total HT29 cell numbers to a much greater extent than the negative and the carrier control. The strongest metabolite predictors of cell proliferation were 4-cresol $r= 0.630$ $p= 0.000$. However, cell proliferation was not correlated with DNA damage-genotoxicity $r= 0.066$, $p=0.290$.

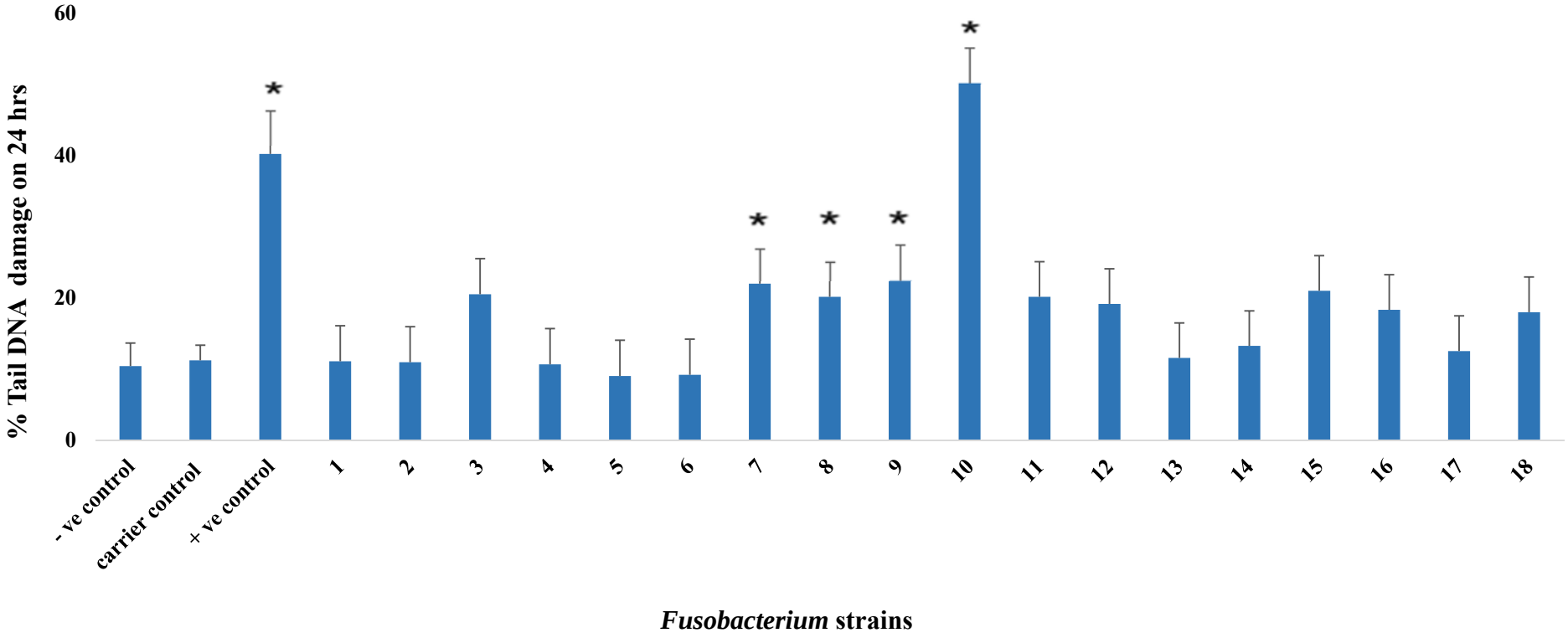

Figure 5.3: Genotoxic effect of different *F. nucleatum* strains supernatants for 24-hour incubation on the DNA strand breaks in HT29 cells. Data shown represent the average of three independent experiment. Values are means ±SEM with three replicates per samples. P values are calculated using one-way ANOVA, * indicate significant difference compare to carrier control with P<0.05

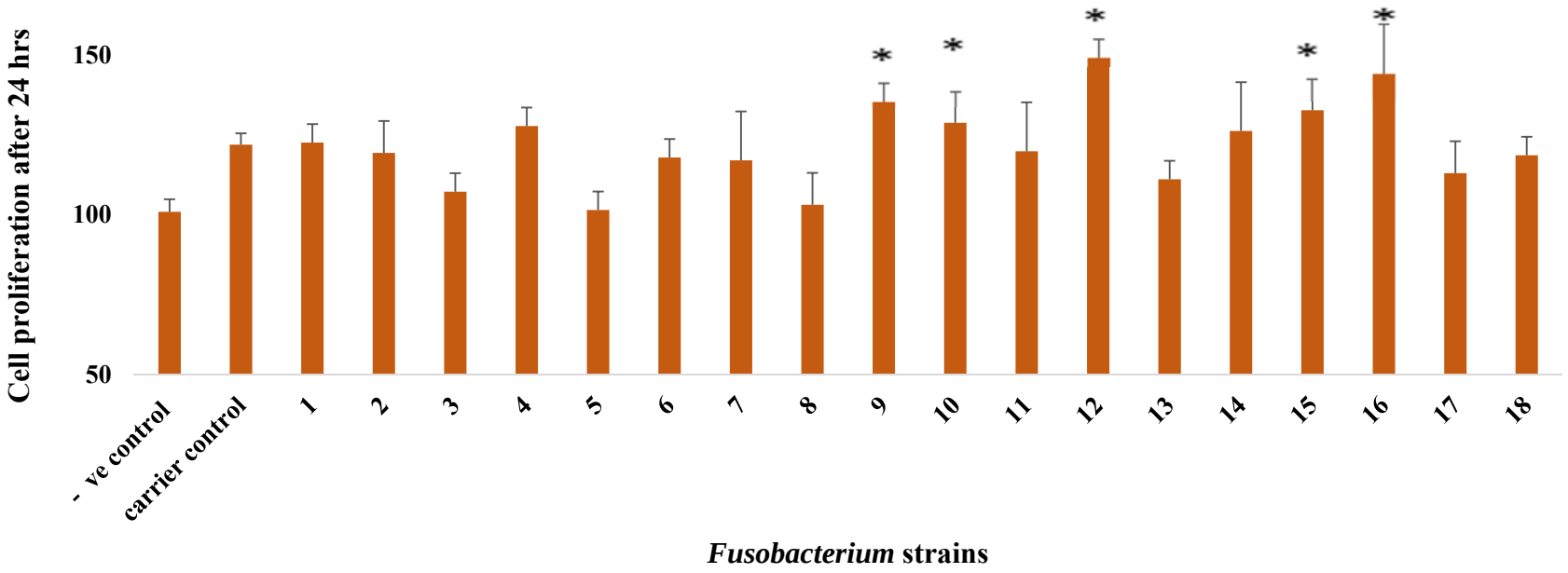

Figure 5.4: Effect of *F. nucleatum* supernatants on HT29 cell proliferation after 24-hour incubation. Data shown represent the average of three independent experiment ±SEM with three replicates per sample. P values are calculated using one-way ANOVA, * indicate significant difference compare to carrier control with P<0.05

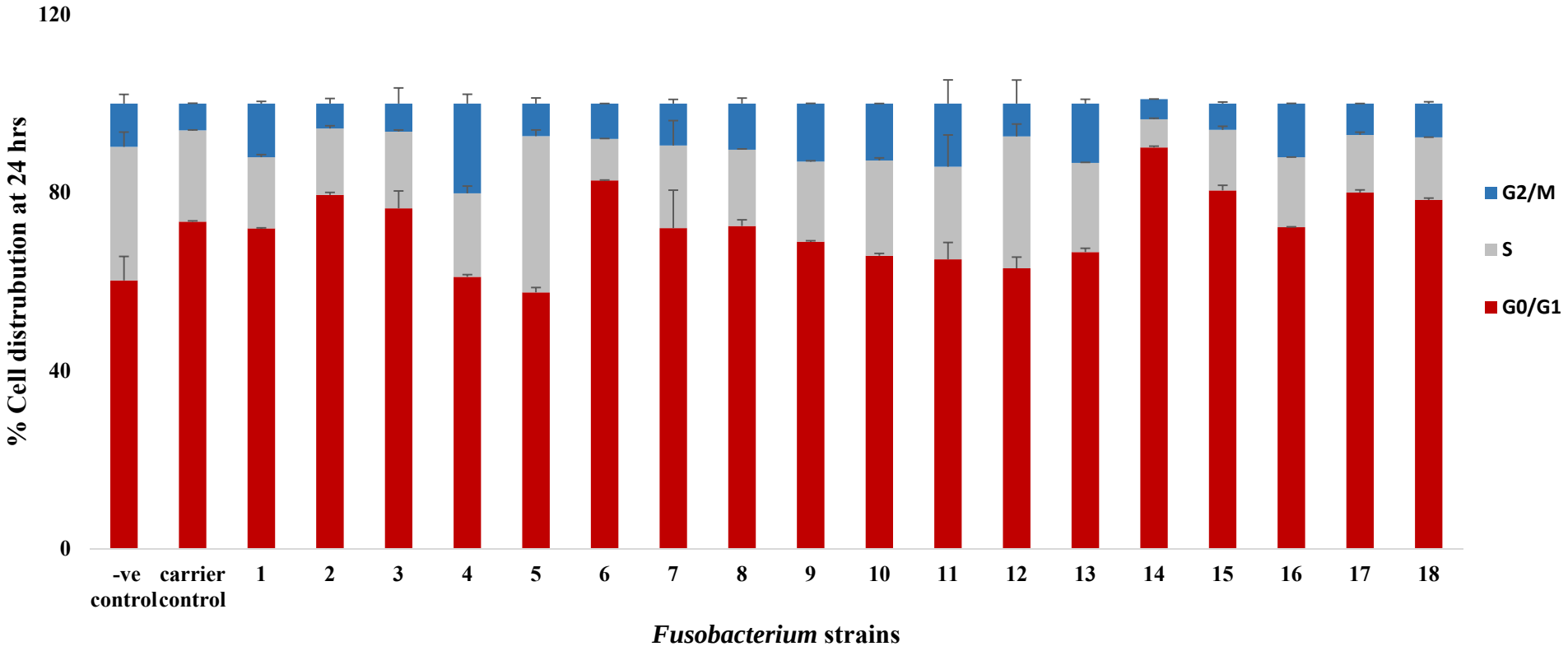

Figure 5.5: Cell cycle analysis of HT29 cells exposed with different types of *Fusobacterium* supernatant for 24-hour incubation. The percentage of cells in each phase of the cell cycle was determined using flow cytometry to quantify DNA content. Values are present in percentage with three replicates per sample.

5.6 Discussion

Recent attention has turned to look at the role of *F. nucleatum* as a key microorganism in CRC. *F. nucleatum* is an invasive anaerobe linked to periodontitis, appendicitis and more recently inflammatory bowel conditions and CRC [11]. *F. nucleatum* strains have been isolated from the mucosa in colon tumours where they are shown to be present at much higher densities than in normal mucosa. Here, we have sought to address the potential influence these bacteria may have on tumour promotion and or initiation. Previous studies have used a single/isolate of *F. nucleatum,* to do similar work [12]. In this study, 18 different *F. nucleatum* CRC isolates used and assessed their potential influence on established *in vitro* models of carcinogenesis.

First, we performed monoculture fermentations using modified basal media, to establish the typical end products of *F. nucleatum* metabolism. Then identified the production of 4-cresol, phenol, indole and SCFAs in these fermentation supernatants and demonstrated the genotoxicity and cytotoxicity of the fermentation supernatants from these isolates. The study observed that the isolates producing the highest concentrations of the metabolite, 4-cresol, induced the highest levels of genotoxic insult in colorectal cell lines. However, there may be other non-identified metabolites and small molecules with the potential to influence host physiology in our supernatants HT29.

The average concentrations of the proteolytic metabolites 4-cresol, phenol and indole were 0.0075 mM, 0.0063 mM and 0.0058 mM respectively, there were significant unexplained differences in the concentration of these metabolites between the isolates. *In vivo* proteins which escape digestion or absorption in the small intestine reach the colon [13] and are metabolised by the dominant proteolytic bacteria which can lead to the production of harmful metabolites [14, 15]. The presence of proteolytic products in our media, albeit at low levels, demonstrates usage of amino acids as substrate by the fusobacteria; additional amino acids may be available within the tumour environment in the form of blood.

Phenol and cresol are commonly identified in colonic fermentations and have been previously implicated as potential toxins. Indole is seen in faecal samples of healthy individuals and in patients with inflammatory bowel disease (IBD) [16]. The fusobacteria did produce SCFAs which are considered beneficial to host physiology, and which may enhance the mucosal barrier, improve immunity and provide metabolic fuel for the colonic epithelia [17]. Depending on the diet, the total concentration of SCFAs typically range between 70 to 140 mM in the proximal colon to 20 to 70 mM in the distal colon [18, 19]. This gradient in concentrations may account for anatomical differences in susceptibility to bowel diseases. The average concentration of SCFA produced by our isolates was acetate 2.1 mM, propionate is 0.43 mM, butyrate 0.46 mM, valerate 0.51 mM and caproic 0.9 mM . However, in general it can be seen that most of the isolates produced SCFAs at levels not significantly different to that observed in unfermented media. Although some *F. nucleatum* isolates produced significant amounts of acetate when compared to the control, this was below what would be expected in a mixed gut culture fermentation with saccharolytic substrates [20] [21]. This is because in mixed culture fermentation (previous chapter) several bacteria are involved in metabolising the substrates whereas in the current chapter only one type of bacteria is involved. Yu et al., found that oral *F. nucleatum* were producing SCFAs as metabolic by-products, from saliva of patients with severe periodontal disease, which they argued increased histone acetylation and eventually contributed to the development of oral Kaposi's sarcoma in herpes patients [22]. In the gut less production of SCFA may lead to imbalance of the T regulatory and T effector cell, which are the function of control gut inflammatory response and diseases [12, 23].

In the current study, SCFA were present in higher concentrations than 4-cresol, indole and phenol. The proteolytic metabolites were produced in relatively low concentration but may still be genotoxic as the samples with higher levels of these proteolytic metabolites led to greater DNA damage in the HT29 cell line **(Figure 5.3)**. Indeed, there were isolate dependent

differences in supernatant genotoxicity which correlated most strongly with the concentration of 4-cresol, r = 0.54 and p< 0.001. However, phenol and indole were not significantly correlated with genotoxicity (r = 0.19 and p= 0.074, r = 0.12 and p= 0.165 respectively).

Furthermore, there were significant differences in the growth curves of HT29 cells post exposure to the fermentation supernatants. Some isolates induced increases in total HT29 cell numbers to a much greater extent than the negative and the carrier control. The regression results showed a significant correlation between the 4-cresol and cell proliferation after 24-hour incubation (r = 0.63, p < 0.000). But, cell proliferation was not correlated with genotoxicity r=0.066, p=0.290. Previously Yang, et al, found that *F. nucleatum* increased proliferation and invasive activities of CRC cell lines compared with control cells. Further, CRC cell lines pre-treated with *F. nucleatum* formed larger tumours, more rapidly, in mice than untreated cells [24].

Several epidemiological studies have shown that *F. nucleatum*, is implicated as a pro-inflammatory pathogen and has been found at higher abundance within IBD patients and may be implicated in human colorectal cancer [25]. A study conducted by Castellarin et al. found *F. nucleatum* within a frozen tumour specimen was at a very high abundance compared with normal controls and therefore, they confirmed it as invasive bacteria [2]. Several other studies have found higher numbers of *F. nucleatum* in faeces of CRC patients compared to healthy controls [26] [27]. Whereas Kostic et al. [28] observed that *F. nucleatum* is enriched in colorectal adenomas and therefore may be involved in early tumorigenesis. However, they also showed that inflammation was not enhanced in *F. nucleatum* colonised Il10$^{-/-}$ mice compared to controls. The result from the current study showed some strains of *F. nucleatum* are not involved in increasing the genotoxic environment, whist others seemed capable of promoting a more genotoxic environment. More information on the location and the stage of the tumour

that the isolates were from could allow us to see if these differences had occurred for a reason; e.g. are *Fusobacterium* from advanced tumours more likely to produce genotoxins?

Considering mechanisms that may be at play, Rubinstein et al., [29] found that FadA stimulates CRC cell growth by binding to E-cadherin and activating Wnt/ß-catenin signaling and differentially regulates the inflammatory and oncogenic responses. However, this stimulation is occurring in colorectal carcinoma cells because FadA binding to other types of cells does not stimulate cell growth.

Importantly the results observed that *F. nucleatum* fermentation products can cause DNA damage and may induce human colonocytes to proliferate. However, these findings are not unique to *F. nucleatum,* and this work would be strengthened by evaluating other bacteria for their metabolic profiles and looking at other colorectal cell lines. Furthermore, the study performed a targeted analysis of fermentation supernatant metabolites, these may simply be correlates of other potential genotoxic, or cell cycle regulating, microbial products, a fuller interrogation of the microbial metabolome would therefore be welcome.

Important questions remain to be answered about the host microbe relationship for *F. nucleatum* in CRC. Notably, the competitive performance of *F. nucleatum* in mixed gut culture conditions with a medium representative of the tumour environment still needs to be established. Additionally research focussing on epigenetic interactions might help explain the observed selective preference of *F. nucleatum* for CIMP phenotype tumours.

5.7 Conclusions

In summary, the study indicated that routine fermentation of *F. nucleatum,* isolated from the surface of colorectal tumours, produce several proteolytic end products, albeit in low concentrations. These have the capacity to influence mammalian cell physiology, either through inducing direct DNA damage, or by influencing cell proliferation. There are inconsistencies in the metabolic behaviour of the *F. nucleatum* isolates studied, and these need further investigation, however the results are intriguing and suggest potential mechanisms of *Fusobacterium* spp. involvement in colorectal carcinogenesis. From this work thus far we are unable to determine whether these isolates are likely to be acting as a driving or a passenger in CRC.

5.8 Acknowledgment

We would like to thank Dr. Alasdair Scott of Imperial College London, for donated us the *F. nucleatum* strains previously isolated from tumour mucosa and Dr. Stephen Elmore, Flavour Centre-University of Reading, for his help to use the GC-MS machine. This work was funded by a His Majesty- Sultan Qaboos-scholarship via Ministry of Higher Education, the Government of the Sultanate of Oman.

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Chapter VI

6. General Discussion and Conclusions

6.1 Discussion

The human intestines are home to billions of bacteria and their fermentation end products, including the genotoxic agent 4-cresol. Variation in microbiome functionality is modifiable by dietary substrates; as such it is likely that diet can be used to modulate host exposure to genotoxins, such as 4-cresol and therefore influence the risk of CRC [1-3]. There are few studies indicating a potential role of 4-cresol to colonic carcinogenesis [4], most previous studies exploring the role of the microbiota focus on sulphate or bile acid faecal-genotoxicity [5, 6], or butyrate-dependent mechanisms using saccharolytic fermentation to protect against cancer [7]. Thus, it seems that genotoxic microbial metabolites present in the colon may be implicated in carcinogenesis, can increase intestinal cell proliferation and or induce DNA damage in the epithelium [8-11]. Importantly, these factors could be impacted on by diet. In the current thesis the role 4-cresol plays in genotoxicty and factors influencing it's production have been considered. The novelty of the current study focuses on less well characterised microbial metabolites present within the faecal stream, whilst considering their production in relation to the activity of an established tumour associated bacterial species.

Within this thesis 4-cresol has been explored across a range of systems; to begin with, an intervention study was performed on a reasonably large cross-section of Omani adults (205). It was found that total self-reported protein intake was positively correlated with urinary 4-cresol excretion ($r=0.571$, $p = 0.030$) **described in Chapter 3**. It is well known in the literature that colonic 4-cresol is absorbed and sulphated by the host prior to excretion. The current study therefore, hypothesised that its excretion in urine would reflect intestinal synthesis, and that this would be dependent on dietary protein intake. As such, the study results confirmed there

to be a correlation between total protein intake and production of 4-cresol this results is consistent with previous findings [12-14]. Several epidemiological studies, and case-control studies and others have tested similar hypotheses, however, the work in this thesis is the first to study these parameters in an Omani population. This study evaluates the macronutrient intake of a large group (205) of study volunteers, monitoring BMI, blood pressure, physical activity along with blood biochemical analysis in (mmol/L) Magnesium, Glucose, Calcium, Phosphorous, Creatinine, Bile, Urea, Total protein, Albumin, Triglyceride, High density lipoprotein, Low density lipoprotein and Cholesterol.

The majority of the studies agreed that there is a positive correlation between high protein intake and production of 4-cresol and risk of having colorectal cancer; whilst other failed to establish a definite conclusion. As the microbiota are producers of 4-cresol faecal microbial composition on a subset from within this cohort (n= 16) showed study subjects who reported consuming a higher protein diet, were found to have higher counts of total bacteria, *Bacteriodes, Clostridium histolyticum* group, *Desulfovibrionales* and *Fusobacterium* (p= 0.024, 0.042, 0.007 and 0.022 respectively) when compared to high carbohydrate consumers. Therefore, competition between bacteria for substrates has a significant influence on the population within and the products that are generated. Thus a high protein intake will increase putrefactive bacteria and their fermentation products such as 4-cresol which may lead to increased CRC risk. This is the first study that has directly linked protein intake with the microbial community and 4-cresol, and as such helps to generate more information on the microbial groups modulated by a high protein diet and the link of these to 4-cresol. Such information is useful for determining the impact of large dietary-style choices of the microbiota, particularly when considering a high protein (meat) intake has been associated with elevated CRC risk.

In previous work by Moore, et al. on faecal specimens which obtained from 22 healthy Japanese from a rural, Japan, and 16 African (both low risk of colon cancer) and 15 Japanese-Hawaiians and 17 Hawaiian and continental U.S. who consumed a western-type diet (both moderate to high risk of colon cancer). They found that the abundance of *Bacteroides* and *Bifidobacterium* was associated with increased risk of colon polyps, whereas *Lactobacillus* and *Eubacterium aerofaciens* were protective [15]. In contrast, in previous case-control studies the abundance of *Clostridium*, *Roseburia*, and other butyrate-producing bacteria and *Eubacteria* spp., may be reduced amongst cancer patients. The results of the current study support this notion that the microbial consortium is important in terms of risk factors of CRC – a factor that can be modulated by diet. As such this highlighting the potential to consider microbial modulation as a way of modulating cancer risk; as well as microbial groups that might be associated with negative effects of high protein consumption.

The absolute production of 4-cresol was explored in faecal culture models of the human colon in order to evaluate potential *in vivo* exposures. The resultant *in vitro* gut fermentation supernatants were used to treat human colorectal cell line based models of carcinogenesis.

In the second part of this thesis, the study focus was to understand the impact of protein on 4-cresol production by the human microbiota and to evaluate its potential contribution to colonic genotoxicity. The novelty of this study in is that 4-cresol is less well characterised in the literature, thus this research focusses on the activities of this lesser studied metabolite. Many studies have looked to the phenols in general and have a very few works about 4-cresol genotoxicity and cytotoxicity. Within this chapter different protein sources were used to investigate their potential to be broken down to yield 4-cresol; this helps to identify whether all proteins are equal when considering dietary risks associated with CRC. Furthermore, in this

chapter two cell lines were used to detect the genotoxicity of 4-cresol on them. Followed by testing the cytotoxicity in cell proliferation and its effect on cell cycle; therefore generating end point data associated with CRC, linking, fermentation metabolites with cancer risk factors.

4-cresol is produced through the microbial metabolism of the amino acid tyrosine which, in the colon, may derive from exogenous or endogenous protein [16]. The batch culture results indicated the highest concentrations of 4-cresol were observed upon fermentation of high tyrosine with FOS (17 mM). This is well in excess of the 4-cresol concentrations reported in faecal samples (58·86 μmol/g) [17]. It is also in excess of the concentrations of 4-cresol which this study found to be cytotoxic. The batch culture fermentation models are limited by the lack of clearance of fermentation metabolites. *In vivo* 4-cresol would be cleared from the colonic lumen through either excretion in faeces or through absorption, they are also limited in that there is no replacement of substrate, thus the supply of tyrosine is quickly exhausted. To overcome that, in this model used perhaps artificially high levels of tyrosine. For these reasons, the study cautiously predict that these very high concentrations are not achievable in the colon of man. Nevertheless, the current data suggest a higher potential exposure to 4-cresol in the colon than previously considered. The other non-animal protein sources tested did not lead to as greater production levels of 4-cresol, indicating the source of protein is important when considering CRC risk.

The optimal media for 4-cresol production contained tyrosine (0.3:100 w/w) and FOS (1.5:100 w/w). This surprisingly produced more 4-cresol than tyrosine alone (0.3:100 w/w), it was hypothesised that the FOS would favour the growth of saccharolytic bacteria and inhibit proteolysis, however the additional substrate may have favoured total bacterial growth and consequently led to more 4-cresol. Furthermore, the high tyrosine diet and high tyrosine diet

with FOS media led to increases in the abundance of *Clostridium histolyticum* group at 24 hours, a group often associated with proteolysis and negative effects. Therefore, mixed substrates somehow led to increased enhancement of this group – this data was not supported by the human study, whereby high carbohydrate was more protective of a high protein diet – i.e. leading to lower 4-cresol that high protein alone. As such, more *in vitro* work to explore how the microbiota are interacting is warranted.

In vivo, high protein intake has previously been shown to stimulate the growth of proteolytic species such as *Clostridium perfringens*, and to reduce faecal counts of beneficial *Bifidobacterium* Anaerobes known to ferment aromatic amino acids include *Bacteroides, Lactobacillus, Bifidobacterium, Clostridium* and *Peptostreptococcus* [18]. The study utilised the fermentation supernatants from this model as treatments applied to cultured colonocytes to assess genotoxicity in the first instance, and influences on cell behaviour in the second instance. These supernatants were applied to cultured cells at a concentration of 10% v/v in carrier media; there was therefore a significant dilution of their metabolic load, nevertheless they showed a range of genotoxicities. In regression analysis the metabolite within these fermentation supernatants that best predicted genotoxicity was indeed 4-cresol ($r= 0.775$, $p = 0.002$).

Importantly the results showed that spiking of weakly genotoxic fermentation supernatants with 4-cresol enhanced their genotoxic potential. This strongly suggests that the 4-cresol within the fermentation supernatant is acting as a direct genotoxin rather than presenting as a proxy for unidentified metabolites. The results confirmed this genotoxicity using 4-cresol as a direct challenge in two different colonic cell lines and using two separate analysts (Grateful for the contribution of Piyarach Kullamathee who made the assessment of Genotoxicity in the Caco-2 model). There was a dose dependent increase in DNA damage with increasing

concentrations of 4-cresol reaching statistical significance at concentrations of 3 mM in both cells line ($p = <0.05$).

Further, the 4-cresol was shown to influence cell cycle kinetics consistently across both cell lines in a dose dependent manner at lower exposures of up to 0.5 mM 4-cresol, the study results observed decreases in the abundance of cells in G_0/G_1 with a compensatory increase in the proportion of cells in S phase. Where at higher concentrations the proportion of cells in G_0/G_1increased significantly relative to the proportion of cells in S phase in both cell lines, suggesting a slight growth promoting effect at lower doses and G_0/G_1growth arrest in response to genotoxic insult at higher doses.

As such, importantly this thesis highlights the importance of 4-cresol in impacting on the cell cycle, and being a major, protein fermentation contributor, to negatively impacting on cell cycle. 4-cresol has not been studied at length in this way, however, this research highlights 4-cresol as a very important component for future investigation in CRC studies.

Finally, this PhD thesis tested the ability of human tumour mucosa derived *Fusobacterium nucleatem* isolates for their ability to both synthesise 4-cresol and to influence *in vitro* models of carcinogenesis. *F. nucleatum*, which was particularly interesting as it is normally considered an oral commensal and not frequently observed in the healthy colon. *F. nucleatum* has begun to attract wider attention as several groups now report its presence on intestinal tumours [19]. This raises the question about its potential role in disease causation. The results suspected that it is perhaps present due to its competitive ability to colonise the environmental niche that is a colonic tumour, therefore, the study was conducted to assess its ability to produce potentially genotoxic metabolites and influence DNA damage.

The 18 isolates were fermented in monoculture using a modified tryptic soy broth. The fermentation supernatants of these strains were analysed by GC-MS to determine the

concentrations of metabolites as in **Chapter 5**, the fermentation supernatants were used to treat HT29 cells line to assess their genotoxicity) via comet assays and their influence on cells proliferation and cell cycle kinetics.

The results of this study found that the isolates were differentially genotoxic, again in a regression analysis; 4-cresol emerged as the biggest predictor of supernatant genotoxicity; however, the absolute concentrations of 4- cresol in these supernatants were much lower than in the mixed culture fermentations described in **Chapter 4**. Some of the *F. nucleatum* isolates produced supernatants, which increased HT29 cell proliferation after 24-hour incubation. There were considerable differences in activity between the isolates studied. Due to a lack of time and resources we were unable to confirm that all of the isolates were indeed *F. nucleatum*, for that we relied on the reporting of our clinical collaborators, given the different metabolite profiles to take this work further we need to address this. This is a highly topical area of research, in an APC mouse model, *F. nucleatum* increased the tumour burden and infiltration of myeloid immune cells and pro-inflammatory markers [20], data which do suggest a role in disease causality.

To take this work forward it is important to next establish whether there are aspects of the tumour environment that favour *F. nucleatum* growth, it would be good to assess this in mixed gut culture models using media enriched to represent the tumour condition. From observations whilst 4-cresol is produced in the isolates studied, there may be other unidentified genotoxins being produced, and therefore a fuller -omic based analysis would be beneficial.

6.2 Conclusions

This work has considered potentially detrimental effects of diet on the metabolic activity of the gut microbiota; from the Omani study, the results found that high protein intakes were associated with elevated urinary 4-cresol, which could be increasing risk factors associated with non-communicable diseases such as CRC. The population described as undergoing the nutrition transition and the study therefore were intended to better understand nutritional issues in that community. Therefore, future nutrition awareness and health education should emphasise in the importance of healthy balanced diets (e.g. including RDA levels of fibre) and active lifestyles

Furthermore, this thesis has increased the knowledge of the activity of 4-cresol in the gut, particularly in relation to DNA damage. This is a unique finding and emphasises the importance of this low concentration microbial metabolite may have a key role in the CRC process. The consumption of protein, specifically of meat origin, has been seen to be a key factor in 4-cresol production; microbial modulation; whilst also being linked to increased genotoxicity. Therefore, this thesis opens the door nicely to further dietary intervention and microbial manipulation studies to reduce 4-cresol levels and potential impact on markers associated with CRC.

Finally, the preliminary work on *F. nucleatum* is topical, at this stage the results are uncertain as to why some but not all of the strains were genotoxic, whether or not they are passengers or drivers of the disease process remains unclear, but it is an exciting avenue of future research. In the immediate future we would hope to explore whether there are aspects of the tumour environment that favour *F. nucleatum* growth.

This work was funded by the Omani Ministry of Health. The Ministry of Health is in an excellent position to use this work to inform dietary intervention programs and guidelines for public health.

6.7 Future work

The bigger picture is one of further development of data surrounding the relationship of 4-cresol production with high protein intake and CRC.

The results from this thesis showed that meat protein enhanced 4-cresol levels, which was seen to be genotoxic. Further exploration of this in an intervention study could explore the faecal microbiota and urinary metabolites of those on a high meat intervention; verses those on a vegetarian, high protein diet; to see the importance of the protein source within a population group. Additional intervention could be to then add a prebiotic to the diet to see if any changes observed, attributable to the diet were then adjusted by a food known to have a positive impact on the microbiota.

The *Fusobacterium* work could be continued to look into if any specific substrates need to be present to increase genotoxic potential of these microorganisms. Furthermore, determining if *Fusobacterium* from different sites of the body and from different cancer stages behave in the same way. This would help to determine if this microbe actually has a role to play in CRC development.

The human observational work described has established an important diet linked biobank for future work, and has helped characterise current food intake in Oman. The study generated a total of, 205 faecal, blood and urine samples stored in -80 °C. These samples require a much more extensive look to investigate different correlations. For example, following on from this thesis a correlation could be sought between microbial populations and 4-cresol levels and dietary patterns from the whole cohort. Indeed, there is great potential for these samples to be used in a variety of ways to explore how different dietary patterns impact on human blood parameters and also microbial metabolites. This thesis is just the beginning and generates many new avenues for future research.

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Appendix 1

CONSENT FORM

Title: Protein Fermentation, Gut Microbiota and Colorectal Cancer

Name of Researchers: Eiman Al Hinai, Dr. Gemma Walton and Dr. Daniel Commane

- I confirm that I have read and understood the information sheet for the above study
- I understand that my participation is voluntary and I am free to withdraw at any time without my medical care being affected
- I agree to take part in the study

Name of the participant:…….

Age:…….

DOB:…….....

Gender: ……..

Study #:……..

Address:……...

Mobile #:…….

Signature date

PATIENT INFORMATION SHEET

Background:

Researchers have suggested that today the global leading causes of death are non-communicable diseases (NCDs) such as Colorectal cancer (CRC), which are rapidly becoming the leading causes of morbidity, and mortality among people. This increased burden of NCDs is could be prevented through primary prevention and early intervention strategies. The increase prevalence of NCDs in the Middle East countries, including Oman, is considered as an alarming phenomenon during adolescence, especially if synergized by adopting a sedentary lifestyle. There is a need to explore the impact of diet and production of 4-cresol in the etiology of CRC among Omani population.

What does it involve?

We would ask you fill in a questionnaire, and if there were any queries, we would ask you to clarify. We would also collect blood, urine and faecal samples from you for preforming clinical, and biochemical analyses

Is it harmful?

Other a simple pin-prick for a blood test – no

Is there any benefit for me?

If we diagnose you with any chronic disease, you will be informed. The results of the study will be published and all the study participants will be acknowledged. Of course your identity would not be disclosed, however, should the study suggest a strong association, positive or negative, with any food / food content, you would be able to discuss the information with your doctor

Is there any benefit for others?

It would help us to work out the causes of non-communicable diseases in Oman

What if I don't want to take part?

It will make no difference to the way we treat you. You will always get the best available treatment

Who can I discuss this with further?

You can discuss it further with Mrs. Eiman Al Hinai by email (alhinaie@yahoo.com)

Appendix 2

Protein Fermentation, Gut Microbiota and Colorectal Cancer

Section 1: Personal Details

- 1.1 Name:……
- 1.2 Date of Birth: …../…../……. Age:……..Years
- 1.3 Gender: M…… F……..
- 1.4 Student number:……..Study number……
- 1.5 Date:…. /…… /2015 Mobile#:……Email:……..
- 1.6 **Demographics Questions**

From which Governorate you are (circle the appropriate letter):

1.6.1 Muscat ☐ 1.6.5 Al Batinah South ☐ 1.6.9 Al Batinah North ☐

1.6.2 Dhofar ☐ 1.6.6 Al Sharqiah South ☐ 1.6.10 Al Sharqiah North ☐

1.6.3 Al Dakheliah ☐ 1.6.7 Al wusta ☐ 1.6.11 Al Dhaherah ☐

1.6.4 Al Buraimi ☐ 1.6.8 Musandam ☐

- 1.7 Marital status:

(1) Single (2) Married ☐

(3) Widowed (4) Divorced

- 1.8 Work:

(1) Working (2) not working ☐

- 1.9 Level of education:

(1) Illiterate (2) read and writes

(3) Primary (4) Preparatory ☐

(5) Secondary (6) University

- 1.10 Income level of the family (Omani Riyals):

(1) 400 or less (2) 400-800 ☐

(3) 800-1200 (4) More than 1200

- Are you related to any in the Institute?
  (1) Yes (2) No

Who is it?.....

Section 2: Anthropometric Measurements

- 2.1 Height ….……cm
- 2.2 Weight: ……...Kg

- 2.3 Body Mass Index (BMI) = kg/m^2 …….
- 2.4 Waist circumference (WC): ……cm
- 2.5 Hip Circumference:……cm
- 2.6 Waist/Hip ratio (WHR):…… (>1 or < 1 or = 1)
- 2.7 Body Fat distribution:
  - ✓ 2.7.1 Visceral fat (using TANITA scale)……..
  - ✓ 2.7.2 Subcutaneous fat (using skin fold caliper)……...
  - ✓ 2.7.3 Body fat % (using TANITA scale)……..

Section 3: Blood pressure measurement:

3.1 Systolic and Diastolic blood pressure……..

Section 4: Life Style Factors:

4.1 Please tick those boxes that relate to your present diet:

4.1.1 Mixed food diet (animal and vegetable sources) □

4.1.2 Vegetarian □

4.1.3 Salt restriction □

4.1.4 Fat restriction □

4.1.5 Starch/carbohydrate restriction □

4.1.6 Calorie restriction □

4.1.7 Other dietary plans, please □

Detail **:**

……
…………

4.2 Physical activity-related energy expenditure:

4.2.1 (P2): In a typical week, on how many days do you do vigorous intensity activities as part of your work?

4.2.2 (P3): How much time do you spend doing vigorous-intensity activities at work on a typical day?

4.2.3 (P5): In a typical week, on how many days do you do moderate intensity activities as part of your work?

4.2.4 (P6): How much time do you spend doing moderate-intensity activities at work on a typical day?

4.2.5 (P8): In a typical week, on how many days do you walk or bicycle for at least 10 minutes continuously to get to and from places?

4.2.6 (P9): How much time do you spend walking or bicycling for travel on a typical day?

4.2.7 (P11): In a typical week, on how many days do you do vigorous intensity sports, fitness or recreational (leisure) activities?

4.2.8 (P12): How much time do you spend doing vigorous-intensity sports, fitness or recreational activities on a typical day?

4.2.9 (P14): In a typical week, on how many days do you do moderate intensity sports, fitness or recreational (leisure) activities?

4.2.10 (P15): How much time do you spend doing moderate-intensity sports, fitness or recreational (leisure) activities on a typical day?

4.3 Smoking:

(1) Smoking (2) not smoking

If yes:

- Smoking duration: ……..
- Types of smoking: ……..

- **4.4 Drinking:**

(1) Drinking (2) not Drinking

If yes:

- Drinking quantity: …….
- Types of Drinks: ……

Section 5: History of Intake of Vitamins and Minerals Supplementation:

5.1 Iron

(1) Yes (2) No

If yes:

5.1.1 What supplementation you are taking? Iron☐ or Iron with other combination ☐

5.1.2 What is the dose of the supplementation?...../ day?

5.1.3 How long you have been taking the supplementation?.....

5.1.4 Reasons for taking the supplemenation?.....

5.2 Calcium

(1) Yes (2) No

If yes:

5.2.1 What supplementation you are taking? Calcium ☐ or Calcium with other combination ☐

5.2.2 What is the dose of the supplementation?...../ day?

5.2.3 How long you have been taking the supplementation?.....

5.2.4 Reasons for taking the supplemenation?.....

5.3 Vitamin D

(1) Yes (2) No

If yes:

5.3.1 What supplementation you are taking? Vitamin D ☐ or with other combination ☐

5.3.2 What is the dose of the supplementation?...../ day

5.3.3 How long you have been taking the supplementation?.....

5.3.4 Reasons for taking the supplemenation?.....

5.4 Vitamin B12

(1) Yes (2) No

5.4.1 What supplementation you are taking? Vitamin B12 ☐ or with other combination ☐

5.4.2 What is the dose of the supplementation?...../ day?

5.4.3 How long you have been taking the supplementation?.....

5.4.4 Reasons for taking the supplemenation?.....

5.5 Multivitamin intake

(1) Yes (2) No

If yes:

5.5.1 What supplementation you are taking? ……

5.5.2 What is the dose of the supplementation?...../ day

5.5.3 How long you have been taking the supplementation?.....

5.5.4 Reasons for taking the supplemenation?.....

5.6 Folate intake

(1) Yes (2) No

If yes:

5.6.1 What supplementation you are taking? Vitamin D ☐ or with other combination ☐

5.6.2 What is the dose of the supplementation?...../ day

5.6.3 How long you have been taking the supplementation?.....

5.6.4 Reasons for taking the supplemenation?.....

(1)< 1 month (2) 1- 2 months

(3) > 2 months (4) Irregular supplementation]

(5) Didn't take supplementation

5.7 Over counter nutritional supplements (Men and Women)

(1) Yes (2) No

If yes:

5.7.1 What supplementation you are taking?.....

5.7.2 What is the dose of the supplementation?...../ day?

5.7.3 How long you have been taking the supplementation?.....

5.7.4 Reasons for taking the supplemenation?.....

5.8 Fish Oil

(1) Yes (2) No

If yes:

5.8.1 What type of fish oil you are taking? Alone ☐ or fish oil with other combination ☐

5.8.2 What is the dose of the supplementation?...../ day?

5.8.3 How long you have been taking the supplementation?.....

5.8.4 Reasons for taking thesupplemenation?.....

Dietary supplements are vitamins, minerals, herbs, and many other products. They can come as pills, capsules, powders, drinks, and energy bars. Some supplements may help to assure that you get an adequate dietary intake of essential nutrients

Section 6: Disease Status:

6.1 Have you been diagnosed for diabetes?

1. Yes 2. No

6.2 Are you diabetic?

1. Yes 2. No

If yes:

What type of medication you are taking?.....

6. 3 Do you have any cardiovascular diseases?

1. Yes 2. No

If yes:

What type of medication you are taking?.....

Section 7: Family History:

7.1 Family history of Diabetes:

1. Yes 2. No

7.2 Family history of Hypertension:

1. Yes 2. No

7.3 Family history of Cardiovascular Diseases (CVD):

1. Yes 2. No

If yes:

- ✓ Types of CVD:

7.3.1 Ischemic Heart Disease: 1. Yes 2. No
7.3.2 Coronary Heart Disease: 1. Yes 2. No
7.3.3 Stroke: 1. Yes 2. No

7.4 Family history of Obesity:

1. Yes 2. No

Appendix 3

Food Frequency Questionnaire

	Vegetables	Serving size	Never	A few times	1-2 / month	1-2 /wk	3-4 /wk	5-6 /wk	1-3/ day	4-5/ day	6+/ day	Quantity
1	Broccoli (القرنبيط)	1/2 cup (40g)										
2	Cabbage (ملفوف)	1/2 cup (40g)										
3	Carrot (جزر)	1/2 cup(40g)										
4	Cauliflower (قرنابيط)	1/2 cup(40g)										
5	Chili (فلفل)	1/2 cup(40g)										
6	Cucumber (خيار)	1/2 cup(40g)										
7	Eggplant (باذنجان)	1/2 cup(40g)										
8	Garlic (ثوم)	yes/ No										
9	Green onion (بصل أخضر)	1/2cup(40g)										
10	Green pepper (فلفل حلو)	1/2cup(40g)										
11	Okra (بامية)	1/2cup(40g)										
12	Lettuce (خس)	1 cup(40g)										
13	Mixed vegetables, raw, cooked (خضرة مشكلة)	1/2 cup(40g)										
14	Onions (raw or cooked) (بصل)	1/2 cup(40g)										
15	Potato, mashed, boiled or baked French fries (بطاطا)	1/2 cup(40g)										
16	Spinach (السبانخ)	1 cup(40g)										
17	Sweet potatoes (فندال)	1/2 cup(40g)										
18	Olive (green-Black) (زيتون)	1/2 cup(40g)										
19	Tomatoes (طماطم)	1/2 cup(40g)										
20	(Sweet) Corn	1/2 cup(40g)										
Fruits												
21	Apple (تفاح)	1/2 cup(40g)										
22	Banana (الموز)	1/2 cup(40g)										
23	Dates (Ratab/tamar)(رطب/تمر)	1/4 cup(20g)										
24	Grapes (عنب)	1/2 cup(40g)										

		Serving size	Never	A few times	1-2 / month	1-2 /wk	3-4 /wk	5-6 /wk	1-3/ day	4-5/ day	6+/ day	Quantity
25	Guava (جوافة)	1/2 cup(40g)										
26	Kiwi (كيوي)	1/2 cup(40g)										
27	Mango (مانجو)	1/2 cup(40g)										
28	Melon (شمام)	1/2 cup(40g)										
29	Orange (برتقال)	1/2 cup(40g)										
30	papaya (فيفاي)	1/2 cup(40g)										
31	Peach (خوخ)	1/2 cup(40g)										
32	Pears (كمثرى)	1/2 cup(40g)										
33	Pomegranate (رمان)	1/2 cup(40g)										
34	Watermelon (بطيخ)	1 piece /(40g)3-4 cm										
Traditional Omani Dishes												
35	Arsiya chicken (عرسية دجاج)	1/2 cup(40g)										
36	Arsiya meat (عرسية لحم)	1/2 cup(40g)										
37	Beans	1/2 cup(40g)										
38	Beriani chicken (برياني دجاج)	1/2 cup(40g)										
39	Beriani fish (برياني سمك)	1/2 cup(40g)										
40	Beriani meat (برياني لحم)	1/2 cup(40g)										
41	Chicken Soup (سوب دجاج)	1/2 cup(40g)										
42	Chickpeas (hummus) (حمص)	1/2 cup(40g)										
43	Harees chicken (هريس دجاج)	1/2 cup(40g)										
44	Harees meat (هريس لحم)	1/2 cup(40g)										
45	Kidney beans (فول)	1/2 cup(40g)										
46	Lentils (عدس)	1/2 cup(40g)										
47	Pasta (spaghetti, macaroni, noodles, (معكرونة)	1/2 cup(40g)										
48	Makboos chicken (مكبوس دجاج)	1/2 cup(40g)										

		Serving size	Never	A few times	1-2 / month	1-2 /wk	3-4 /wk	5-6 /wk	1-3/ day	4-5/ day	6+/ day	Quantity
49	Makboos meat (مكبوس لحم)	1/2 cup(40g)										
50	Meat Soup (سوب لحم)	1/2 cup(40g)										
51	Pizza	1 pc										
52	Qabooli chicken (قبولي دجاج)	1/2 cup(40g)										
53	Qabooli fish (قبولي سمك)	1/2 cup(40g)										
54	Qabooli meat (قبولي لحم)	1/2 cup(40g)										
55	Saloona chicken (صالونة دجاج)	1/2 cup(40g)										
56	Saloona fish(صالونة سمك)	1/2 cup(40g)										
57	Saloona meat(صالونة لحم)	1/2 cup(40g)										
58	Samosa (سمبوسة)	2 small / 1 big piece(60g)										
59	Thareed chicken (ثريد دجاج)	1/2 cup(40g)										
60	Thareed meat (ثريد لحم)	1/2 cup(40g)										
61	White Rice (boiled or cooked w fat) (رز أبيض)	1/2 cup(40g)										
62	Grains (wheat, oats, etc…)	1/2 cup(40g)										
Breads												
63	Brown Toast bread(توست أسمر)	1 pc /25g										
64	Burger bread (خبز برجر)	1/2 bun(40g)										
65	Chapati bread (خبز شباتي)	1slice(40g)										
66	Lebnani bread -brown(خبز لبناني أسمر)	1slice(40g)										
67	Lebnani bread-white (خبز لبناني أبيض)	1slice(40g)										
68	Parata (باراتا)	1/2 slice(40g)										
69	Rekhal (رخال)	1 slice(40g)										
70	Salalah bread (خبز صلالة)	1 slice(40g)										
71	Tanoor Bread (خبز تنور)	1/2 slice(40g)										

		Serving size	Never	A few times	1-2 / month	1-2 /wk	3-4 /wk	5-6 /wk	1-3/ day	4-5/ day	6+/ day	Quantity
72	Unspecified bread (أنواع أخرى)	1 slice (40g)										
73	White Toast bread(توست أبيض)	1 slice(40g)										
Meats, Milk, Dairy products and Nuts												
74	Almonds (لوز)	1/2 oz (14g)										
75	Cashew (كازو)	1/2 oz (14g)										
76	Peanuts (الفول السوداني)	1/2 oz (14g)										
77	Pistachio (فستق)	1/2 oz(14g)										
78	Fish (cooked) (سمك مطبوخ)	2oz (56g)										
79	Sea foods (squid, prawn, etc…)	½ cup (40g)										
80	Tuna (canned) (تونة معلبة)	2 oz(56g)										
81	Meat (lamb) (Mutton)(لحم الضأن)	2 oz(56g)										
82	Luncheon meats: salami, turkey, mortadella	1 slice(40g)										
83	Saudages, not dogs…..	1 item (30g)										
84	Chicken (دجاج)	2 oz(56g)										
85	Cheddar (جبنة شيدر)	2 oz(56g)										
86	Cream cheese(جبن القشدة)	2 oz(56g)										
87	Sliced cheese (شرائح الجبن)	2 oz(56g)										
88	Milk (whole milk) (حليب كامل الدسم)	1 cup (120g)										
89	Low fat or skimmed milk	1 cup (120g)										
90	Milk w chocolate (حليب بالشوكلاته)	1 cup(120g)										
91	Milk w fruits (حليب بالفواكه)	1 cup(120g)										
92	Yogurt (plain) (روب)	1 cup(120g)										

		Serving size	Never	A few times	1-2 / month	1-2 /wk	3-4 /wk	5-6 /wk	1-3/ day	4-5/ day	6+/ day	Quantity
93	Labneh	2 oz(56g)										
94	Egg (scrambled, boiled, omelet)(بيض)	1 egg (50g)										
Beverages												
95	Coffee (Omani) (brewed) (قهوة)	1 cup(120g)										
96	Instant coffee (Nescafe) (قهوه التحضير سريعة)	1 cup(120g)										
97	Tea w milk (بالحليب شاي)	1 cup(120g)										
98	Tea w/o milk (حليب بدون شاي)	1 cup(120g)										
99	Soft Drinks	1 cup(120g)										
100	bottled or canned fruit juices	1 cup(250g)										
Sandwiches												
101	Egg sandwich (بيض سدويشة)	1 sandwich										
102	Cheese sandwich (جبن سندويشة)	1 sandwich										
103	Chicken sandwich (دجاج سندويشة)	1 sandwich										
104	Falafel sandwich (فلافل سندويشة)	1 sandwich										
105	Cheese burger (برجر سندويشة بالجبن)	1 sandwich										
106	Chicken fillet (دجاج فيليه سندويشة)	1 sandwich										
107	Fish fillet (سمك فيليه سندويشة)	1 sandwich										
Dessert & snacks												
108	Apple pie (تفاح فطيرة)	2 oz (56g)										
109	Popcorn	½ cup (40g)										
110	Biscuit (بسكويت)	15g (piece)										

		Serving size	Never	A few times	1-2 / month	1-2 /wk	3-4 /wk	5-6 /wk	1-3/ day	4-5/ day	6+/ day	Quantity
111	Cake (sponge) (كعك اسفنجي)	2 oz(56g)										
112	Cheese cake (كعكة الجبن)	80g										
113	Croissant (كروسون)	1 croissant(120g)										
114	Custard (كستر)	150g										
115	Date pi (فطيرة تمر)	80g										
116	Donuts (دونت)	2 oz(56g)										
117	Luqaimat (لقيمات)	2 oz(56g)										
118	Omani Halwa (حلوى عمانية)	1 oz=3TAS (28g)										
119	Pancake (فطيرة)	40g										
120	Potato chips (شرائح البطاطا)	30g										
121	Pudding (بودينغ)	150g										
122	Sweets/ chocolate/ candy (حلويات)	46g										
123	Arabic sweet, baklawah, konafah,mamaol….	1 items (40g)										
124	Honey and Jam	1 TES (5g)										
125	Breakfast Cereals, cornfleks, suger coated cereal….etc	½ cup (40g)										
Fast Food												
126	Pizza (بيتزا)	1 pc										
127	KFC/ Pizza Hut/ McDonald's	1 sandwich										
128	fatiyer	1 pc										