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The effects of substituting red and processed meat with mycoprotein on markers of colorectal cancer risk and systemic health

D N Farsi

PhD

2022

The effects of substituting red and processed meat with mycoprotein on markers of colorectal cancer risk and systemic health

Dominic Nathan Farsi

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Research undertaken in the Department of Applied Sciences, University of Northumbria

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ABSTRACT

Mycoprotein is a meat alternative made from *Fusarium venenatum*. Mycoprotein, as well as other meat alternatives, are used to replace meat by health and environmental conscious consumers, however these products are not direct nutritional replacements for meat, and their health effects are not fully understood. Mycoprotein is high in protein, fibre and low in saturated fat, which make it an interesting meat alternative with potential benefits to health beyond meat displacement.

The work in this thesis compares the effects of substituting a high red and processed meat diet with mycoprotein on gut and cardiometabolic health. The original aim was to investigate whether the putative mechanisms linking red and processed meat consumption to colorectal cancer risk might be attenuated by replacing meat with mycoprotein.

In a dietary intervention study in healthy male adults, I show that substituting red and processed meat with mycoprotein positively influences gut health, by lowering intestinal genotoxicity, and by increasing the relative abundance of beneficial gut bacteria.

In a secondary analysis of the dietary intervention study data, I also show that mycoprotein consumption may improve cardiometabolic disease risk related endpoints.

Finally, using nationally representative data, I show that at a population level, displacement of meat with mycoprotein and other meat alternatives would influence nutritional intake and potentially nutritional status in the UK population. Notably, it would allow consumers to achieve the dietary guidelines around fibre but raises addressable concerns about certain nutrients.

This work is timely, given the current focus on agricultural systems in the context of climate change. My data supports arguments around the potential healthfulness of choosing mycoprotein, which may help reduce uncertainty amongst potential consumers and thereby increase public confidence in mycoprotein as a replacement for meat. The findings may be of interest to the nutrition and public health community who have struggled to increase fibre consumption in the population and have concerns about the high meat content of the western diet. The findings may also be of interest to those working in the burgeoning meat alternative space. Mycoprotein has considerable potential as part of a healthier, greener food future.

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Last, but certainly not least, thank you to my parents, for their continued support and for giving me a strong platform to pursue all my endeavours, whether it entailed method, or just the madness.

DECLARATION

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others.

Any ethical clearance for the research presented in this commentary has been approved. Approval has been sought and granted through the researcher's submission to Northumbria University's Ethics Committee on 24/03/2019.

Name: Dominic Nathan Farsi

Date: 30/03/2022

CHAPTER I

INTRODUCTION

1.1. Mycoprotein

Amidst concerns for both environmental and human health, recommendations encourage a manoeuvre away from a dependence on animal-based foods to dietary patterns that emphasise plant-based sources (1, 2). However, changing dietary behaviours is a challenge owing to strongly held culinary traditions, taste preferences, and social and cultural norms (3-5). Furthermore, although there is evidence of health perturbations from animal-meat consumption, it represents and remains a major source of nutrients in the diet (6, 7).
The emergence of meat-alternatives represents a convenient way to reduce meat consumption, as consumers can replace meat with meat-alternatives (8, 9). One example is mycoprotein; sold under the brand name "Quorn" (Marlow Foods) since 1985 (10).

Mycoprotein is produced via the continuous fermentation of *Fusarium venenatum*, a naturally occurring fungus, using an aerobic fermentation system supplemented with carbohydrate and nutrient substrates required for growth (11). The mycelium of the fungus is then heat-treated to reduce ribonucleic acid (RNA) content to approved levels. The RNA-reduced biomass is then subjected to centrifugation after which the suspended hyphae are recovered, and the yielded supernatant is mycoprotein (12). The final phases of production of steaming, chilling and freezing of mycoprotein result in a high degree of fibrosity through fibre assembly, resembling a meat-like texture (13). This production method is also environmentally favourable; with a significantly reduced carbon and water footprint in comparison to beef and chicken (10, 14).

Mycoprotein is high in both fibre, protein and low in fat (by dry weight, typically 45% protein and 25% fibre) (**Table 1.1.**) (13). Due to its fibre content, mycoprotein would be classified as a high fibre food according to European Commission standards (i.e., providing at least 6 g of fibre per 100g) (15). This fibre is largely insoluble in the small intestine and comprises two-thirds β-glucan and one-third chitin, creating a "fibrous chitin–glucan matrix" (13, 16). This composition is due to the fungal cell wall structure which mycoprotein derives from, which constitutes a protective layer of chitin, β-glucans, and mannoproteins on its surface (17) (**Figure 1.1.**). The fungal β-glucan in mycoprotein differs from other β-glucans such as those found in oats, due to its unique branching (β(1,3), β(1,6) in mycoprotein vs β(1,3), β(1,4) in oats) (**Figure 1.2.**). Consumption of sufficient quantities of oat products has been

shown to reduce blood cholesterol and cardiovascular disease risk, mediated by the ability of oat β-glucan to influence bile acid and cholesterol metabolism, as well as impact the gut microbiota (18). Considering the difference in structure; fungal β-glucans in mycoprotein may elicit different effects in digestion, metabolism, and the gut microbiota. To date, there is limited data on the gastrointestinal effect of fungal β-glucans, although there is evidence that they possess immunomodulating properties (19).

Due to its unique fibre composition, mycoprotein is an interesting alternative to meat, beyond that of meat displacement. An area of health where meat replacement is of considerable importance is gut health and colorectal cancer (CRC) risk.

Figure 1.1. ***Fusarium venenatum*** **cell wall.**
The fungal cell wall contains a cell membrane with various membrane proteins, a protective layer of chitin, as well as glucans (-1,3, -1,6), and mannoproteins on its surface.

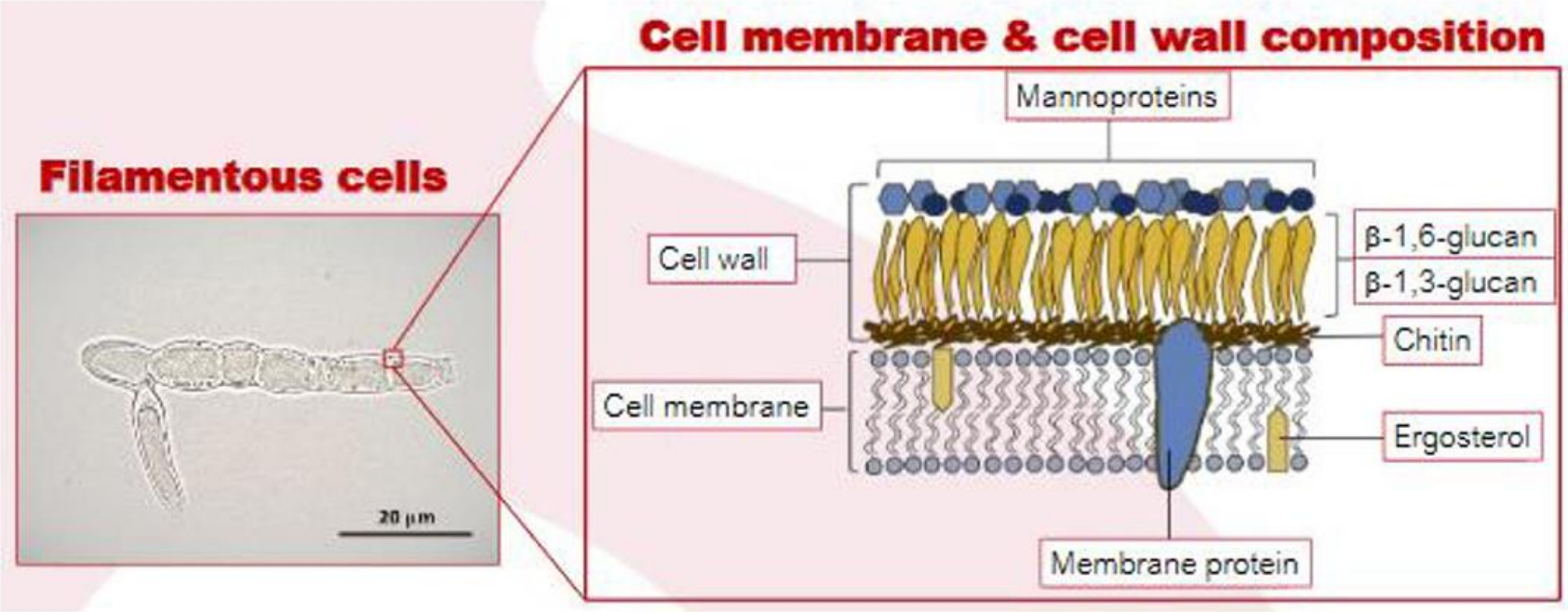
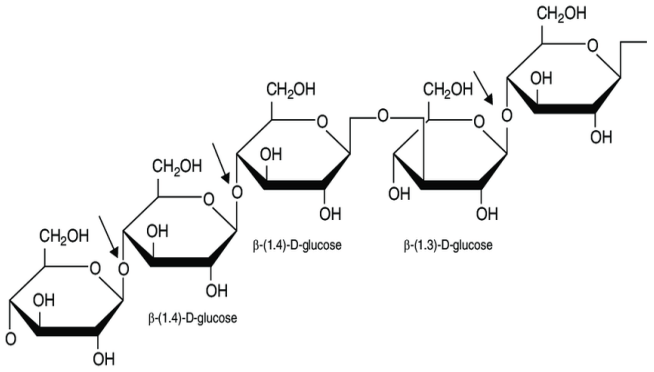


Figure 1.2. Difference in structure of fungal and oat β-glucan.

Oat β-glucan
(Linear β1,3/β1,4-glucan)



Fungal β-glucan
(Short β1,6 branched, β1,3-glucan)

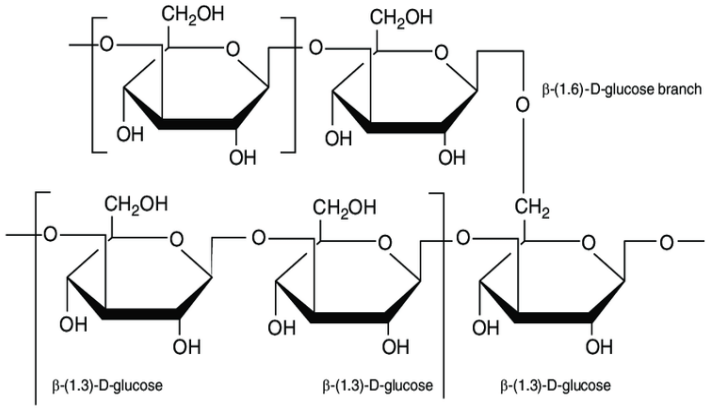


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Table 1.1. Nutritional content of mycoprotein with reference to other protein sources (fungal, plant and animal based).

(Adapted from (20)).

Nutrient	Fungal-based protein sources		Plant-based food proteins		Animal-based food proteins	
	Mycoprotein*	**Mushrooms (shitake)**	**Tofu, soya bean**	**Chickpeas**	**Chicken (whole, roasted)**	**Beef mince (topside, roasted w/ lean and fat)**
Energy (kcals/100g)	85	55	73	129	138	244
Protein (g/100g)	11	1.6	8.1	8.4	16.6	32.8
Carbohydrate (g/100g)	3	12.3	0.7	18.3	0	0
Fat (g/100g)	2.9	0.2	4.2	3	7.9	12.5
Of which saturates (g/100g)	0.7	0.1	-	0.29	2.2	5.2
Fibre (AOAC) (g/100g)	6	N	-	7.1	0	0
Vitamin B6 (mg)	0.1	N	0.07	0.38	0.21	0.51
Vitamin B9 (ug)	114	N	15	35	6	20
Vitamin B12 (ug)	0.72	0	0	0	N	3
Calcium (mg)	48	3	N	48	7	7
Phosphorous (mg)	290	29	95	141	140	210
Iron (mg)	0.39	0.4	1.2	1.9	0.4	2.6
Magnesium (mg)	49	14	23	44	16	24
Zinc (mg)	7.6	N	0.7	1.1	0.9	5.8
Potassium (mg)	71	120	63	281	200	370

*Data provided by Marlow Foods (wet weight)

N, negligible; NR, not reported.

Nutritional composition data extracted from the McCance and Widdowson's Dataset.

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1.2. Red and processed meat consumption and colorectal cancer risk

Nutrition and intestinal function are interrelated, with diet a predominant factor in both the induction and prevention of many gastrointestinal disease states such as diverticulitis, ulcerative colitis, inflammatory bowel disease, and notably, CRC (21). Dietary risk factors include a high consumption of red and processed meat (22, 23) and a low consumption of fibre and certain nutrients (24-30). Taking this evidence into consideration, in 2015, the International Agency for Research on Cancer categorised processed meat as a Group 1 carcinogen ("carcinogenic to humans") and red meat as a Group 2A carcinogen ("probably carcinogenic to humans") (31). Since this decision, further prospective studies have been published with similar associations, amongst which includes an analysis from the UK Biobank study demonstrating that consuming an additional 55g/day of red and processed meat increased risk of CRC by 20% (32). Certain components in meat are considered as explanatory factors, including endogenous compounds such as haem iron as well as components formed through meat processing including heterocyclic aromatic amines and polycyclic hydrocarbons (33-36). It is therefore desirable to find suitable foods which can replace red and processed meat in the diet for the benefit of gut health and long-term CRC risk.

1.3. A microbial factor in colorectal cancer?

There is evidence that the association between red and processed meat and CRC is stronger for distal regions of the colon (30). This body region is inhabited by a large microbial population, postulating a microbial influence in the argument.

The human gastrointestinal (GI) tract harbours a complex and abundant microbial community, reaching as high as 10^{11} bacteria/ml in the colon (37), which can be grouped into about 1000 bacterial species (38, 39). The collective prokaryote community outnumbers eukaryote cells in the human body by a factor of 10 and their collective genome is 100 times more abundant than the host genome (40). Germ-free mice demonstrate the importance of host-microbe interactions to healthy host physiology; in these models, the colon architecture is visibly aberrant, there is an under-developed immune system (41), poor *WNT* signalling mediated differentiation of epithelial cells (42) and a functionally impaired epithelial barrier (43).

The relative abundance of microorganisms in the colon is a distinguishing characteristic, microbial communities present elsewhere in the GI tract are much smaller and show lower diversity. Concomitantly, the comparative incidence of colorectal to oesophageal and stomach cancers is approximately 30:1:2 (44, 45). Notably, microbes differ in mucosal and/or faecal samples between CRC patients and healthy controls (46-48) as well as in tumour tissue verses the adjacent healthy mucosa

(49-52). Furthermore, microbial patterns may be distinct in different stages of CRC (53), as well as between molecular subtypes (54).

However, it has become increasingly clear that the compositional balance of the large intestinal microbiota, as well as pro-carcinogenic actions of specific pathogens are not the only microbial influences in CRC; but the collective metabolic activity of this microorganismal community may strongly influence protection against, and predisposition to, the development of CRC (55, 56). Undigested dietary residues enter the large intestine and are metabolised by the resident microbiota to produce a plethora of metabolites that can confer benefits and/or detriments to intra, as well as extraintestinal health (57).

Dietary fibre constitutes a spectrum of non-digestible food components including non-starch polysaccharides, oligosaccharides, lignin, and analogous polysaccharides which escape digestion by host enzymes in the upper gut, and are subsequently metabolized by the microbiota in the cecum and colon (57). Short chain fatty acids (SCFA) are the main products of saccharolytic fermentation of dietary fibre in the large intestine, including predominantly, acetate, propionate, and butyrate, which are produced in the approximate molar ratio of 60:20:20, reaching a combined concentration of over 100 mM in the intestinal lumen (58-60). SCFA are rapidly absorbed and enter the periphery contributing to an array of physiological processes that can impact host immunity (61, 62), metabolic health (63, 64), and brain function (65).
Focussing on the gut, SCFA maintain intestinal homeostasis by supporting epithelial cell proliferation (66, 67). Butyrate is the favoured energy source of colonocytes; accounting for at least 70% of energy requirements (68), and contributes to the maintenance of a healthy colonic epithelium by increasing transcription of tight junction proteins, contributing to barrier integrity (69, 70), as well as regulating inflammatory pathways that are important in determining gut health (71-73).
Of the SCFA, butyrate is arguably the most interesting microbial product with respect to colonic health owing to an apparent dual role, often referred to as the "butyrate paradox". Butyrate induces proliferation in healthy colonocytes while sensitizes cancer cells to terminal differentiation and apoptosis (74-76), thereby maintaining colonic homeostasis through sustaining a strong mucosa under normal conditions, and mitigating oncogenic events in the colon.
SCFA can be quantified in stool samples, enabling prediction of endogenous production in the gut. Human dietary interventions with high fibre diets evidence increased SCFA excretion (77-79). A diet rich in fibre may shift metabolism in the gut to a saccharolytic environment with greater SCFA production and putative benefits to colonic health. In line with this, contrary to red and processed meat, dietary patterns high in fibre are associated with reduced risk CRC (80-82).

In contrast, microbial products from undigested proteins and peptides include nitroso compounds (NOC), branch chain fatty acids (BCFA), ammonia, phenol, p-cresol, indole and hydrogen sulphide

(83). These metabolites can be leveraged as nitrogen sources for bacterial cross-feeding, or they may be taken up by colonocytes and transported into the bloodstream (84); however, their accumulation in the colonic lumen is associated with increased epithelial cell toxicity (85-87). In congruence, the abundance of microbial metabolism genes involved in amino acid and sulphur metabolism are enriched in stool of patients with preneoplastic polyps (53). This is consistent with the long-standing assumption that a gut microbial environment favouring proteolytic over saccharolytic fermentation may increase CRC risk (88).

The "Western diet" is characterised by a high content of protein derived from animal based foods such as red and processed meat (89), somewhere between 6 and 18 g of protein per day is thought to reach the colon (90, 91). With decreasing availability of fermentable carbohydrate in the distal colon, there is a shift towards the production of potentially carcinogenic proteolytic end products (39).This assortment of toxic compounds can be recovered from non-invasive body samples, NOC, ammonia, BCFA as well as hydrogen sulphide can be traced in stool samples, whereas phenols, indoles and p-cresol; fermentation end products of aromatic amino acids, are not well recovered in stool, but rather enter the hepatic circulation to be detoxified in the liver and eventually excreted in urine (92). Quantifying the concentration of these compounds in stool and urine may indicate harmful effects in the gut elicited by dietary practices, with relevance to gut health and CRC risk.

High red and processed meat dietary interventions in human volunteers leads to increased excretion of the above toxic compounds, predominantly ammonia and NOC (93-96). Furthermore, this correlates with increased faecal water genotoxicity (97-100); underlining the causal link between meat intake, large intestinal fermentation, and CRC risk.

It is important to note that a proportion of these interventions did not have the metagenomic and metabolomic techniques now at disposal in nutrition research (101, 102). Future dietary interventions could use such techniques to capture a greater coverage of metabolite excretion, shifts in microecology and activity, and correlating with faecal water genotoxicity.

1.4. Mycoprotein to benefit gut health?

Since its establishment, research has arisen concerning the health effects from mycoprotein consumption. In recent years, this work has intensified, exploring varied aspects of health (**Table 1.2.)**

A consistent finding has been improvements in blood lipid profile, after both acute and chronic intake (103-110). These effects on blood lipids may be related to mycoproteins fibre content (111). Increased fibre intake is inversely associated with lower blood lipids in both observational studies (112, 113), and clinical trials (114), translating to a lower risk of cardiovascular disease (115). Mechanistically, these profound effects in blood lipids could be interrelated to the composition and activity of the gut

186 microbiota. As mentioned, SCFA produced from saccharolytic fermentation in the colon have an array
187 of physiological effects; in this instance, SCFA have been evidenced to inhibit cholesterol synthesis in
188 the liver, primarily through propionate (116).

189 Despite mycoproteins fibre composition, as well as the importance of fibre in the gut, there have been
190 no investigations to date concerning the effects of mycoprotein on gut health.

Table 1.2. Previous studies that have investigated the health effects of mycoprotein consumption

Reference	No. of participants	Participants	Type of study	Type of intervention	Duration of intervention	Study findings
Udall et al (1984)(110)	100	Healthy adults	Double-blind crossover trial	Mycoprotein-based cookie supplementation (20 g dw per day) vs control cookies	30 d	→ 6.9% ↓ in PC → No changes in BW or other blood markers (glucose, urea, nitrogen, sodium, potassium, calcium, phosphorus, uric acid, creatinine, lactic acid dehydrogenase, alkaline phosphatase, amylase, SGOT, total protein, albumin, TGs, CBC) → No changes in urine markers (pH, glucose, protein, ketones, white and red blood cells)
Turnbull et al (1990) (105)	17 (9 mycoprotein, 8 control)	Healthy adults with total cholesterol between 5.2 and 6.2 mmol/L	Randomized controlled parallel group trial	Mycoprotein (≈ 191 g Quorn per day) vs meat during a fully controlled diet	3 wk	→ 13% ↓ in PC → 9% ↓ in plasma LDL-C (12% ↑ in control group) → 12% ↑ in plasma HDL-C (11% ↓ in control group) → 53% ↓ in TGs (in both groups) → No differences in BW or BP → No changes in fasting insulin and glucose → No changes in Apo A-I or Apo B

Table 1.2. (continued)

Turnbull et al (1992) (104)	21 (11 mycoprotein, 10 control)	Healthy adults with total cholesterol > 5.2 mmol/L	Blinded randomized controlled parallel group trial	Mycoprotein-based cookie supplementation (26.9 g dw per day) vs control cookies	8 wk	→ 7.9% ↓ in PC → 12.6% ↓ in plasma LDL-C → No changes in plasma HDL-C or TGs → No changes in Apo A-I or Apo B → No differences in BW
Turnbull et al (1993) (117)	13	Healthy females (nonrestrained eaters)	Randomized controlled crossover trial	Energy-matched mycoprotein-based meal vs chicken-based meal	2 d	→ 24% ↓ in 24-h energy intake on day of meal → 16.5% ↓ in 24-h energy intake on day after meal → ↓ in prospective food consumption and desire to eat 3 h after meal
Burley et al (1993) (118)[2]	18	Healthy adults	Randomized controlled crossover trial	Energy-matched mycoprotein-based meal vs chicken-based meal	2 d	→ 18% ↓ in energy intake in subsequent meal → ↓ in 24-h energy intake on day of meal (resulting from no compensation after reduction in subsequent meal) → No differences in 24-h energy intake on day after meal → No overall differences in eating rate or motivation to eat. Significant ↓ in hunger 4 h after meal

Table 1.2. (continued)

Nakamura et al (1994) (108)	15	Healthy males	Randomized parallel group trial	Mycoprotein-based cookies/crisps supplementation (18 g or 24 g dw per day)	8 wk	→ 4.3% ↓ in PC in 24-g mycoprotein group
Ishikawa (1995) (107)	37	Hypercholesteremic patients, with total cholesterol > 220 mg/dl	Double-blind randomized controlled parallel group trial	Mycoprotein-based cookie supplementation (12 g or 24 g dw per day) vs control cookies	4 wk	→ ↓ in PC
Homma et al (1995) (106)	52	Healthy males	Randomized crossover trial	Mycoprotein-based crisps supplementation (18 g or 24 g dw per day)	4 wk	→ 6.7% ↓ in PC in 24-g mycoprotein group
Turnbull & Ward (1995) (119)	19	Healthy adults	Double-blind randomized controlled crossover trial	Mycoprotein-based milkshake (20 g dw) vs control milkshake	120 min	→ ↓ in glycemia (13% at 60 min) → ↓ in insulinemia (19% at 30 min and 36% at 60 min)

Table 1.2. (continued)

Williamson et al (2006) (120)	42	Overweight premenopausal women	Randomized controlled crossover trial	Mycoprotein-based preload meal vs tofu or chicken-based preload meals before lunch	1 d	→ 12.3% ↓ in energy intake at lunch 20 min after mycoprotein preload when compared with chicken preload → No difference in intake at dinner (no compensation) → No differences in subjective ratings of hunger or satiety
Ruxton & McMillan (2010) (109)	31 (21 mycoprotein, 10 control)	Healthy adults	Controlled parallel group trial	Mycoprotein-based diet (≥ 88 g ww per day; 21 g dw per day) vs animal-based diet	6 wk	→ ↓ in PC in individuals with baseline cholesterol ≥ 4.19 mmol/L → No changes in TC, LDL-C, HDL-C, TGs, glucose, BP, BMI, or WC for sample as a whole
Bottin et al (2016) (121)	Part A: 36 Part B: 14	Overweight and obese adults	Single-blind randomized controlled crossover trial	Part A: Energy-matched mycoprotein-based preload meal (44 g, 88 g, or 132 g ww) vs chicken-based meal (equivalent amount of chicken and macronutrient matched at each protein content)	180 min	→ 10% ↓ in energy intake at lunch after high mycoprotein preload when compared with high chicken preload → 9% ↓ in 24-h energy intake following mycoprotein ingestion → 8%, 12%, and 21% ↓ in insulin iAUC after low, medium, and high mycoprotein preload, respectively.

Table 1.2. (continued)

				Part B: Macronutrient-matched mycoprotein-based meal (132 g ww) vs chicken-based meal		→ 21% and16% ↓ in insulinogenic and disposition indices, respectively, following mycoprotein ingestion → 9% ↑ in Matsuda Index following mycoprotein ingestion → No differences in appetite ratings → No differences in postprandial glucose concentrations → No differences in plasma GLP-1 or PYY → No differences in gastric emptying → No differences in resting energy expenditure and substrate utilization
Dunlop et al (2017) (122)	12	Healthy men	Single-blind randomized controlled crossover trial	Mycoprotein-based drinks (20 g, 40 g, 60 g, and 80 g dw) vs milk protein drink	240 min	→ Equivalent postprandial AA bioavailability between protein-matched amounts of mycoprotein and milk protein. Slower but more sustained hyperinsulinemia and hyperaminoacidemia compared with milk when protein matched. Dose-response effects on all parameters until 60–80 g of mycoprotein consumed

Table 1.2. (continued)

Monteyne et al (2020) (123)	20	Healthy resistance-trained men	Double-blind randomized controlled parallel group trial	Mycoprotein based drink (70g dw) vs milk protein drink, following bout of unilateral resistance-type exercise Test drinks matched in leucine content (2.5g)	240 min	→ Smaller and less sustained hyperaminoacidemia compared with milk. → Slower but more sustained hyperinsulinemia compared with milk → ↑ in FSRs greater in rested and exercised muscle compared with milk (Mycoprotein: from 0.025 ± 0.006 to 0.057 ± 0.004%·h^{-1} in rested, and 0.024 ± 0.007 to 0.072 ± 0.005%·h^{-1} in exercised muscle vs Milk: from 0.036 ± 0.008 to 0.052 ± 0.006%·h^{-1} in rested, and 0.035 ± 0.008 to 0.056 ± 0.005%·h^{-1} in exercised muscle)
Coelho et al (2020) (103)	20	Healthy adults	Randomized controlled parallel group trial	Mycoprotein-based diet vs animal-based diet (total energy intake specific to individual requirements, protein 1.2g/kgBW/day, 30% energy from fat, 50-55% energy from carbohydrate)	7 d	→ No differences in fasting insulin and glucose → No differences in insulin and glucose iAUC → No difference in IS → ↓ in 45 plasma lipid markers after mycoprotein diet compared to control, including TC, FC, VLDL, LDL, HDL, DHA.

Table 1.2. (continued)

Monteyne et al (2020) (124)	19 Healthy older adults (55-75yr)	Randomized controlled parallel group trial	Individualized energy, high protein mycoprotein-based diet (protein 1.8g/kgBW/day, 23-28% energy from fat, 42-52% energy from carbohydrate) vs animal-based diet (protein 1.8g/kgBW/day, 23-26% energy from fat, 49-54% energy from carbohydrate)	3 d	→ 12% and 13% ↑ in daily MPSRs in leg that had conducted daily bout of unilateral resistance-type leg extension exercise during mycoprotein and control dietary periods respectively. No differences between diets.

Abbreviations and symbols: AA, amino acid; APO, apolipoprotein; BMI, body mass index; BP, blood pressure; BW, body weight; CBC, complete blood count; DHA, docosahexaenoic acid; dw, dry weight; FC, free cholesterol; FSRs, fractional protein synthetic rates GLP-1, glucagon-like peptide 1; HDL-C, high-density lipoprotein cholesterol; iAUC, incremental area under the curve; IS, insulin sensitivity; LDL-C, low-density lipoprotein cholesterol; MPSRs, muscle protein synthesis rates; PC, plasma cholesterol; PYY, peptide YY/peptide tyrosine tyrosine; SGOT, serum glutamic oxaloacetic transaminase; TC, total cholesterol; TGs, triglycerides; VLDL, very low density lipoprotein; WC, waist circumference; ww, wet weight; ↑, increase; ↓, decrease.

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1.5. Thesis hypothesis

Both mycoprotein's high fibre content, and the composition of that fibre, are suggestive of potential benefits in gut and systemic health. In contrast there is a strong association between red and processed meat consumption and CRC risk, thus, it is both important and timely that any benefits associated with replacing meat with mycoprotein are clarified.

I hypothesise that consuming mycoprotein in place of red and processed meat will elicit a positive influence in the gut and modulate markers indicative of reducing long term CRC risk and benefiting gut health.

1.6. Thesis outline

This thesis is structured over 8 chapters. Chapter 2 presents a rationale for the various methodologies used in the project. Chapter 3 is a literature review which has been submitted for publication in Mutation Research Reviews. This review was written as I planned the main dietary intervention study in this project; its purpose was to evaluate the suitability of available risk biomarkers for use in CRC prevention trials. Four experimental chapters follow suit; the first chapter presents the primary outcome measures from the randomised crossover dietary intervention trial, which investigated the effects of replacing a high intake of red and processed meat with mycoprotein on faecal genotoxicity and other measures of gut health. In the second experimental chapter, I leverage samples collected from the intervention trial to explore the wider effects of consuming mycoprotein on outcome measures relevant to cardiovascular and metabolic health. The following chapter is dedicated to exploring the rich metabolomic and metagenomic datasets obtained from the human intervention, asking questions which were not set *a priori* and exploring relationships between microbial communities and metabolites which could reveal future avenues of research. In chapter 7, a modelling analysis considers the implications for nutritional intake, if meat were to be replaced with mycoprotein, amongst other meat alternatives in the United Kingdom. Here, I used a substitution model based on the National Diet and Nutrition Survey Rolling Programme data set. This chapter, which has been published in the British Journal of Nutrition, was not intended at the outset of the project; the work was carried out whilst awaiting the easing of coronavirus restrictions. This chapter is well placed within the current area of nutrition, with respect to the emerging, but under researched meat alternative food group, as well as an increased awareness that reducing the amount of meat consumed is required to mitigate both environmental damage and health perturbations.

The thesis closes with a general discussion in which I contextualise the study findings, address the limitations of my work and consider how mycoprotein research should develop going forward.

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CHAPTER II

GENERAL METHODOLOGY

2.1. Randomised crossover control clinical trial

An investigator-blind randomised crossover control trial was used to test the hypothesis that consuming mycoprotein in place of red and processed meat will provide benefits to gut health. The advantage of using a crossover design is that participants are exposed to all treatments, separated by washout periods, thus acting as their own controls as opposed to a parallel-group design, in which participants are required to be matched for numerous biological traits to enable reciprocally robust findings and conclusions to be drawn. This allows for smaller cohorts to be recruited in comparison to parallel-group studies (5).

The crossover study comprised two separate two-week dietary phases, separated by a four-week washout period (**Figure 2.1.**). Each day during the two-week dietary phases, participants consumed 240 grams (uncooked weight) of either red and processed meat or mycoprotein; in the form of Quorn products. Participants were randomly allocated to the order in which they received the diets. At the beginning and conclusion of each dietary phase, blood, urine, and stool was collected from participants following an over-night fast. In addition, anthropometric measurements including body weight, blood pressure, body fat percentage, waist and hip circumference were taken. During each dietary period, participants completed a one-day food diary documenting food and beverages consumed to capture differences in nutritional intake between the diets.

The quantity of meat was chosen as previous literature demonstrates this can raise the endogenous production of metabolites deemed to be carcinogenic but is also proven to be a tolerable level of intake (6-8). It is also a quantity of meat that members of the public are currently consuming. In data obtained from the National Diet and Nutrition Survey Rolling Programme (NDNS-RP), red and processed meat consumption for parts of the population far exceed the dosage used in the current work (**Table 1**). The reasoning for matching the substrates for weight, as opposed to an alternative, such as energy or protein content, was to simulate a scenario where consumers replace meat in their dish with a meat alternative like Quorn. In this instance, consumers may not consider energy or protein content in this replacement, unless health conscious and meticulous over nutritional intake. Both the quantity of foods used, as well as the replacement methodology, allow the findings of this work to be relatable to the general population.

Figure 2.1. Mycomeat study design

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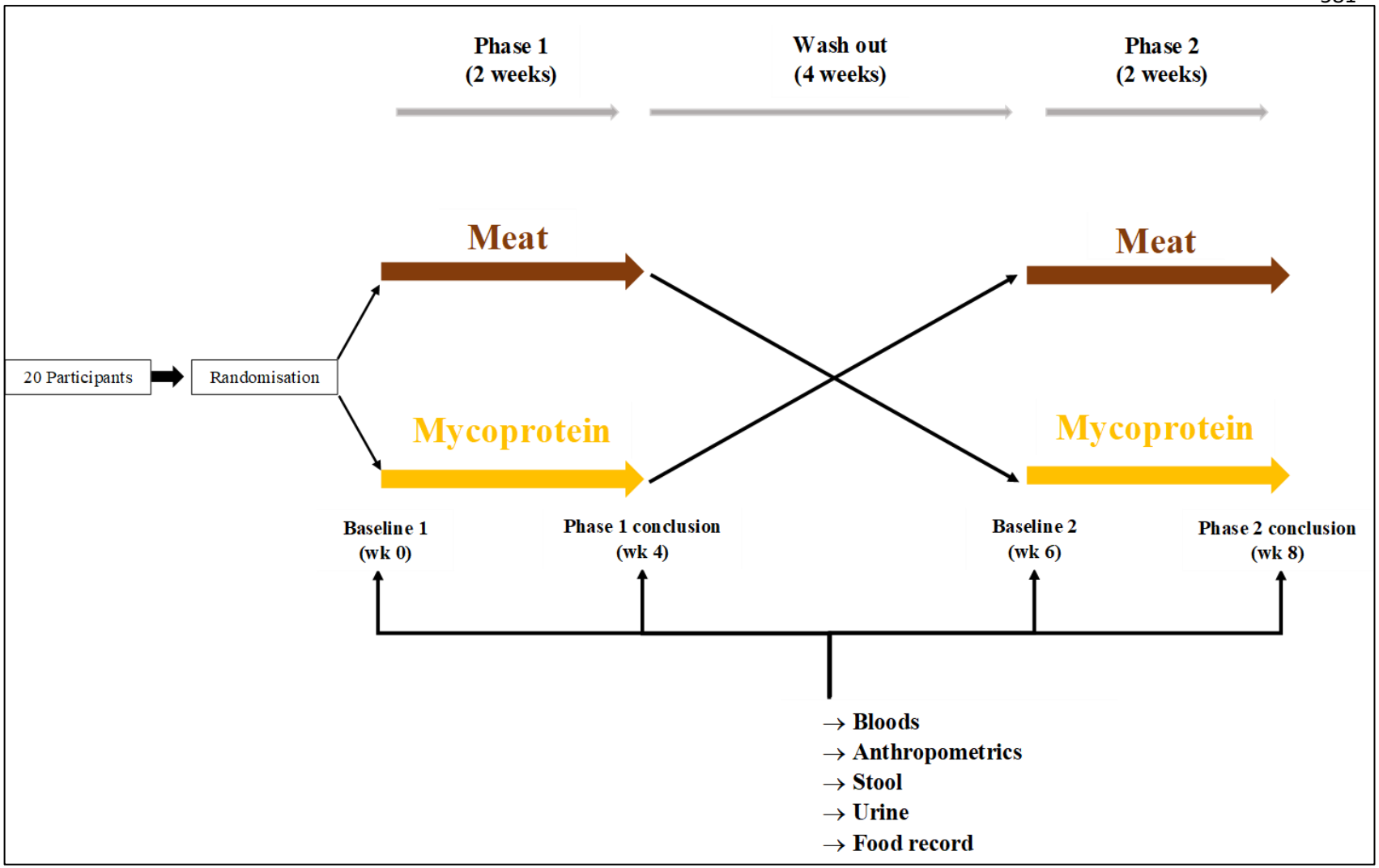

Table 2.1. Red and processed meat intake in the adult UK population

Population group	Mean intake (g/day)	Range (g/day)
Male, 19-64yr	102.53	0 – 359.03
Female, 19-64yr	65.27	0 – 313.57
Male, 65yr+	94.86	0 – 289.96
Female, 65yr+	53.90	0 – 174.07
Total population	70.09	0 – 359.03

Data obtained from the National Diet and Nutrition Survey Rolling Programme, survey year 9 (2017-2018)

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2.2. Primary study outcomes

2.2.1. CometChip; High throughput single cell gel electrophoresis assay (Comet assay)

To assess faecal genotoxicity, the CometChip was used; a high-throughput version of the single cell gel electrophoresis assay (also referred to as the comet assay).

DNA damage is a critical factor in the CRC pathway. Gut microbial products and food components are thought to be principally responsible for the damage that initiates disease progression (9).

The comet assay is a rapid, sensitive and relatively simple method for measuring DNA damage (10). The most commonly used method is to separate faecal water from homogenized faeces by high-speed centrifugation (typically 2 h at 14,000–70,000 g), and subsequently expose enterocyte cell lines (Caco-2, HT 29, or HT 29 clone 19A) to faecal water at 5–50% dilutions for 30 min at 37°C (9). Cells are then isolated and embedded in agarose on a microscope slide prior to lysis with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at a high pH leads to broken DNA strands migrating towards the anode and forming structures which resemble a comet (11).
Visual analysis of the slides by fluorescence microscopy incorporating DNA staining gel allows the extent of DNA damage to be determined by the intensity of the comet tail relative to the head; reflecting the number of DNA breaks (12) (**Figure 2.2.**).

Although the comet assay has been routinely used for research involving DNA damage and repair as well as genotoxicity testing, it has been reported as having poor reproducibility due to sample-to-sample, person-to-person, and lab-to-lab variability (3, 13). Image acquisition and data analysis are laborious, rendering the assay low throughput. To minimize sample-to-sample variation and to reduce comet assay labour, the CometChip was developed by the Engelward laboratory and Bhatia laboratory at MIT (14-16). The CometChip is based on the same underlying principles as the traditional comet assay mentioned above **(Figure 2.3.)**. The high-throughput capability is achieved by capturing cells within an array of agarose microwells. This eliminates overlapping comets, enabling a greater number of analysable comets per mm^2, and allows the assay to be performed in a 96-well format (14). In addition, cells are on the same plane, facilitating the analysis of dozens of comets from a single image. These characteristics, combined with automated analysis, mean the CometChip assay drastically reduces the analysis duration (3). Since it is a high-throughput version of the comet assay, the CometChip can be used to detect the same classes of DNA damage. The high-throughput nature, time efficiency, and greater reproducibility of this form of the comet assay is reasoning for its incorporation.

636 The comet assay's relevance to oncogenesis remains to be confirmed, however faecal samples are a
637 non-invasive proxy for toxic exposures in the colorectal mucosa; thus faecal genotoxicity may be seen
638 as a potential intermediate between dietary genotoxins in the colon and colorectal cancer (17).
639 Incorporating this methodology has the potential to predict the impact that dietary patterns high in red
640 and processed meat and high in mycoprotein may have upon colorectal cancer risk.

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Figure 2.2. The traditional Comet Assay

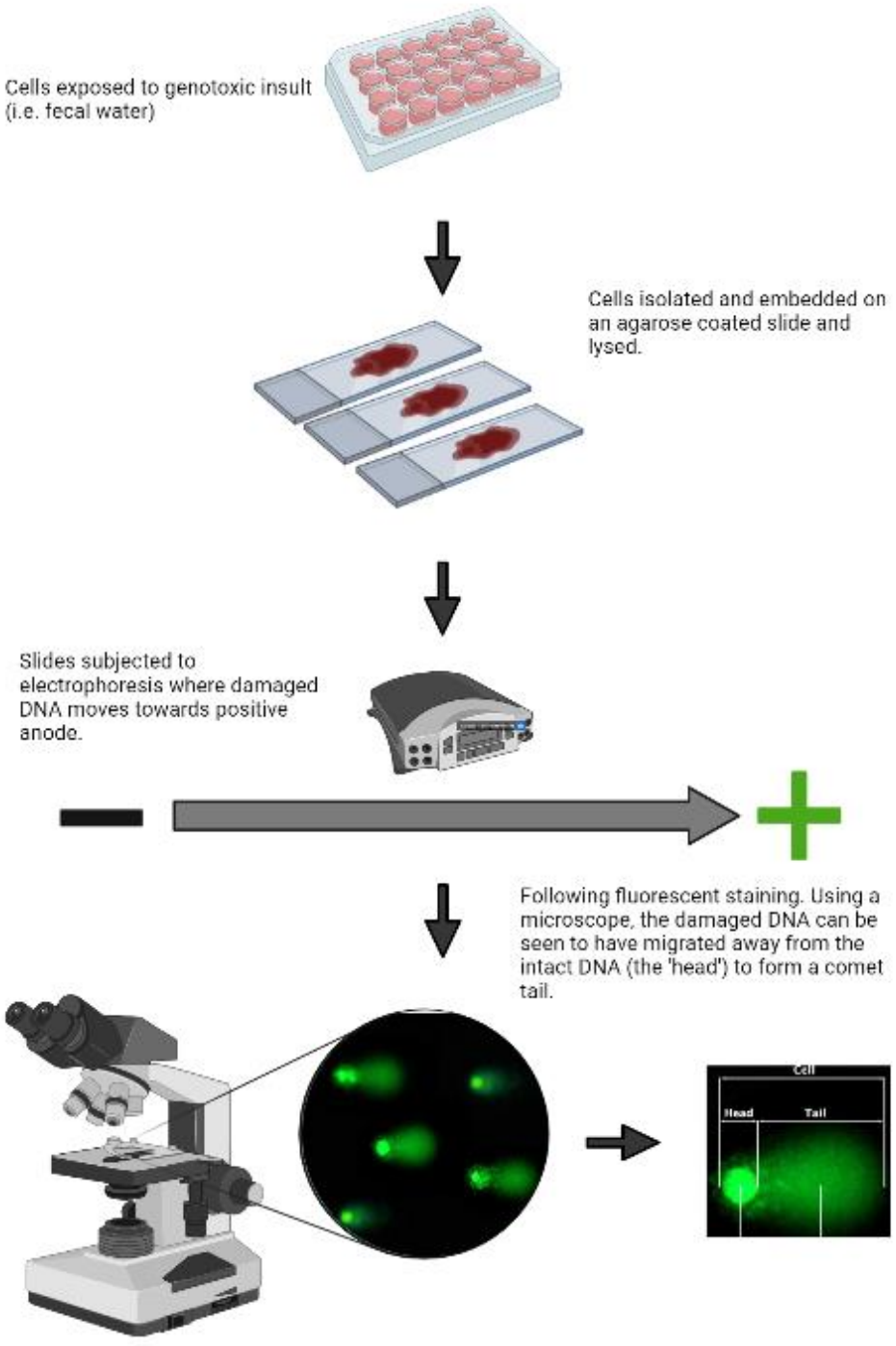

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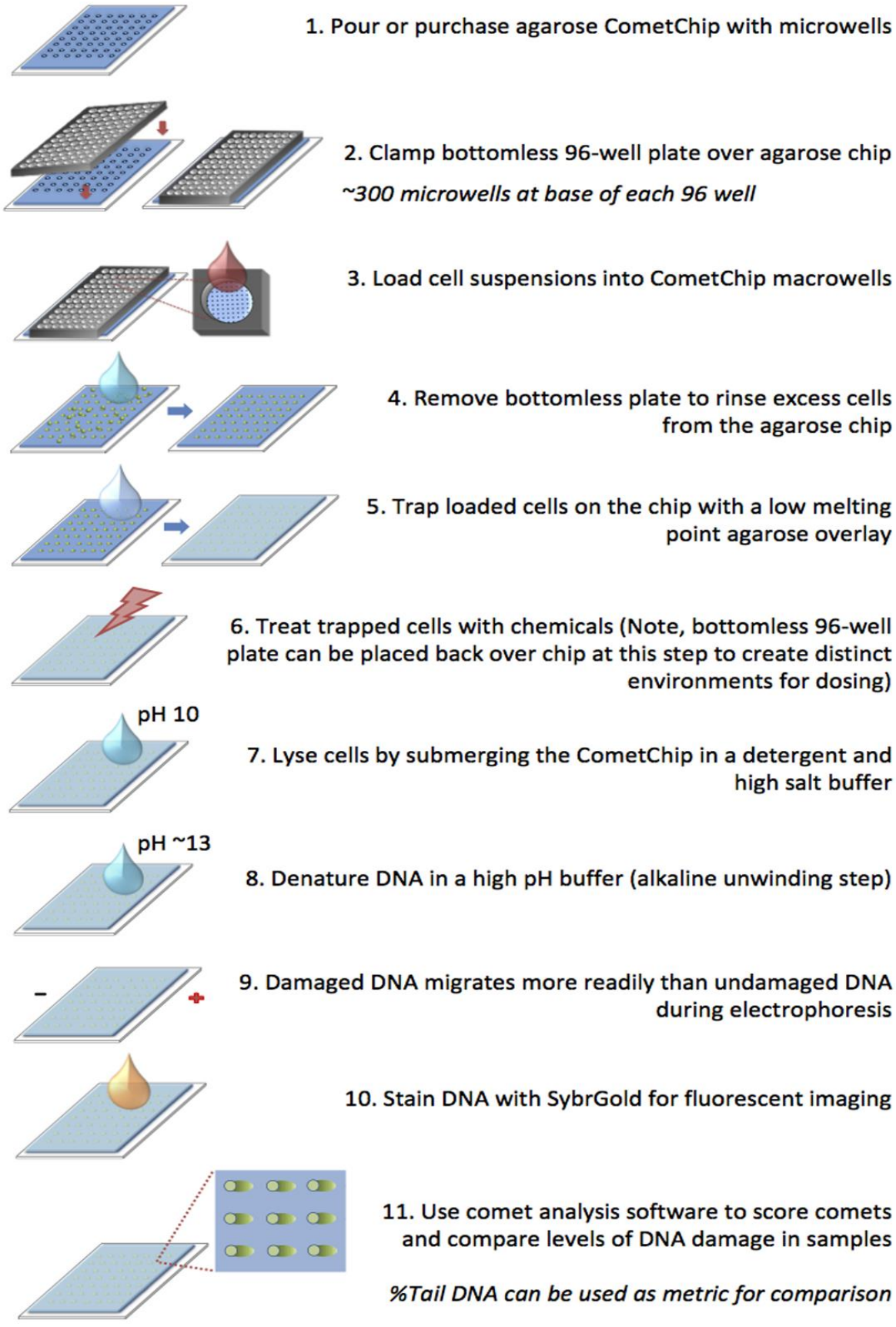
Figure 2.3. The CometChip (3).



2.2.2. MTT Assay

The MTT assay was incorporated to validate the concentration of faecal water to be used in the genotoxicity assays. If faecal water is too concentrated, cells are destroyed and the degree of genotoxic damage in cells cannot be adequately quantified.
The MTT assay is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals, by cellular dehydrogenases (**Figure 2.4.**) (18, 19). This reaction proceeds in viable cells with active metabolism; when cells die, they lose the ability to convert MTT into formazan (18, 20); therefore, colour formation serves as a useful and convenient marker of only viable cells. The insoluble formazan crystals are dissolved using a solubilization solution and the resulting solution is quantified by measuring absorbance at 500-600 nanometres using a multi-well spectrophotometer (21, 22). The darker the solution, the greater the number of viable, metabolically active cells.

Figure 2.4. MTT to Formazan reaction

A) The yellow MTT is converted by the mitochondrial reductase into the purple formazan.

B) A microtiter plate after an MTT assay. Increasing amounts of viable cells results in increased purple colouring.

Figure adapted from (1)

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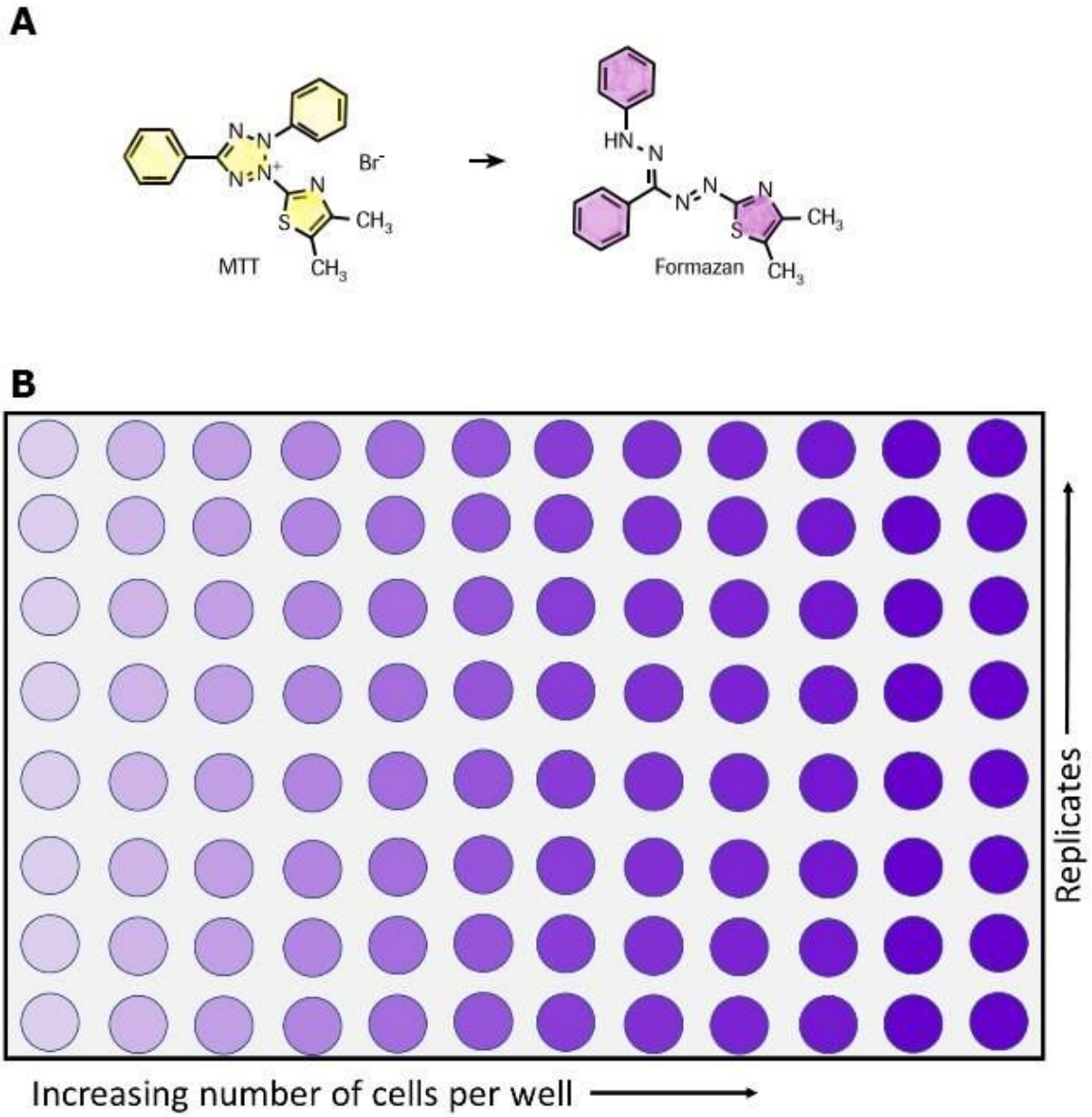

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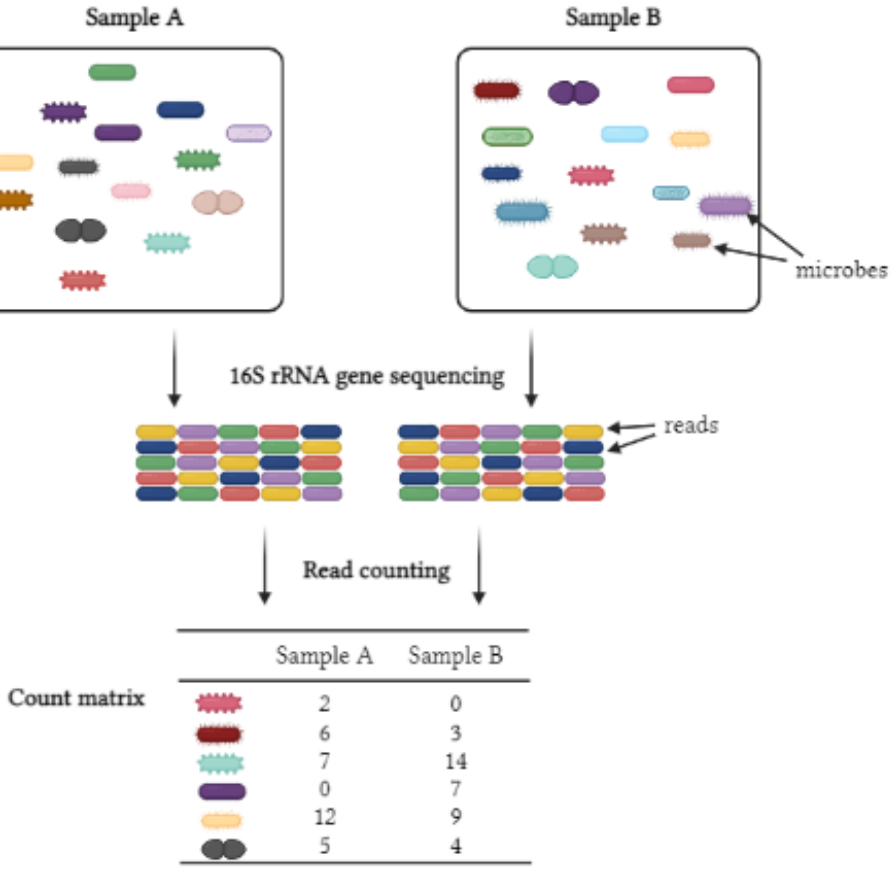
2.2.3. 16S ribosomal RNA gene sequencing

16S ribosomal RNA (rRNA) gene sequencing was carried out to characterise diet effects on gut microbial composition, using an Illumina 16S metagenomics amplicon sequencing pipeline. The use of the 16S rRNA gene as a phylogenetic marker has proven to be an efficient and cost-effective strategy for microbiome analysis (23, 24).
The 16S rRNA gene is used as target gene because it is present in all prokaryotes and contains regions that are highly conserved between species. The conserved regions serve as amplification targets for polymerase chain reaction (PCR) primers to extract one or more variable regions of the 16S rRNA gene. The latter are then subjected to next-generation sequencing (NGs), producing thousands of sequenced fragments, called 'reads'. Finally, the reads are mapped to known 16S rRNA gene sequences of different microbial taxa, such as genera or phyla, or clustered according to their sequence similarity into operational taxonomic units (OTUs) (25).
The number of NGS reads assigned to each taxon/OTU; referred to as a 'count', is used as a proxy of the abundance of that taxon/OTU in the original sample (**Figure 2.5.**).

The ability to capture a large coverage of microbial abundances enables indices of diversity to be investigated, providing valuable mathematical tools to detect changes in microbial composition between different conditions (2).
In the human intervention, measures of diversity implemented were Shannon index, inverse Simpson index, and Fisher's index. The number of species is a measure of richness, the more species present in a sample, the 'richer' the sample. Evenness is a measure of the relative abundance of the different species making up the richness of an area - as the richness and evenness increase, so does the diversity. Shannon's diversity is a widely used metric to characterise species diversity, accounting for both richness and evenness of a community. Simpson's diversity index is another measure of diversity that considers both richness and evenness. The value of Simpson's index of diversity (D) ranges from 0 to 1; the greater the value, the greater the sample diversity. Simpson's index is usually expressed as its inverse (1/D), and the meaning is the same; the higher the diversity, the greater the value (26). Fisher's index represented the first attempt to describe mathematically the relationship between the number of species and the number of individuals in those species by implementing a logarithmic series model (27).

Employing this methodology enabled the opportunity to gather an understanding of the microbial dynamics in the gut when consuming diets high in red and processed meat or mycoprotein.

Figure 2.5. Schematization of 16S rRNA gene sequencing data generation.
16S rRNA gene sequencing count data are generated through three main steps. First, microbial communities of a set of samples are subjected to 16S rRNA gene sequencing. The reads are then assigned to the different species of microbes. Finally, the reads assigned to each species are counted and summarized in a count matrix, representing the abundances of the different species in the samples. Figure adapted from (2).



2.2.4. Gas chromatography time of flight mass spectrometry (GC-TOF-MS)

Using gas chromatography time of flight mass spectrometry (GC-TOF-MS), the quantification of short chain fatty acids (SCFA) in faecal samples was assessed. SCFAs are the main metabolites produced in the large intestine through the microbial fermentation of indigestible polysaccharides such as dietary fibre and resistant starch, playing a pivotal role in maintaining colonic homeostasis. In addition, a substantial body of evidence demonstrates these metabolites exert beneficial effects in many peripheral sites in the body, impacting metabolic, endocrine, immune and neurological heath (28-32).

Gas chromatography (GC) is an analytical technique used to separate the chemical components of a sample mixture followed by detecting their abundance (33). GC is the useful methodology for characterisation of metabolites such as SCFA, due to their low molecular weight and volatility; chemical properties suited for GC analysis (33). Hyphenation of GC with mass spectrometry (MS) allows identification and quantification of a wide range of GC-amenable compounds in complex matrices (34).

MS is based on ionization and fragmentation of sample molecules in the gas phase. Since molecules fragment in a unique manner, the resulting ion fragmentation pattern can be used to obtain structural information for a given molecule. This structural information is then used to generate large libraries of molecules that can then be identified in sample matrices (35). There are different strategies in MS, however time of flight (TOF) may have superior sensitivity over time in comparison to other methods (36).

The combination of these factors: identification of small, volatile compounds, as well as sensitive recognition and characterisation of specific molecular structures in complex matrices, is reasoning for incorporating this methodology for the analysis of microbial metabolites.

2.2.5. Untargeted metabolomics performed via ultra-high performance liquid chromatography (UHPLC) coupled with high resolution mass spectrometry (HRMS)

To determine changes in the excretion of gut metabolites in urine and faecal samples in a non-targeted facet, ultra-high performance liquid chromatography (UHPLC) coupled with high resolution mass spectrometry (HRMS) was used.

UHPLC is predominately chosen to identify, quantify and separate components of a mixture by using high pressure to push solvents through the column. In UHPLC, particle sizes less than 2um can be used, providing better separation than high performance liquid chromatography (HPLC) where particle size is limited to 5um. These smaller particles require higher pump pressures, rendering this technique very efficient with fast analysis and higher resolution (37). Therefore, UHPLC has the advantages of improved resolution, higher peak efficiency, fast separations and reduced solvent consumption

compared with conventional HPLC, opening up new possibilities for improving the analytical methods for complex sample matrices, such as ingredients, foods and biological samples (38).

Coupling of UHPLC with mass spectrometry (MS) brings further advantages in terms of selectivity, sensitivity, and high throughput for the analysis of complex samples (39). UHPLC coupled to triple quadrupole tandem MS or high-resolution MS is an important tool for both targeted and nontargeted analysis (40). This enables a better means of identifying and quantifying metabolites in complex matrices such as those biological samples collected in the human intervention. The MS used was an Orbitrap ID-X Tribrid Mass Spectrometer (OTMS) (41). This system is a mass analyser technology dedicated for small molecule analysis, as well as the determination of low abundance molecules in complex samples (41-43). By combining quadruple and linear ion trap mass analyser technologies, this enables data acquisition with less experimental setup and interpretation time (41-43). The OTMS can capture these low-abundance metabolites that may not be targeted *a priori*, allowing for a greater coverage of microbial products in urine and faeces when consuming the tested diets. Using an untargeted approach is gaining appreciation in many fields, including nutrition, as it provides opportunity to discover novel metabolites that could be relevant to health (44-47).

Further benefits can be drawn from the ability to annotate specific compounds in biological samples, as a range of metabolites of interest *a priori* can likewise be identified in these large datasets that may not been infeasible to measure and quantify separately due to time restraints. Overall, this allows a greater spectrum of metabolites to be obtained that can contribute towards the conclusions of the present thesis.

2.2.6. Nitric oxide analyser for N-nitroso compound quantification

Quantification of nitrate, nitrate, and total N-nitroso compounds (NOCs) in faeces was assessed using a Nitric Oxide Analyser (NOA). Although nitrite and nitrate are not carcinogens themselves, exposure to high levels may have a genotoxic risk for humans due to the ability to react with NOC precursors in the gastrointestinal tract, mainly amines and amides, thereby subsequently forming NOCs (48). Many NOCs are known to be carcinogenic (49) and are implicated as a plausible link between red and processed meat consumption and colorectal cancer (50). NOCs can be endogenously produced during digestion of red and processed meat (51, 52), as well as ingested from consuming processed meat due to the use of nitrites in the production of such products (53). Nitrates and nitrites can be recovered in human faeces, urine and saliva which can infer the level of endogenous production following exposures such as diet (54-56). The NOA is a high-sensitivity detector for measuring nitric oxide (NO) based on a gas-phase chemiluminescent reaction between nitric oxide and ozone. The principle of chemiluminescent NO detection is based on the rapid reaction of NO in the gas phase with ozone (O3), which yields NO2* in an excited state. As the excited electron returns to its ground state, a photon is emitted and is detected as chemiluminescence (hv) (57):

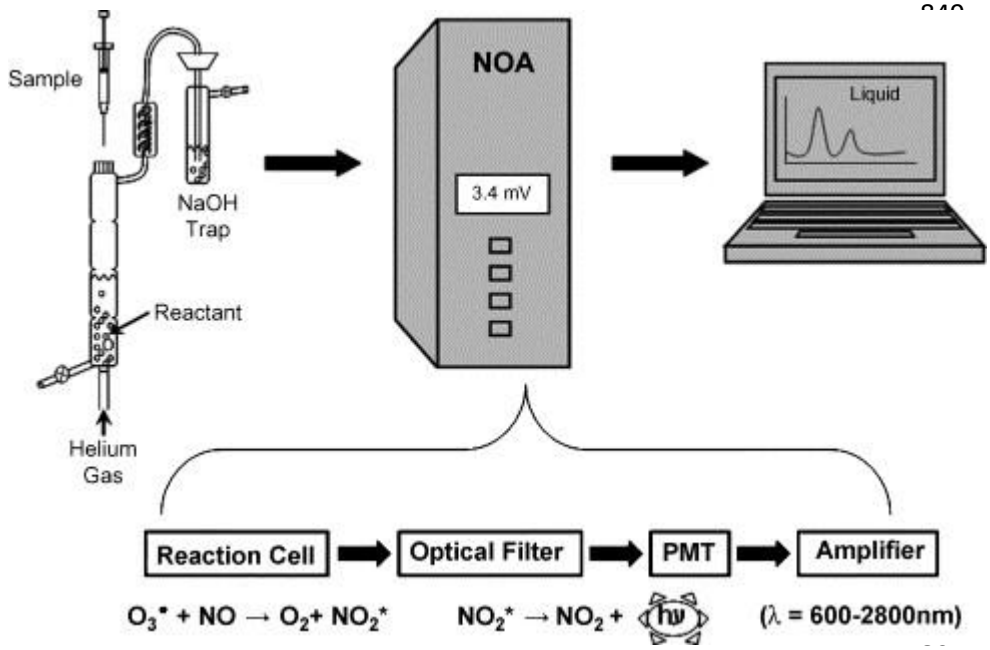
$NO + O3 \rightarrow NO2^* + O2$

$NO2^* \rightarrow NO2 + h\nu$

This emitted light is detected and amplified by a photomultiplier tube (PMT), to generate an electrical signal (**Figure 2.6.**).

Although many methods and analytical techniques are employed to measure NO and its metabolites, in recent years, gas-phase ozone-based chemiluminescence has emerged as the most commonly used assay for NO detection due to its specificity, sensitivity, versatility and dependable operating performance (4). This is reason for its widespread use in dietary meat interventions; previous work deciphering the effects of high meat diets have incorporated NOA and demonstrated increased excretion of nitrates, nitrites, and total NOCs in stool following such dietary practices (51, 56, 58, 59).

Figure 2.6. NOA mechanism and apparatus set up.
Samples are injected into the glass purge vessel containing reductive solutions which is bubbled with helium gas. NO in the headspace is carried through a NaOH-trap into the NOA, which generates ozone to react with NO. The reaction of NO with ozone produces chemiluminescence which is detected by the photomultiplier tube and the signal is recorded by data recording software (4).



2.3. Secondary study outcomes

2.3.1. Roche COBAS c702 analyser for blood biochemical analysis

Blood biomarkers with relevance to cardiometabolic health were incorporated as secondary endpoints for the intervention. Incorporating secondary outcome measures, as dictated *a priori* by study specific aims, has the advantage of increasing the scope of hypotheses tested (60). In the context of the current work; alongside the strong ties of meat consumption and colorectal cancer, associations also exist between high meat diets and cardiovascular disease, with data suggestive that substituting plant protein for animal protein may confer benefits to blood biochemistry (61), which can the lower risk of cardiovascular disease (62, 63). It is therefore of interest to clarify the effects on blood biochemistry elicited by the human intervention. Concentrations of total cholesterol, triglycerides and glucose in blood were quantified in conjunction with The Department of Blood Sciences at Newcastle Royal Victoria Infirmary Hospital, using a Roche COBAS c702 Clinical Analyser (64). This is a high throughput clinical chemistry module capable of up to 2000 tests/hour that performs photometric assay tests for a wide range of analytes (64). This enables the ability to produce rapid and reliable results; which is reasoning for its preferential use in large clinical settings such as hospital blood science laboratories and the inclusion in this work.

2.3.2. Randox Daytona blood lipoprotein analysis

To clarify excursions in blood lipoprotein fractions, a Randox Daytona Clinical Analyser was used (65). Perturbations in concentrations of blood lipoprotein fractions is a hallmark of cardiovascular disease (66). Diets containing meat may shift lipoprotein characteristics to an atherogenic profile, while nonmeat dietary practices provide more desirable effects (67, 68). The Randox Daytona is a fully automated analyser with a broad spectrum of analytes that can be quantified in a range of biological samples (65). Due to the provision of reliable and accurate results, this equipment is routinely used in assessment of nutritional status across many interventions (69-71), and provides reasoning for incorporation in the current work.

2.3.3. Anthropometric measurements

Secondary outcome measures from the intervention also comprised anthropometric measurements of body weight and body fat, using a Tanita machine; waist and hip circumference, measured using a tape measure, as well as blood pressure using a blood pressure monitor.

These endpoints all have relevance to cardiometabolic disease, with increased body weight (72), body fat (73), and waist circumference (74) widely appreciated as risk factors. Although the intervention was short in duration and therefore, body composition was not expected to be altered significantly, excursions in these measurements may be indicative of a larger effect if chronically consuming such dietary patterns.

With respect to blood pressure, high blood pressure is one of the most important risk factors for cardiovascular disease (75), which is evidenced to be positively influenced by increased fibre intake (76). It may be that mycoproteins fibre content could positively influence blood pressure indices.

2.3.4. Flame photometer for urinary sodium and potassium

Urinary excretion of sodium and potassium was measured using a flame photometer. Meat alternatives, such as Quorn, can sometimes be high in sodium, which is one of the reasons for concerns surrounding the widespread consumption of this food group (77). Increased sodium intake is associated with an increase in blood pressure and risk of hypertension (78, 79), which manifests in an increased risk of cardiovascular disease (80). It is therefore of interest to discover if the high sodium content of meat alternatives would raise the excretion of sodium in biological samples such as urine. As indicated above, another secondary outcome measure was blood pressure, which along with urinary sodium excretion, will capture a greater picture with relevance to sodium intake and the effects on blood pressure.

A flame photometer is an analytical instrument used for the determination of sodium and potassium, among other ions in body fluids. The principle of flame photometry is based on the thermal dissociation of atoms in these compounds when exposed to a flame, with some of the atoms produced in this process excited to a higher energy level. The atoms then emit radiations when returning to the ground state. These radiations generally lie in the visible region of a spectrum, with each of the compounds having a specific wavelength, allowing for the different compounds to be captured individually. The intensity of the emission is directly proportional to the number of atoms returning to the ground state – and the light emitted in turn, is proportional to the concentration of the sample (81, 82).

In the analysis of sodium and potassium, the flame photometer enables the rapid and accurate measurement of these compounds over a large range of values (83). This is reasoning for its incorporation in the current work, alongside other advantages including the low cost compared with other techniques such as atomic absorption or atomic emission spectrophotometry - where the emitted light is analysed with a monochromator, as well as how flame photometry does not suffer from spectral and ionization interferences because ionization only occurs at higher temperatures (81).

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CHAPTER III

LITERATURE REVIEW

The potential of risk biomarkers to be employed as clinically relevant endpoints in colorectal cancer prevention trials.

Abstract

Colorectal cancer (CRC) is a leading cause of morbidity and mortality. Epidemiological evidence implicates dietary and lifestyle exposures; however, the precise aetiology of the disease is not well defined. Experimental evidence from well controlled trials in human volunteers are needed to further elucidate links between diet and disease. Intervention studies exploring factors influencing CRC risk are limited by the availability of well-validated and practical endpoints that might be applied as attenuable markers of early CRC risk.

In this review, I evaluate the current panel of risk biomarkers available for use in human intervention studies evaluating CRC. I discuss these risk markers as being either i/ markers capturing biological transition, i.e., polyp recurrence, the induction of aberrant crypt foci, crypt cell behaviour, and aberrant epigenetic measures; ii/ markers capturing risk-exposures, including toxicological and metabolite assessments of stool and other samples; and iii/endpoints that might be characterised as biological indicator markers, such as changes in the gut microbiome. I discuss the application of these risk markers in intervention study models and evaluate their relative strengths and weaknesses.

Going forward, I recommend validation of risk biomarkers against each-other, and where possible, leveraging existing cohort samples against cancer outcomes. I strongly advocate integration of these markers into future prospective cohort studies. I note with some concern that the existing biomarker panel largely assumes common aetiology underpinning the various molecular sub-types of colorectal cancer and argue that this must be factored into future biomarker design and validation programs.

3.1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide [1], with a lifetime risk approaching 5% [2]. Observational studies associate CRC occurrence with ageing, inflammatory disease, sedentary lifestyles, excessive adiposity, western dietary patterns, and the composition and activity of the gastrointestinal microbiota [3]. Observational and prospective cohort studies upon which most of our understanding of diet and cancer is based are limited by flawed dietary data collection tools, such as food frequency questionnaires. This methodology may adequately evaluate overall patterns of food consumption associated with cancer risk; however, these tools rarely have the power to capture accurate data on infrequently consumed food items or ingredients with specific causative or chemo-preventative qualities. Experimental data from animal models have identified a number of such food items with the capacity to influence the carcinogenic process, including various strains of probiotics, certain prebiotics and a variety of non-nutritive food components such as curcumin and various phenolics [4-6].

The ultimate goal of these studies is to support dietary or chemo-preventative public health policy and to advocate the incorporation, or removal of, foods that may ameliorate, or exacerbate, risk [7]. Ethically, public health strategies affecting lifestyle can only be justified with robust evidence. Proving causality, or indeed protective effects, in humans ultimately requires experimental evidence from randomised control trials with clinically relevant endpoints. Using cancer incidence itself to assess the efficacy of putative chemoprevention strategies is highly impractical due to the long duration of the carcinogenic process and the large numbers of study volunteers required. There are additional ethical barriers to demonstrating disease causation with high-risk exposure interventions. For these reasons, various attenuable surrogate endpoints have been proposed for use as cancer risk biomarkers in intervention studies [8]. Schatzkin et al. characterised a surrogate endpoint biomarker as a "biological event which takes place between a carcinogenic or external exposure and the subsequent development of cancer" [9]. Broadly speaking, the panel of risk biomarkers may be broken up into i/ 'biomarkers of phenotypic, or molecular, biological transition in the cancer pathway' which generally require invasive tissue sampling, but which may be strongly related to cancer onset; ii/ 'biomarkers of exposure' which are often easier to collect and assume exposures to mutagens and tumour promoters are closely linked to long term cancer risk; iii/ 'indicator markers', which may change consequential to neoplastic transformation, but are not necessarily reflecting processes that affect the cancer pathway, this type of marker has been well developed for diagnostics but are not well used in the assessment of risk.

Here I review the suitability of the currently available panel of risk biomarkers for use in CRC prevention/causality human intervention studies. The purpose of this review is to inform future intervention trials, to identify the gaps in biomarker identification and to encourage research investment in this area. Well validated CRC risk biomarkers would facilitate future chemoprevention trials as well

as investigations assessing risk exposures by enabling ethically appropriate studies of manageable sample sizes and durations [10, 11].

3.2. Biomarkers of phenotypic, or molecular, biological transition in the cancer pathway

3.2.1. Polyp recurrence

A colorectal polyp is an intermediate neoplastic staging post in the developmental trajectory of major subsets of CRC; and they are thus routinely removed to mitigate disease development. [12]. Individuals who have polyps identified at endoscopy are at an increased risk of developing further polyps; it is hypothesised that field effects occur in macroscopically normal colorectal tissue, inducing what may be termed the 'polyp former phenotype' [13]. Due to this risk of polyp return, patients undergo regular screening programs post-polypectomy, creating a window of opportunity for chemoprevention trials; with treatment efficacy assessed by following patients for polyp recurrence.

This model has been used with different levels of success; Sandler et al. [14] tested a daily dose of 325mg aspirin over a mean duration of 12.8 months (n = 517), reporting significantly fewer polyps at follow up with the treatment compared to a control group (mean (± SD) = 0.30 (± 0.87) vs. 0.49 (± 0.99), P = 0.003). In an earlier study assessing the effects of 1.2g/day of calcium (n = 832), Baron et al [15] reported a significant reduction in the recurrence of colorectal polyps at 4 years compared to the placebo treatment (RR (95% CI) = 0.76 (0.60-0.96), P = 0.02). In a later trial however, the same group failed to replicate these findings using a daily supplementation of either 1.2g calcium (RR (95% CI) = 0.95 (0.85–1.06)), 1000IU vitamin D3 (RR (95% CI) = 0.99 (0.89–1.09)), or a combined treatment (RR (95% CI) = 0.93 (0.80–1.08)) [16]. Ishikawa [17] investigated the impact of a daily probiotic (1g preparation of *Lactobacillus casei)* and prebiotic supplement (25g wheat bran biscuit; containing 7.5g wheat bran) in a 48 month trial (n = 380). At the conclusion of the study, risk of polyps was significantly lower following the probiotic supplementation (OR (95% CI) = 0.65 (0.43-0.98)); whereas the prebiotic treatment resulted in an increased risk (OR (95% CI) = 1.31 (0.87-1.97)).

Critics of polyp recurrence studies argue that they target late-stage events in the cancer process, evidencing either an anti or pro cancerous effect in tissue already transformed into the polyp former phenotype. This may explain inconsistencies between the findings from polypectomy clinical trials and the observed anticancer effects of various treatments in animal models and suggested protective effects of these treatments from the epidemiological literature. For example, Colbert et al. [18] observed no protective effects on polyp recurrence for physical activity, a finding in stark contrast to the conclusions

of a meta-analysis, on the effects of physical activity on CRC risk in prospective cohort trials, published by the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) [19].

From a practical perspective, clinical polyp recurrence trials are highly invasive and may require several years for a thorough evaluation of treatment effect, with typical intervention periods ranging from 1 and 5 years [14-17, 20-22]. Additionally, complex nutritional and pharmacological based interventions can be demanding for both the investigators and participants, threatening both high participant attrition rates and compromised compliance [23, 24].

3.2.2. Aberrant crypt foci

Aberrant crypt foci (ACF) are microscopic lesions whereby individual crypts of Lieberkühn appear larger in diameter compared to the neighbouring crypts; these lesions are considered an early event in the adenocarcinoma sequence [25, 26]. Treating rodents with chemical mutagens induces detectable ACF in a relatively short time frame [27, 28], which can be attenuated using chemo preventative strategies [29-32]. Notably, these precancerous foci have been identified in the human colon prior to polyp development [33]; thus representing an early event in the disease pathway that could be employed as a risk biomarker.

Past clinical trials have used colorectal ACF to assess the chemo preventative efficacy of various treatments, with mixed results. Hosono [23] significantly reduced ACF number from baseline using a daily dose of 250mg metformin in a study lasting just 1 month (n = 25, P = 0.007). Likewise, Carroll [34] observed a 40% reduction in the number of ACF in a 30-day study, phase IIa clinical trial using 4g/day curcumin, relative to a placebo treatment (P = < 0.005). Takayama et al. [35] assigned patients (n =179) to 2 months sulindac (300mg/day), resulting in a significant reduction in ACF number compared to a placebo group (P = 0.0075). This result contrasts with findings from Limburg et al. [36], who observed no significant reduction in ACF number from baseline using the same dose of sulindac albeit in a significantly smaller and therefore less well powered cohort (n = 77, P = 0.60). The same trial also failed to attenuate ACF development using other agents, including atorvastatin (20mg/day, P = 0.30) and an inulin extract supplement (2 x 6g/day, P = 0.92).

ACF are dependent on several factors like age as well as habitual dietary intake; with increased ACF identified in older age groups and those following low fibre diets [37]. In animal models, age has also been found to mitigate efficacy of chemo preventative agents [38]. Histological classification may also be a critical factor, many colorectal ACF are hyperplastic in nature and undergo fast yearly turnovers [12]; only persistent dysplastic lesions are associated with malignancy [39-41]. These factors could explain the mixed results in previous studies, as well as cast doubts on its reliability as a risk biomarker for use in human intervention trials.

That being said, it has been evidenced that ACF can be attenuated over a relatively short time frame, and this, coupled with the centrality of ACF to the adenocarcinoma sequence, makes numeration of ACF an attractive risk biomarker. The drawback is the requirement of an endoscopy, on at least two occasions (more would be required in a cross over design); limiting it to trials comprising at-risk individuals in clinical settings, where endoscopic examinations are integral to the treatment process. Additionally, ACF require further validation to predict CRC; preferably using well planned longitudinal cohort models. In the interim, case-control studies are feasible and may yield valuable information on the relation of ACF to CRC risk [42].

3.2.3. Crypt cell cycle kinetics

Complete cell renewal of the colonic epithelium is a 5 day process; involving tight control over cell proliferation, differentiation and apoptosis, across the entire length of the crypts of Lieberkühn [43, 44]. During this *wnt* signalling regulated process; stem and transit cells with high proliferative capacity reside at the base of the crypts, differentiated cells are located in the middle/upper portion, while apoptotic cells populate close to the luminal surface where they can be shed from the epithelium in the normal process of cell turnover [43-46]. Case-control comparisons of normal mucosa from patients with CRC, suggest an upward expansion of proliferative cell types, from the base through to the upper third of the crypts [43, 47-50] (**Figure 3.1.**). This expanding proliferative zone may be an early event in CRC and the associated increase in proliferative capacity may explain ACF and subsequent polyp development [43, 51]. It should also be noted that in addition to indicating dysfunctional *wnt* signals, the increased proliferative capacity may also indicate increased risk of subsequent genetic mutations due to copy error [52].

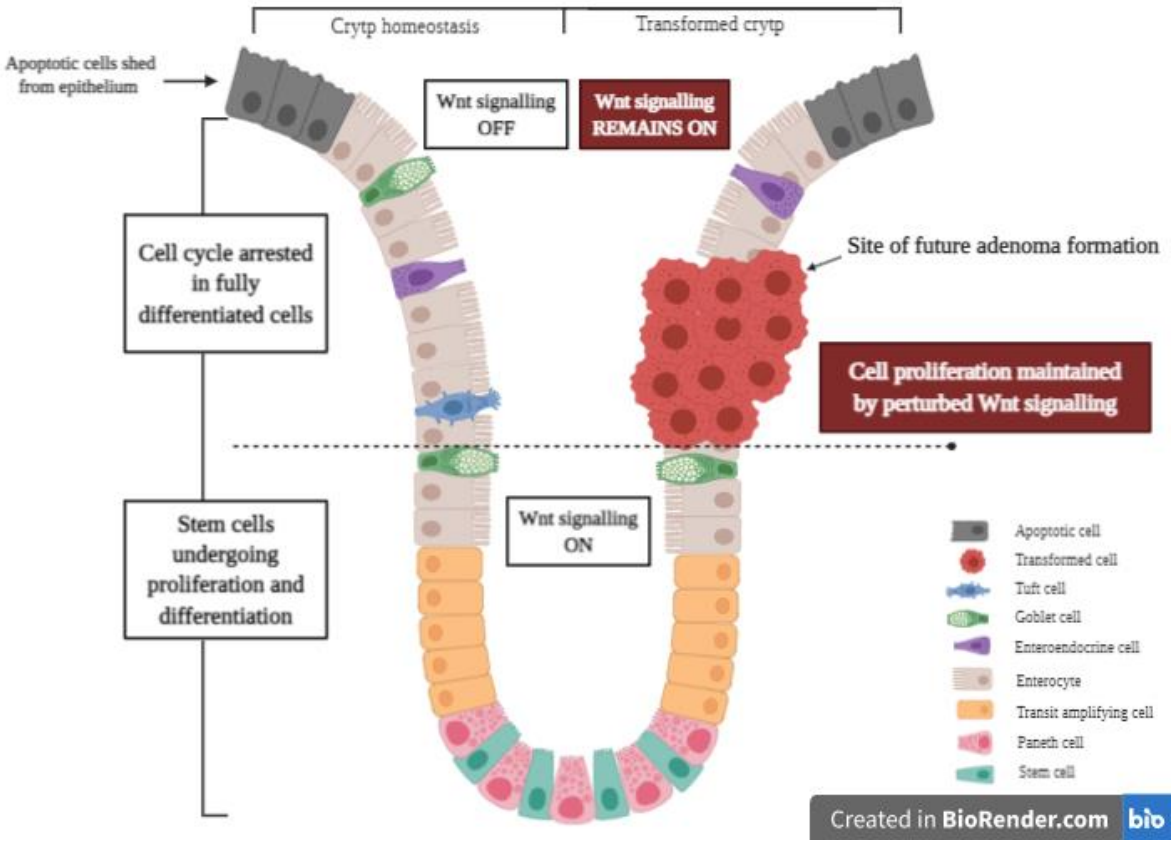
Importantly, crypt cell kinetics can predict polyp occurrence [49, 53], and has been used successfully to evaluate various prophylactic interventions. Anti and colleagues [54] identified significantly reduced cell proliferation in rectal mucosal tissue using different doses of fish oil (2.5g, 5.1g, and 7.7g/day) in a 30 day double-blind study design ($P = 0.02$). Likewise, at the end of a 12 month trial, Holt et al [55] reported a significant reduction in proliferation indices of recto-sigmoid tissue compared to a control group, when participants in the intervention group increased their calcium intake to 1200mg a day by consuming low fat dairy foods ($P = < 0.05$). Khosravian [56] reported a significant reduction in rectal cell proliferation at the conclusion of a 3 month trial where participants supplemented with 2mg of folate each day ($n = 11$, $P = 0.006$). In addition, Dronamraju et al [57] significantly reduced colonic cell proliferation compared to a placebo treatment over a 4-week period using a daily supplementation of 30g resistant starch ($n = 10$, $P = 0.028$).
In contrast, several interventions using nutritional components; assumed to be protective against CRC, were unable to positively modulate crypt cell cycle kinetics. These investigations included; calcium (doses ranging from 1.5-2.0g/day) [58-60], and fibre supplementation (13.5g/day) [61]; consumption

of oil-rich fish (300g/week) [62]; prebiotic (25g/day high-amylose maize starch) and probiotic formulations (5g/day *Bifidobacterium lactis*) [63]; as well as 6 months adherence to a Mediterranean style diet [64].

There is a strong biological argument linking aberrant crypt cell kinetics to CRC; higher rates of cell proliferation in the upper portions of a crypt may indicate poorly functioning *wnt* signalling, itself a prominent feature in the adenocarcinoma sequence [65]. The findings that crypt cell cycle kinetics can predict future polyp occurrence [49, 53], as well as the capability to attenuate the degree of crypt cell turnover within a short timeframe [54-57, 66], are justification for its use as a risk biomarker. The limitations include the degree of analyst subjectivity [7]; and as is the case for other tissue-based biomarkers, the acquisition of samples from healthy volunteers may be problematic. Therefore, like ACF, studies with crypt cell kinetics are limited to endoscopy study.

Figure 3.1. Perturbation in crypt cell cycle regulation observed in CRC.

A consistent finding in CRC is that wnt-signalling is perturbed in the crypt, leading to disruption in the compartmentalisation of cell type and regulated process of cell renewal. Cells continue to exhibit higher proliferative capacity in crypt regions where fully differentiated cells would normally reside, leading to uncontrolled cell expansion (CRC, colorectal cancer).



3.2.4. Epigenetics

Epigenetic mechanisms involved in cellular regulation include DNA methylation, post-translational modifications to histone proteins, as well as small non-coding RNAs, notably microRNAs (miRNAs); all three influence the cancer process and notably, all are attenuable by nutritional and pharmacological exposures [67-71].

Whilst epigenetic expression in colonic tissue may be of most direct relevance to CRC, many cells and tissues release some of their constituents into the bloodstream, including fragmented cell free DNA, creating opportunities to capture changes in the epigenome that may reflect CRC risk [72]. Additionally, colonocytes shed from the intestinal epithelium may be present in faecal waste, offering scope to also measure epigenetic aberrations in stool samples [73]. Thus, both blood and stool could provide less invasive sample sources for the assessment of epigenetic abnormalities.

3.2.4.1. DNA methylation

DNA methylation involves the covalent addition of a methyl group at the 5^{th} carbon of a cytosine ring, where this cytosine is then followed by a guanine; termed a CpG dinucleotide [68, 74]. This reaction is catalysed by DNA methyl transferases and often has a suppressive effect on gene expression [69, 74].

Abnormalities in DNA methylation lead to compromised DNA integrity and stability, changes in gene expression, development of mutations and chromosomal modifications [68, 75, 76]. In the development of cancers, including CRC, altered DNA methylation is recognised to play a causal role by silencing tumour suppressor genes and increasing oncogene expression [76-78]. DNA methylation, sampled from both blood and stool can distinguish patients with CRC, and importantly, preneoplastic adenomas, from those absent from disease [73, 78-82].

There is a dearth of instances where blood and stool DNA methylation have been considered as CRC endpoints in intervention trials. Kok et al [83] randomly assigned elderly participants with mildly elevated homocysteine levels (n = 92; age: 65-75yrs) to a daily supplementation of 400µg folic acid and 500µg vitamin B12, or a placebo, during a 2 year intervention. The study reported 6 differentially methylated regions that were statistically significant between the intervention and placebo group in blood DNA (P = < 0.05). In a clinical trial substantially shorter in duration, Hariri et al [84] observed significant decreases in methylation at a specific promoter region in blood DNA within an 8 week period, by way of a daily probiotic soy milk drink (containing 2 x 10^7 cfu/ml of *Lactobacillus plantarum A7*) in a cohort of subjects with type II diabetes (n = 40, P = < 0.01). A 1 year weight loss trial performed by Martín-Núñez et al [85] reported a significant reduction in blood DNA methylation in participants post weight loss, compared to a control group (n = 155, P = < 0.001). These clinical trials do not associate specifically with CRC; however, they demonstrate that DNA methylation can be influenced in less invasive sample sources, using treatments applicable to lifestyle exposures. Future clinical trials

should target DNA methylation in genes implicated in the CRC process, using non-invasive sample sources such as blood and stool. Positively modulating these risk biomarkers in a context relevant to CRC would provide greater validity for their future use.

3.2.4.2. Histone modifications

Histone proteins can be modified through a variety of mechanisms including methylation, acetylation, phosphorylation, biotinylation, ubquitination, sumoylation, and ADP ribosylation [86]; with the influence on transcriptional activity dependent on the mechanism used and location of modification [87]. Due to the influence on transcriptional events, irregularities in these modifications are thought to contribute to both upregulated oncogene expression and silencing of tumour suppressors in cancer [88]. Unsurprisingly, previous case control comparisons have reported abnormalities in circulating histone proteins in persons with CRC [89-92]. To build on the biological plausibility and reported differences in CRC, evidence of previous success in modulating histone markers in clinical trials would provide further support for use as a risk biomarker. To date however, evidence of the effect of nutrition interventions on histone modifications pertinent to cancer comes primarily from animal models and cell line studies [93]. The investigations included caloric restriction [94], diets high in fat [95], and low in protein content [96]; as well as individual compounds such as curcumin [97], epigallocatetchin-3-gallate [98] and genistein [99]. Nonetheless, these studies succeeded in modulating histone markers. The lack of clinical trials may be due to investigators using other biomarkers that arguably have a greater influence on human health; as well as requiring less laborious analytical procedures [75]. Going forward; well controlled clinical trials ought to be performed to build on the findings from the animal and cell line models; to provide evidence that histone markers are similarly capable of modulation in humans.

3.2.4.3. MicroRNAs

Post-transcriptional regulation of gene expression by miRNAs influences a plethora of cellular processes including differentiation, proliferation, apoptosis, growth, migration and survival, by inhibiting translation and triggering degradation of target messenger RNA [100-104]. In cancer, miRNAs may also contribute to initiation, invasion, metastasis and chemotherapeutic resistance [105-109].

Several studies have demonstrated that CRC can be associated with dysregulated miRNA expression in both blood [110-130] and stool samples [110, 111, 115, 124, 131-137]. Further, there is some evidence that plasma miRNA expression (specifically miRNA 21) can predict future CRC occurrence [138].

Nutritional and lifestyle interventions, whilst not specific to CRC, have successfully attenuated circulating miRNA expression. These studies include the manipulation of endogenous zinc status (n = 9) [139], daily supplementation of vitamin C (n = 46) [140] (both within healthy cohort populations); a

resveratrol supplementation trial in a cohort of diabetic and hypertensive patients (n = 35) [141]; as well as a weight loss intervention trial using energy restricted diets differing in glycaemic index (n = 103) [142]. Whilst in need of substantial further validation, miRNA expression holds promise as a future CRC risk biomarker due to its biological relevance, differential expression in CRC, as well as ability to predict future CRC. Clinical trials that aim to modify the expression of miRNAs implicated in CRC would be of benefit to help establish miRNAs as validated CRC risk biomarkers.

3.3. Biomarkers of exposure

Exposure based biomarkers have less direct biological association with the cancer endpoint but may be useful indicators of future disease risk, they are also often dysregulated under CRC conditions and importantly, can be highly sensitive to intervention strategies.

3.3.1. DNA damage

DNA lesions result from both endogenous processes and exogenous exposures. If these injuries are not repaired correctly, as a consequence of defective DNA repair systems or an overwhelming degree of insult, it can result in mutations that threaten cell and organism viability [143, 144]. DNA damage in key genes is considered an initiating event in carcinogenesis [145]; to date however, the significance of in situ DNA damage as a predictor of future CRC risk is uncertain. With greater validation against CRC, DNA damage would represent a reliable biomarker to monitor over the course of a clinical trial; owing to the capacity to modulate using lifestyle exposures such as diet [146].

Strategies to capture endogenous DNA damage vary in their relevance to CRC; the choice of method will be influenced by cohort characteristics and whether invasive options are appropriate. DNA damage can be quantified directly in colonic tissue using biomarkers associated with the DNA damage response, such as γ-H2AX [147, 148] ; however, this type of measure suffers the same drawbacks as other invasive tissue-based methods. Alternatively, systemic DNA damage can be determined in urine and blood samples by quantifying 8-hydroxy-deoxyguanosine; a metabolite produced in the cellular repair of guanosine [149]. Additionally, DNA strand breaks can be detected in peripheral blood mononuclear cells using the single cell gel electrophoresis assay (also referred to as the comet assay) [150]. It must be stressed that systemic damage is not specific to colonic exposures, and these measures are very poorly validated against CRC.

Representing greater relevance to the colonic environment, stool may contain sloughed off intestinal cells that could be used to quantify DNA damage; however, current methodologies for isolating human cells from the stool matrix are laborious and have not been regularly implemented [151, 152]. Another method often employed is to use stool samples *ex vivo* as inducers of DNA damage in cultured

epithelial cells (often referred as faecal water genotoxicity) [153]. This approach assumes that the genotoxic potential of stool reflects intestinal lumen exposures; it is likely that the genotoxicity of stool is a good reflection of recto-sigmoid exposure. Although this may give an indication of the level of toxicity that colonocytes are exposed to *in situ*, its direct relevance to endogenous DNA damage is uncertain.

Overall, greater validation against CRC is required in order to implement endogenous DNA damage as a risk biomarker on its own accord. In the interim, validation against biomarkers with a stronger link to CRC (such as ACF) might enable greater confidence in studies using endogenous or ex vivo DNA damage as an endpoint.

3.3.2. Metabolomics

Metabolomics, and/or targeted quantification of specific metabolites, can be used to capture both aberrant host cell activity [154] and toxicological, or cancer relevant exposures, in response to diet and intestinal microbial activity. Metabolites from blood [155-158], urine [159-162], and stool samples [163-168]; can distinguish CRC patients from those in good health. However, metabolomics approaches have not yet been shown to predict future CRC incidence.

Toxicological assessment of specific intestinal metabolites has linked them to CRC initiation via their genotoxic and mutagenic properties, whilst others may be linked to tumour promotion through influencing cell cycle signalling, inflammation and tissue turnover [169-171]. Faecal metabolites for consideration as risk endpoints, or for use in a panel of relevant genotoxic and inflammation associated endpoints, might include ammonia, 4-cresol, phenol, indole, nitroso-compounds, secondary bile acids, and hydrogen sulfide [172]. In contrast, the short chain fatty acids, and butyrate in particular, have demonstrable anti-cancer efficacy in mechanistic studies. The promise of metabolomic approaches lies in the capacity to use non-invasive sampling methods [173]; as well as the proven sensitivity of the faecal, urinary and stool metabolomes through interventions applicable to lifestyle exposures and chemoprevention [174, 175].

Similar to DNA damage, future work should seek to clarify a metabolite profile, or specific genotoxic metabolites, that can predict CRC risk using well planned cohort and cross-sectional studies combining metabolite evaluation alongside risk biomarkers with greater validity against CRC.

3.4. Indicator biomarkers

3.4.1. Composition and select marker species of the gut microbiota

Comparative studies show an enrichment of certain microbial species in stool samples from CRC patients; strains frequently associated with CRC include, *Fusobacterium nucleatum*, *Streptococcus*

galloylyticus, Polyketide-synthase^{+}ve *Escherichia coli*, and *Bacteroides fragilis*toxin^{+}ve *Bacteroides fragilis, Bilophilus wadswarthia, Parvimonus micra* and *Atopobium parvulum* [168, 176-183], with an apparent reduction in the abundance of other strains and genera (**Figure 3.2.**). The presence of an intestinal tumour may be strongly selecting for these strains; a tumour likely creates a lactate rich environment with a disrupted mucous layer, and the tumour may bleed, become inflamed and disrupt normal colonic flow. There is however, strong mechanistic evidence to suggest that these organisms are not all bystanders in CRC; they variously produce genotoxins and cyclomodulins, as well as metabolites that elicit inflammation and proteins that suppress the immune response to tumours. That said, their usefulness as potential early indicator markers of future CRC risk requires a better understanding of the temporal changes in the microbiome along the carcinogenic pathway. The adenoma and aberrant crypt foci associated faecal microbiome has, to this point, been significantly less well characterised than CRC. Frustratingly, the limited data available suggests the possible enrichment of a different set of strains in early pre-neoplasia such as *Alistipes putredinis*, *Anaerostipes hadrus* and *Bacteroides ovatus.* Further, the faecal microbial changes associated with the serrated polyp pathway have been even less well characterised but may be different again, for example, Peters et al. noted an enrichment of *Lactobaclillus zeae* and a general reduction in the abundance of *Lachnospiraceae* and the *Anaerostipes* [181]. There is now a clear and pressing need for prospective data linking microbiota in health to subsequent CRC development, if the microbiome is to be used to characterise risk. Furthermore, its utility as a diet-related risk biomarker has not been well explored. The overall gut microbiome is very sensitive to dietary intervention, however to our knowledge the specific targeting of these cancer associated strains for removal through dietary means has not yet been demonstrated. The microbiome holds significant potential as an indicator biomarker for studies of CRC risk, although as of 2021 its time has perhaps not yet come.

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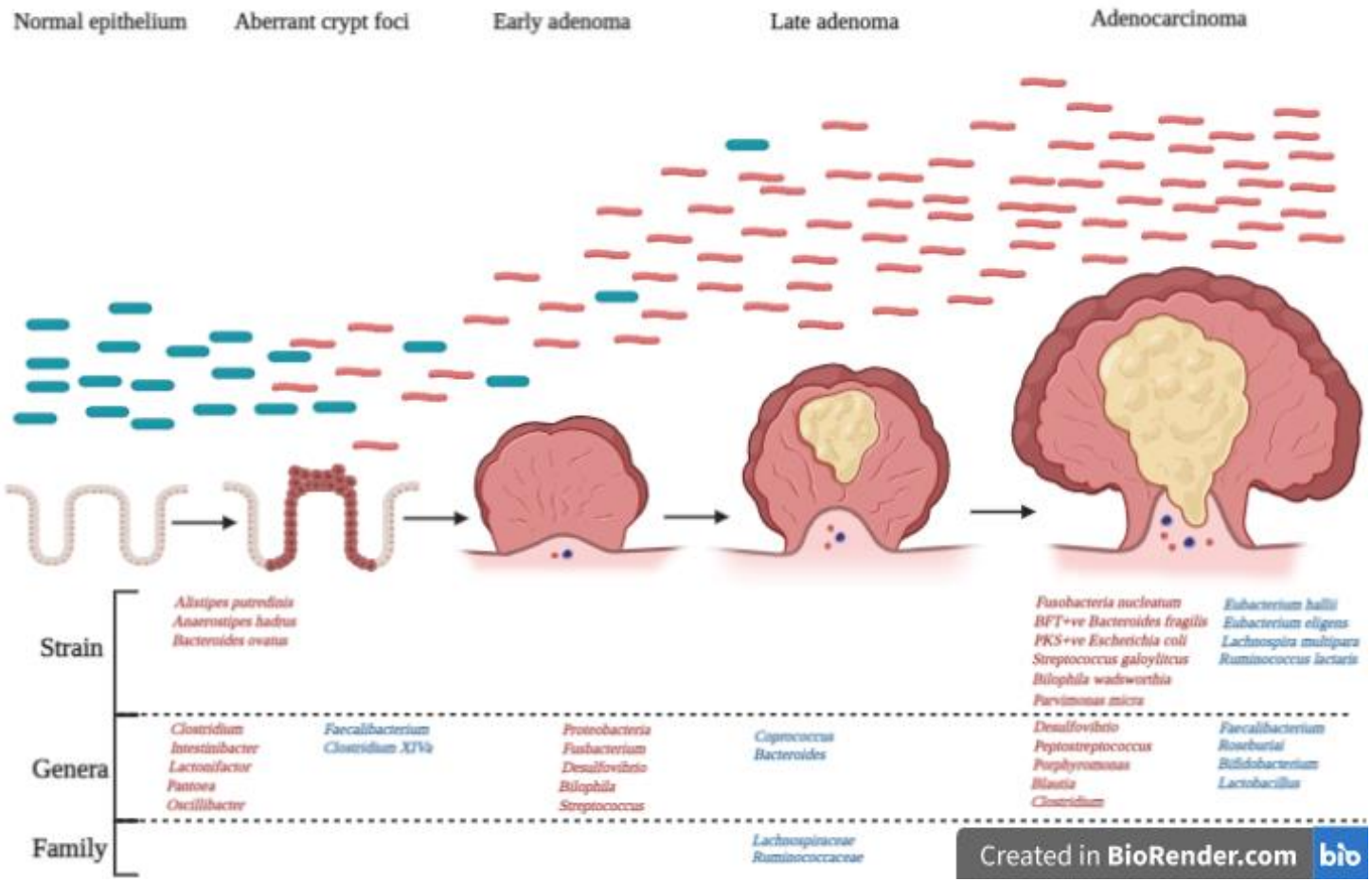
Figure 3.2. Changes in faecal microbial composition along CRC pathway.
Taken from investigations comparing patients with ACF, adenoma, and CRC to healthy controls. Orange indicates an enrichment in stool samples while blue designates an under representation. Bacteria defined at family, genera, and strain level. (ACF, aberrant crypt foci; CRC, colorectal cancer).



3.5. Discussion

3.5.1. Considerations when choosing risk biomarkers

Within a clinical setting, the optimal CRC risk biomarker should be both predictive of CRC development and amenable to intervention early in the carcinogenic pathway; enabling treatment interventions to be implemented in a timeframe to mitigate disease manifestation [7, 10]. Away from the clinical setting, biomarkers suitable for assessing risk exposures in dietary, or lifestyle interventions, comprising healthy cohorts, should ideally be non-invasive [184]. The collection of biopsy material is traumatic and poses potential risk for the person undergoing the procedure; as well as being an expensive and demanding process involving valuable clinician time. Furthermore, the bowel preparation necessary for endoscopy is highly disruptive to an intestinal microbiota whose activity can significantly influence study outcomes [185]. It is therefore suitable for this practice to be reserved for the clinical care setting. For intervention study purposes, a balance must be found between the relative strengths of biomarkers available from intestinal biopsy material, in contrast to the weaknesses in the available panel of non-invasive risk biomarkers.

3.5.2. Future directions

Colorectal cancer has a high personal, social and economic impact, set to increase with the ageing population. It is clear from prospective cohort and migrant studies that lifestyle exposures play a significant part in disease burden, but these exposures remain under characterised. Thus, there is a pressing need for high quality evidence from dietary intervention studies, and therefore, for the development of modifiable risk biomarkers as endpoints for these studies; as to date, none of the currently available risk biomarkers have been fully validated against CRC. Colorectal polyps are viewed by some as the next best endpoint for intervention in place of cancer incidence. However, assessing CRC risk exposures via polyp recurrence requires an endoscopy setting, which are intrusive and laborious in nature. These studies are further confounded by adherence over the duration required, and due to the fact that participants, by definition, represent a high-risk group; investigators could not ethically assess foods or other agents, which may exacerbate their risk. Ideal risk biomarkers (unlike polyp recurrence) would be non-invasive; enabling the implementation of shorter, less expensive studies, that do not cause substantial intrusion on participants' lifestyles; in the same way that LDL cholesterol or plasma TAG might be considered important markers in studies of CVD risk. The panel of biomarkers considered in the present review vary in respect to the nature of sample acquisition and validation against disease outcome; biomarkers that provide greater validation against CRC also carry a higher level of invasiveness in order to analyse (**Table 3.1., Figure 3.3.**).

There remains a need to capture temporal information on the role of individual exposures through the CRC pathway. For example, an agent that stops, or promotes, early stages in carcinogenesis might have

1563 little effect on late-stage risk biomarkers. Going forward, leverage of retrospective sample specimens
1564 such as stool, urine and blood from existing prospective cohort studies might help identify early CRC
1565 risk exposure markers. These findings might be strengthened by validating putative risk/exposure-
1566 biomarkers against each other, or against colorectal polyps and ACF.

1567 To date, relatively little attention has been given to the molecular subsets of CRC when considering risk
1568 biomarkers. CRC subtypes show some geographic preference through the colon, indicating distinct
1569 aetiologies [186], thus pre-cancerous risk markers might also need to be different. Attention to these
1570 disease subtypes will need to be integrated into future studies of CRC risk.

1571 ### 3.5.3. Conclusions

1572 Validated risk biomarkers expressed at early stages of disease initiation following biological
1573 perturbation, as well as risk biomarkers indicative of insult, or protection, elicited by exogenous
1574 carcinogenic exposures and chemo preventative agents respectively, would enable the early opportunity
1575 for intervention, to mitigate disease progression in the former, while providing the chance to remove
1576 risk exposures, or incorporate protective agents, to prevent disease initiation in the latter (**Figure 3.4.**).
1577 The outcome would be an amelioration in the high incident of a disease that elicits considerable
1578 detriment across the globe.

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Table 3.1. Overview of putative risk biomarkers

Biomarker	Advantages	Limitations
Polyp recurrence	▪ Known precursor to CRC, representing reliable biomarker that demonstrates risk, ▪ Enables identification of at-risk persons in clinical settings as well as assessment of risk exposures contribution to CRC	▪ Invasive ▪ Requires colonoscopy ▪ Lengthy timeframe required for attenuation ▪ Unethical for individuals with polyps to be included in studies investigating risk exposures, potentially increasing their disease risk.
Aberrant crypt foci	▪ Biological relevance to CRC ▪ Early expression in CRC, enabling timeframe to attenuate in clinical settings as well as identify risk exposures that may contribute to initiation of CRC.	▪ Invasive ▪ Requires colonoscopy ▪ Requires colon tissue biopsy ▪ Inconsistent evidence of effective attenuation ▪ Unreliable biomarker to follow up treatment/risk exposure effects due to fast yearly turnovers, sporadic expression, and dependency on extraneous factors such as age ▪ Requires further validation to predict CRC
Cell cycle kinetics	▪ Biological relevance to CRC ▪ Evidenced to predict CRC ▪ Early expression in CRC, enabling timeframe to attenuate in clinical settings as well as identify risk exposures that may contribute to initiation of CRC. ▪ Evidence of positive attenuation within short timeframe	▪ Invasive ▪ Requires colonoscopy ▪ Requires colon tissue biopsy ▪ Degree of analyst subjectivity in measurement may lead to inconsistent findings when used by different personnel
Epigenetic risk markers	▪ Non-invasive ▪ Biological relevance to CRC, specific genes/loci dysregulated epigenetically in CRC ▪ Capable of positive attenuation by interventions applicable to lifestyle exposures	▪ Limited evidence of positive attenuation in epigenetic markers associated specifically with CRC ▪ Further clarification needed for expression to predict CRC
In situ DNA damage	▪ Non-invasive	▪ Poorly validated to predict CRC risk

	▪ Clear differences in CRC vs healthy ▪ Capable of positive attenuation by interventions applicable to lifestyle exposures	
Metabolomics	▪ Non-invasive ▪ Clear differences in CRC vs healthy ▪ Capable of positive attenuation by interventions applicable to lifestyle exposures	▪ Poorly validated to predict CRC risk
Microbiome	▪ Non-invasive ▪ Clear differences in CRC vs healthy ▪ Capable of positive attenuation by interventions applicable to lifestyle exposures	▪ Poorly validated to predict CRC risk
Faecal water genotoxicity	▪ Non-invasive ▪ Capable of positive attenuation by interventions applicable to lifestyle exposures	▪ Poorly validated to predict CRC risk

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Figure 3.3. Spectrum of candidate risk biomarkers in relation to CRC validation and invasiveness of sample acquisition.

Biomarkers with greater validity to CRC require more intrusive means to obtain samples, whereas biomarkers which are more feasible to obtain for both practitioner and patient do not carry as great an association with CRC (CRC, colorectal cancer).

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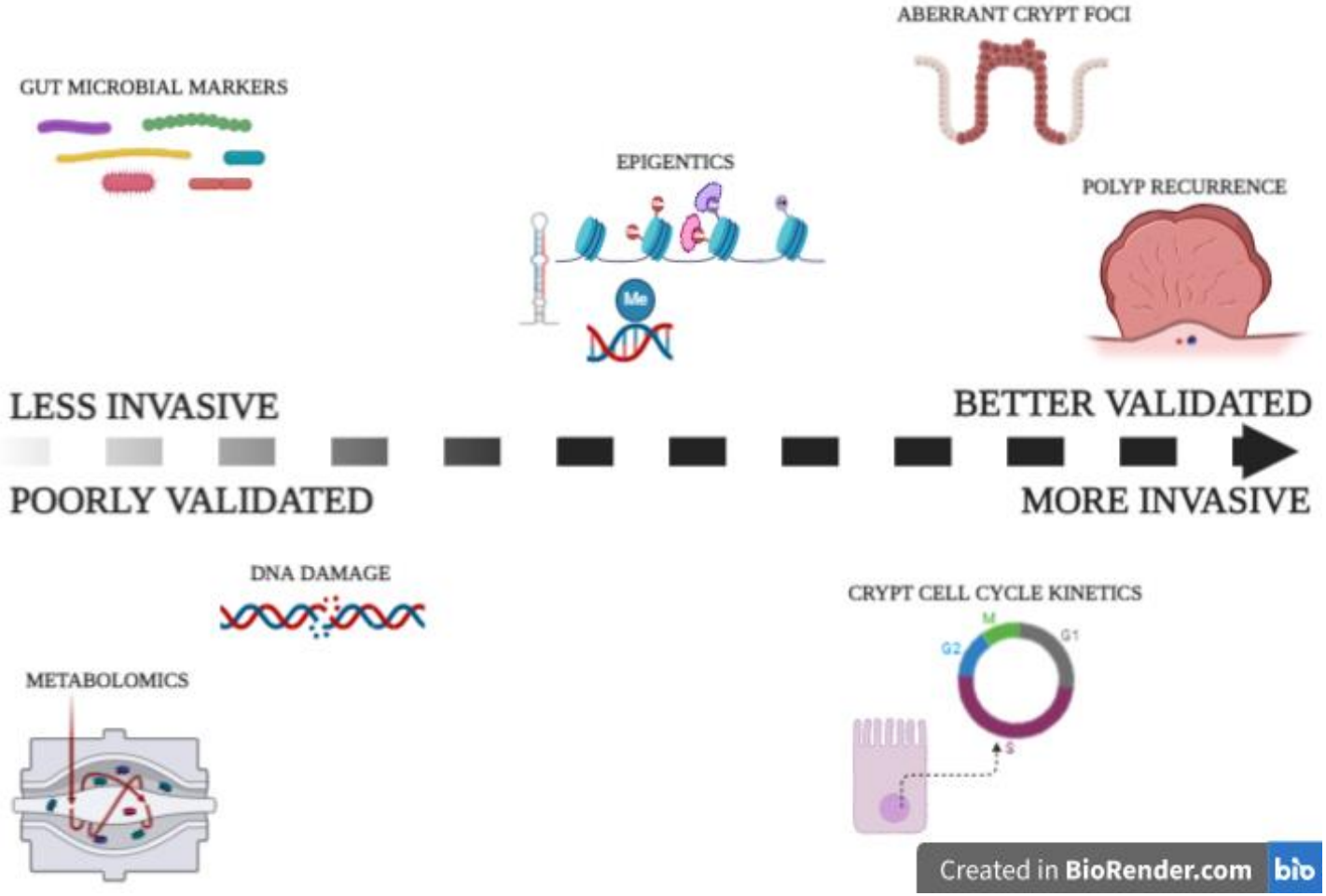

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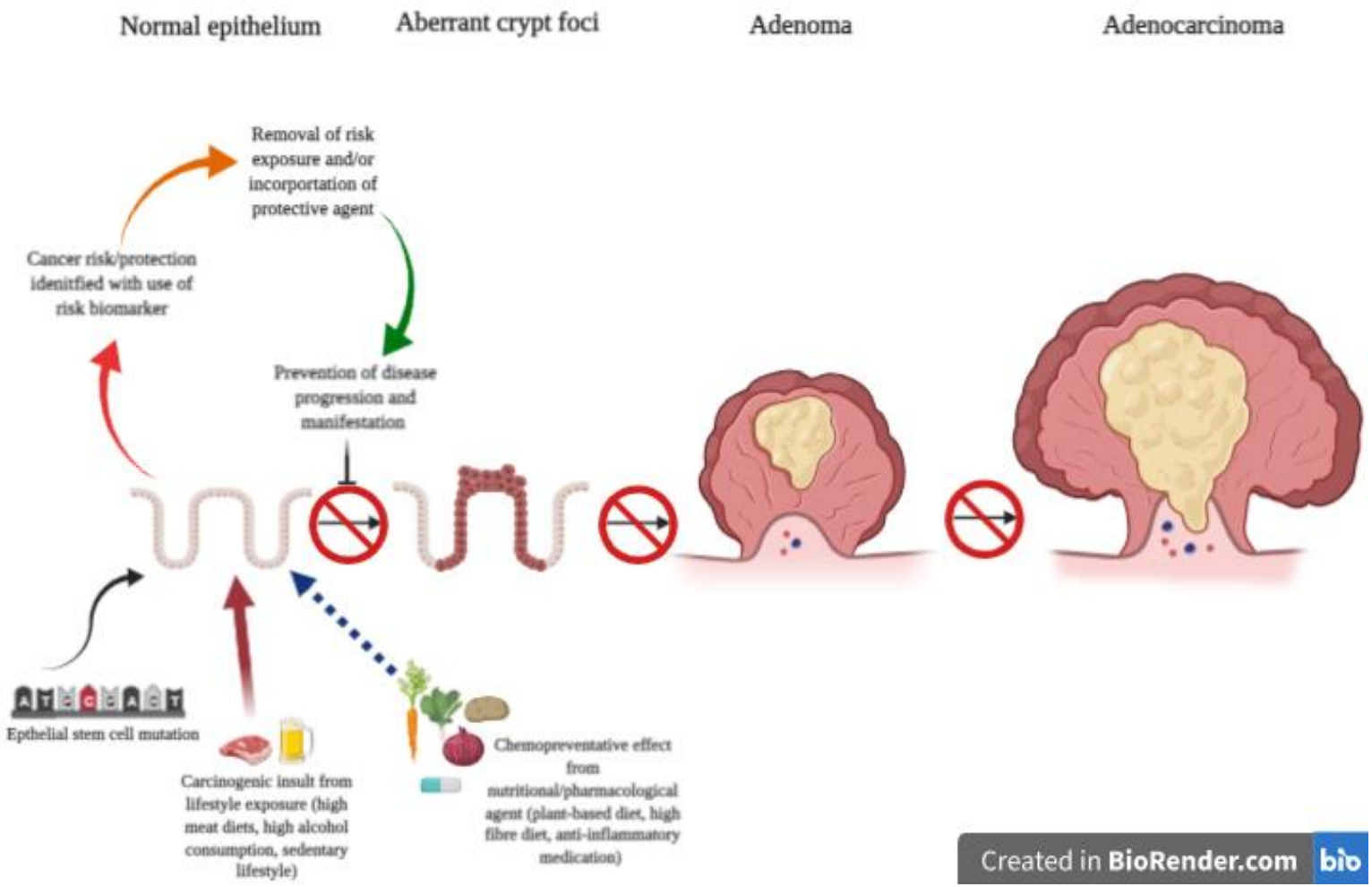
Figure 3.4. The potential of fully validated CRC risk biomarkers to alleviate disease burden.
Validated CRC risk biomarkers would enable identification of either exposures conferring risk or protection, providing opportunity to either remove exposures in the former, or incorporate in the latter. Ultimately this would enable CRC to be halted at early stages of progression (CRC, colorectal cancer).



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CHAPTER IV

EXPERIMENTAL CHAPTER I

The effects of replacing meat with mycoprotein on markers of colorectal cancer risk and gut health, Mycomeat, an investigator blind randomised cross over study.

Abstract

The high meat, low fibre western diet is damaging to intestinal health. Mycoprotein, produced from *Fusarium venanatum,* has been sold for decades as a culinary alternative to meat that is high in protein, fibre and low in fat. Hitherto, the effects of mycoprotein in the human bowel have not been investigated.

The present study evaluated the effects of replacing red and processed meat with mycoprotein on measures of gut health. An investigator-blind, randomised, cross-over dietary intervention trial was performed in healthy adult males (n = 20). Primary endpoints were faecal genotoxicity and genotoxins, secondary endpoints were markers related to gut health, including changes in gut microbiome composition and excreted metabolites related to both microbial and host metabolism.

Higher meat intake increased faecal genotoxicity and nitroso-compound excretion, whereas mycoprotein decreased faecal genotoxicity and nitroso-compounds. Meat intake increased the presence of putrefactive bacteria (*Oscillobacter* and *Alistipes*) whereas mycoprotein consumption increased the abundance of lactate and butyrate producing genera (*Lactobacilli* and *Roseburia*), as well as increasing the abundance of mucin degrading *Akkermansia*. Metabolomic pathway analysis showed an increase in sphingolipid metabolism in stool, and higher taurine, hypotaurine and bile metabolism in urine with the meat diet. Mycoprotein, in turn, was associated with higher fatty acid metabolism.

This work further evidences the harmful effects of a high red and processed meat intake, it also demonstrates that mycoprotein may be a beneficial alternative to meat in the context of gut health.

4.1. Introduction

Epidemiological data consistently associate red and processed meat with an increased risk of colorectal cancer (CRC) (1-5). Mechanistic explanations from dietary intervention trials show meat consumption increases both faecal water genotoxicity, and the excretion of faecal genotoxins, such as nitroso-compounds (NOC). (6-10). Further, higher meat consumption may displace plant foods, and thus fibre, from the diet, leading to a reduced exposure to anti-carcinogenic gut microbial metabolites, including butyrate (11).

This association between meat consumption and CRC risk is reflected in public health recommendations from both EatLancet and the IARC to reduce meat consumption (12, 13). Reducing meat intake at a population level is challenging due to strongly held culinary traditions (14, 15). Meat-alternatives – i.e., products designed to functionally mimic meat, yet made from ingredients devoid of animal flesh – may facilitate meat reduction without requiring drastic changes to culinary practice (16, 17). Mycoprotein is a culinary meat alternative produced from the biomass of *Fusarium venenatum*. It is high in both protein and fibre, and low in energy and fat (18-20). From a gut health perspective, the fibre fraction of mycoprotein is an interesting combination of 1,3/1,6 β-glucan, chitin and lesser studied mannoproteins (21, 22). In mixed-culture fermentations with faecal inoculate, the fibre fraction of mycoprotein induces a significant increase in the production total short chain fatty acids (23). Further, when compared to myofibrillar protein, the fermentation of intact mycoprotein yields lower concentrations of ammonia (24). Several intervention studies have also shown cardiometabolic benefits associated with replacing meat with mycoprotein (25, 26), that may be attributable to its fibre composition; however, to date, there have been no dietary intervention trials exploring the effect of substituting meat with mycoprotein on CRC risk markers or other facets of gut health.

The objective of this investigator-blind, randomised-crossover dietary intervention study (The Mycomeat Study) was to compare the effects of a diet high in red and processed meat with a diet high in mycoprotein on biomarkers of CRC risk and gut health, i.e. faecal water genotoxicity, faecal genotoxins, gut microbial composition, and metabolite excretion.

4.2. Methods

Procedures for this study were followed in accordance with the declaration of Helsinki and was approved by the Northumbria University Ethics Committee (reference number 15274). All study participants provided written informed consent.

4.2.1. Study design

The Mycomeat Study was a single-site, investigator-blind, randomised crossover trial (NCT03944421) among male adults assigned to 1 of 2 sequences; 2 weeks of red and processed meat (Meat) followed by 2 weeks of mycoprotein based foods (Mycoprotein), or vice versa., separated by a 4 week washout (**Figure 4.1.**). A sample size of 20 to reach a statistical power of 80% at an α-level of 0.05 was established based on a repeated measures analysis of the primary endpoint, faecal water genotoxicity. This power calculation was informed by a previous report that found 300g/day of red meat for 7 days increased faecal water genotoxicity compared to participants habitual diets (before intervention 9.6 ± 13.8, after intervention 16.7 ± 18.2) (6).

4.2.2. Participants

The target population comprised metabolically healthy male participants, recruited from the North-East of England, UK, they were recruited via on-campus poster advertisement and using a database of previous study participants. Inclusion criteria were: age 18-50yr; BMI 18-30 kg/m^2; and willingness to refrain from use of pre- and probiotics and vitamin supplements as well as alcoholic beverages for the duration of the study. Exclusion criteria included: gastrointestinal disease; use of medications that may affect gastrointestinal motility; an unwillingness to consume all key study foods; antibiotic, prebiotic, or probiotic use within 3 months before the start of the intervention; use of tobacco or recreational drugs and history of coronary artery disease, diabetes, or other chronic disorders. Participants were also excluded who had been enrolled in previous dietary trials within 3 months of participating in the present study. All participants took part in a screening visit where they were characterised by blood parameters within standard clinical cut off values to confirm study eligibility.

4.2.3. Intervention

The Mycomeat Study consisted of 2 study phases, each lasting 2 weeks: Mycoprotein and Meat (**Figure 4.1.**). During study phases, participants were provided with, and instructed to consume 240g (uncooked weight) of either red and processed meat products, or the equivalent by weight of mycoprotein, in the form of Quorn™ products. The meat dose was selected to represent high intakes for the UK population and as a reflection of previous intervention trials (7, 9, 27-30). All Mycoprotein products were supplied by Quorn™, and all meat products were sourced from the Asda supermarket chain (UK), both Mycoprotein and Meat products were distributed on-site at the research facility.
To promote compliance, a variety of meat products were provided based on a 7-day meal rotation and included the following: beef steak, pork sausages, ham slices, gammon steak, bacon, beef mince and hot dogs. For Mycoprotein, the equivalent Quorn™ products were included: peppered steak, sausages, deli ham slices, gammon steak, bacon, mince, and hot dogs. As stated, the study foods were matched for weight (240g uncooked), the total energy content of the meat and Quorn™ products were also closely matched to avoid introducing additional effects from energy intake between the diets. The nutritional composition of the study foods and differences in nutrients are included in **Table 4.1**.

At the beginning of each study phase, pre-frozen study foods sufficient for the duration (14 x 240g packs; equating to 240g per day for 14 days) were provided to the participants, along with instructions for appropriate storage, preparation, and cooking. The supplied meat products and instructions were prepared externally and provided in concealed packaging, in this way the investigators were blinded to diet order. During each phase of the study (**Figure 4.1.**), 1-day food diaries were collected at baseline and during each study period, from which the energy and macronutrient intake was calculated using Nutritics nutrition analysis software (version 5.66 Education) (31).

4.2.4. Sample collection

At baseline and the conclusion of each intervention period, participants visited the Brain, Performance and Nutrition Research Centre (BPNRC, Northumbria University, Newcastle, UK) having fasted overnight (**Figure 4.1.**). At each visit, a first spot urine sample, and a stool sample (collected ≤10 hr prior to visiting the research centre) were obtained from participants. Urine samples were immediately put on ice, then aliquoted into sterile 1.5-ml Eppendorf tubes and stored at -80 °C until further analysis. Stool samples were put on ice at collection and then aliquoted into sterile 2-ml tubes and stored at -80 °C for gut microbial analysis. The remainder of the sample was weighed, diluted 1:1 with PBS (Sigma) and homogenised in a stomacher (Seward Stomacher 400 Circulator) at 200bts/min for 2 minutes. The homogenates were transferred into polypropylene tubes and centrifuged at 65,000 x g, for 2 hours, at 4°C using an ultra-speed centrifuge system (Thermo Scientific Sorvall LYNX 6000 Superspeed Centrifuge). The supernatants were then filtered through a 0.44µm Polyethersulfone (PES) syringe filter prior to a second filtration through a 0.22µm PES syringe filter (Fisher Scientific). The supernatants, representing the faecal water fraction, were aliquoted into sterile 1.5-ml Eppendorf tubes and stored at -80 °C for analysis of faecal water genotoxicity and metabolites.

Figure 4.1. Mycomeat study design.

Study design was a randomised crossover trial comprising two diet phases (2 wk) separated by a washout (4 wk)

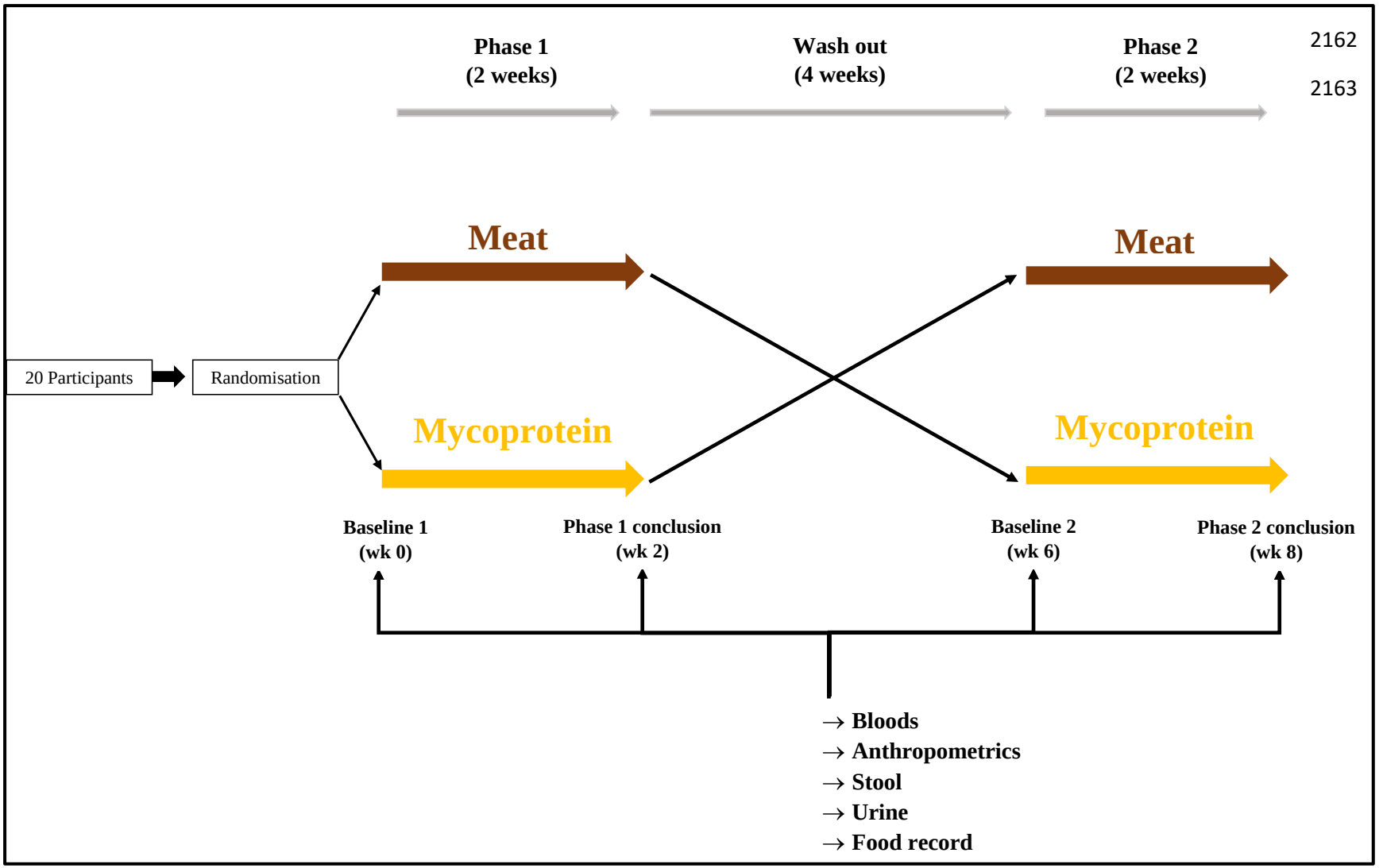

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Table 4.1i. Nutrient profiles for Mycoprotein and Meat products[1,2]

Product[2]	Calories, kcals	Carbohydrates, g	Protein, g	Total fat, g	Saturates, g	Fibre, g	Sodium, mg
Mycoprotein products							
Peppered Steak	269	12.0	28.1	11.8	5.3	13.0	1248
Sausage	475	25.4	26.9	26.6	3.4	13.2	1152
Meat Free Ham Deli Slices	295	12.5	39.8	6.5	2.9	13.0	864
Gammon Steaks	298	4.1	36.2	12.5	6.2	12.7	1440
Bacon Style Slices	514	13.0	24.2	37.7	2.6	13.4	1536
Mince	252	10.8	34.8	4.8	1.2	13.2	288
Hot Dogs	410	8.6	35.5	23.0	1.7	13.0	1248
Meat products							
Beef Steak	367	0	49.0	18.6	8.4	0	188
Pork Sausage	390	13.2	28.8	23.0	8.3	4.2	768
Cold Cut Ham	257	1.2	40.8	9.6	3.8	0	1536
Gammon Steak	360	0.9	52.2	16.4	6.7	0	1800
Bacon Rashers	272	0	30.7	16.3	6.6	0	1536
Beef Mince	200	0	31.9	6.7	3.4	0	134
Hot Dogs	540	1.2	31.2	45.6	19.7	0	1056

[1]Nutritional data for Mycoprotein products obtained from Quorn Foods and Meat products from Asda Supermarket.

[2] Products included in 7 day rotation. Each product repeated twice over the 14 day diet phases.

[2] All products were supplied as 240g uncooked servings.

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Table 4.1ii. Difference in nutritional composition between Mycoprotein and Meat products[1,2].

	Meat products	Mycoprotein products	Difference	P^2
Energy				
Content in daily portion, *kcal*	341.00 ± 107.00	359.00 ± 102.00	+18.00 ± 123	0.65
Protein				
Content in daily portion, *g*	37.80 ± 9.20	32.20 ± 5.56	-5.60 ± 9.17	0.07
Contribution to energy total, *%*	48.10 ± 15.60	39.40 ± 14.00	-8.70 ± 10.60	0.13
Carbohydrates				
Content in daily portion, *g*	2.36 ± 4.64	12.30 ± 6.28	+9.94 ± 3.35	< 0.001
Contribution to energy total, *%*	2.48 ± 4.76	13.90 ± 5.64	+11.42 ± 5.04	< 0.001
Fat				
Content in daily portion, *g*	19.50 ± 12.30	17.60 ± 11.50	-1.90 ± 12.60	0.68
Contribution to energy total, *%*	47.70 ± 14.80	40.10 ± 16.80	-7.60 ± 11.2	0.22
Saturated Fat				
Content in daily portion, *g*	8.11 ± 5.25	3.33 ± 1.76	-4.78 ± 5.80	0.01
Fibre				
Content in daily portion, *g*	0.60 ± 1.53	13.10 ± 0.22	+12.50 ± 1.49	< 0.001
Sodium				
Content in daily portion, *mg*	987.06 ± 631.66	1093.36 ± 398.67	+106.03 ± 522.33	0.60

[1]Nutritional data for Mycoprotein products obtained from Quorn Foods and Meat products from Asda Supermarket.

[2] Daily averages for the intervention periods. Data is expressed as means ± SD. Differences between the supplied intervention food was tested with independent t-tests. The difference column shows the difference between the Mycoprotein products compared to the Meat products.

[2] $P > 0.05$ is considered NS.

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4.2.5. Faecal water genotoxicity

The human colon adenocarcinoma cell line Caco-2 was used to test faecal water genotoxicity using a CometChip (Trevigen), a high throughput version of the standard alkaline comet assay (32).

Caco-2 cells were purchased from the ECACC and used between passage 10 and 22, they were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.1 mM of nonessential amino acids, 100 U/mL of penicillin, 0.1 g/mL of streptomycin, and 20% fetal bovine serum (FBS) (all Lonza UK). In the final passage prior to experimental procedures the FBS was reduced to 10% of culture medium and experiments were carried out with this as carrier control.

The comet assay was performed using the CometChip protocol (Trevigen, US). To ensure adequate blinding, an independent analyst prepared 10% (v/v) faecal extract in carrier media, prepared by passing the previously prepared faecal water through a 0.22 micron filter immediately prior to treating cells. 10 % v/v faecal extract preparations were shown to be non-cytotoxic in a 24 hour MTT assay (viability was maintained at over 90%) and thus this concentration was deemed appropriate for genotoxicity. Caco-2 cells were split at 80% confluence, adjusted to 1 x 10^5 cells per ml and loaded on to the CometChip in 100 µl aliquots. After allowing cells to embed, excess media was removed and 100 µl of treatment added. After 30 minutes the treatment media was removed, the chip was washed with PBS and sealed in low melting point agarose. The comet assay was completed under alkali conditions. Sybr Gold stained comets were visualised under fluorescence (LEICA DM5000 Fluouresence Microscope) at 5 X magnification. Image acquisition was completed by a second investigator with no knowledge of treatments.

Comet tails were assessed using the automated CometChip software (Trevigen, US) which counted a minimum of 150 cell nuclei per well. Data represents a mean of four wells per treatment. 50 µM H_2O_2 and a carrier control were included as inter assay controls.

4.2.6. Nitroso compounds

To determine nitrite concentrations, 50µl of faecal water was injected into a purge vessel containing 8 ml glacial acetic acid and 2 ml aqueous potassium iodide (50 mg/ml). Nitrogen was bubbled through a glass frit to mix the sample and transfer released nitric oxide to a Sievers NOA 280 analyser (Sievers, Boulder, CO, USA) via a condenser, a NaOH (1 mol/L) trap and a polypropylene filter (0.2 µm; Whatman, USA). The signal was processed using the instrument software. After every 6 injections, the purge vessel was emptied and refilled with fresh reagents. For quantification, known standards of sodium nitrite (1 to 10000 nmol) were injected into the purge vessel filled with 8 ml glacial acetic acid and 2 ml aqueous potassium iodide (50 mg/ml).
For nitrate determination, the faecal water was incubated 2-fold with methanol for 30 minutes, followed by centrifugation at 14,000g at 4 °C for 5 minutes. 50µl of the supernatant was injected into the purge

vessel containing 8ml vanadium (III) chloride solution (~0.4g vanadium (III) chloride in 50ml 1M hydrochloric acid). The purge vessel was fitted with a water jacket to allow heating of the reagent to 96°C and cold-water condenser (6°C), using a circulating bath. Thereafter, the purge vessel was replenished with reagents as described above. The samples were quantified by comparing the area to the area of known standards of sodium nitrate (1 to 10000 nmol). Results are expressed as nanomoles of total NOC.

4.2.7. Microbial composition analysis by 16S ribosomal RNA sequencing

Stool aliquots in sterile 2-ml tubes were thawed prior to microbial DNA extraction using a Fast DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. Sequencing libraries were prepared for targeted sequencing of the V4 region of the 16S rRNA gene from PCR amplicons as per the Schloss standard operating procedure (SOP) (33) using primers 515F and 806R (34). Libraries were sequenced on the Illumina MiSeq platform (CA), using V2 (2×250), chemistry. Extraction kit and sequencing negative controls were prepared and sequenced simultaneously with all samples. Paired end reads were trimmed, merged, and processed by alignment to the SILVA database, followed by de novo clustering into operational taxonomic units (OTUs), and taxonomic assignment in Mothur, following the MiSeq SOP (33).

4.2.8. Metabolomics

Frozen aliquots of urine were thawed prior to normalisation through dilution with LC-MS grade water to the lowest specific gravity measured by refractometry as previously described (35). Urine samples were thawed and centrifuged at 13,000g at 4°C for 10 minutes. An aliquot of the supernatant (100 µL) was pipetted onto the lens aperture of a digital refractometer (Hanna Instruments HI-96801) previously calibrated with LC-MS grade water, to measure specific gravity. Once all specific gravity measurements had been recorded, all urine samples were diluted to the lowest urinary density measured (E.G. a lowest density of $d = 1.5$; a sample with a density of 6.0 was diluted 4-fold). Samples were ordered for pipetting according to increasing specific gravity to reduce potential experimental error. Once all the samples had been diluted, they were then centrifuged at 13,000g at 4°C for 10 minutes. It was checked that all samples were accurately diluted to the lowest specific gravity (1.5), after which 225µL of the supernatant was transferred to 9mm glass screw thread vials (Thermo Scientific), followed by the addition of 75µL acetonitrile and stored at −80°C until the time of analysis.

For faecal water, frozen aliquots of sample were thawed before centrifugation at 18,000*g* at 4°C for 30 minutes. 225µL of the supernatant was transferred to 9mm glass screw thread vials (Thermo Scientific), followed by the addition of 75µL acetonitrile and stored at −80°C until the time of analysis.

Hydrophilic Liquid Interaction Chromatography (HILIC) metabolite profiling of the urine and faecal water samples were performed on an Vanquish Liquid chromatography chromatographic separation

system connected to an IDX High Resolution Mass Spectrometer (Thermo Scientific). The HILIC positive and negative data sets were processed via Compound Discoverer 3.2 according to the following settings: Untargeted Metabolomic workflow with online database: mass tolerance 10ppm, maximum shift 0.3 min, alignment model adaptive curve, minimum intensity 500K, S/N threshold 3, compound consolidation, mass tolerance 10ppm, RT tolerance 0.3 min. Database matching was performed at MS2 level using Thermo scientific m/z cloud with a similar index of 80% or better.

4.3. Statistical analysis

All statistical analyses and visualisations were performed in RStudio (36). Data was assessed for normality by visualising Q-Q plots and performing Shapiro-Wilks tests before statistical analysis. Changes from baseline within study phases and differences between study phases in all measures, excluding metagenomic and metabolomic data was assessed using mixed effects models. In all models, age, BMI, habitual alcohol intake and treatment order were included as fixed effects, with the participant as the random effect. For faecal nitroso compounds, there was insufficient sample at a single timepoint for three of the volunteers, these volunteers were retained in the analysis on an intention to treat basis, with the absent data point being replaced with the treatment mean.

For gut microbial analysis, Bray-Curtis dissimilarity was performed to assess the beta-diversity within and between study phases, with ordination plots generated for visualisation. Differences within and between study phases in beta-diversity were compared using a permutational multivariate analysis of variance (PERMANOVA). Alpha diversity was measured using Shannon index, inverse Simpson index, Fisher's index and species richness using CHAO1 estimates.

As microbiome sequencing data can contain a considerable number of zeros, geometric mean of pairwise ratio (GMPR) was applied, a normalization method for zero-inflated data (37). Using the normalised data, the abundances of bacterial taxonomy (i.e., phyla, genus) was determined to elucidate the diets influences on specific phylotypes as well as overall composition. Changes in alpha diversity metrics and bacterial phylotype abundance within and between study phases were compared using generalised mixed-effects models including participant as a random-effects factor. The models were adjusted for weight and habitual alcohol intake; factors which can impact the microbiome, as well as the interaction of the randomisation order to determine any carry over effects. P values were adjusted using the Benjamini and Hochberg false discovery rate (38).

For faecal and urinary metabolomics, multivariate statistical analysis was performed using MetaboAnalyst 5.0 (39). Partial least squares discriminant analysis (PLS-DA) was performed to see the changes in metabolite profiles within study phases, as well as differences between study phases. The variable importance in projection (VIP) >1.5 was taken to identify the features significantly

differentiating within and between study phases, then the fold change ratio was obtained for each feature. Hierarchical cluster analysis heat maps was obtained using ward clustering algorithm and Euclidean distance calculation to further confirm the results of PLS-DA and to show the distribution of metabolites among all individuals. Taking the VIPs >1.5 between diets, pathway analyses were performed to reveal which pathways were enriched in the Meat and Mycoprotein diets. Within the metabolomic datasets, potential genotoxins of interest were identified, these included the bile acids apocholic acid and 7-ketodeoxycholic acid in stool, and p-cresol sulphate in urine. To assess the differences within and between study phases on the excretion of these genotoxins, mixed effects models were implemented as outlined above.

For all statistical analyses, a $P < 0.05$ was defined significant.

4.4. Results

4.4.1. Participants

Table 4.2. presents the baseline characteristics of the participants who completed the study and **Figure 4.2.** depicts the flow of The Mycomeat Study. Participant enrolment began on 1 June 2019 and continued through to 1 December 2019. The date of final data collection was 29 January 2020.

4.4.2. Diet

Mean self-reported intake of total energy, protein, fat, saturated fat and sodium were not significantly changed from baseline by either intervention arm. Mycoprotein was associated with higher self-reported fibre intake, both from baseline (+16.99 ± 2.79g/day, $P < 0.001$), and in comparison to Meat (+16.73 ± 3.65g/day, $P < 0.001$) (**Table 4.3.**).

Table 4.2. Participants' baseline characteristics (n = 20)[1]

Age, *y*	30.4 ± 7.92
Anthropometric parameters	
Weight, *kg*	80.6 ± 10.90
BMI, kg/m^2	24.0 ± 2.87
Waist circumference, *cm*	86.9 ± 8.16
Body fat, *%*	15.5 ± 5.56
Trunk fat, *%*	16.3 ± 6.90
Systolic blood pressure, *mmHg*	126 ± 12.2
Diastolic blood pressure, *mmHg*	71.5 ± 9.27
Blood parameters	
Total cholesterol, *mmol/L*	4.32 ± 0.83
HDL Cholesterol, *mmol/L*	1.57 ± 0.43
LDL Cholesterol, *mmol/L*	2.29 ± 0.88
Triglycerides, *mmol/L*	0.89 ± 0.46
Glucose, *mmol/L*	4.86 ± 0.45
Dietary intake	
Energy, *kcal/d*	2515.59 ± 754.85
Protein, *g/d*	126.44 ± 45.42
Protein, *% of energy*	20.10 ± 7.22
Carbohydrate, *g/d*	281.20 ± 81.41
Carbohydrate, *% of energy*	44.71 ± 12.94
Fat, *g/d*	98.37 ± 46.75
Fat, *% of energy*	35.19 ± 16.73
Saturated fat, *g/d*	35.94 ± 21.22
Saturated fat, *% of energy*	12.86 ± 7.59
Fibre, *g/d*	26.16 ± 7.79
Sodium, *mg/d*	2853.02 ± 1243.93

[1]Data are presented as means ± SDs.

Figure 4.2. Participant flowchart.
Meat, red and processed meat study phase; Mycoprotein, mycoprotein study phase.

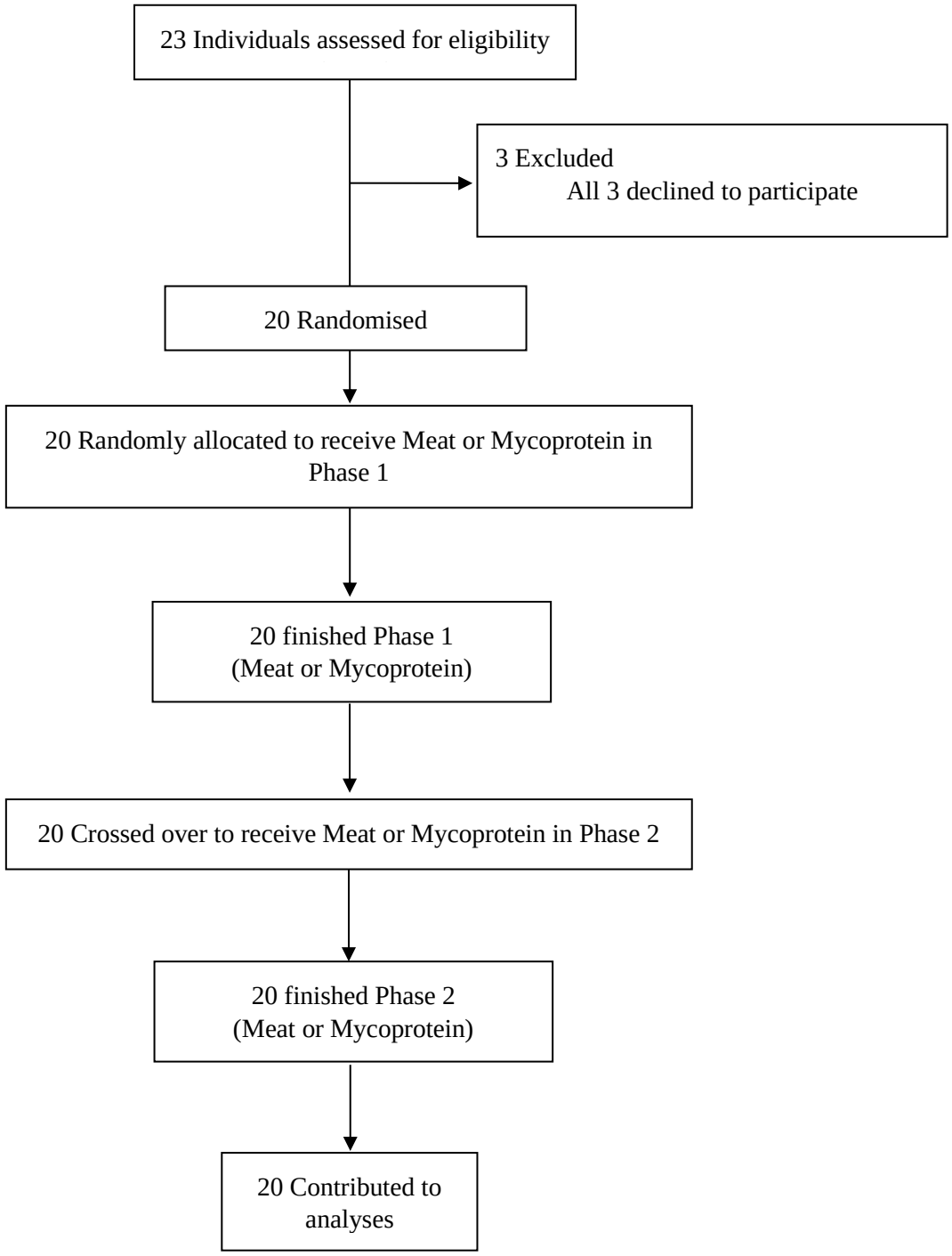

Table 4.3. Difference in participants nutritional intake during study phases, including provided study food[1,2]

Nutrient	Meat	Mycoprotein	Difference[2]	*P*[3]
Energy, *kcal/d*	2355.18 ± 553.32	2567.44 ± 796.97	+212.26 ± 242.63	0.47
Protein, *g/d*	117.83 ± 31.26	115.03 ± 35.86	-2.80 ± 11.92	0.78
Carbohydrate, *g/d*	243.82 ± 48.81	298.20 ± 104.37	+54.38 ± 28.86	0.06
Fat, *g/d*	100.43 ± 39.95	98.98 ± 37.04	-1.45 ± 13.51	0.68
Saturated fat, *g/d*	36.96 ± 19.32	36.92 ± 14.64	-0.04 ± 6.01	0.79
Fibre, *g/d*	26.52 ± 8.40	43.26 ± 8.69	+16.74 ± 2.92	< 0.001
Sodium, *mg/d*	2714.25 ± 979.59	3229.12 ± 1405.28	+514.87 ± 424.95	0.32

[1]Data calculated from participants 1-day food record completed during study phases using Nutritics nutritional analysis software.

[2]Values are presented as means ± SDs.

[2] Differences between nutrient intake during Mycoprotein compared to Meat presented as least square means ± SEs.

[3]*P* values were calculated for differences between study phases using mixed effects models. A $P < 0.05$ was considered significant.

4.4.3. Stool weight

There were notable differences between the study phase effects on stool weight. There was a non-significant increase from baseline after Mycoprotein (+32.63 ± 15.70g, $P = 0.12$), while following Meat, there was a significant decrease (-51.01 ± 13.40, $P < 0.01$), with the difference in the diet effects highly significant (82.64 ± 20.20g, $P < 0.01$) (**Figure 4.3.**).

4.4.4. Faecal water genotoxicity

Mean faecal water genotoxicity reduced from baseline after Mycoprotein, with this reduction reaching statistical significance (-8.28 ± 3.60 % DNA in tail, P = 0.05). While following Meat, there was a non-significant increase in mean genotoxicity (+4.91 ± 2.65 %DNA in tail, P = 0.09)**.** The difference in the effects between the diets also reached significance (13.19 ± 4.41 %DNA in tail, P = 0.01) (**Figure 4.4.**).

4.4.5. Potential genotoxins

The study diets had distinctive effects on faecal NOC. Following Meat, there was a non-statistically significant increase from baseline ($P = 0.20$), whereas there was a significant reduction with Mycoprotein ($P = 0.007$). The difference in the effects between the diets was also statistically significant ($P = 0.01$) (**Figure 4.5A.**).

As well as NOCs, other potential genotoxins were identified within both the urinary and faecal metabolomics datasets. In urine, p-cresol sulphate was identified; while in stool, the bile acids apocholic acid and 7-ketodeoxycholic acid were found. Following Mycoprotein, there was significant reduction in the urinary p-cresol sulphate excretion ($P = 0.002$), while there was an increase after Meat ($P = 0.40$), the difference in the diet effects also reached statistical significance ($P = 0.02$) (**Figure 4.5B.**). Apocholic acid was increased in stool from baseline after Mycoprotein ($P = 0.27$) whereas it was reduced following Meat ($P = 0.27$), with the difference in diet effects not significant ($P = 0.15$) (**Figure 4.5C.**). 7-ketodeoxycholic acid followed a similar pattern, with an increase following Mycoprotein, ($P = 0.14$) while there was a significant reduction after Meat ($P = 0.009$), the differences in diet effects did not reach significance ($P = 0.07$) (**Figure 4.5D.**).

Figure 4.3. Changes in stool weight following Mycoprotein and Meat.
Assessed using mixed effects models. Error bars represent standard deviation. *Significant change from baseline; †Significant difference between study phase effects.

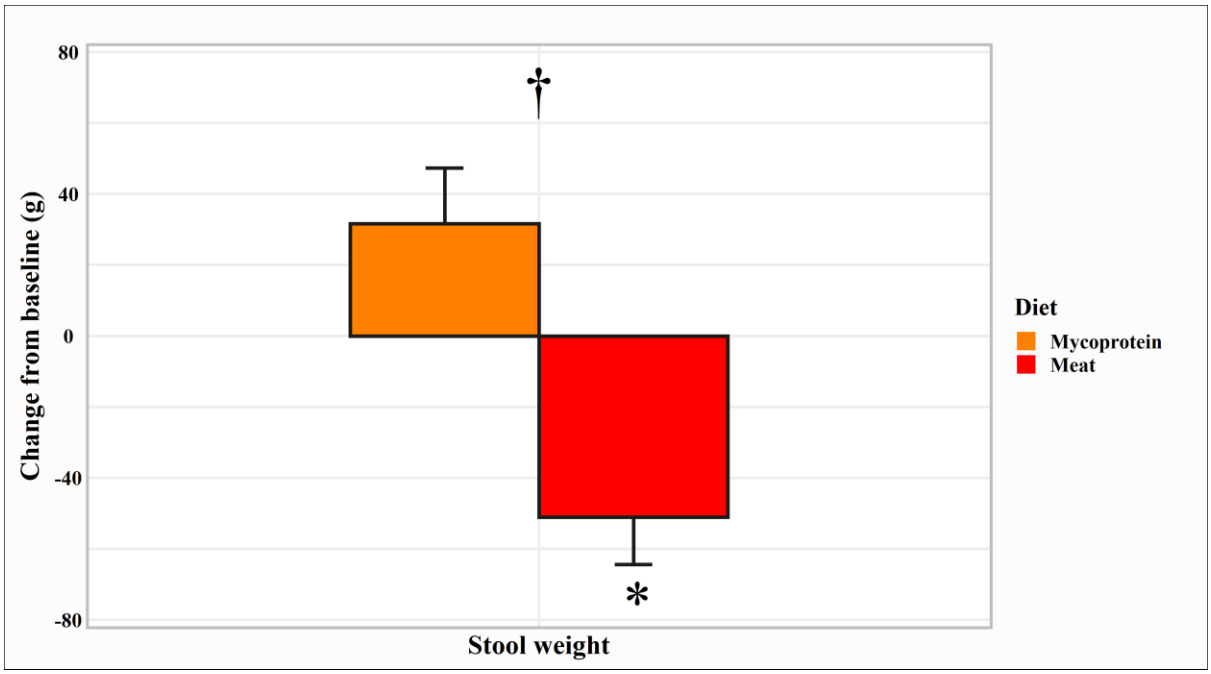

Figure 4.4. Effects of 2-wk dietary interventions on faecal genotoxicity.
Assessed by percentage (%) DNA in tail following exposure to faecal water. 50 uM H_2O_2 and a carrier control were included as inter assay positive and negative controls respectively. Error bars represent standard deviations. *Significant change from baseline; †Significant difference between study phase effects.

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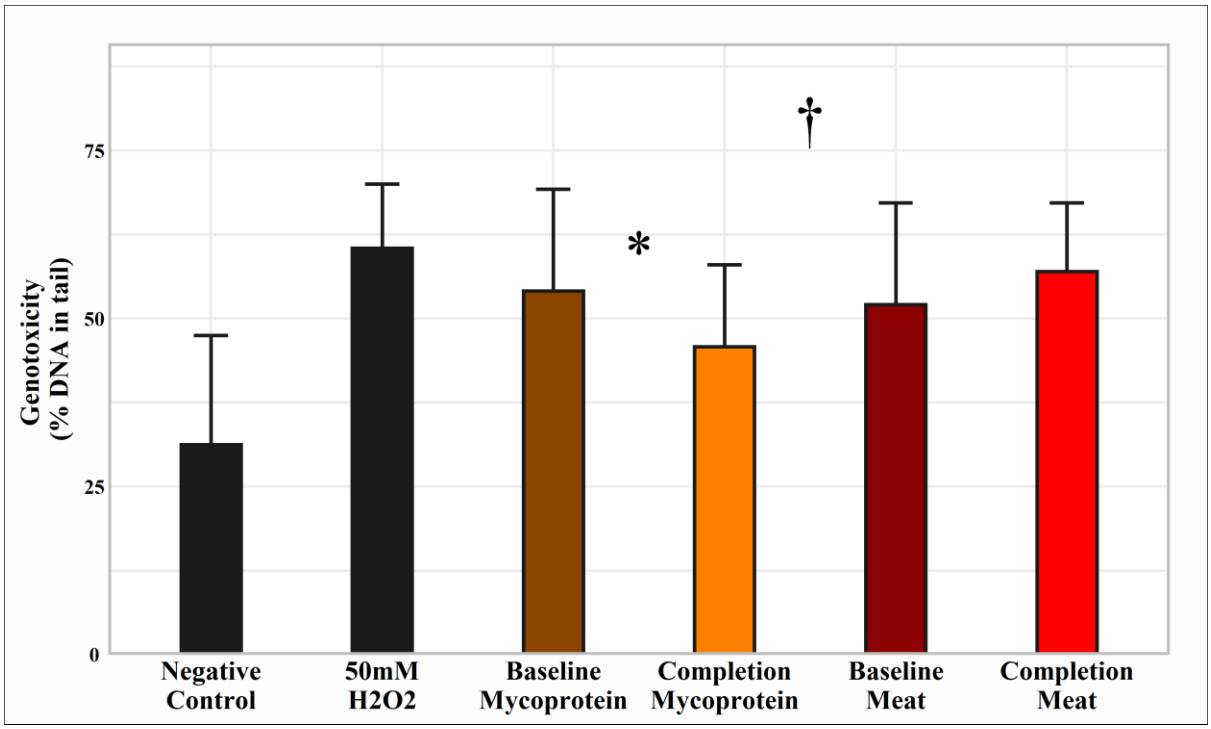

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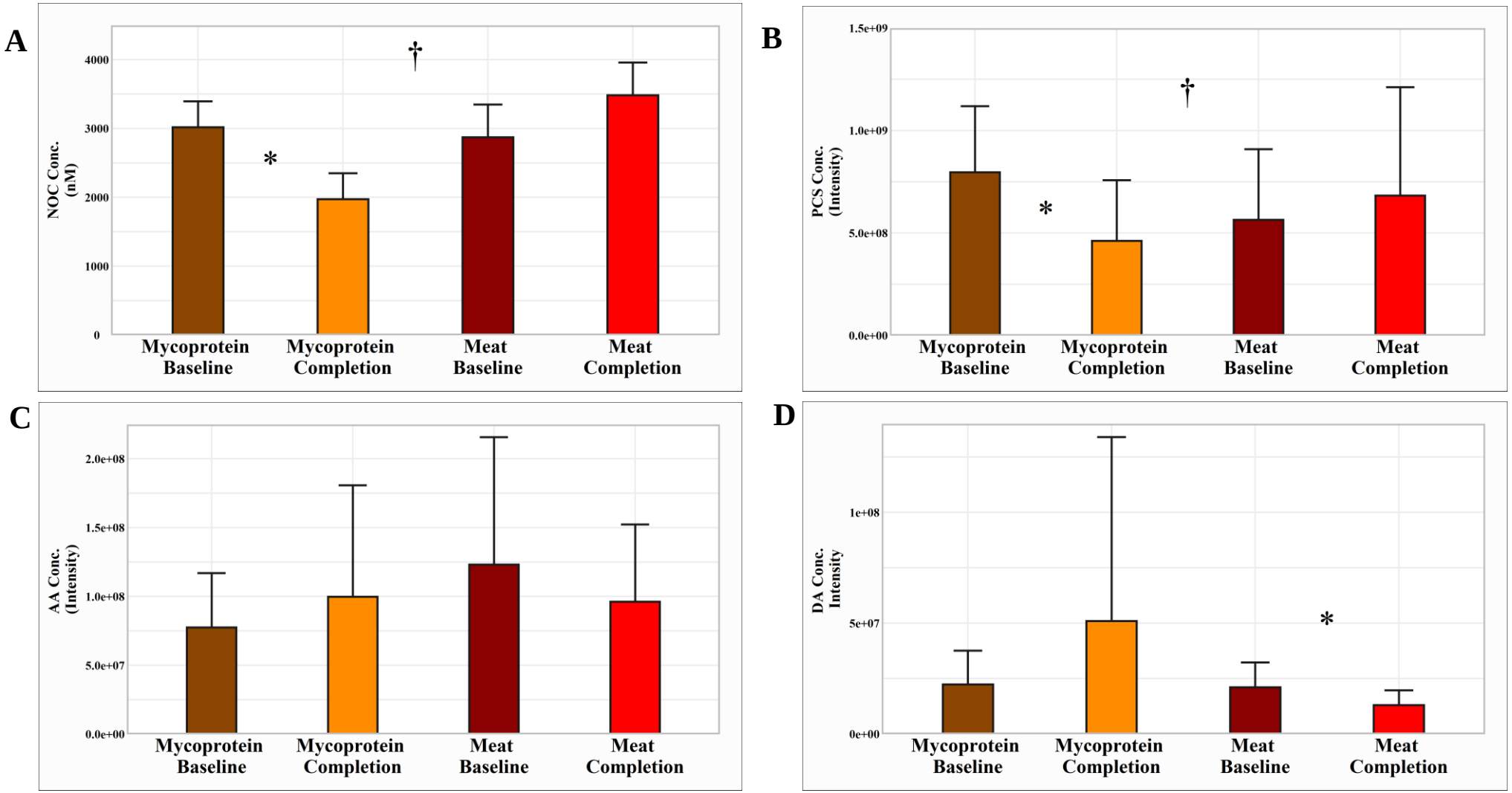
Figure 4.5. Effects of 2-wk dietary interventions on potential genotoxins.

Results presented as peak intensity in LC-MS analysis. **A**) Faecal nitroso compounds (NOC). **B**) Urinary P-cresol sulphate (PCS). **C**) Faecal Apocholic acid (AA). **D**) Faecal Deoxycholic acid (DA). Error bars represent standard deviations. *Significant change from baseline; †Significant difference between study phase effects.



4.4.6. Microbial composition

Both study phases reduced alpha diversity (i.e., the variation of microbes within each participants sample) from baseline, none of which reached significance. Changes between study phases was also non-significant (**Figure 4.6A., 4.B.).** Comparison of β diversity (i.e., the variation of microbial communities between the samples post meat and mycoprotein) between diets, revealed no significant changes nor patterns in diversity over the course of each intervention period, as well as between intervention periods (**Figure 4.6C.**).

Bacterial phylotype analysis revealed significant changes in the relative abundances of bacterial communities.

There were significant increases in the bacterial phyla *Proteobacteria* ($P = 0.004$) and *Verrucomicrobia* ($P = 0.02$) following Mycoprotein, while *Verrucomicrobia* was significantly reduced after Meat ($P = 0.04$), the difference between the diets in the effects on *Verrucomicrobia* was also significant ($P = 0.03$) (**Figure 4.7A.**).

Both diets elicited significant influences on a number of genera, with expansions and reductions observed following Meat and Mycoprotein. *Akkermansia* significantly increased in abundance following Mycoprotein ($P = 0.02$), while there was a reduction in this genus after Meat, the difference between the study phases was significant (P = 0.03). Mycoprotein and Meat caused differing effects in the abundance of *Roseburia,* increasing after Mycoprotein while it was significantly reduced following Meat ($P < 0.001$), the difference between the dietary phases was highly significant ($P < 0.001$). In a similar facet to *Akkermansia*, the study phases had opposing influences on the abundance of *Faecalibacterium* which significantly increased after Meat ($P = 0.006$) whereas there was a slight reduction following Mycoprotein, the difference between diet phases was significant ($P = 0.02$). *Oscillibacter* was significantly increased after both study phases (Mycoprotein, $P = 0.05$; Meat, $P = 0.003$), there was a greater increase following Meat, with the difference between the diets also reaching significance ($P = 0.004$). Other notable effects in genera were a significant reduction in *Ruminococcus* following Meat ($P = 0.03$) and a significant increase in *Lactobacillus* after Mycoprotein ($P = 0.05$). The genera significantly affected within and between study phases are shown in **Figures 4.7B., C, and D.**

In total, the analysis identified 392 OTUs in the samples; of these 392, the abundance of 19 OTUs were significantly influenced by Mycoprotein, while 25 were significantly affected by Meat, with changes in 20 OTUs significantly different between diets. The OTUs significantly different following both Mycoprotein and Meat as well as the OTUs significantly different between the study phases are included in **Table 4.4.**

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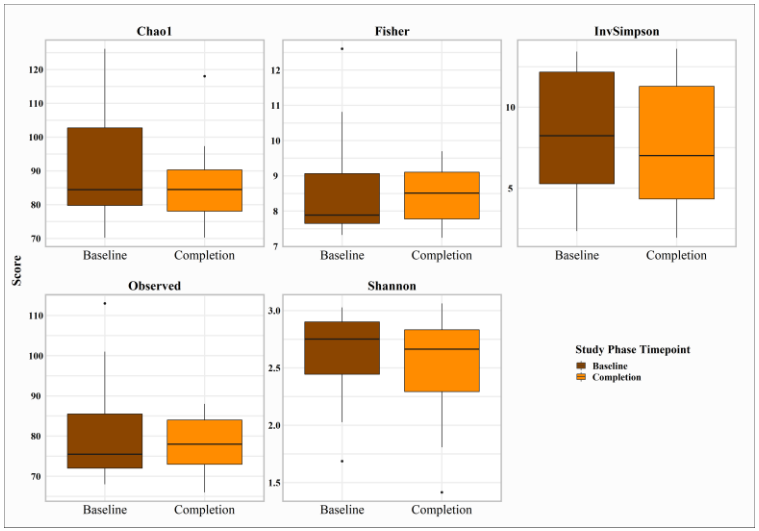

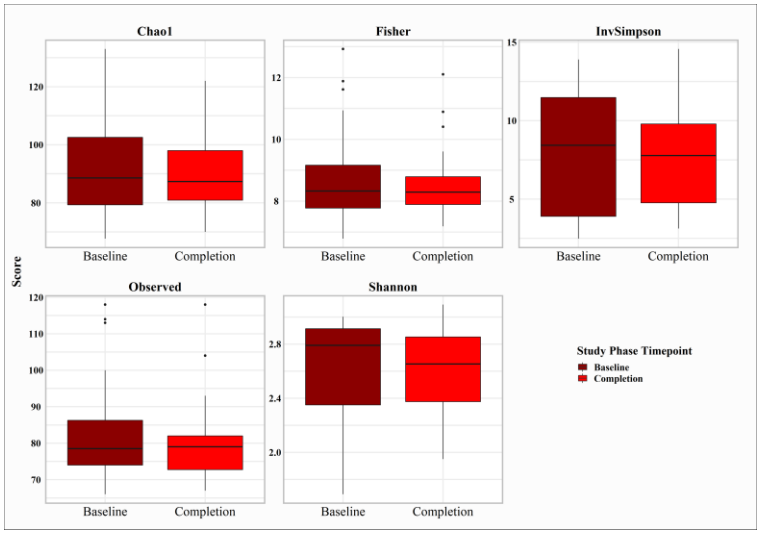

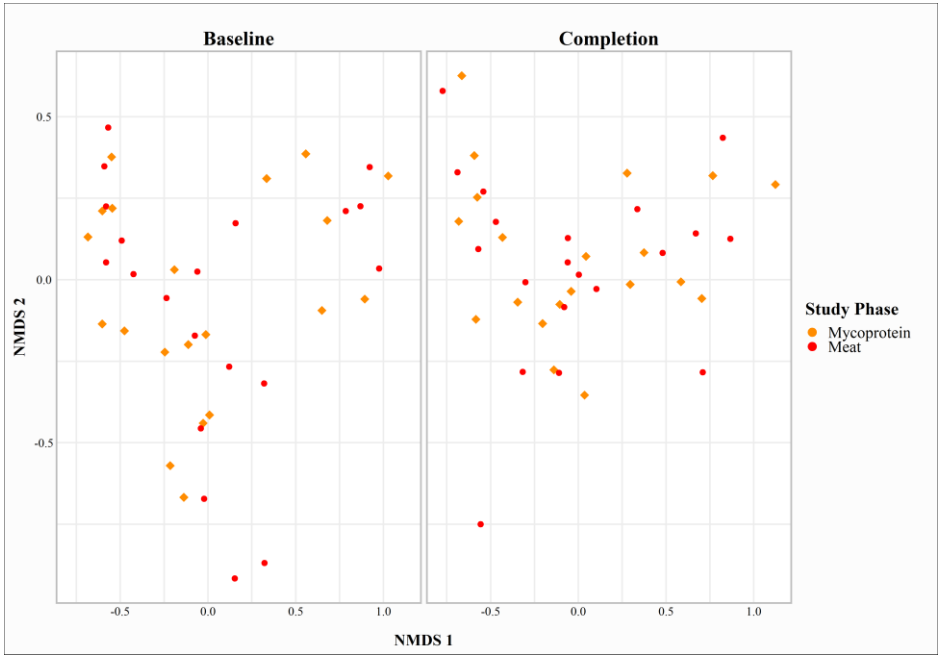

Figure 4.6. Influence of 2-wk dietary interventions on gut microbial alpha and beta diversity.

A) Alpha diversity changes, Mycoprotein **B**) Alpha diversity changes, Meat.

Alpha diversity metrics presented include Chao estimate, Fisher's Test, Inverse Simpson Index, Observed species and Shannon Index. **C**) Beta diversity at baseline and completion for both study phases, presented as Bray Curtis distances. Analyses revealed neither diet significantly influenced beta diversity.

Table 4.4. Operational Taxonomic Units (OTUs) significantly affected by the mycoprotein diet. Table includes OTU number as well as bacterial genera assigned to OTU. For OTUs where genera not assigned, the closest available known taxa is assigned (i.e. family).

No. Operational Taxonomic Unit (OTU)	Bacterial Taxonomic Classification	Change in relative abundance from baseline[1]	*P*[2]
1	*Bacteroides spp.*	+1.58	0.04
13	*Polaromonas spp.*	+1.80	0.03
15	*Parabacteroides spp.*	-1.54	0.01
19	*Prevotella spp.*	-1.48	0.006
25	*Alistipes spp.*	+1.11	0.001
27	*Lachnospiraceae family (unclassified)*	+0.60	0.009
31	*Oscillibacter spp.*	+0.04	0.05
33	*Akkermansia spp.*	-1.66	0.007
34	*Barnesiella spp.*	+0.12	0.008
42	*Phascolarctobacterium spp.*	-0.07	0.01
44	*Parabacteroides spp.*	+0.56	0.002
50	*Bacteroides spp.*	+0.67	0.002
57	*Ruminococcaceae family (unclassified)*	-0.046	0.03
91	*Clostridium Cluster IV (unclassified)*	+0.001	0.01
95	*Lactobacillus spp.*	+0.02	0.05
102	*Pedobacter spp.*	+0.01	0.02
107	*Prevotella spp.*	-0.02	0.01
110	*Bacteroidetes phylum (unclassified)*	-0.01	0.007
163	*Clostridiales family (unclassified)*	-0.002	0.003

[1]Results presented as least square mean change from baseline for the study phases.

[2]*P* values were calculated for changes within each study phase using generalised mixed effects models. Differences regarded as significant at a $P < 0.05$.

Table 4.5. Operational Taxonomic Units (OTUs) significantly affected by the meat diet. Table includes OTU number as well as bacterial genera assigned to OTU. For OTUs where genera not assigned, the closest available known taxa is assigned (i.e. family).

No. Operational Taxonomic Unit (OTU)	Bacterial Taxonomic Classification	Change in relative abundance from baseline[1]	P^2
2	*Faecalibacterium spp.*	+3.77	0.02
5	*Bacteroides spp.*	-2.14	0.04
9	*Alistipes spp.*	+1.94	0.01
10	*Roseburia spp.*	-2.01	< 0.01
12	*Bacteroides spp.*	+0.85	0.03
14	*Sutterella spp.*	-1.29	0.02
15	*Parabacteroides spp.*	-2.01	< 0.01
21	*Bacteroides spp.*	+1.71	0.03
27	*Lachnospiraceae family (unclassified)*	-0.65	0.03
31	*Oscillibacter spp.*	+0.70	0.001
37	*Prevotellaceae family (unclassified)*	+0.06	0.02
40	*Ruminococcus spp.*	-0.46	0.02
45	*Paraprevotella spp.*	-0.20	0.005
47	*Alphaproteobacteria class (unclassified)*	+0.16	< 0.01
50	*Bacteroides spp.*	+012	0.04
58	*Bacteroidales order (unclassified)*	+0.001	0.05
60	*Faecalibacterium spp.*	-0.29	0.05
65	*Lachnospiraceae family (unclassified)*	+0.07	0.01
67	*Prevotella spp.*	-0.03	0.04
74	*Bacteroidales order (unclassified)*	+0.03	0.007
91	*Clostridium Cluster IV (unclassified)*	-0.001	0.01
115	*Clostridiales family (unclassified)*	+0.008	0.007
210	*Bacteroidetes phylum (unclassified)*	-0.001	0.02
238	*Lachnospiraceae family (unclassified)*	+0.009	0.02

[1]Results presented as least square mean change from baseline for the study phases.

[2]*P* values were calculated for changes within each study phase using generalised mixed effects models. Differences regarded as significant at a $P < 0.05$.

Table 4.6. Operational Taxonomic Units (OTUs) affected significantly different between the meat and mycoprotein diets.

Table includes OTU number as well as bacterial genera assigned to OTU. For OTUs where genera not assigned, the closest available known taxa is assigned (i.e. family).

No. Operational Taxonomic Unit (OTU)	Bacterial Taxonomic Classification	Difference in change in relative abundance between diets[1]	P^2
3	*Faecalibacterium spp.*	-4.03	0.02
11	*Roseburia spp.*	+2.22	0.001
13	*Bacteroides spp.*	-3.00	0.01
15	*Sutterella spp.*	+1.35	0.03
20	*Prevotella spp.*	-2.86	< 0.001
28	*Lachnospiraceae family (unclassified)*	+1.25	< 0.001
32	*Oscillibacter spp.*	-0.66	0.004
34	*Akkermansia spp.*	+1.66	0.02
35	*Barnesiella spp.*	+0.12	0.02
45	*Parabacteroides spp.*	+0.64	0.001
48	*Alphaproteobacteria class (unclassified)*	-0.23	0.001
49	*Alphaproteobacteria class (unclassified)*	-1.66	0.05
51	*Bacteroides spp.*	+0.58	0.02
66	*Lachnospiraceae family (unclassified)*	-0.07	0.007
75	*Bacteroidales order (unclassified)*	-0.02	0.03
92	*Clostridium Cluster IV (unclassified)*	+0.001	0.001
108	*Prevotella spp.*	-0.02	0.008
116	*Clostridiales order (unclassified)*	-0.008	0.007
156	*Clostridiales order (unclassified)*	+0.02	0.02
239	*Lachnospiraceae family (unclassified)*	-0.009	0.04

[1]Difference between diets calculated by subtracting change following Mycoprotein by change following Meat.

[2]*P* values were calculated for differences between study phases using generalised mixed effects models. Differences regarded as significant at a $P < 0.05$.

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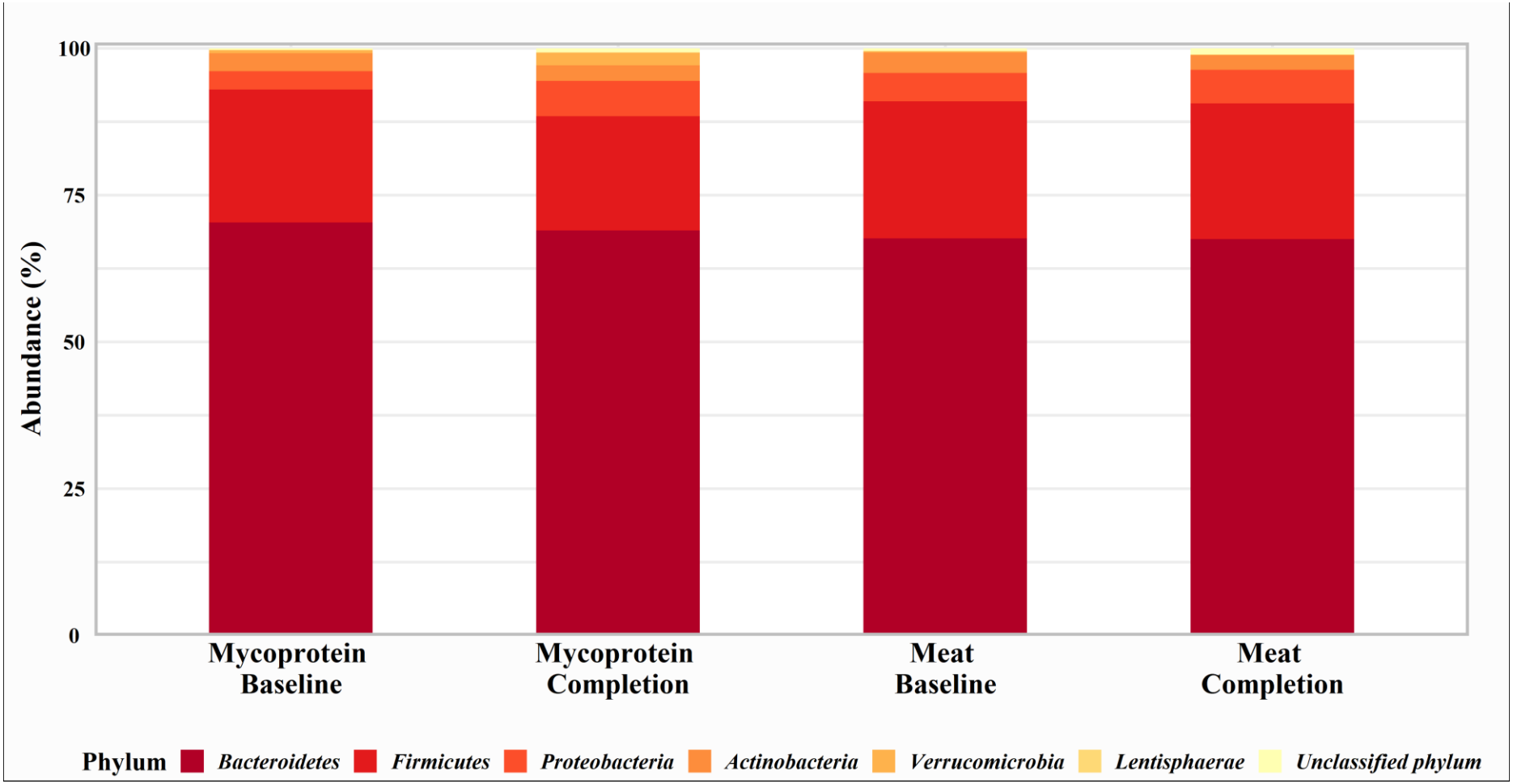
Figure 4.7. Effects of 2-week dietary interventions on gut microbial composition.
A) Effects of 2-wk dietary interventions on relative abundance of gut microbial phylum.



2432

Figure 4.7. Effects of 2-week dietary interventions on gut microbial composition.
B) Genera significantly different in abundance between study phases

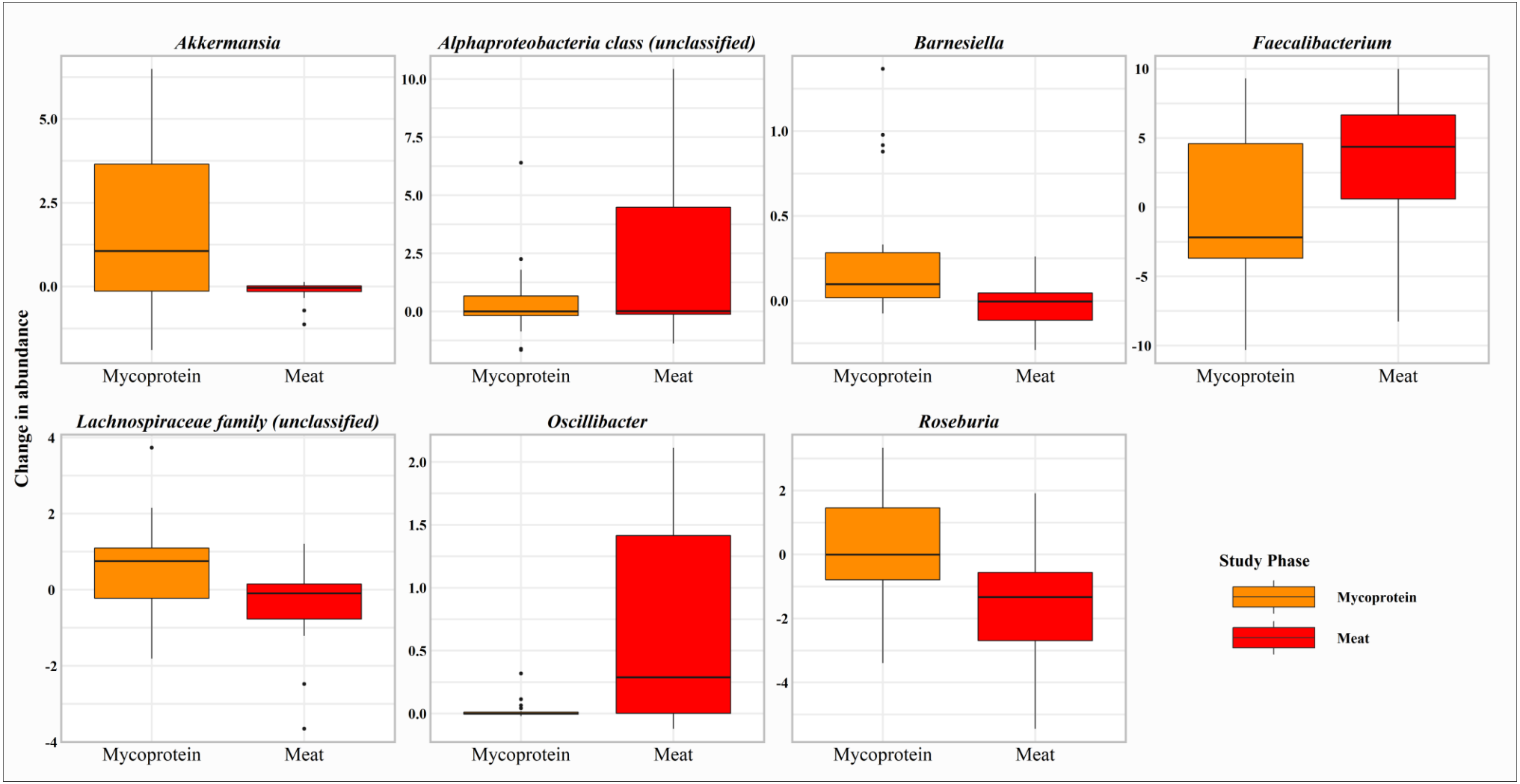


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Figure 4.7. Effects of 2-week dietary interventions on gut microbial composition.
C) Genera significantly changed in abundance from baseline after the Mycoprotein study phase.

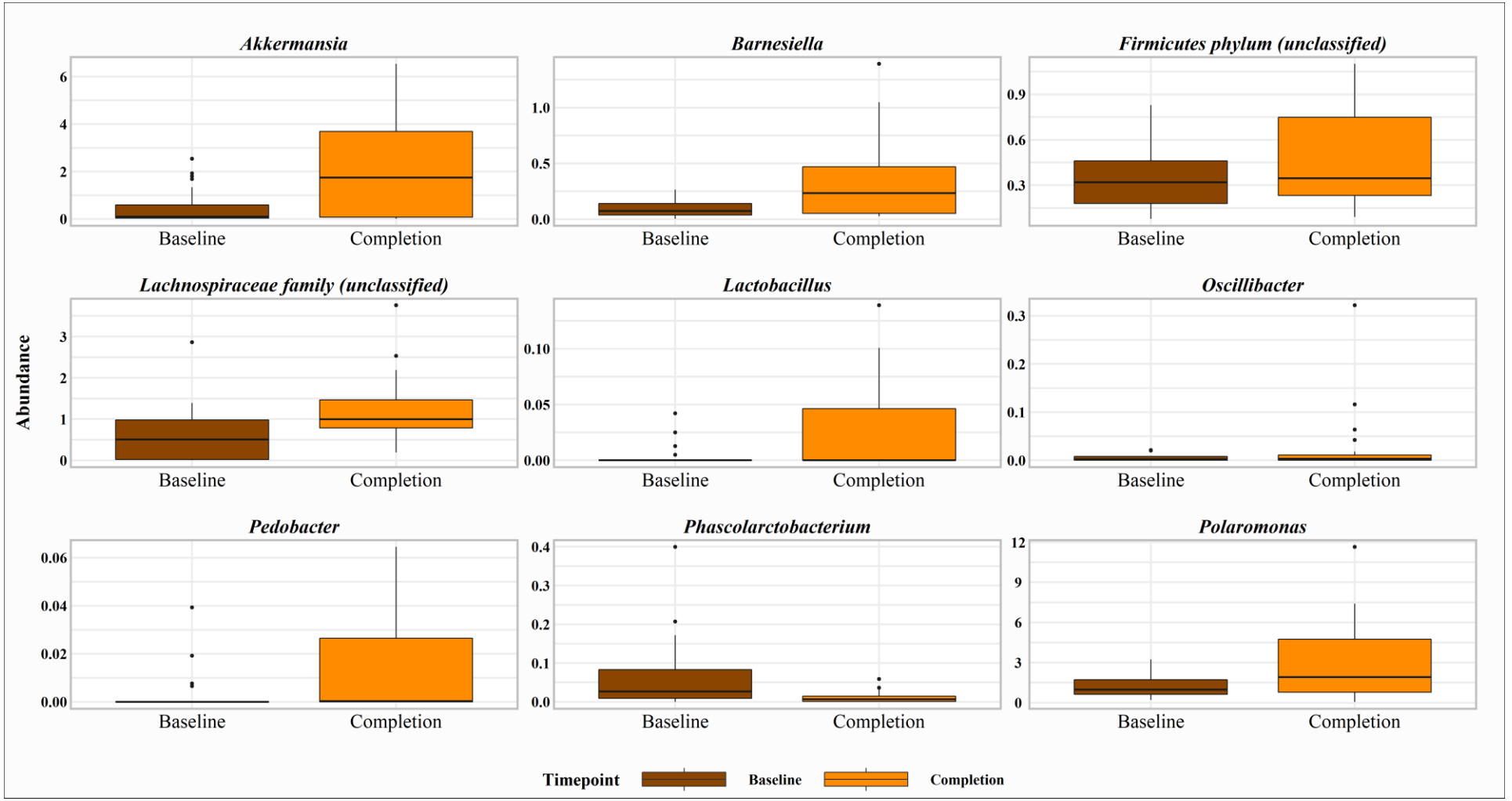
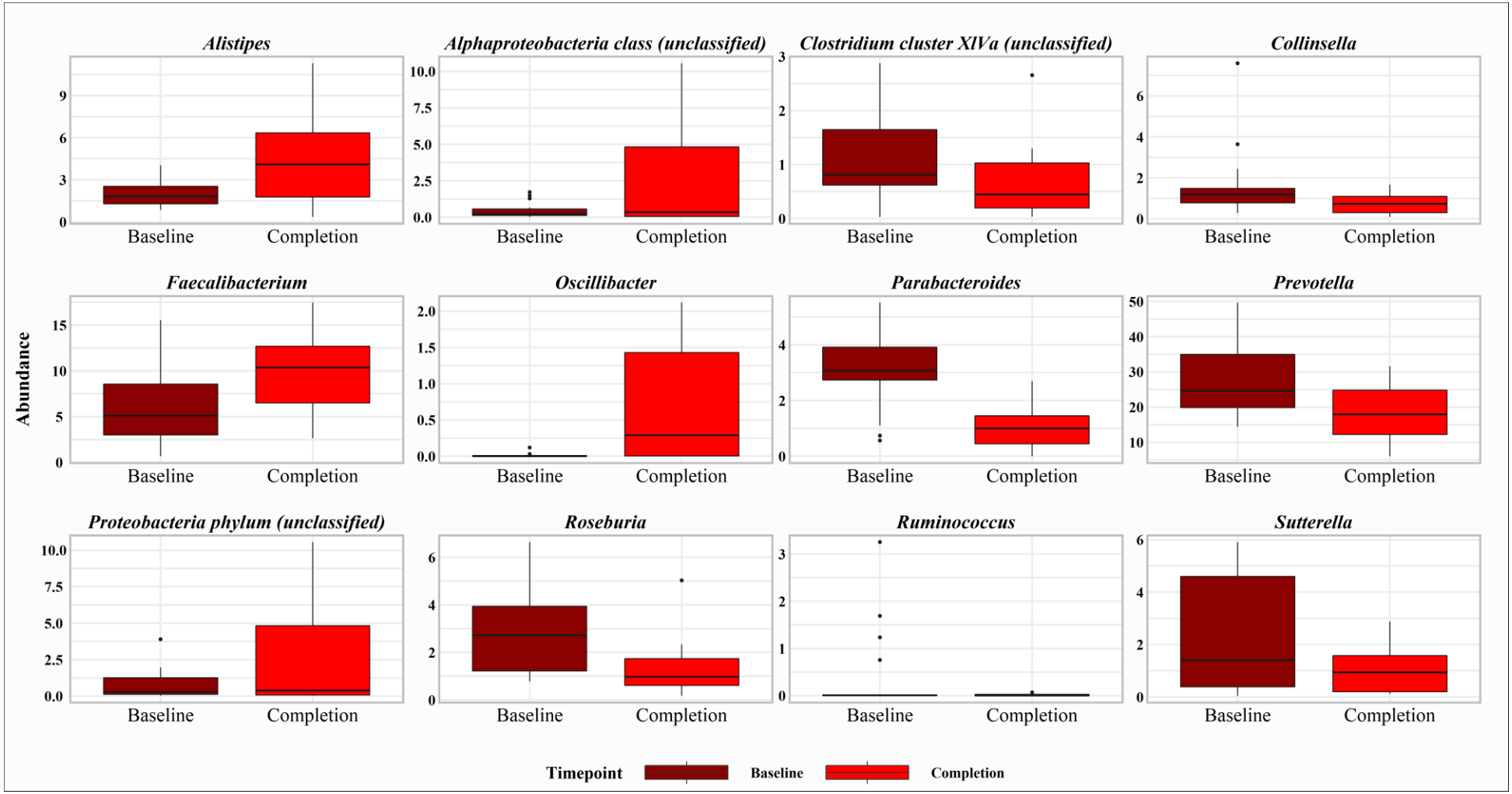


Figure 4.7. Effects of 2-week dietary interventions on gut microbial composition.
D) Genera significantly changed in abundance from baseline after the Meat study phase.



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4.4.7. Metabolomics

A total of 1,214 faecal metabolites were detected within the samples.

When comparing faecal metabolites within study phases, PLS-DA plots did not reveal clear clustering of metabolic profiles for either Mycoprotein (**Figure 4.9A.**) or Meat (**Figure 4.9B.**). However, there were a number of metabolites which had significant fold changes from baseline during study phases (**Figure 4.9C.** and **Figure 4.9D.**). The metabolites considered VIP >1.5 for differences between baseline and post diet on both Meat and Mycoprotein are included in **Table 1 of Appendix A**, with the fold change during study phase.

PLS-DA plot of faecal metabolites between the study phases revealed a small distinction between metabolic profiles of the diets by the primary and secondary components (**Figure 4.10A.**). Together, these two components accounted for ~16.9% of the variability (7% and 5.9% for the first and second components, respectively). There was no clear clustering between study phase metabolite composition, but there were many metabolites that differed in abundance between the diets (**Figure 4.10B.**). The metabolites considered a VIP >1.5 are included in **Table 2 of Appendix A**, with the fold difference between the diets.

Pathway analyses using the metabolites with VIP >1.5 revealed that thiamine metabolism was enriched as well as multiple pathways of fatty acid beta-oxidation and carnitine synthesis following Mycoprotein, whereas after Meat, sphingolipid metabolism and purine metabolism were enriched (**Figure 4.10C.** and **D**).

In urine, a total of 1,302 metabolites were detected within the samples.

PLS-DA plots capturing urinary metabolites within study phases demonstrated an absence of clear clustering of metabolic profiles for either Mycoprotein (**Figure 4.11A.**) or Meat (**Figure 4.11B.**). Within the study phases, there were a number of metabolites which had significant fold changes from baseline (**Figure 4.11C.** and **Figure 4.11D.**). The metabolites considered a VIP >1.5 for differences between baseline and completion of study phases are included in **Table 3 of Appendix A**, with the fold change during study phase.

Comparison of urinary metabolites between study phases employing PLS-DA revealed a small distinction between metabolic signatures of the diets by the primary and secondary components, however, there was not a defined clustering (**Figure 4.12A.**). Together, these two components accounted for ~11.8% of the variability (6.5% and 5.3% for the first and second components, respectively). There were many metabolites that differed in abundance between the diets (**Figure 4.12B.**). The metabolites considered a VIP >1.5 are included in **Table 4 of Appendix A**, with the fold difference between the diets.

Pathway analyses using the metabolites with VIP >1.5 revealed that thiamine metabolism was enriched after Mycoprotein, followed by ammonia recycling, beta-alanine, histidine and glycine and serine metabolism. Whereas taurine and hypotaurine metabolism was enriched the greatest after meat, followed by beta oxidation of very long chain fatty acids, phospholipid, steroid and bile acid biosynthesis. Both study phases led to an enrichment in the oxidation of branch chain fatty acids (**Figure 4.12C.** and **D**).

Figure 4.8. Influence of study diets on faecal metabolites profiles.
Partial least square discriminant analysis of faecal metabolites profiles at baseline and completion for **A)** Mycoprotein **B)** Meat.

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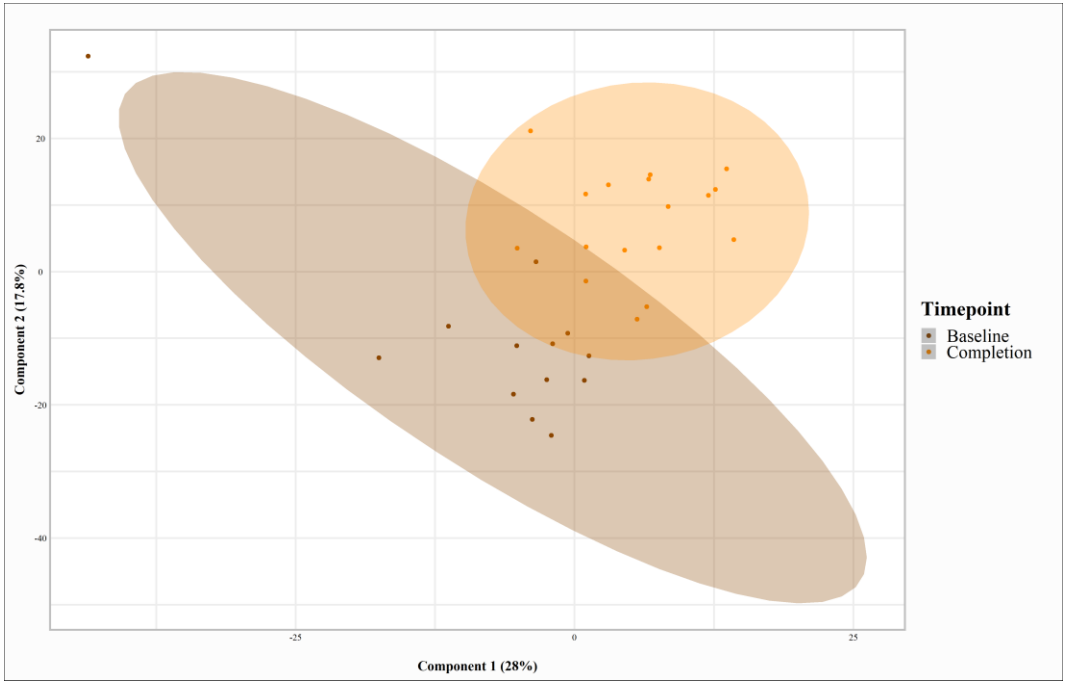
A

B



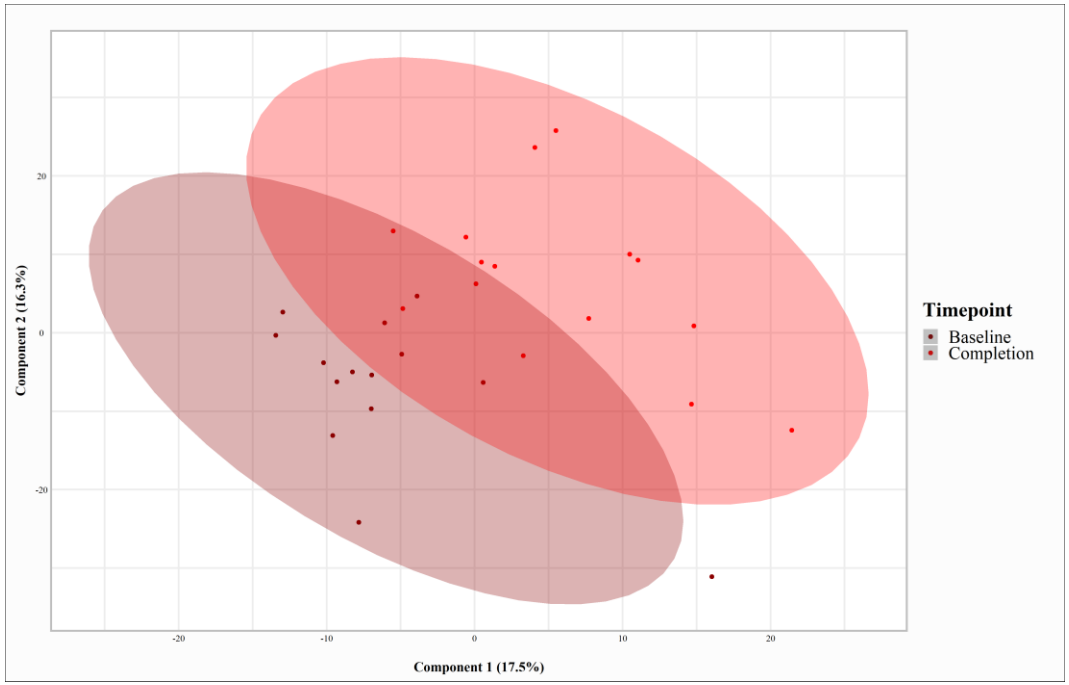


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Figure 4.8. Influence of study diets on faecal metabolites profiles.

Heat map of the distribution of faecal metabolites among all individuals at baseline and completion for **C)** Mycoprotein **D)** Meat.

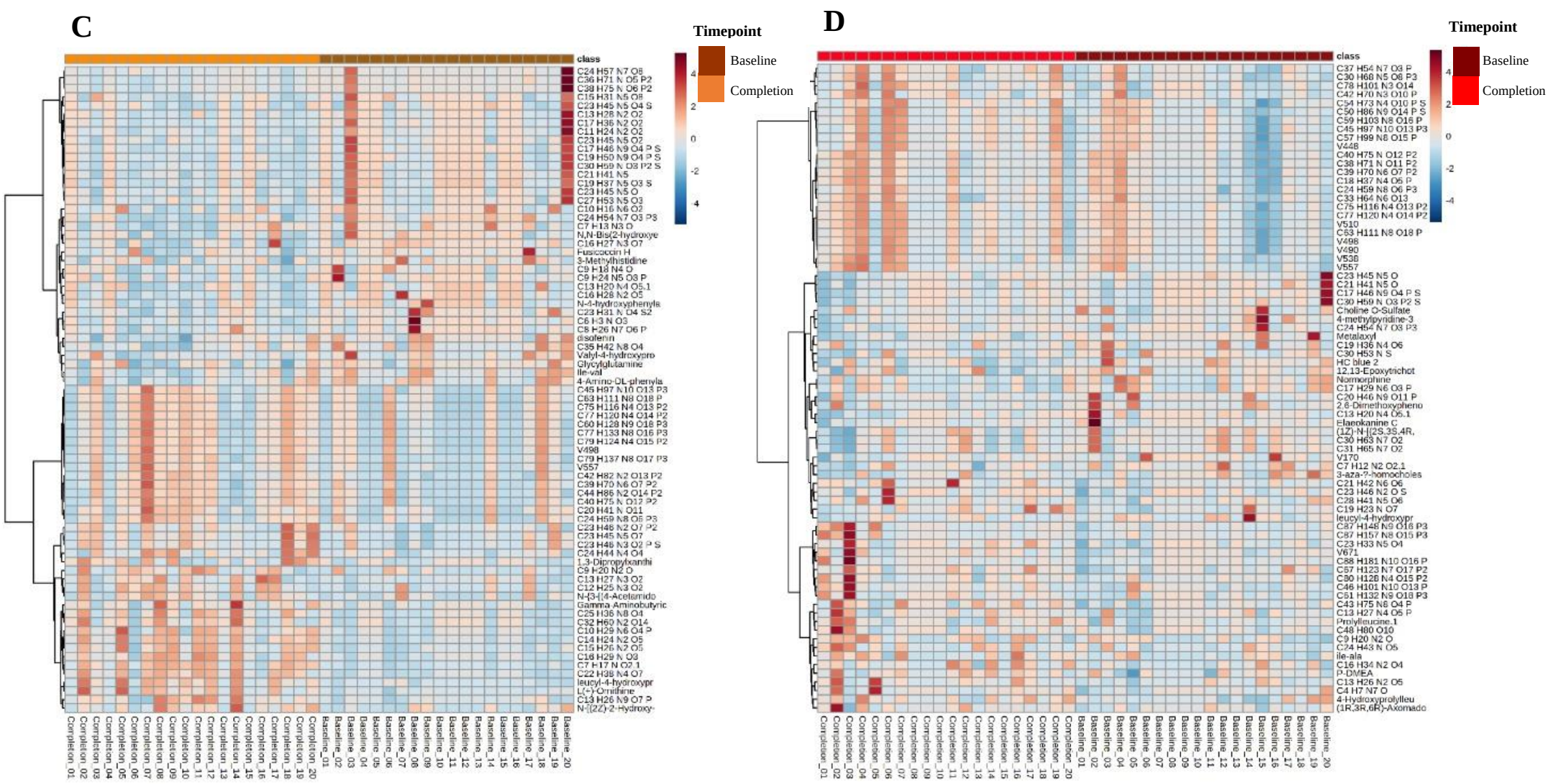


Figure 4.9. Comparison between study diets influence on faecal metabolites profiles.
A) Partial least square discriminant analysis (PLS-DA) of faecal metabolites profiles at study phase completion. **B)** Heat map of the distribution of faecal metabolites among all individuals at study phase completion**. C)** Metabolic pathways enriched following Mycoprotein and **D)** Meat. Metabolites defined as variable importance in projection (VIP) >1.5 between diets included in pathway analysis.

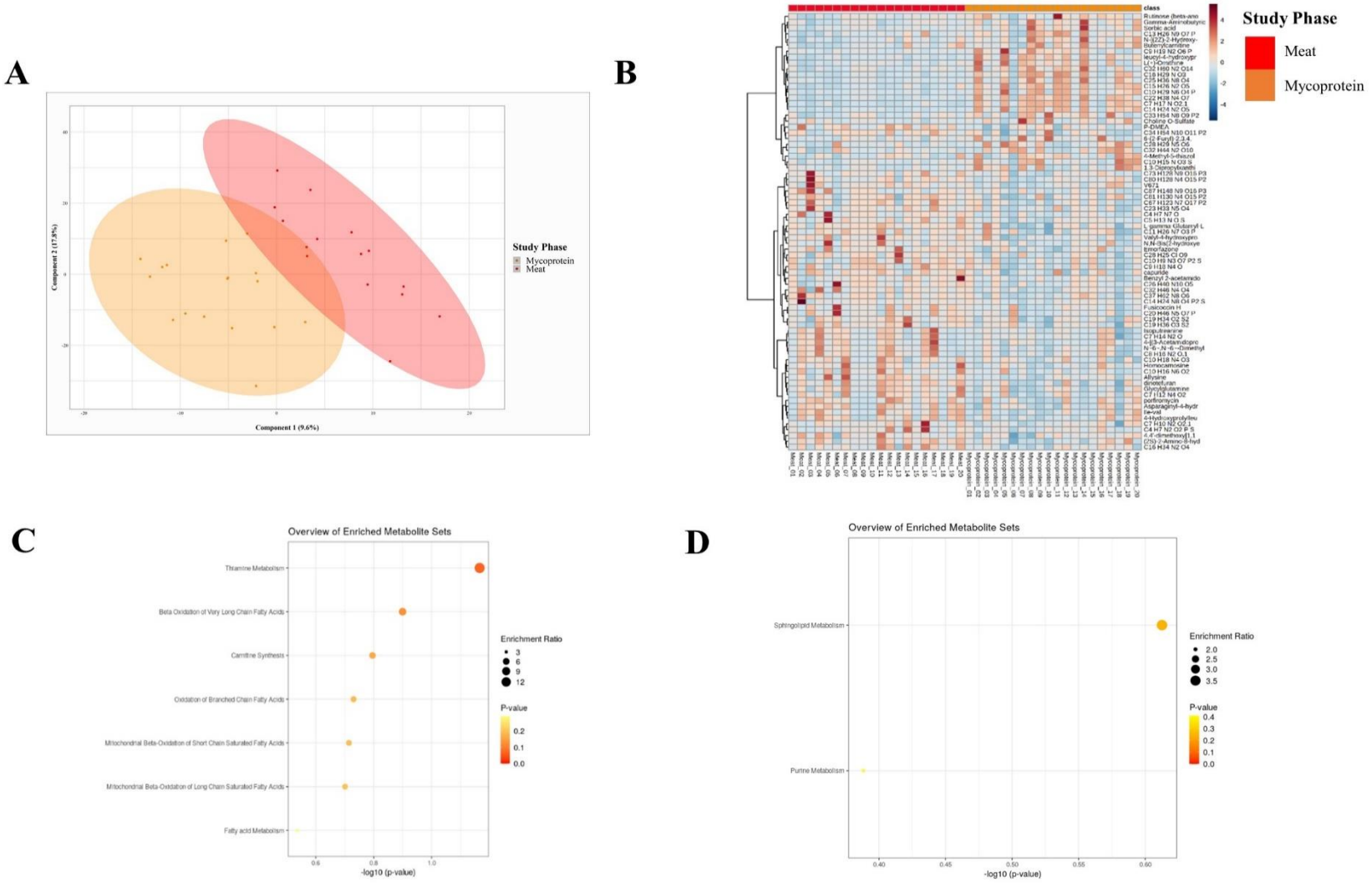
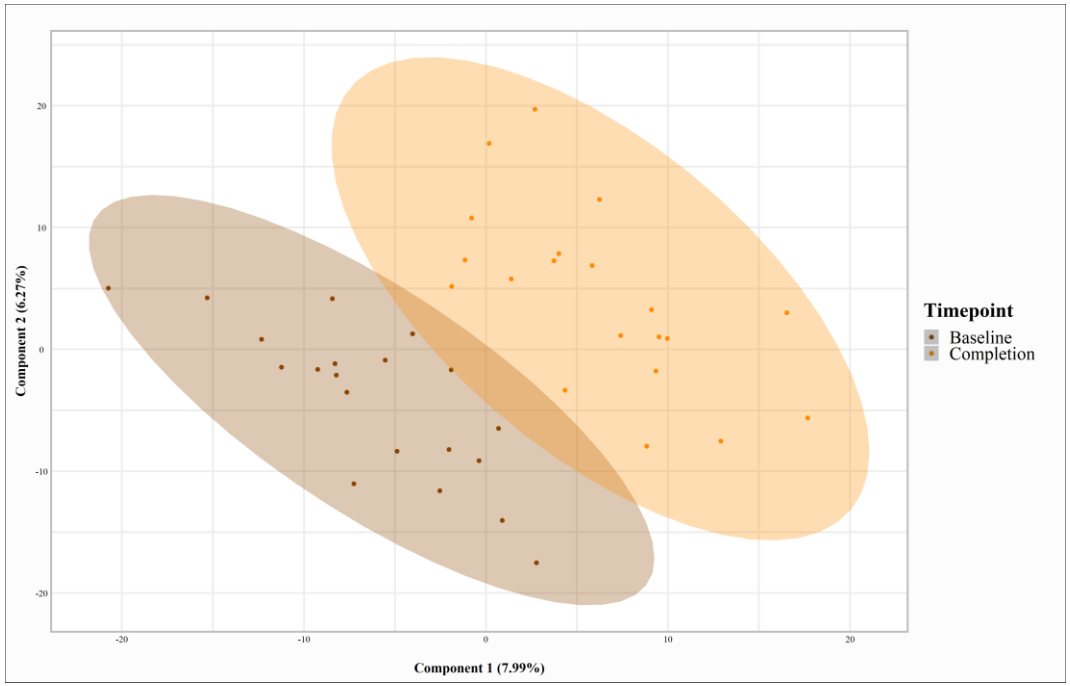


Figure 4.10. Influence of study diets on urinary metabolites profiles.
Partial least square discriminant analysis of faecal metabolites profiles at baseline and completion for **A)** Mycoprotein **B)** Meat.

A



B

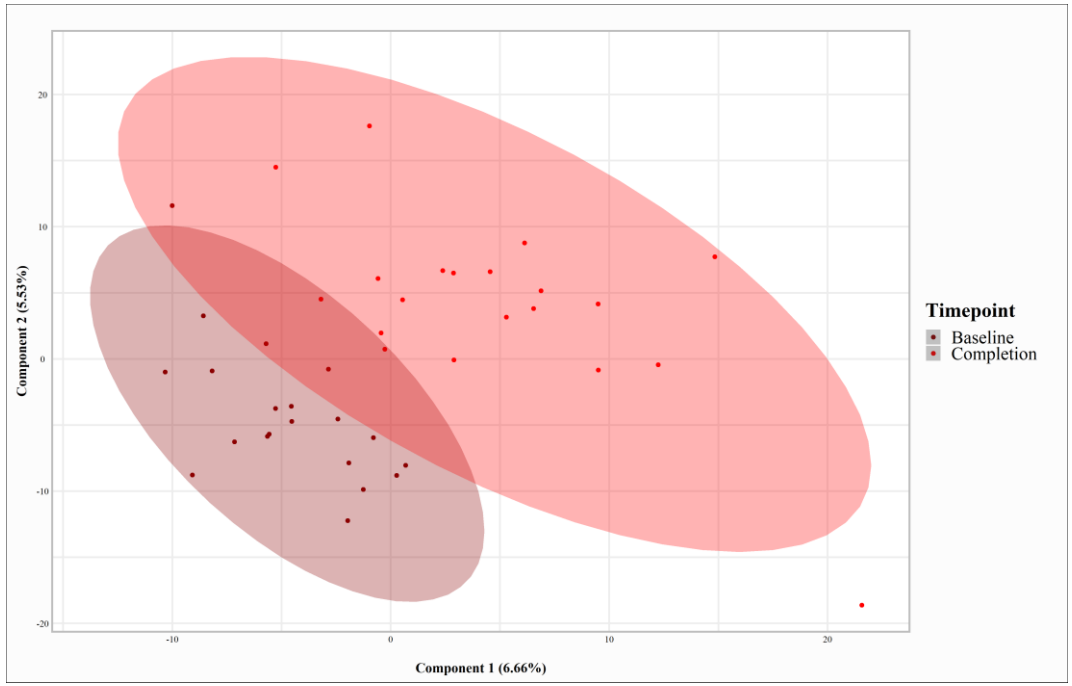


Figure 4.10. Influence of study diets on urinary metabolites profiles.

Heat map of the distribution of faecal metabolites among all individuals at baseline and completion for **C)** Mycoprotein **D)** Meat.

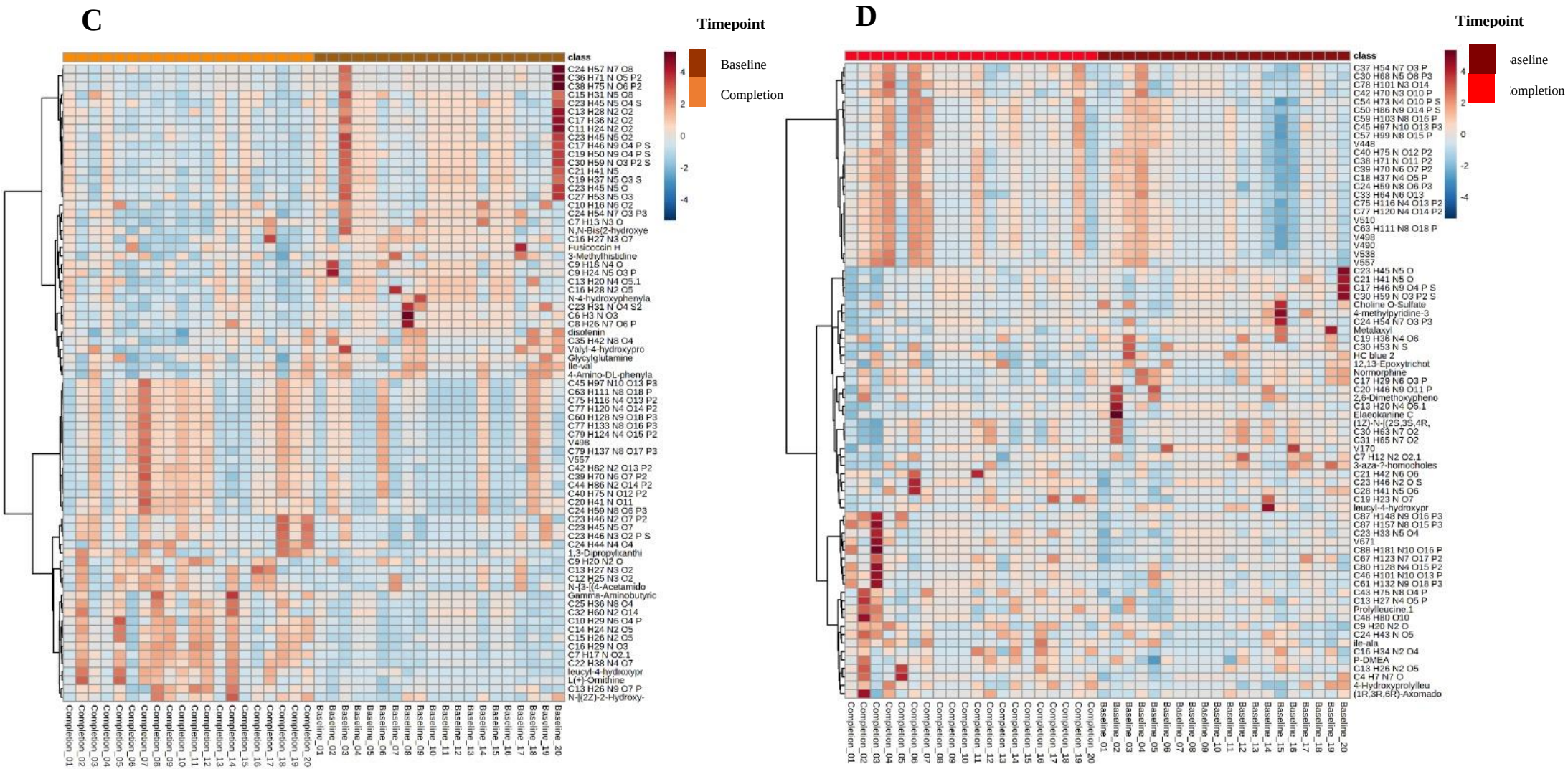
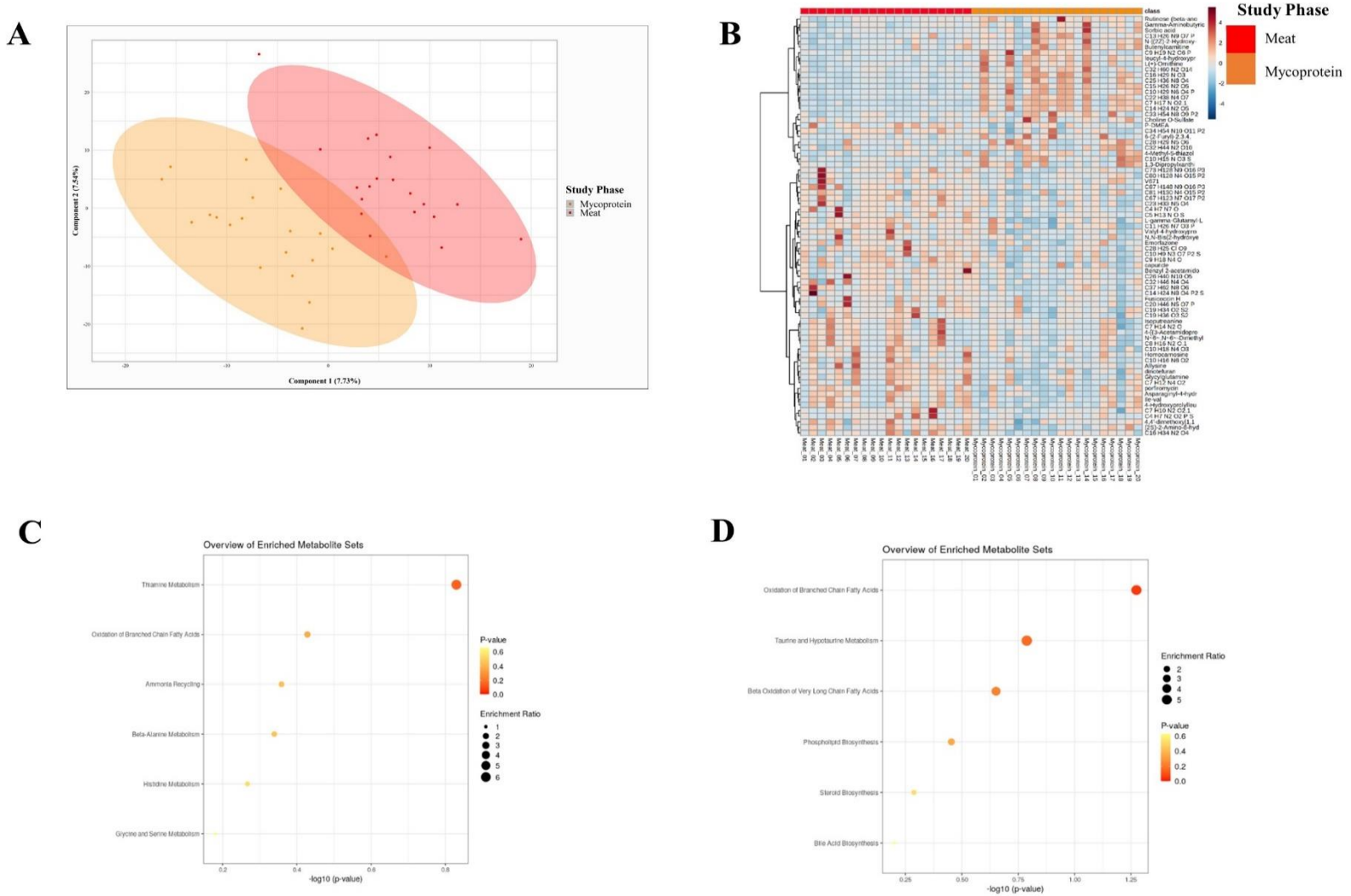


Figure 4.11. Comparison between study diets influence on urinary metabolites profiles.
A) Partial least square discriminant analysis of faecal metabolites profiles at study phase completion**. B)** Heat map of the distribution of faecal metabolites among all individuals at study phase completion. **C)** Metabolic pathways enriched following Mycoprotein and **D)** Meat. Metabolites defined as variable importance in projection (VIP) >1.5 between diets included in pathway analysis.



4.5. Discussion

This intervention involved changes in the composition of volunteers' diets across both study arms. In the high-meat arm of the study, volunteers' average daily meat consumption increased from baseline by 21g, whilst on the mycoprotein arm of the study, individuals did not consume any meat and increased their consumption of mycoprotein by an average 240g per day from baseline. Importantly, there were no significant differences in self-reported energy, fat, or protein intake between the study arms, but fibre intake was significantly increased on the mycoprotein arm of the study. This increase in fibre intake might in part explain the observed increase in stool weight with mycoprotein.

The primary aim of this work was to establish the impact of replacing a high red and processed meat diet with mycoprotein on faecal water genotoxicity and other markers related to intestinal health. Faecal water genotoxicity is poorly validated against tumour incidence in homo sapiens. However, it has been used as a non-invasive surrogate risk marker in a number of short-term intervention studies exploring dietary components, gut health and CRC risk (6, 7, 40, 41). Further, it has been shown to predict subsequent tumour incidence in rodent models (42), and is used on the well-founded assumption that DNA damage is an initiating event in carcinogenesis (43), and therefore that high genotoxic exposures are probably a significant risk factor for neoplasia.

I observed that high red and processed meat intake increased faecal water genotoxicity, this corroborates previous findings (6, 7). I also observed that substituting meat for mycoprotein significantly reduced genotoxicity, both relative to the high meat arm of the study, and also compared to baseline (i.e. a standard western diet). This was a novel finding which suggests that consuming mycoprotein may be protective against DNA damage, either via a displacement effect on harmful constituents of the diet, or independently via the introduction of antigenotoxic factors within the gut.

There are many potential contributors to genotoxicity in filter-sterilised aqueous faecal supernatants, i.e. activated dietary chemical residues, such as nitroso compounds, haem, or heterocyclic amines; host derived bioactives, such as bile acids or their microbial metabolites; microbial fermentation products such as cresol, phenols, or hydrogen sulfide; or hitherto poorly-studied genotoxic peptides such as *Bacteroides fragilis* toxin or colibactin (44-46). Nitrates and nitrites are a feature of various elements of the diet including as additives in processed meats; in addition to dietary haem, these compounds react with amines in the acidic conditions of the stomach to form NOCs (47, 48); faecal NOCs are established mutagens (49-51) that have previously been shown to increase with meat consumption (27). Consistent with earlier studies, I observed that faecal NOCs were increased from baseline on the high-meat arm of our study, however they were also reduced from baseline following the mycoprotein arm of the study. Self-reported mean intake of meat at baseline was 219g day; The observed reduction in faecal NOCs with the mycoprotein is probably a consequence of the displacement effect on the participants meat-rich habitual 'western' diet. The close

association between the genotoxicity data and the faecal NOC's is strongly suggestive of the role of NOCs as the main predictor of faecal genotoxicity.

The intestinal microbiome may exert both pro- and anti-carcinogenic activities that are attenuable by diet. Certain microbial strains have demonstrable anti-carcinogenic and anti-genotoxic activities. Further, microbially produced short chain fatty acids (SCFAs) feed intestinal epithelial cells, and may promote barrier function, and the production of a healthy protective mucus layer as a barrier between epithelial cells and luminal mutagens (52-56).
In this trial, there were treatment specific effects on the composition and activity of the faecal microbiome. I analysed faecal microbial composition via 16S rRNA based amplicon sequencing, so I cannot comment on the effects of the intervention on total microbial cell numbers other than to note the 80 gram difference in stool weight between the study arms which might hint at increased biomass with mycoprotein. Alpha-diversity was reduced following both diets, this is probably due to the displacement of other foods by the intervention, thereby limiting the breadth of fermentable substrates. (57)

Both diets significantly affected the relative abundance of various bacteria across taxonomic ranks. The most notable findings with the mycoprotein diet were increases in the abundance of the genera *Lactobacillus*, *Roseburia*, *Rumminococcus,* and *Akkermansia*. The *Lactobacilli* are lactic acid producing bacteria widely used as probiotics. In *in vivo* models, *Lactobacilli* consistently exert significant protection against chemically induced tumours (58). *In vitro* studies suggest that one of the mechanisms for this anticancer effect may be potent antigenotoxic activity including via binding and chemical modification of NOCs (59). It is also evidenced that some of the *Lactobacilli* enhance intestinal barrier function by improving tight junction integrity (60) and enhancing colonic mucin production (61), conferring a further layer of defence against genotoxic insult. While this study, to our knowledge, is the first to report increases in *Lactobacilli* following mycoprotein consumption, further evidence akin to our findings could suggest a putative 'prebiotic' effect of mycoprotein analogous to well-regarded prebiotics such as inulin which likewise increases *Lactobacilli* abundance in the gut (62). The dominant butyrate producing *Roseburia* were increased in relative abundance following mycoprotein consumption but decreased on the meat diet. *Roseburia* have been associated with suppression of gut inflammatory processes (63), and their presence is reduced in both inflammatory bowel disease (64), and CRC (65). Butyrate is a potent anti-neoplastic agent in the gut, it is also an inducer of intestinal mucin production (66). Thus, the mycoprotein-linked observed increases in the SCFA producing *Lactobacilli* and *Roseburia* may help explain the parallel increase in the relative abundance of mucin-degrading *Rumminococcus* and *Akkermansia* through a potential form of indirect cross feeding. Future studies might attempt to demonstrate this proposed interaction.

Akkermansia produces propionic and acetic acid from the degradation of mucins, these SCFA can be utilised by the host for energy or leveraged by other species to produce butyrate (67). Propionate in

particular triggers host GPR43 receptors in the gut inducing anorectic pathways which shut down food seeking behaviour (68), this may help explain observed associations between *Akkermansia* and a reduced risk of obesity (69) and metabolic dysfunction (70). Thus hypothetically, our observed increase in the abundance of *Akkermansia*, might help explain a previous study showing that mycoprotein consumption reduces *ad libitum* energy intake in a test meal, relative to chicken (71).

In contrast, the meat diet was associated with an increase in the relative abundance of bile resistant putrefactive bacteria, including *Alistipes* and *Oscillibacter*. The increased abundance of the *Alistipe*s and *Oscillobacter* in the high-meat arm of the study may be an adaptive response to bile, or due to a greater proclivity for digestion-resistant myofibrillar protein arriving in the gut from meat versus mycoprotein. Acute feeding studies with mycoprotein lead to very high muscle protein synthesis rates, suggesting a high bioavailability of mycoprotein (72), which might mean less total protein reaching the colon. Metabolically, in silico analysis indicates putrefactive pathways for *Oscillobacter* involving the generation of the bioactive polyamines, putrescine and spermidine (73), both of which have been associated with suppression of inflammation and an increase in apoptosis in the gut (74). However, an increased abundance of *Oscillobacter* has been associated with weight gain, metabolic dysfunction and a leaky gut (75). In contrast, the putrefactive capacity of *Alistipes* extends to the production of histidine, tetrahydrofolate, and the toxins, indole and phenol. Observational data are conflicting, with studies showing that *Alistipes* may be variously associated with both health and disease (76). The meat arm of the study also led to a relative increase in the glycan utilising, and butyrate producing *Faecalibacterium*. This was not observed in the mycoprotein arm of the study, which was surprising, as the mycoprotein arm was associated with a significant increase in total glycan intake (+16.99g/ day).

I applied untargeted metabolomics to both stool and urine samples using a Thermo IDX LC-MS. This instrument has integrated peak annotation against an established spectral library; in total 1,214 metabolites were detected in stool, and 1,302 metabolites in urine, using the library I was able to identify 41% of the total compounds. Given the high abundance of unidentified compounds that differ between the diets I intend to review this data set as the library develops further. I noted changes in individual metabolites differing between treatment arms, and I observed differences in the enrichment of metabolic pathways in both sample sets.

In stool, mycoprotein was associated with an increase in thiamine metabolism, oxidation of fatty acids, notably short and branched chain fatty acids, which may be indicative of greater microbial fatty acid production.

In contrast, the meat arm of the study was associated with increased sphingolipid metabolism, characterised by a reduction in ceramide. Sphingolipids are important mammalian signalling molecules with various biological activities; Ceramide induces anti-proliferative and pro-apoptotic signals in gut epithelial tissue (77), importantly increased sphingolipid metabolism may be a response to DNA damage (78).

In urine, meat was associated with increases in the metabolism of taurine and hypotaurine and a slight increase in bile acid metabolism. In contrast, mycoprotein led to an increase in the metabolism of histidine, beta-alanine, glycine and serine. These differences in amino acid pathways may be reflective of the differential amino acid profile of the meat and mycoprotein study food products. The combination of increases in taurine and hypotaurine, as well as bile acid metabolism may be indicative of a greater production of bile acids during the meat study phase, this may stem from a greater intake of fat when consuming the meat products, although our food record data does not suggest large differences in fat intake. It may also be that animal fat digestion stimulates a greater production of bile acids in comparison to the fat content of the mycoprotein products. While bile acids have long been known to facilitate digestion and absorption of lipids in the small intestine as well as regulate cholesterol homeostasis (79), they are implicated in the promotion of CRC via multiple mechanisms (80, 81). An enrichment of these pathways may be indicative of a negative effect in the host. Glycine metabolism, which was enriched after the mycoprotein diet, has been observed to be reduced in obesity and metabolic disorders (82). Whereas histidine metabolism can have unique roles in proton buffering, metal ion chelation, as well as scavenging of reactive oxygen and nitrogen species (83). This theoretically could form part of the reasoning for the observed lower load of NOCs following the mycoprotein diet. These differences in urinary metabolites may also be suggestive of opposing influences of the diets on gut health.

I identified other potential genotoxins within the metabolomics dataset, these included the bile acids apocholic acid and 7-ketodeoxycholic acid in stool, and p-cresol sulphate in urine. P-cresol sulphate is a product of tyrosine gut bacterial fermentation in the large intestine (84), followed be sulfation by the host's cells (85). Studies have demonstrated that p-cresol reaches genotoxic concentrations within *in vitro* gut fermentation models, and notably, p-cresol concentrations can predict the genotoxicity of the gut fermentation supernatant (86). I found that the urinary levels were increased following the meat diet, whereas substitution with mycoprotein led to a significantly lower level compared to both the meat diet, as well as from baseline values. This observation for the meat diet may be further reasoning for the associations between high meat intakes and CRC risk, with an increased production of genotoxic gut metabolites such as p-cresol.

The bile acid apocholic acid is understood to display carcinogenic activity (87), while 7-ketodeoxycholic acid is a secondary bile acid metabolite. 7α-dehydroxylating bacteria convert primary into secondary bile acids in the gut (88), which have the potential to induce oxidative stress and DNA damage (89, 90). These bile acid metabolites are implicated in playing a significant role in tumorigenesis, mediated through the gut microbiota (81, 91). I found that these bile acids were reduced following the meat diet, whereas substitution with mycoprotein led to increased levels. This result is unexpected, with respect to the significant reduction in genotoxicity, as well as the genotoxins NOCs and p-cresol sulphate following the mycoprotein diet, which would suggest a similar pattern might have

been found in the genotoxic bile acids. This finding may be due to chance, as such discrepancies have occurred in previous work. Although implicated in CRC, one study observed 7-deoxycholic acid to be lower in abundance in the stool of CRC patients compared to control (92). Bile acid metabolism is also a large component in cholesterol homeostasis (93), the increased abundance of bile acids in stool may be indicative of mycoprotein's ability to bind and sequester bile acids in the gut (94), which is a potential mechanism for the observed reductions in cholesterol observed with mycoprotein consumption (95-97), which I have also found (Chapter V).

Another finding from the present study was the effect that the diets had on stool weight. With stool weight increasing following the mycoprotein diet and reducing significantly after the meat diet. Stool weight has been inversely correlated with CRC risk (98). Taking this into account, the effects seen in the current study would suggest beneficial and detrimental effects for the mycoprotein and meat diets respectively.

The present study has several strengths. I used a randomised crossover design, which allows each participant to act as their own control. This has benefits over a parallel design, where cohort characteristics must be stringently matched and greater numbers required to achieve an equivalent statistical robustness of results. Our study was also investigator blinded, removing any potential form of bias from our results. In addition, the meat and mycoprotein food products implemented in the investigation are foods regularly consumed in the general public, bringing greater relevance of our results to a real-life context. Another strength is the mass spectrometry platform used, which is a state-of-the-art machinery that uses automated data acquisition and encompasses an increased profiling efficacy in comparison to alternative platforms. As such, this allowed the ability to obtain a greater number of identified metabolites with distinguishable fragmentations, which in turn, enabled a richer dataset to determine metabolic pathways which were enriched by the high meat and mycoprotein diets.

That said, our study also had limitations. The cohort was exclusively males; the reason for this was the fact that this study was novel, and I decided to keep a greater control over introducing a variety of external factors. Due to this, our findings may be more robust and applicable to the general UK population if also tested in a mixed gender cohort. In addition, I advised participants to avoid certain foods and supplements, however, I did not control dietary intake other than the supplied study food products. Other dietary factors during the intervention periods may have influenced our findings. Regarding 16S rRNA sequencing, although this methodology can bring wide coverage of bacterial taxonomies in samples, it is limited to the level of genus. Therefore, determining changes and detailed compositional profile at the species level is not possible. I also collected single day samples, the stool microbiome profile can oscillate daily, meaning this may not be the full picture of the microbial changes elicited by the diets in the current study.

In conclusion, our work has demonstrated that substituting red and processed meat for the meat alternative mycoprotein reduces faecal genotoxins, the genotoxic load, as well as increases the abundance of butyrate producing, and mucin utilising bacterial genera in the human gut.

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CHAPTER V

EXPERIMENTAL CHAPTER II

The effects of substituting meat for mycoprotein on biomarkers of cardiometabolic health: an analysis of secondary endpoints from The Mycomeat Study.

Abstract

Diets high in red and processed meat increase risk of cardiovascular disease (CVD). These associations combined with environmental concerns feed public health recommendations to reduce meat consumption. Meat alternatives may help reduce barriers to pursuing a plant based diet. Mycoprotein is a fibre-rich meat alternative produced from *Fusarium venenatum*. Here, I report on *a priori* defined secondary endpoints, related to cardiometabolic health, from an investigator-blind, randomised, crossover, dietary intervention trial in which healthy male volunteers (n = 20) supplemented their habitual diet with 240g per day of either red and processed meat, or the weight equivalent of mycoprotein.

I observed that 2 weeks of daily mycoprotein consumption induced a significant but small reduction in waist circumference compared to meat (-0.95 ± 0.42cm, $P = 0.04$). It also induced reductions in both total cholesterol (-0.33 ± 0.13cm, $P = 0.02$) and LDL cholesterol (-0.37 ± 0.14cm, $P = 0.02$), but an increase in plasma TAG (+0.13 ± 0.09cm, $P = 0.09$). In contrast, meat intake caused non-significant increases in total (+0.04 ± 0.09cm, $P = 0.66$) and LDL cholesterol (+0.02 ± 0.08cm, $P = 0.95$). Neither intervention induced significant shifts in urinary TMAO. Mycoprotein consumption was also associated with reductions in systolic (-2.41 ± 1.89cm, $P = 0.23$) and diastolic blood pressure (-0.80 ± 1.23cm, $P = 0.43$), whilst both were increased following the high meat arm. Neither intervention arm significantly affected urinary sodium, with increases following both diets, while potassium excretion was reduced after both the mycoprotein and meat diet, with a significant reduction following meat. Glucose was not affected in this study.

These observations, reported in a metabolically healthy group, are in line with previous studies showing potential cardiovascular benefits from mycoprotein consumption. Future studies might investigate these effects in high risk cohorts and seek to establish mechanism of action.

5.1. Introduction

Observational studies suggest that animal meat consumption, particularly red and processed meat, is associated with an increased risk of cardiovascular and metabolic disease (CVD) (1-5). In contrast, adherence to a plant-based dietary pattern appears to confer a cardioprotective effect (6, 7). Notably, findings from one prospective cohort study suggest a 10% increase in energy intake from animal protein translates into an 8% increase in CVD mortality, while a 3% increase in energy from plant protein reduces risk by 12% (8). These observations are reciprocated by findings from clinical trials that report favourable effects in CVD risk markers when meat consumption is curtailed (9-12), including lower LDL cholesterol and apolipoprotein B levels (13); as well as a reduction in the urinary excretion of microbially produced TMAO (14).

Public health approaches have had limited success in inducing meaningful population level transitions to plant-based eating, in part due to strongly held culinary traditions, taste preferences, and social and cultural norms (15-18). Plant-based alternative meats – i.e., products designed to mimic meat, yet made entirely from plant-based, sustainable sources – offer an effective approach to curtail meat consumption without drastically altering meal patterns and dietary habits (19, 20). Mycoprotein is an alternative, sustainable protein source derived from the continuous cultivation of *Fusarium venanatum*, which is both high in protein and fibre, while low in fat and energy (21-23). With processing, it can resemble the texture and flavour of meats. Previous chronic dietary intervention trials consistently demonstrate that replacing meat with mycoprotein based foods induce a clinically meaningful decrease in total and LDL cholesterol but with differing effects on HDL reported between these trials (24-26). The heterogeneity between studies on HDL outcomes may be reflective of the baseline metabolic and cardiovascular health of the study cohorts, it is important therefore that further studies characterise the cardiometabolic response to displacing meat with mycoprotein in differing population groups.

In the current paper, I present an analysis of *a priori* defined secondary endpoints (cardiometabolic risk markers) from The Mycomeat Study. This was a randomised crossover dietary intervention trial in a cohort of metabolically healthy males. The primary endpoints described previously, showed beneficial effects of consuming a diet high in red and processed meat (henceforth Meat) with those of consuming a diet where this quantity of meat was replaced with mycoprotein based foods (henceforth Mycoprotein) on faecal genotoxicity and markers of gut health.

5.2. Methods

5.2.1. Study setting and participants

The Mycomeat Study has been described in detail elsewhere (Chapter IV). Procedures were followed in accordance with the declaration of Helsinki and the study was approved by the Northumbria University Ethics Committee (reference number 15274). The participants involved in the study provided written informed consent.

The Mycomeat Study was an investigator-blind randomised crossover trial (NCT03944421) conducted in Newcastle, UK. Study participants were metabolically-healthy male adults recruited from the North East of England, UK, via on-campus poster advertisement and using a database of previous study participants. Inclusion criteria included: age 18-50yr; BMI < 30 kg/m^2; Blood HbA1c <58mmol/mol (<6.5%) (not diagnosed with diabetes); Fasting total cholesterol <7.8 mmol/l; Triglycerides <2.3 mmol/l; Normal liver function (assessed by measuring liver enzymes in the screening blood sample), Blood pressure lower than BP <140/90 mmHg. Not suffering cardiovascular diseases; not suffering from chronic gastrointestinal problems (e.g., Inflammatory Bowel Disease, Irritable Bowel Syndrome, coeliac disease); No history of alcohol misuse based on self-reported alcohol intake and measurement of liver enzymes in the screening blood sample; and a willingness to refrain from use of pre- and pro-biotics, vitamin supplements as well as alcoholic beverages for the duration of the study. Exclusion criteria comprised: gastrointestinal disease; use of medications that may affect gastrointestinal motility; an unwillingness to consume all key study foods; antibiotic, prebiotic, or probiotic use within 3 months before the start of the intervention; use of tobacco or recreational drugs and history of coronary artery disease, diabetes, or other chronic disorders. In addition, any volunteers who had been enrolled in previous dietary trials within 3 months of participating in the current study were also excluded. Participants took part in a screening visit where blood measurements were taken and assessed by standard clinical cut off values to confirm study eligibility.

5.2.2. Study design and interventions

The Mycomeat Study consisted of 2 phases, each lasting 2 weeks, separated with a 4 week washout (**Chapter IV, Figure 4.1.**). During study phases, participants were provided with 240g/day (uncooked weight) of either red and processed meat (Meat) products or mycoprotein-based products (Mycoprotein) to consume each day of the 2 week study phase. The meat dose represented high intakes and was also a similar dosage as prescribed in previous intervention trials investigating similar themes (meat, gut health and colorectal cancer risk) (27-32). All Mycoprotein products were supplied by Quorn Foods^{TM}, and all Meat products were obtained from Asda supermarket chain. Researchers distributed both Mycoprotein and Meat products to participants at the research facility. To aid adherence to the interventions, the study food products followed a 7-day rotation (Chapter IV). As outlined, study foods were matched for weight (240g uncooked), however, I opted for a selection of products which were matched closely for total energy content to avoid introducing additional influences from energy intake. As well as the Mycoprotein and Meat substitution, participants were provided guidance to avoid

additional high-protein, fibre, or probiotic supplements, and to refrain from alcohol consumption for the duration of the trial. Apart from this guidance, participants were otherwise asked to maintain their usual diet during the study. During each phase of the study (**Chapter IV, Figure 4.1.**), participants completed a 1-day food record, from which energy and macronutrient intake was calculated using Nutritics nutrition analysis software (version 5.66 Education) (33).

5.2.3. Sample collection and anthropometric measurements

At baseline and the conclusion of each intervention period, participants visited the Brain, Performance and Nutrition Research Centre (BPNRC, Northumbria University, Newcastle, UK) in an overnight fasted state (**Chapter IV, Figure 4.1.**). Anthropometric measurements of body weight, blood pressure, hip and waist circumference, body fat percentage by bioimpedance scale (Tanita BC-418) were taken. Venous blood samples were collected in serum SST and fluoride oxalate tubes for analysis of blood lipids and glucose, and EDTA tubes for the analysis of lipoprotein particle sub fractions (Fisher Scientific). In addition, at each visit, a urine (morning spot) and stool sample (collected at participants homes ≤10 hr prior to visiting the research centre; for primary end point measurements) were obtained from participants. Urine samples were stored on ice during the study visit before the sample was aliquoted into sterile 1.5-ml tubes and stored at -80 °C until further analysis.

5.2.4. Blood biochemical measurements

Blood collected in serum SST and fluoride oxalate tubes was analysed on the same day for fasting serum triglycerides, total cholesterol, and plasma glucose via routine automated clinical biochemistry at the Blood Sciences Department of The Royal Victoria Infirmary Hospital, Newcastle, UK. Blood that was collected in EDTA tubes was kept at 4°C prior to centrifugation at 1900g at 4°C for 10min, and the plasma collected was stored in aliquots (0.5ml) at -80°C until analysis. Plasma HDL cholesterol was measured by enzymatic endpoint analysis using enzyme reagent kits on a clinical chemistry analyser (Randox Daytona Series). LDL cholesterol was estimated using the Friedewald equation (34).

5.2.5. LC-MS quantification of TMAO

Urine samples were specific gravity normalised and prepared as previously described (Chapter IV).

Trimethylamine n oxide (TMAO) was then quantified by Hydrophilic Liquid Interaction Chromatography (HILIC) in positive mode, performed on an Vanquish Liquid chromatography chromatographic separation system connected to an IDX High Resolution Mass Spectrometer (Thermo Scientific). The HILIC positive data set were processed via Compound Discoverer 3.2 according to the following sittings: Untargeted Metabolomic workflow with online database: mass tolerance 10ppm, maximum shift 0.3 min, alignment model adaptive curve, minimum intensity 500K, S/N threshold 3, compound consolidation, mass tolerance 10ppm, RT tolerance 0.3 min. Database matching were

performed at MS2 level using Thermo scientific m/z cloud with a similar index of 80% or better. TMAO intensity was then identified in the overall metabolite dataset.

5.2.6. Urinary sodium and potassium

Gravity normalised urine samples were thawed prior to dilution to 1:100 with distilled water. Sodium and potassium concentrations were determined with a flame photometer (PFP-7, Jenway, UK). Standards of sodium (2ppm – 8ppm) and potassium (5ppm – 40ppm) were used to quantify levels prior to transforming into concentrations. Results are expressed in μmol.

5.2.7. Assessment of CVD risk

CVD risk was calculated using the Framingham Risk Score (FRS) based on the following risk factors, age, gender, systolic blood pressure, smoking habit, total cholesterol and HDL cholesterol (35). Results are presented as % risk of CVD in the next 10 years.

5.3. Statistical analysis

This study was powered according to the primary endpoint, change in faecal genotoxicity, described previously (Chapter IV). Recruitment and testing were ended once the trail was fully recruited according to the prior power calculation.

Data was assessed for normality by visualising Q-Q plots and performing Shapiro-Wilks tests before statistical analysis was performed.
Changes from baseline within study phases and differences between study phases in all measures was assessed using mixed effects models. In all models, age, BMI and habitual alcohol intake were included as fixed effects, with the participant as the random effect. For measures related to BMI (body weight, body fat, trunk fat, and BMI itself), BMI was excluded from the model to avoid collinearity. The interaction of the order at which the participants received the diets was also included to capture the impact of diet order on study outcomes.

A P value of > 0.05 was considered significant. All statistical analyses and visualisations were performed in RStudio (36).

5.4. Results

5.4.1. Participants

Twenty participants completed the study, the mean age at baseline was 30.4 years, and the average BMI was 24 **(Chapter IV, Table 4.2.)**, the flow of The Mycomeat Study is depicted in **Chapter IV, Figure 4.2.** Participant enrolment began on 1 June, 2019 and the date of final data collection was 29 January, 2020.

5.4.2. Diet

Comparing nutritional intake during study phases, fibre intake was significantly greater during Mycoprotein compared to Meat (+16.74 ± 2.92g/day, $P < 0.001$). Apart from the difference in fibre intake, total energy, carbohydrate, and sodium intake were greater during Mycoprotein, whereas protein, fat, and saturated fat were higher during meat. All these differences were not significantly different between the study phases (**Table 5.1.**).

5.4.3. CVD risk markers

Over the course of the study, there were no significant effects by either of the diet phases on body weight (Meat, +0.26 ± 0.22kg; $P = 0.33$; Mycoprotein, -0.17 ± 0.27kg; $P = 0.58$), BMI (Meat, +0.09 ± 0.06kg/m^2; $P = 0.22$; Mycoprotein, -0.04 ± 0.08kg/m^2; $P = 0.63$), or body fat (Meat, +0.33 ± 0.25%; $P = 0.42$; Mycoprotein, +0.18 ± 0.39%; $P = 0.82$). Following Meat, waist circumference slightly increased (+0.80 ± 0.39cm) while there was a decrease after Mycoprotein (-0.15 ± 0.31cm), with the difference between study phases reaching significance ($P = 0.04$). Following Mycoprotein, both total cholesterol (-0.33 ± 0.13mmol/L, $P = 0.02$) and LDL cholesterol (-0.32 ± 0.10mmol/L, $P = 0.005$) were significantly lower from baseline. In addition, these changes in both total cholesterol and LDL cholesterol were significant compared to Meat (TC, $P = 0.02$: LDL, $P = 0.01$). There were marginal increases in both systolic and diastolic blood pressure after Meat (SBP, +1.70 ± 1.40mmHg; $P = 0.27$, DBP, +1.77 ± 1.23mmHg; $P = 0.23$), whereas there were slight reductions following Mycoprotein (SBP, -2.41 ± 1.89mmHg; $P = 0.23$, DBP, -0.80 ± 1.23mmHg; $P = 0.43$); the difference between dietary phases also failed to reach significance (SBP, $P = 0.11$; DBP, $P = 0.16$). HDL cholesterol, triglycerides, as well as glucose were not significantly affected by either diet phase and there was no significant difference between Meat and Mycoprotein (**Table 5.2.**).

The study diets effected urinary TMAO excretion differently, with an increase following the Mycoprotein ($P = 0.34$), while there was a reduction after Meat ($P = 0.54$). None of the effects were statistically significant, and the difference in effects also did not reach significance ($P = 0.14$) (**Figure 5.1**).

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Table 5.1. Difference in self-reported nutritional intake between diets, including provided study food[1]

Nutrient	Meat	Mycoprotein	Difference[2]	*P*[3]
Energy, *kcal/d*	2355.18 ± 553.32	2567.44 ± 796.97	+212.26 ± 242.63	0.47
Protein, *% of energy*	20.28 ± 3.85	18.21 ± 3.38	-2.07 ± 1.28	0.13
Carbohydrate, *% of energy*	42.23 ± 6.24	46.30 ± 6.85	+4.07 ± 2.32	0.07
Fat, *% of energy*	37.27 ± 7.31	34.53 ± 7.70	-2.74 ± 2.65	0.27
Saturated fat, *% of energy*	13.95 ± 3.78	12.49 ± 3.81	-1.46 ± 1.32	0.21
Fibre, *g/d*	26.52 ± 8.40	43.26 ± 8.69	+16.74 ± 2.92	< 0.001
Sodium, *mg/d*	2714.25 ± 979.59	3229.12 ± 1405.28	+514.87 ± 424.95	0.32

[1]Values are presented as means ± SDs.

[2] Differences between nutrient intake during Mycoprotein compared to Meat presented as least square means ± SEs.

[3]*P* values were calculated for differences between study phases using mixed effects models. A $P < 0.05$ was considered significant.

Table 5.2. Effects of 2-wk dietary interventions on CVD risk markers[1]

Variable	Mean change from baseline, Meat	P^3	Mean change from baseline, Mycoprotein	P^3	Overall difference between Meat and Mycoprotein	P^3
Anthropometric parameters[2]						
Weight, *kg*	+0.26 ± 0.22	0.33	-0.17 ± 0.27	0.58	-0.43 ± 0.33	0.23
BMI, *kg/m²*	+0.09 ± 0.06	0.22	-0.04 ± 0.08	0.63	-0.13 ± 0.10	0.24
Body fat, *%*	+0.33 ± 0.25	0.42	+0.18 ± 0.39	0.82	-0.15 ± 0.48	0.76
Trunk fat, *%*	+0.62 ± 0.39	0.09	+0.41 ± 0.51	0.82	-0.21 ± 0.65	0.76
Waist circumference, *cm*	+0.80 ± 0.39	0.05	-0.15 ± 0.31	0.67	-0.95 ± 0.42	0.04†
Systolic blood pressure, *mmHg*	+1.70 ± 1.40	0.27	-2.41 ± 1.89	0.23	-4.11 ± 2.47	0.11
Diastolic blood pressure, *mmHg*	+1.77 ± 1.23	0.23	-0.80 ± 1.23	0.43	-2.57 ± 1.73	0.16
Blood parameters						
Total cholesterol, *mmol/L*	+0.04 ± 0.09	0.66	-0.33 ± 0.13	0.02*	-0.37 ± 0.14	0.02†
HDL Cholesterol, *mmol/L*	+0.05 ± 0.05	0.34	-0.04 ± 0.05	0.42	-0.09 ± 0.07	0.21
LDL Cholesterol, *mmol/L*	+0.02 ± 0.08	0.95	-0.32 ± 0.10	0.005*	-0.34 ± 0.13	0.01†
Triglycerides, *mmol/L*	-0.06 ± 0.08	0.42	+0.13 ± 0.09	0.09	+0.19 ± 0.11	0.09
Glucose, *mmol/L*	+0.13 ± 0.13	0.29	+0.15 ± 0.14	0.48	+0.02 ± 0.20	0.92

[1]Values are presented as least square means ± SEs. Differences between variables at the beginning and end of each diet are shown. The overall difference column shows the differences between variables at the end of Mycoprotein compared with the end of Meat. *P* values were calculated for changes within and differences between study phases using mixed effects models.
[2]For measures related to BMI (body weight, body fat, trunk fat, and BMI itself), BMI was excluded from the model to avoid collinearity.
[3]A $P < 0.05$ was considered significant.
*Mean change significantly different from baseline.
† Mean change significantly different between Mycoprotein and Meat dietary periods.

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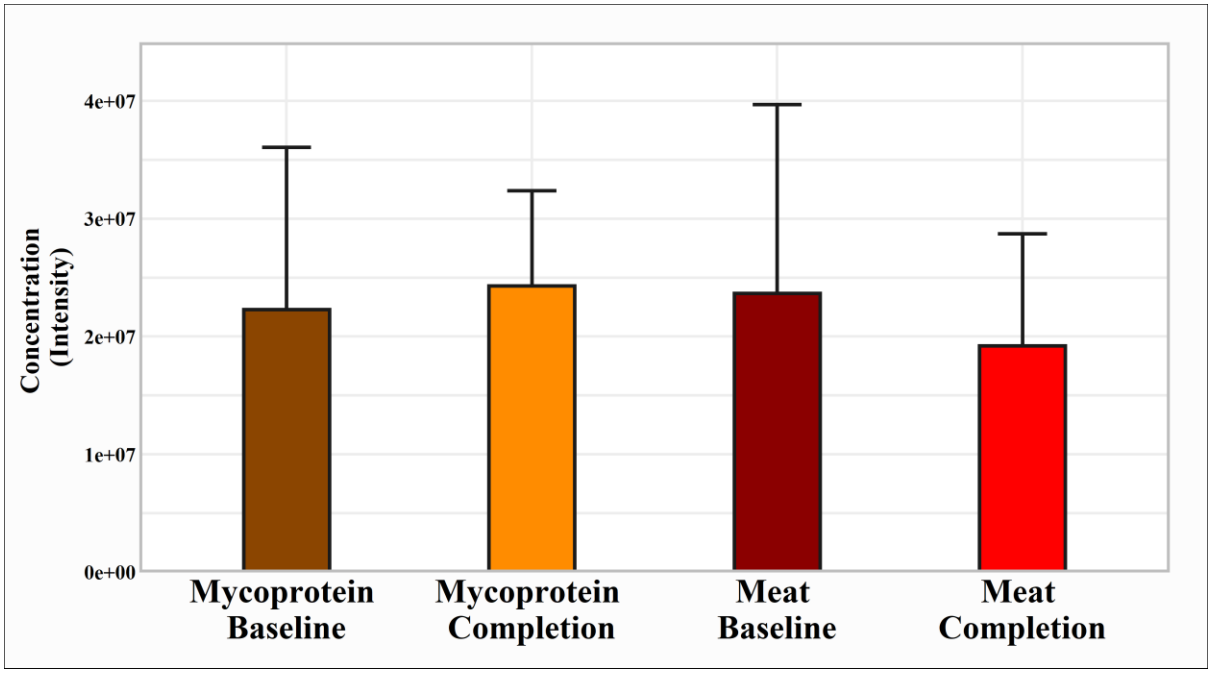
Figure 5.1. Effects of 2-wk dietary interventions on urinary TMAO.
Results presented as peak intensity in LC-MS analysis. Error bars represent standard deviations.



5.4.4. Urinary sodium and potassium

Urinary excretion of sodium increased from baseline after both Meat and Mycoprotein, neither reached significance (Meat, $P = 0.58$; Mycoprotein, $P = 0.25$), the difference in effects between study phases was also not significant ($P = 0.71$) (**Figure 5.2A.**). In contrast, potassium was reduced from baseline after Mycoprotein, not reaching statistical significance ($P = 0.32$), while it reduced significantly following Meat ($P = 0.007$), the difference between study phases also reached significance (P = 0.02) (**Figure 5.2B.**).

5.4.5. CVD risk

Table 5.3. presents the mean CVD risk at each study timepoint. Following Mycoprotein, CVD risk reduced from baseline by an average of 0.29% ($P = 0.19$) while there was a marginal increase of 0.05% in risk after Meat ($P = 0.99$). The difference in diet effects on CVD risk was not significant (difference, 0.34, $P = 0.24$).

Figure 5.2. Effects of 2-wk dietary interventions on A) urinary sodium. B) urinary potassium.
Results presented in mM. Error bars represent standard deviations. *Significant change from baseline; †Significant difference between study phase effects.

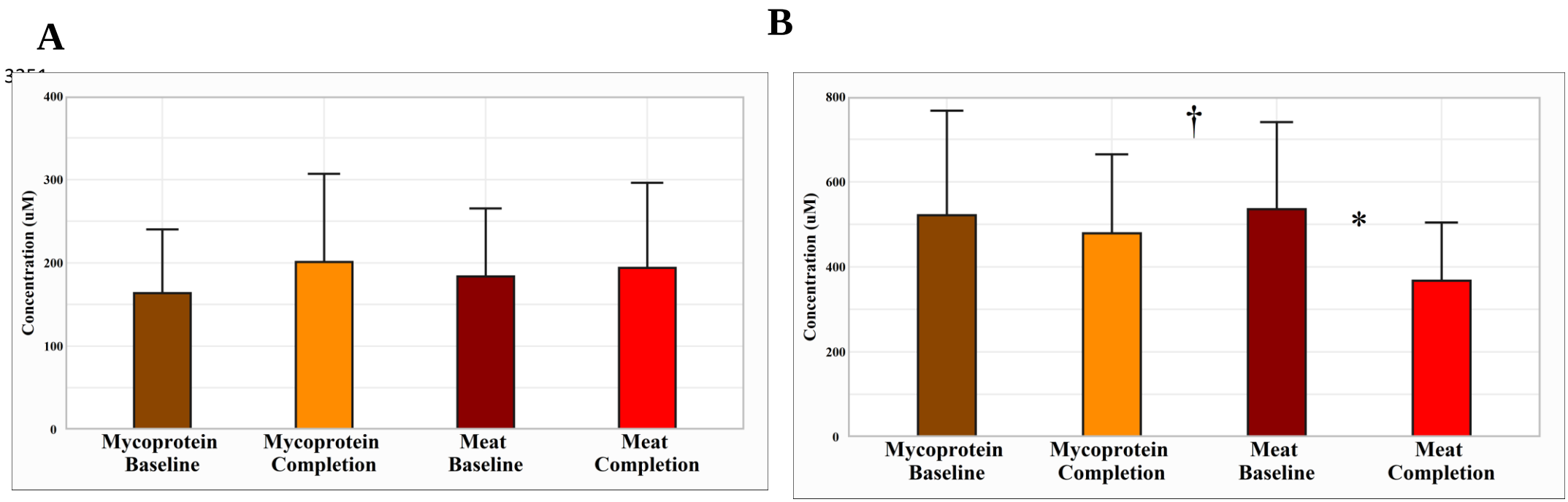


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Table 5.3. Effects of 2-wk dietary interventions on 10 year CVD risk[1,2]

	Mycoprotein baseline	Mycoprotein completion	Change	*P*[3]	Meat baseline	Meat completion	Change	*P*[3]	Difference in diet effects[4]	*P*[3]
CVD risk	2.03	1.74	-0.29	0.19	1.86	1.91	0.05	0.99	-0.34	0.24

[1]Results presented as %c CVD risk. The difference in diet effects column shows the Mycoprotein phase effects compared with at the Meat phase effects. *P* values were calculated using mixed effects models.

[2]CVD risk was calculated using the Framingham Risk Score (FRS) based on the following risk factors, age, gender, systolic blood pressure, smoking habit, total cholesterol and HDL cholesterol.

3 A $P < 0.05$ was considered significant.

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5.5. Discussion

I investigated the impact of replacing a two-week red and processed meat diet, with the weight equivalent of the meat alternative mycoprotein, on markers of CVD risk in metabolically healthy male adults. In this study, the level of meat intake that volunteers were asked to consume was high relative to the UK mean, but at a level that a minority of the population do consume, and at a level of intake that is associated, in epidemiological studies, with an increased risk of cardiometabolic disease (37). I observed that the mycoprotein diet led to a reduction total cholesterol and LDL fractions and reduced both systolic and diastolic pressure, such changes are deemed beneficial for long term cardio-metabolic health.

Elevated blood concentrations of both total cholesterol and LDL fractions are considered risk factors for compromised cardio-metabolic health (38, 39). Previous work has demonstrated that mycoprotein consumption over periods ranging from 1-8 weeks can reduce both total cholesterol and LDL concentrations (24-26). These examples differ from the present study, as they were either investigator-controlled diets (24, 26) or a free-living design where participants consumed mycoprotein in a cookie matrix (25). I have found that under free living conditions, consuming mycoprotein as part of a habitual diet, and in a wholefood matrix that is regularly consumed by global consumers, there were significant reductions in both total blood cholesterol and plasma LDL fractions.

Mycoprotein is high in fibre and increased total fibre intake from baseline and relative to meat during the intervention (**Table 5.1.**). Fibre has well established anti-hyperlipidaemic effects (24-26). The intakes of total and saturated fat were similar between study phases. Thus, fibre is the most plausible mechanistic link between the diets and the outcomes described. Mycoprotein is comprised of an interesting fibre composition of a β-glucan/chitin complex, with this β-glucan (β1-3, β1-6) structurally different to that found in oats and barley (β1-3, β1-4) (40, 41). β-glucan in oats is a well-established cholesterol lowering agent (42), the differential structure of mycoproteins fungal β-glucan may elicit different effects. Future work could disentangle this potential fibre type specific effect of mycoprotein by matching treatments/diets for fibre/ β-glucan content. Nevertheless, focussing on quantity, higher fibre intake resulting in blood cholesterol reductions agrees with clinical trials (43) evidencing lower blood lipids as fibre intake increases, translating to reduced risk of CVD as evidenced in the epidemiological data (44). It is evidence akin to this which is reasoning for dietary guidelines advising the UK population to intake 30g fibre daily (45); which many fail to attain (46). Our study provides evidence that a reasonable dietary substitution could help increase fibre intake leading to improvements in cardio-metabolic health.

Putative mechanisms for the reductions in blood cholesterol could be the capability of mycoprotein to sequester bile acids during digestion, reducing the reuptake, and necessitating free cholesterol to be

taken up into bile acid synthesis (47). Another potential mechanism may be related to gut bacterial fermentation of mycoprotein's fibre matrix in the large intestine, resulting in the production of short-chain fatty acids (SCFAs), including acetate, propionate and butyrate (48). SCFAs are credited with an array of roles in human physiology, and many health effects elicited by the gut microbiota can be attributed to these metabolic by-products (49). In the context of our findings, SCFAs (propionate in particular) have been evidenced to be potent inhibitors of hepatic cholesterol synthesis (50). To substantiate this, mycoprotein has been evidenced to be fermented into the SCFA butyrate and propionate *in vitro* (51).

It is important to stress that our cohort included metabolically healthy participants. The influence elicited by the mycoprotein diet is indicative of the capability to confer significant health benefits in the general population, especially at-risk groups. Future investigations should consider recruiting high-risk cohorts (i.e., elevated blood cholesterol, LDLR/ApoB polymorphisms) to assess whether mycoprotein consumption could have similar effects as the present study. Research seeks to find facets to improve health without dependency on pharmacological interventions, if comparable results to our study are reciprocated in high-risk cohorts, there may be a role for mycoprotein beyond a sustainable culinary meat alternative.

To our knowledge, the effects of mycoprotein on BP have not been investigated before. I report reductions in both systolic and diastolic BP following mycoprotein consumption, these reductions did not reach statistical significance, however an average 2.41mmHg reduction in systolic BP within a 2 week timeframe in healthy adults is indicative of a positive impact. Further, when comparing to an average 1.70mmHg increase following the meat diet, this equates to an average difference of 4.11mmHg over the 2 weeks. I return to mycoproteins fibre as the putative mechanism, as fibre has been attributed with a BP lowering effect (52). BP is regarded as one of the most important risk factors for CVD (53), therefore future research could consider an intervention dedicated to exploring mycoproteins BP effects.

Our study found that mycoprotein did not elicit different effects on blood glucose compared with red and processed meat, with slight increases following both 2 week interventions (+0.13 ± 0.13mmol/L after Meat vs +0.15 ± 0.14mmol/L after Mycoprotein). Our findings agree with a previous study which observed insulin sensitivity and glucose were not altered significantly by chronic mycoprotein consumption over 1 week (24). Earlier work found a reduction in both glycemia and insulinemia by mycoprotein when taken in powder form with an oral-glucose-tolerance test (54). This was developed with a single meal design where mycoprotein improved insulin sensitivity but did not influence blood glucose compared to chicken in an overweight and obese cohort (55). Mechanistic work has demonstrated the fungal cell wall of mycoprotein can entrap α-amylase and thereby reduce starch hydrolysis (56). It could be that a slower rate of digestion and a lower bioaccessibility of glycogen could play a role in a reduced insulin response without altering glucose concentrations. I stress that glucose

homeostasis was not a primary endpoint in our intervention, further work is warranted to determine whether this previous evidence of improvements in glycaemia and insulinemia by mycoprotein can be translated into chronic consumption of mycoprotein as wholefoods, potentially in a compromised cohort.

Following the meat diet, I found a significant increase in waist circumference compared to the mycoprotein diet. This was unexpected, given the characteristics of our cohort and short duration of the intervention period. In addition to the increase in waist circumference, there were also non-significant increases in other anthropometric measures (weight, BMI, body fat). These effects were evidenced during a two-week timeframe in a metabolically healthy cohort; chronic consumption of such a dietary pattern over a sustained period could result in the development obesity and increased risk of CVD (57). Increased waist circumference itself is considered a risk factor for CVD (58). Our findings highlight dietary patterns consisting of large quantities of red and processed meat may contribute to compromised cardio-metabolic health and increased risk of CVD.

TMAO has previously been found to predict cardiovascular mortality (59), and urinary and blood levels increase when consuming higher quantities of meat (14). Although there is debate surrounding the significance to CVD, as TMAO has also been found to be a marker of fish consumption (60). Unlike meat, fish consumption has not been evidenced to carry risk for CVD (5), and may even confer protection in high-risk populations (61). In the present study, urinary levels of TMAO reduced following the high meat diet, whereas they increased after the mycoprotein diet. The reasons for our findings are unclear.

The Framingham Risk Score (FRS) is a well-known risk profile algorithm to assess CVD risk (62), and is regularly implemented in research (63, 64). The mycoprotein diet resulted in an average reduction of 0.29% in CVD risk based on FRS. While this is a marginal reduction, it does clarify that based on our findings, mycoprotein consumption elicited a positive influence on risk markers of CVD.

A noteworthy finding in our study was that self-reported energy intake, and subsequently, overall nutrient intake, was greater during the mycoprotein study phase. Mycoprotein has been hypothesised and researched for its potential positive influence in increasing satiety and reducing energy intake (65, 66). However, although our study is an isolated case, I did not find this to be the case.

Sodium intake increased from baseline during the mycoprotein diet, which was expected to a degree, meat alternatives such as Quorn can sometimes be high in sodium (67), and although I included processed meat within the meat arm of the study, the Quorn products on average, were still marginally higher in sodium (**Table 5.1ii.**). The higher sodium intake was reciprocated in urinary sodium excretion, which also increased from baseline on the mycoprotein diet compared to the meat diet. Interestingly, although increased sodium intake and excretion is associated with increased BP and CVD risk (68), as mentioned, BP was reduced following the mycoprotein diet. This is arguably an area of research which

warrants further research within a study design dedicated to the interplay between sodium intake, excretion and BP.

In contrast, potassium excretion was reduced from baseline following both diets, with a significant reduction after the meat diet. While I didn't collect extensive dietary intake data during study phases, it could be postulated this is indicative of the interventions displacing fruit and vegetable intake, which correlate with urinary potassium (69, 70).

The strengths of our study include the use of a randomised crossover design; a crossover methodology brings benefits as each participant acts their own control, as opposed to a parallel design, where different groups characteristics require careful matching and greater participant numbers to achieve a similar degree of statistical validity. Our study was also investigator blinded, removing any form of bias from the results. Another strength of the study was that the meat and mycoprotein food products included are regularly consumed in the general public; this brings greater relevance of the study results to a real life context. The LC-MS platform used to identify TMAO is a highly efficient metabolite profiling system in comparison to alternative platforms with the ability to obtain a greater number of identified metabolites with distinguishable fragmentations, which in turn, enabled the sensitive quantification of TMAO.

Our study also had limitations, our cohort only included males as this study is novel in its nature and I aimed to avoid introducing a variety of external factors, our findings may be more robust and applicable to the general UK population if also tested in a mixed gender cohort. As well as this, although participants were given guidelines to avoid certain foods and supplements, I did not control dietary intake other than supply the study products, other dietary factors during the intervention periods may have influenced our findings. Our study duration of 2 weeks was also arguably too short in duration to see major changes in body weight, I did observe a small reduction in body weight after the mycoprotein diet, despite the fact participants consumed more total energy. This is not so much a weakness, rather a question that deserves further research, what would be the impact of a longer-term consumption pattern of mycoprotein on body weight?

The current study provides evidence that a diet high in red and processed meat can be adequately substituted with mycoprotein, a widely available meat alternative, and this substitution improves biomarkers pertaining to cardiometabolic health.

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CHAPTER VI

EXPERIMENTAL CHAPTER III

Metabolomic and microbial community predictors of genotoxicity and cardiovascular disease risk markers; a secondary interrogation of Mycomeat study data.

Abstract

The gut microbiome and metabolome can serve as both indicators of health status, but also disease predictors. The present work leveraged metagenomic and metabolomic datasets obtained from a randomised crossover dietary intervention trial comparing a high red and processed meat diet, with a weight equivalent mycoprotein diet. These datasets were interrogated to determine associations with the intervention primary and secondary endpoints, faecal genotoxicity and cardiovascular risk markers respectively. In addition, correlations between the gut microbiome and metabolome were assessed.

Analysis found putrefactive, bile acid tolerant bacterial genera to be predictors of genotoxicity which were also increased following the meat diet in the intervention (*Alistipes* and *Oscillibacter*), while *Clostridium XIVa*, a cluster in which butyrate producing species belong, was a negative predictor. Acylcarnitines were associated with negative effects on cardiovascular disease risk markers, while *Roseburia* with positive influences. Correlating the microbiome with the metabolome, *Prevotella* was inversely correlated with putative meat consumption markers in stool, while *Bacteroides* was positively correlated.

Findings from this analysis corroborate with pre-existing data associating the gut microbial community with diet, and the implications for both gut and cardiometabolic health. The analysis poses avenues for future research including the interplay between the gut microbiota, mycoprotein digestion and cardiometabolic health, the relevance of putative animal meat dietary metabolites to colorectal cancer risk, as well as the establishment of novel faecal genotoxins.

6.1. Introduction

The human gut contains a complex microbial ecosystem made up of trillions of microbes referred to as the gut microbiota. Shifts in the composition of this gut microbial community can have major impacts on host health (1-3). However, while compositional shifts undoubtably elicit effects in health and disease, the metabolic activity of the microbiome is perhaps a more important and clinically relevant factor. This is highlighted by the capability of microbial metabolites to shape host phenotype (4). Further, the metabolic potential of the gut microbiome may differ between individuals, resulting in different and perhaps personalised metabolic responses to diet (5, 6).

The microbiome and metabolome are being investigated as independent and co-dependent predictive tools in understanding disease risk, including metabolic syndrome (7, 8), specific health perturbations such as T2D (9), Polycystic Ovary Syndrome (10), as well as cancer (11). In the context of the present thesis, this includes predictors for the early detection of (12), and the early onset of colorectal cancer (13), in addition to predictors of adenoma reoccurrence (14).
The ability of the microbiome and metabolome to predict disease may in future allow for personalised interventions to mitigate risk. An intriguing aspect is that both the microbiome and metabolome are malleable by diet (15, 16), postulating the concept that lifestyle-based interventions could aid to offset disease risk.

Previous chapters have presented the primary and secondary end points from a randomised crossover trial which investigated the effect of consuming a high mycoprotein diet (240g/day) in place of a weight equivalent high red and processed meat diet.
In the present chapter I leverage data obtained from that intervention and explore *a posteriori* questions about the interconnectivity of the microbiome and the metabolome with the gut and cardiovascular disease related endpoints of the intervention, as well as relationships between the microbiome and metabolome.

The aim of this chapter is to further interrogate associations between datasets. If strong metabolomic or microbial community predictors of genotoxicity and/ or CVD risk markers can be identified, there may be opportunities to change how I approach future studies, and further, it may pose new research questions on the role of gut microbial activity in disease.

6.2. Methods

Sample collection and metagenomic and metabolomic data processing have been described in detail in the preceding chapters on the primary and secondary end points (Experimental Chapters I & II). For the following analyses, the metabolites identified as VIP >1.5 between the diets were included from the

metabolomics datasets, while analyses were performed using the microbiome sequencing data at the taxonomic level of genus.

6.2.1. Metabolic and microbial predictors of genotoxicity and CVD risk

To identify faecal and urinary metabolic and microbial predictors of genotoxicity and CVD risk markers, linear regressions were implemented to find estimates for the strength of associations. Potential genotoxins, identified in stool or urine from the human intervention samples, were also included as predictor variables against genotoxicity to determine if any relationships existed. These genotoxins were faecal nitroso compounds (NOCs), the faecal secondary bile acids apocholic acid and 7-ketdeoxycholic acid, and urinary p-cresol sulphate in urine. The association between these potential genotoxins with genotoxicity was also assessed by linear regression.

Multivariate predictor analysis: Independent principal components analysis with orthogonal rotation were performed on both the faecal and urinary metabolomics datasets, as well as the gut microbiota metagenomic dataset. 8 Principal components (PC) were retained from both the faecal and urinary datasets, in addition with 3 PC from the metagenomic dataset, based on the inflection points of scree plots. The retained PC were then included as predictor variables in independent linear regressions with genotoxicity and CVD risk markers as outcome variables. The analysis incorporated a P-value threshold of <0.05 to define significance.

6.2.2. Correlation between microbiome and metabolome

To interrogate the metagenomic and metabolomic datasets and identify correlations between metabolites and microbial genera, a sparse partial least squares regression (sPLS) was performed in canonical mode. To visualise the sPLS results, cluster image heat maps were acquired, and correlation estimates obtained. The sPLS and visualisations were performed using the mixOmics R package (17).

6.3. Results

6.3.1. Genotoxic candidates as predictors of faecal genotoxicity

The present analyses did not find any significant relationships between pre-described genotoxins; faecal NOCs and bile acids, as well as urinary p-cresol sulphate. There was a weak negative association between urinary p-cresol sulphate and faecal genotoxicity ($r = -0.19$, $P = 0.08$). Faecal NOCs ($r = -0.091$, $P = 0.48$) and the bile acids apocholic acid ($r = 0.11$, $P = 0.33$) and 7-ketodeoxycholic acid ($r = 0.10$, $P = 0.38$) were not good predictors of faecal genotoxicity (**Figure 6.1.**).

6.3.2. Metabolic predictors of genotoxicity

Significant estimates for the relationship between faecal metabolites and genotoxicity are included in **Table 6.1**. Both allysine (r = 0.50, *P* = 0.03) and P-DMEA (r = 0.35, *P* = 0.03) were statistically significant predictors of genotoxicity, while none of the metabolites were inversely associated with genotoxicity. Regression analyses identified no significant predictors of genotoxicity from the urinary metabolite dataset.

In the multivariate analysis, PC7 in stool was a weak, but statistically significant, predictor of higher genotoxicity (r = 0.30, *P* = 0.03). While in urine, a weak positive estimate was found for PC7 (r = 0.31, *P* = 0.05), whereas PC5 (r = -0.21, *P* = 0.04) and PC6 (r = -0.31, *P* = 0.009) were negatively associated (**Table 6.2.**). Loading plots of the retained PC are included in **Figure 6.2.** showing the metabolites with the greatest contributions towards the PC. With respect to the significant correlations with genotoxicity, the greatest contributor to PC7 in stool was DL-leucine. In urine, an unknown metabolite with the formula C20 H42 O11 was the largest contributor to both PC5 and 6, with acetylcadaverine a large contributor in PC5 and acetyl- β-methylcholine in PC6, while prolylleucine was the metabolite which contributed the greatest to PC7.

6.3.3. Microbial predictors of genotoxicity

Estimates for the relationship between microbial genera and genotoxicity identified as significant are included in **Table 6.3.**. The analysis identified *Alistipes* (r = 1.33, *P* = 0.04), *Oscillibacter* (r = 12.06, *P* = <0.001) and *Faecalibacterium* (r = 1.36, *P* = <0.001) as positive predictors of genotoxicity, while *Clostridium XlVa* (r = -5.06, *P* = 0.008) as a negative predictor.

In the multivariate analysis, both PC2 (r = 0.44, *P* = 0.03) and PC3 (r = 0.76, *P* = 0.03) were statistically significant predictors of genotoxicity (**Table 6.4.**). Loading plots of the retained PC are shown in **Figure 6.3.** that show the microbial genera with the greatest contributions towards the PC. *Prevotella* was the greatest contributor to PC2, with *Bacteroides* the next greatest, while an unclassified genus of the family *Ruminococcaceae* was the largest contributor to PC3, with *Bacteroides* again the next largest.

Figure 6.1. Relationship between potential genotoxins and genotoxicity.
A) Faecal nitroso compounds. **B)** Urinary p-cresol sulphate. **C)** Faecal apocholic acid. **D)** Faecal 7-ketodeoxycholic acid.

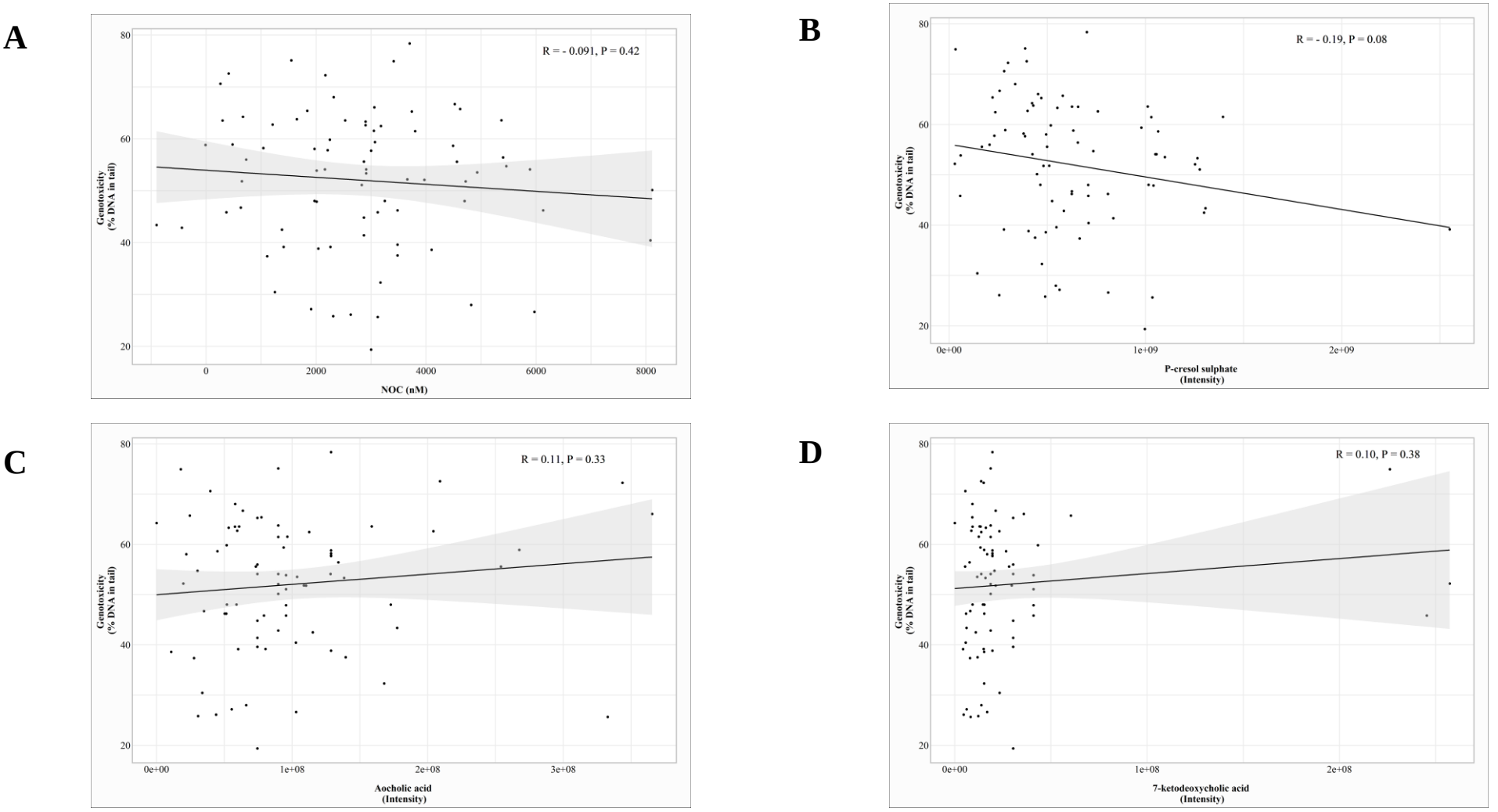


Table 6.1. Significant relationships between faecal metabolites and genotoxicity[1].

Metabolite	Estimate	P[2]
Allysine	0.50	0.03
P-DMEA	0.35	0.03

[1]Estimates obtained by implementing linear regressions.

[2]P-values <0.05 were considered significant.

Table 6.2. Significant relationships between metabolic principal components (PC) and genotoxicity[1].

	Stool	
PC	**Estimate**	**P[2]**
PC7	0.30	0.03
	Urine	
PC	**Estimate**	**P[2]**
PC5	-0.21	0.04
PC6	-0.31	0.009
PC7	0.31	0.05

[1]Estimates obtained by implementing linear regressions.

[2]P-values <0.05 were considered significant.

Table 6.3. Significant correlations between microbial genera and genotoxicity[1].

Genus	Estimate	P[2]
Alistipes	1.33	0.04
Clostridium XlVa	-5.06	0.008
Faecalibacterium	1.36	<0.001
Oscillibacter	12.06	<0.001

[1]Estimates obtained by implementing linear regressions.

[2]P-values <0.05 were considered significant.

Table 6.4. Significant relationships between microbial principal components (PC) and genotoxicity[1].

PC	Estimate	P[2]
PC2	0.44	0.03
PC3	0.76	0.03

[1]Estimates obtained by implementing linear regressions.

[2]P-values <0.05 were considered significant.

Figure 6.2. Loading plots of retained metabolic principal components (PC).

A) In stool samples. PC retained from visualising scree plots.

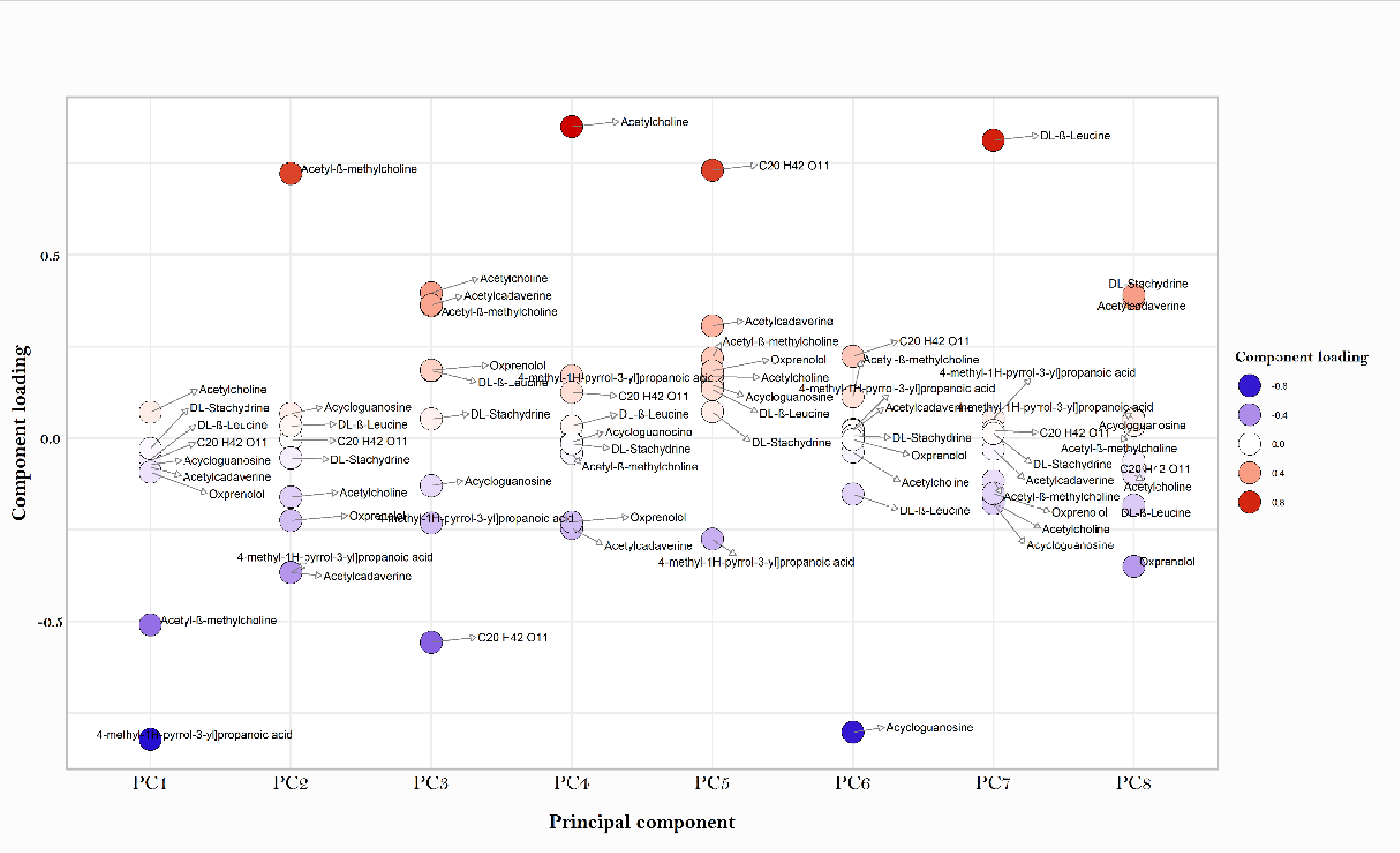
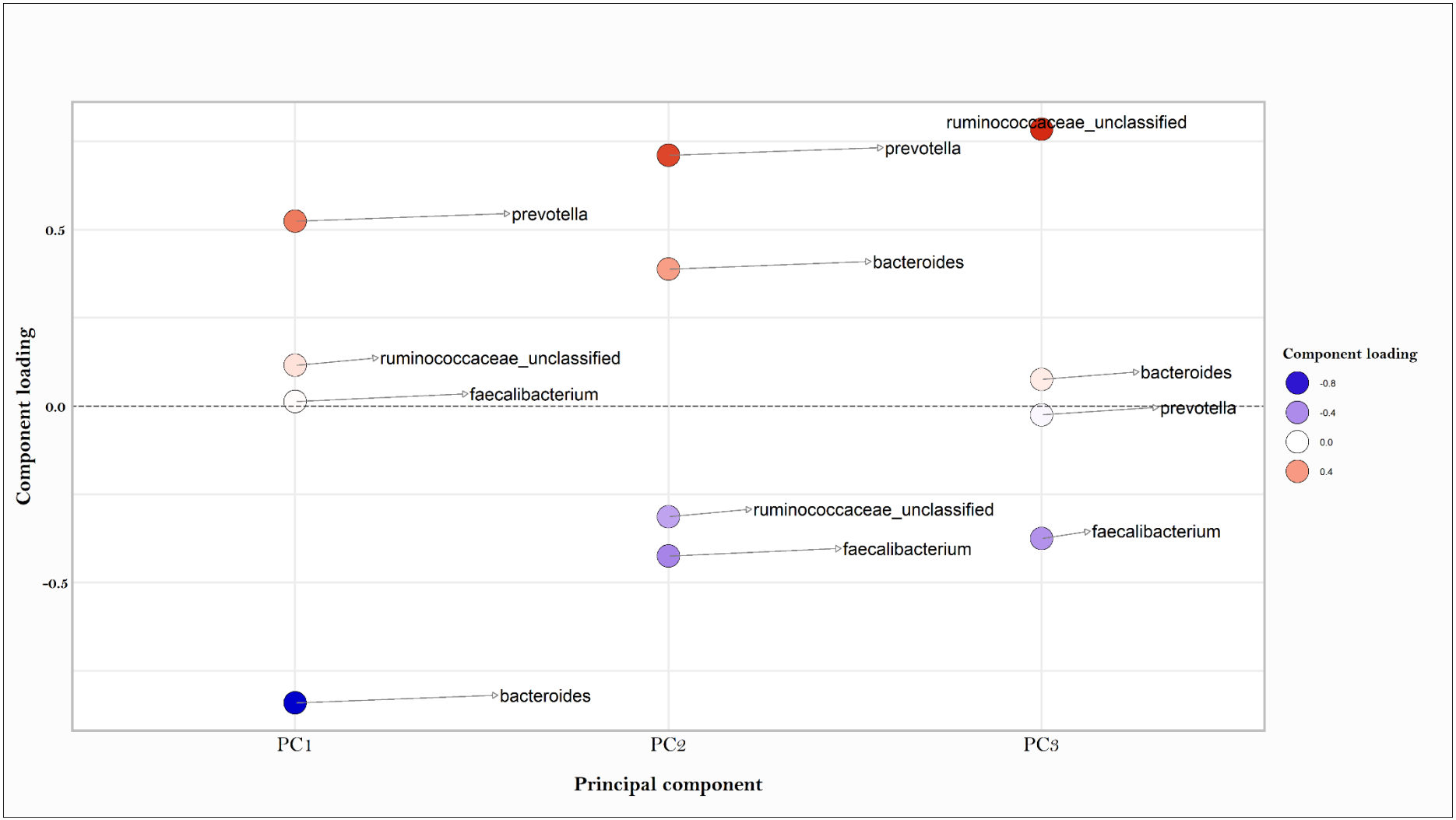


Figure 6.2. Loading plots of retained metabolic principal components (PC).
B) In urine samples. PC retained from visualising scree plots.

Figure 6.3. Loading plots of retained microbial principal components (PC).
PC retained from visualising scree plots.



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6.3.4. Metabolic predictors of CVD risk markers

Estimates for the relationship between faecal and urinary metabolites and CVD risk markers identified as significant are included in **Table 6.5**. and **6.6.**

6.3.4.1. Associations between faecal metabolites and CVD risk markers

Analysis of faecal metabolites defined fusicoccin H as a predictor of both lower SBP (r = -0.66, *P* = 0.04) and DBP (r = -0.63, *P* = 0.04), whereas 4-hydroxyprolylleucine a predictor of greater DBP (r = 0.20, *P* = 0.04). Gamma-aminobutyric-acid (GABA) (r = -0.20, *P* = 0.05), N-[(2Z)-2-Hydroxy-2-octenoyl]glycine (r = -0.80, *P* = 0.05) and sorbic acid (r = -0.62, *P* = 0.05) were all predictors of reduced total cholesterol, ISOXYL reduced HDL (r = -0.55, *P* = 0.03), and butenylcarnitine (r = -0.49, *P* = 0.01), GABA (r = -0.24, *P* = 0.02), N-[(2Z)-2-Hydroxy-2-octenoyl]glycine (r = -0.90, *P* = 0.02) and sorbic acid (r = -0.73, *P* = 0.02) reduced LDL.

6.3.4.2. Associations between urinary metabolites and CVD risk markers

Within the urinary metabolites, N-acetylleucylleucine (r = -0.51, *P* = 0.04) was determined to be a predictor of lower DBP, 3-methylglutarylcarnitine (r = -0.33, *P* = 0.04), mevalonic acid (r = -0.16, *P* = 0.05) and N-[(2Z)-2-Hydroxy-2-octenoyl]glycine (r = -0.75, *P* = 0.02), predictors of lower total cholesterol, whereas acetyl-ß-methylcholine (r = 0.05, *P* = 0.004), l-histidinal (r = 0.90, *P* = 0.03), miglitol (r = 0.40, *P* = 0.04), propionylcarnitine (r = 0.10, *P* = 0.04) and TDIQ (r = 0.54, *P* = 0.02) were predictors of increased cholesterol. Balenine was defined as a predictor of lower HDL (r = -0.57, *P* = 0.05), (3S6S)-3-(Hydroxymethyl)-6-{4-[(3-methyl-2-buten-1-yl)oxy]benzyl-25-piperazinedione (r = 0.13, *P* = 0.04), acetyl-ß-methylcholine (r = 0.05, *P* = 0.003), acetylcholine (r = 0.18, *P* = 0.02), miglitol (r = 0.46, *P* = 0.02), propionylcarnitine (r = 0.12, *P* = 0.01) and TDIQ, (r = 0.58, *P* = 0.01) predictors of higher LDL, while N-[(2Z)-2-Hydroxy-2-octenoyl]glycine was a predictor of reduced LDL (r = -0.72, *P* = 0.03).

6.3.4.3. Associations between identified principal components in faecal metabolites and CVD risk markers

In stool, PC8 was a correlate of lower weight (r = -0.47, *P* = 0.001), BMI (r = -0.13, *P* < 0.001), waist circumference (r = -0.28, *P* = 0.007) and HDL (r = -0.01,*P* = 0.02).

6.3.4.4. Associations between identified principal components in urinary metabolites and CVD risk markers

When regressing PC scores in urine, PC5 was correlated with both higher weight (r = 0.18, *P* = 0.03) and waist circumference (r = 0.14, *P* = 0.02), PC7 with lower SBP (r = -0.29, *P* = 0.02), while PC2 was associated with higher total cholesterol (r = 0.01, *P* = 0.03) and LDL cholesterol (r = 0.01, *P* = 0.005). When reviewing the metabolites which contributed greatest to the PCs, DL-stachydrine and

acetylcadaverine were the largest contributors to PC8 in stool, while in urine, D-carnitine was greatest in PC2. In PC5, an unknown metabolite with the chemical formula C12 H33 N7 O7, and to a lesser extent N-phenylacetylglutamine were large contributors, and as already mentioned, in PC7, prolylleucine contributed the greatest (**Figure 6.2.**).

Table 6.7. includes the significant estimates between the PC in stool and urine with CVD risk markers.

6.3.5. Microbial predictors of CVD risk markers

Estimates for the relationship between microbial genera and CVD risk markers identified as significant are included in **Table 6.8**. Analysis revealed both *Succinivibrio* (r = -233.27, *P* = <0.001) and *Sutterella* (r = -1.72, *P* = 0.03) as predictors of lower SBP. *Succinivibrio* was also predictor of lower DBP (r = -153.33, *P* = 0.01), along with *Clostridium cluster XlVa* (r = -3.29, *P* = 0.02) and an unclassified genus of *Betaproteobacteria* (r = 0.42, *P* = <0.001) and *Sutterella* (r = 0.06, *P* = 0.05) were predictors of higher TAG. Regressions determined that the following genera were predictors of lower blood markers; *Succinivibrio* of total cholesterol (r = -10.88, *P* = 0.02), *Clostridium cluster XlVa* (r = -0.15, *P* = 0.04) and *Sutterella* of HDL (r = -0.06, *P* = 0.03), and *Roseburia* of LDL (r = -0.11, *P* = 0.04).

Regression of the retained microbial PC found no significant relationships with CVD risk markers.

Table 6.5. Significant correlations between faecal metabolites and CVD risk markers[1].

Weight		
Metabolite	**Estimate**	**P[2]**
4-Hydroxyprolylleucine	0.24	0.02
Homocarnosine	-0.24	0.03
L(+)-Ornithine	-0.15	0.002
leucyl-4-hydroxyproline	-0.17	0.002
BMI		
Metabolite	**Estimate**	**P[2]**
4-Hydroxyprolylleucine	0.69	0.01
L(+)-Ornithine	-0.41	0.002
leucyl-4-hydroxyproline	-0.47	0.002
P-DMEA	0.86	0.01
Waist		
Metabolite	**Estimate**	**P[2]**
Homocarnosine	-0.16	0.04
L(+)-Ornithine	-0.12	0.001
leucyl-4-hydroxyproline	-0.13	0.001
porfiromycin	0.50	0.02
SBP		
Metabolite	**Estimate**	**P[2]**
Fusicoccin H	-0.66	0.04

DBP

Metabolite	Estimate	P[2]
4-Hydroxyprolylleucine	0.20	0.04
Fusicoccin H	-0.63	0.04

Total Cholesterol

Metabolite	Estimate	P[2]
Gamma-Aminobutyric-acid (GABA)	-0.20	0.05
N-[(2Z)-2-Hydroxy-2-octenoyl]glycine	-0.80	0.05
Sorbic acid	-0.62	0.05

HDL

Metabolite	Estimate	P[2]
ISOXYL	-0.55	0.03

LDL

Metabolite	Estimate	P[2]
Butenylcarnitine	-0.49	0.01
GABA	-0.24	0.02
N-[(2Z)-2-Hydroxy-2-octenoyl]glycine	-0.90	0.02
Sorbic acid	-0.73	0.02

[1]Estimates obtained by implementing linear regressions.

[2]P-values <0.05 were considered significant.

Table 6.6. Significant correlations between urine metabolites and CVD risk markers[1].

BMI

Metabolite	Estimate	P[2]
(5E)-7-methylidene-10-oxo-4-(propan-2-yl)undec-5-enoic acid	-0.92	0.02
2-(610-Dimethyl-8-oxospiro[4.5]dec-6-en-2-yl)-2-hydroxypropyl hexopyranoside	-0.73	0.02
5-(2	-0.32	0.02
Arbusculin A	-0.47	0.02
gamma-thiomethyl glutamate	-0.31	0.009
N-Acetyl-L-cysteine	-0.87	0.02

Waist

Metabolite	Estimate	P[2]
5-Methylangelicin	-0.32	0.03
Acronycidine	-0.12	0.04
Arbusculin A	-0.14	0.01
Methoxyresorcinol	-0.99	0.05
val-his	0.66	0.02

DBP

Metabolite	Estimate	P[2]

N-Acetylleucylleucine	-0.51	0.04

Cholesterol

Metabolite	Estimate	P
3-Methylglutarylcarnitine	-0.33	0.04
Acetyl-ß-methylcholine	0.05	0.004
l-Histidinal	0.90	0.03
Mevalonic acid	-0.16	0.05
Miglitol	0.40	0.04
N-[(2Z)-2-Hydroxy-2-octenoyl]glycine	-0.75	0.02
Propionylcarnitine	0.10	0.04
TDIQ	0.54	0.02

HDL

Metabolite	Estimate	P^2
Balenine	-0.57	0.05

LDL

Metabolite	Estimate	P^2
(3S6S)-3-(Hydroxymethyl)-6-{4-[(3-methyl-2-buten-1-yl)oxy]benzyl-25-piperazinedione	0.13	0.04
Acetyl-ß-methylcholine	0.05	0.003

Acetylcholine	0.18	0.02
Miglitol	0.46	0.02
N-[(2Z)-2-Hydroxy-2-octenoyl]glycine	-0.72	0.03
Propionylcarnitine	0.12	0.01
TDIQ	0.58	0.01

[1]Estimates obtained by implementing linear regressions.

[2]P-values <0.05 were considered significant.

Table 6.7. Significant correlations between metabolic principal components (PC) and CVD risk markers[1].

Stool		
Weight		
PC	**Estimate**	**P[2]**
PC8	-0.47	0.001
BMI		
PC	**Estimate**	**P[2]**
PC8	-0.13	< 0.001
Waist		
PC	**Estimate**	**P[2]**
PC8	-0.28	0.007
Glucose		
PC	**Estimate**	**P[2]**
PC7	-0.01	0.04
Triglycerides		
PC	**Estimate**	**P[2]**
PC2	0.03	0.04
HDL		
PC	**Estimate**	**P[2]**
PC8	-0.01	0.02
Urine		
Weight		
PC	**Estimate**	**P[2]**
PC5	0.18	0.03

Waist		
PC	**Estimate**	**P[2]**
PC5	0.14	0.02
SBP		
PC	**Estimate**	**P[2]**
PC7	-0.29	0.02
Total Cholesterol		
PC	**Estimate**	**P[2]**
PC2	0.01	0.03
LDL		
PC	**Estimate**	**P[2]**
PC2	0.01	0.005

[1]Estimates obtained by implementing linear regressions.

[2]P-values <0.05 were considered significant.

Table 6.8. Significant correlations between microbial genera and CVD risk markers[1].

BMI		
Genus	**Estimate**	**P[2]**
Succinivibrio	34.60	0.05
Waist		
Genus	**Estimate**	**P[2]**
Dialister	-11.43	0.02
SBP		
Genus	**Estimate**	**P[2]**
Succinivibrio	-233.27	<0.001
Sutterella	-1.72	0.03
DBP		
Genus	**Estimate**	**P[2]**
Clostridium cluster XlVa	-3.29	0.02
Succinivibrio	-153.33	0.01
TAG		
Genus	**Estimate**	**P[2]**
Betaproteobacteria (unclassified)	0.42	<0.001
Sutterella	0.06	0.05

Cholesterol

Genus	Estimate	P[2]
Succinivibrio	-10.88	0.02

HDL

Genus	Estimate	P[2]
Clostridium cluster XlVa	-0.12	0.04
Sutterella	-0.06	0.03

LDL

Genus	Estimate	P[2]
Roseburia	-0.11	0.04

[1]Estimates obtained by implementing linear regressions.

[2]P-values <0.05 were considered significant.

6.3.6. Correlation between microbiome and metabolome

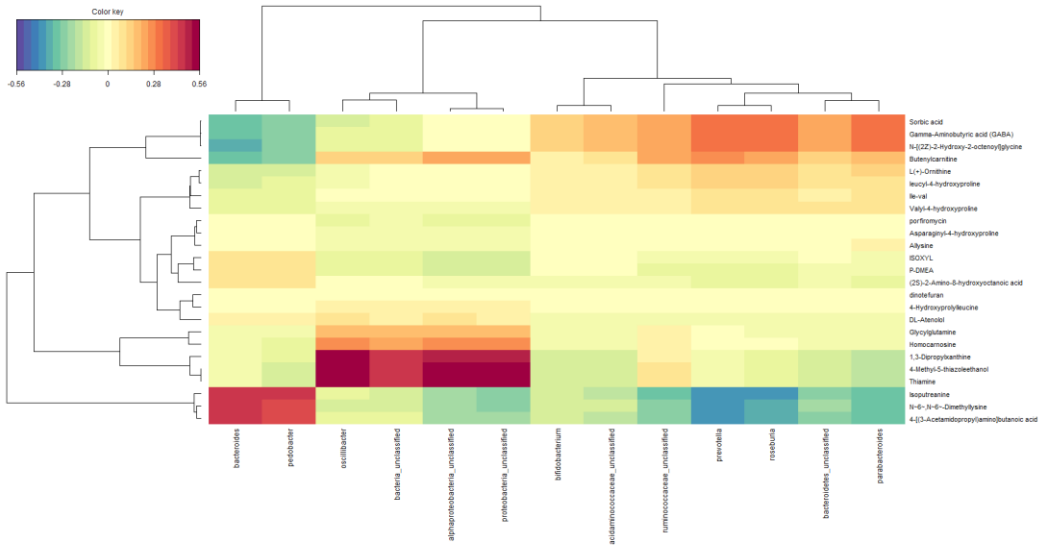
Correlation coefficients of significance between microbial genera and faecal and urinary metabolites are presented in **Table 1** and **2** of **Appendix B,** respectively, and cluster image heatmaps are displayed in **Figure 6.4**.

Within the faecal metabolome and gut microbiota, there were negative correlations between isoputreanine and the bacterial genera *Roseburia* (-0.279) and *Prevotella* (-0.276), while *Bacteroides* was positively correlated (0.296). *Roseburia* and *Prevotella* were positively associated with sorbic acid (*Roseburia*, 0.273; *Prevotella*, 0.229), GABA (*Roseburia*, 0.273; *Prevotella*, 0.273) and N-[(2Z)-2-Hydroxy-2-octenoyl]glycine (*Roseburia*, 0.276; *Prevotella*, 0.232), whereas *Bacteroides* was negatively associated with these metabolites (sorbic acid, -0.230; GABA, -2.30; N-[(2Z)-2-Hydroxy-2-octenoyl]glycine, -2.33). *Oscillibacter* was positively associated with thiamine (0.439) 1,3-dipropylxanthine (0.417) and 4-methyl-5-thiazoleethanol (0.439).

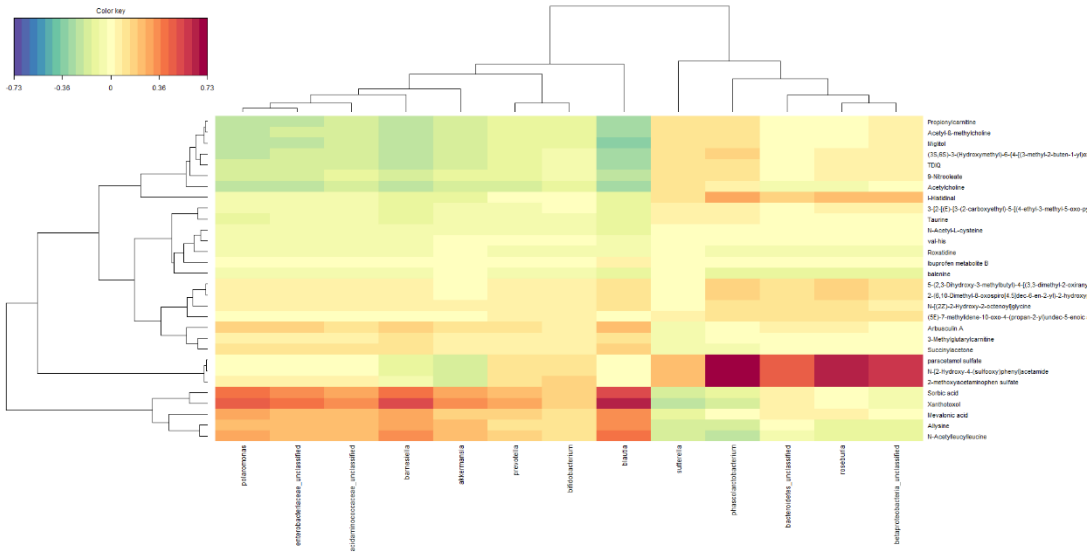
From the sPLS of the urinary metabolome and gut microbiota, *Blautia* was identified to be negatively correlated with propionylcarnitine (-0.326), miglitol (-0.325), acetyl-ß-methylcholine (-0.321) and acetylcholine (-0.327), while *Blautia* was positively associated with sorbic acid (0.489) and xanthotoxol (0.560). *Roseburia* and *Phascolarctobacterium* were positively correlated with paracetamol sulfate (*Roseburia*, 0.548; *Phascolarctobacterium*, 0.627) and 2-methoxyacetaminophen sulfate (*Roseburia*, 0.552; *Phascolarctobacterium*, 0.632).

Figure 6.4. Associations between microbiome and metabolome.
Clustered image heat maps of associations between **A)** faecal metabolites and microbiota, **B)** urinary metabolites and microbiota. Tested using a sparse partial least squares regression.

A



B



6.4. Discussion

This was a secondary analysis of data obtained from a human dietary intervention described in the previous experimental thesis chapters. These analyses were not set *a priori* to the intervention, however important questions have been asked of the breadth of the intervention data; notably, 1/ can faecal genotoxicity be predicted based on an interrogation of the faecal metabolome, and/or the microbial community structure. 2/ are there relationships between an individual's metabolome and their microbial community structure. 3/ can cardiovascular disease risk be predicted based on the metabolome of the microbial community structure of otherwise healthy individuals.

In the faecal genotoxicity chapter, several well-established intestinal genotoxins were identified, some of which, in particular NOCs and p-cresol, were differentially attenuated in the dietary intervention according to treatment arm. However, none of these were found to be good predictors of faecal genotoxicity in this regression analysis. Results demonstrated that there were very minor negative associations between faecal NOCs and urinary p-cresol sulphate with genotoxicity. This was unexpected, given that analysis of the effects of the intervention showed a commonality of outcomes by treatment between genotoxicity and NOCs and p-cresol (see Chapter IV). Previous work has shown that faecal NOCs are closely related to genotoxicity (18), and that p-cresol can predict the genotoxicity of gut model fermentation supernatants (19). In addition, secondary bile acids are well studied genotoxins that have been linked to colorectal tumorigenesis (20).

Intestinal genotoxicity is likely a function of the entirety of the complex biochemical milieu, therefore, identifiable metabolites from the faecal metabolome were explored as to whether they could predict genotoxicity in a regression model. Both allysine and P-DMEA were identified in faecal water as very weak predictors of genotoxicity in this model. The relevance of these associations to gut health and CRC risk are not clear, to my knowledge, previous work has not demonstrated any significance of these metabolites as biomarkers of CRC risk. Previous studies have found allysine, as well as its oxidation product, α-amino adipic acid, to be markers of diabetes risk (21). Neither compound is an established genotoxin, and thus they may be co-correlates of other chemical, or perhaps unidentified, biological genotoxins.

The microbial community can produce peptide based genotoxins which have not been characterised, however, microbial genera were explored as to whether they might predict faecal genotoxicity. *Alistipes*, *Oscillibacter* and *Faecalibacterium* were identified as predictors of genotoxicity, while *Clostridium XIVa* was determined to be a predictor of lower genotoxicity. These associations agree with the observed changes in genotoxicity and microbial community structure in the dietary intervention, *Alistipes*, *Oscillibacter* and *Faecalibacterium* were in greater abundance following the meat diet, which also increased genotoxicity, whereas *Clostridium XIVa* was in greater abundance after the mycoprotein diet, where there was a reduction in genotoxicity (Chapter IV).

With respect to the characteristics of the microbial genera, *Alistipes* and *Oscillibacter* are bile resistant putrefactive bacteria, Alistipes is implicated in gut inflammation and CRC (22), while both *Alistipes* and *Oscillibacter* have been found to be both increased and implicated in uraemia in chronic kidney disease (23). *Clostridium XIVa*, in contrast, is a microbial cluster that includes a number of beneficial butyrate producing microbes. These may play a probiotic role through providing energy for intestinal epithelial cells, strengthening intestinal barrier and interacting with the immune system (24, 25). The positive association between *Faecalibacterium* and genotoxicity is unexpected, as this genus is a glycan degrading SCFA producer thought to benefit the host (26, 27). However, this genus was also enriched on the meat diet compared to the mycoprotein diet. The microbial interactions in the gut when degrading mycoprotein is an area of warranted future research to clarify and understand the findings of the present work.

In the dietary intervention study, CVD risk markers were included as secondary endpoints. Thus, the metabolome and gut microbial community profile were analysed to determine whether they might predict cardiovascular risk. The faecal metabolite, fusicoccin H was found to be both a predictor of lower SBP and DBP. This metabolite is an organic compound produced by the fungus *Fusicoccum amygdali;* a parasite mainly found in almond and peach trees. The production leads to the death of plants (28). The reason for its appearance in the data is unclear, as it was also found to be greater on the meat diet than the mycoprotein diet, despite mycoprotein's fungal origins.
Faecal GABA, N-[(2Z)-2-Hydroxy-2-octenoyl]glycine and sorbic acid were all predictors of both reduced total cholesterol and LDL fractions.
GABA has previously been demonstrated to reduce the percentage of cholesterol esters in human monocyte-derived macrophages through its action on GABA receptors and subsequent gene regulation (29). Sorbic acid is a common food preservative, which has been shown to deregulate hepatic lipid metabolism (30).

In urine, propionylcarnitine was a predictor of both higher total cholesterol and LDL fractions. Increased excretion of acylcarnitines has been associated with a western dietary pattern (31). However, evidence also associates propionylcarnitine with obesity and insulin resistance (32, 33), and increased risk of CVD and diabetes (34-36).
Interestingly, N-[(2Z)-2-Hydroxy-2-octenoyl]glycine, as was the case in stool, was a predictor of both reduced total and LDL cholesterol.

There were also microbial genera that predicted CVD risk markers; notably, *Succinivibrio* predicted both lower BP and total cholesterol. *Succinivibrio* is a succinate producer (37), and has been found to be increased in abundance in the gut when consuming diets with a high fibre content. Some examples include an enrichment in children living in a rural village in Burkino Faso compared to children in urbanised areas (38), as well as a higher frequency in the Hadza hunter gatherers and traditional

Peruvian populations (39, 40). Such observations may be indicative that *Succinivibrio* encompasses metabolizers of starch, hemicellulose, and xylan which are derived from high-fibre dietary patterns, similarly to *Prevotella* and *Treponema* (41). Enrichments of *Succinivibrio* in individuals consuming high-fibre diets is within context of the present results, as high-fibre intakes are also associated with lower BP and total cholesterol (42-44). In contrast with this data, *Succinivibrio* has also been observed to show positive associations with faecal and plasma lipopolysaccharide (LPS) and be in greater abundance in coronary heart disease (45). Such discrepancies may be reflective of the fluidity of the gut microbiota and the fine balance of this ecosystem in health and disease. Succinate may serve as an example, the predominate metabolite produced by *Succinivibrio* is also associated with both disease and health. Succinate acts as a primary cross-feeding metabolite essential for the maintenance of a healthy resident gut microbiota. In this instance, the increase in succinate in some pathological conditions may be reflective of dysbiosis but being exploited by pathogens (45).

A genus which has considerable literature demonstrating positive influences on health is *Roseburia,* which was found to predict lower LDL cholesterol in the present analysis. *Roseburia* is understood to include certain strains which colonise the mucin layer and represents a major source of butyrate to colonocytes (25). It is unsurprising that it's abundance is reduced in CRC (46). This association corroborates with other findings from the study which found an enrichment of *Roseburia* following the mycoprotein diet, in conjunction with a significant reduction in blood LDL cholesterol. As for this prediction of lower LDL cholesterol, certain strains of *Roseburia* also produce propionate (47), which has been shown to be a cholesterol lowering agent (48).

The analysis also revealed that *Sutterella* was a predictor of lower SBP, higher TAG and lower HDL. This is interesting as these associations coincide with the general pattern that was seen on mycoprotein diet; a reduction in SBP, HDL, and an increase in TAG. *Sutterella* has been associated with autism (49), down syndrome (50) and inflammatory bowel disease (51), however there is evidence that *Sutterella* is a widely prevalent commensal in the gut, and while members of this genera have mild pro-inflammatory capacity, they do not contribute significantly to disrupting epithelial homeostasis (52).

Regressing PC scores bring benefits to analyses, with large datasets such as the metagenomic and metabolomic in the present analysis, there may be a number of variables which are highly correlated or collinear, incorporating PCs into a regression can help focus on the meaningful components in the overall dataset which hold the greatest explanatory power. However, it is also important to note that when reviewing PCs, the metabolites which are highlighted as the major contributors are not necessarily the reasoning for the correlations observed. It may be that other metabolites and microbial members within the PCs are responsible for the observed correlations.

In the PCs that were retained in the analysis, there were significant associations with both genotoxicity and CVD risk markers. PC7 in stool was correlated with both increased genotoxicity, as well as lower blood glucose. These correlations are mixed, increased genotoxicity is undesirable with respect to gut health, however reduced blood glucose would be deemed positive for cardiovascular health. This was similar for PC7 in urine, also associated with increased genotoxicity, as well as favourable cardiovascular effects, including both lower systolic blood pressure and blood glucose. The main metabolic contributor to this PC was prolylleucine, a metabolite previously shown to increase in plasma in conjunction with a bifidogenic effect elicited by human milk oligosaccharide consumption in a cohort of irritable bowel syndrome patients (53).
Both PC5 and 6 in urine displayed inverse associations with genotoxicity; the former also associated with higher weight and waist circumference. The main contributor in both these PCs was phenylacetylglutamine, which interestingly is a major nitrogenous metabolite that accumulates in uraemia (54). In addition, it is evidenced to increase in plasma after cigarette smoke exposure, in ischemic heart failure patients, hypertension, cardiovascular risk (55), as well as be an early urinary marker of kidney function decline along with two other known toxins, indoxyl sulphate and p-cresol sulphate (56). These associations along with the main metabolites identified in the PCs raise the prior point that there may well be other metabolites that correlate well within the PCs that are contributory towards the identified associations with genotoxicity and CVD markers.

There were associations indicative of compromising cardiovascular health for PC2 in both stool and urine. In stool, there was a positive association with triglycerides, while in urine, there were correlations with both higher total and LDL cholesterol. Both D-carnitine and acetyl-l-carnitine were the largest contributors to PC2 in urine, which may be an influence of the meat diet, as both total and LDL cholesterol increased following the meat diet in the intervention.
The PC8 in stool had a number of inverse associations with CVD markers, including weight, BMI, waist circumference, as well as HDL cholesterol. The metabolites with the greatest contributions to this PC were the previously mentioned prolylleucine, as well as N-acetylcadaverine; the acetylated form of the polyamine cadaverine, a secondary metabolite produced by both bacterial and host metabolism from the decarboxylation of lysine (57). Bacteria use diamines, like cadaverine to buffer the pH of their environment (58), and while increased concentrations can be toxic (59), protein derived polyamines have multifaceted roles in intestinal physiology (60), whether such characteristics have relevance with the above associations is unclear.

Regressing the retained PCs from the microbiome data demonstrated positive relationships with genotoxicity for PC2 and 3. The microbial genera with the greatest contributions to these PC were *Prevotella*, and an unclassified genus belonging to the *Ruminococcaceae* family, respectively. As delineated above, the genus *Prevotella* has been well characterised to be enriched in the gut of individuals consuming dietary patterns higher in carbohydrates and fibre (41, 61, 62), while species

within *Ruminococcaceae* are specialised in degrading complex carbohydrates including cellulose and resistant starch, predominant constituents of plant based foods (15, 62). While the regression analysis found these correlations, in the intervention, faecal water genotoxicity increased following the meat diet, in conjunction with reductions in the abundance of both *Prevotella* and *Ruminococcaceae*. When reviewing the loading contributions, a pattern emerged that *Bacteroides* was the second greatest contributor to both PC. This genus is enriched in western dietary patterns with high fat and animal protein intake (61-63), and such dietary patterns can elicit greater faecal water genotoxicity (63). It could be postulated that this *Bacteroides* factor is the driver behind the positive correlations between the microbial PC and genotoxicity in our data.

When dealing with metagenomic and metabolomic datasets, it is often the case that variable number outweigh sample number which can introduce problems for classical correlation analyses. These include selecting a set of important variables among many variables, as well as collinearity between the variables (64, 65).
To address this, a sPLS regression was implemented to identify correlations between the metabolic profile and microbial composition of the human dietary intervention. Within the faecal metabolome and microbiota, inverse association were found between *Prevotella* and *Roseburia* with isoputreanine, while a positive association was found for *Bacteroides*. Although there is limited data, isoputreanine may be a marker of meat consumption, specifically chicken and pig. If this is the case, these associations corroborate with the previous point concerning well known data demonstrating dietary patterns which include protein and animal fats enrich the abundance of *Bacteroides*, while diets which comprise more carbohydrates, especially fibre, increase the abundance of *Prevotella* (41). Within the limited literature on isoputreanine, this is an organic acid which has been found to be associated with CRC risk in blood samples (66). Although this is an isolated result, it arguably builds the picture further with respect to the gut microbial response to dietary patterns, as well as the ties between high intakes of meat and CRC.

sPLS of the urinary metabolites and microbiota identified negative associations between *Blautia*, and propionylcarnitine. As mentioned above, acyl carnitines are associated with metabolic perturbations, whose enrichment in samples may be indicative of risk to metabolic health. *Blautia* is a genus which is gaining understanding of possessing potential probiotic properties such as producing short chain fatty acids (SCFA) (67), the production of bacteriocins which can inhibit the colonization of pathogenic bacteria in the intestine (68). In addition, *Blautia* has previously been shown to have a significant negative relationship with visceral fat accumulation (69), as well as it's abundance to increase in combination with improvements in glucose and lipid homeostasis (70). The latter is in context with the present results with respect to the negative relationship with acyl carnitines which can be enriched in metabolic perturbations. The sPLS also found positive relationships between *Roseburia* and *Phascolarctobacterium* with paracetamol sufate and 2-methoxyacetaminophen sulfate, both metabolites from paracetamol metabolism. While not necessarily associated with the main thesis aims,

it does raise the question whether paracetamol administration elicits changes in the gut microbiota, or whether changes in the gut microbiota are reflective of pain which ultimately leads to administration of analgesic agents.

Th present analysis has strengths. The microbiome and metabolome datasets which were integrated comprised multiple data points, providing a wealth of data to explore. The metabolomics data was generated using a modern technological metabolomics platform which encompasses increased profiling efficacy in comparison to alternative platforms. This produced a large, high quality metabolite data to leverage in the present work. All the data explored originated from a well-controlled investigator-blind randomised crossover trial, contributing robust statistical data, a by-product of using such a study design, to the analyses.

However, there were some weaknesses, the data was derived from a relatively small cohort, in addition, the microbiome data was obtained from 16S ribosomal RNA amplicon sequencing. While this methodology has advantages, it is limited in the depth of sequencing, rendering an inability to characterise microbial communities any further than the taxonomic level of genus. While in the metabolomics dataset, there were unidentified peaks representing metabolites of interest that may have been additive to the overall work.

There are questions arising from the present analysis which arguably require exploration. With respect to the interplay between the gut microbiota, mycoprotein and cardiometabolic health, I found favourable associations between the genera *Roseburia* and *Succinovibrio* with blood pressure, as well as both total and LDL cholesterol, three predominant CVD risk markers. The results from the intervention demonstrated increases in these genera following the mycoprotein diet (Chapter IV) as well as reductions in the CVD risk markers (Chapter V). Both these genera are enriched when consuming diets high in fibre and plant polysaccharides, delineating the utilisation of mycoprotein in the gut by *Roseburia* and *Succinovibrio* could be considered in future mechanistic work to determine whether these genera are specific degraders of mycoprotein and its constituents, or whether the effects observed are due to the overall dietary intake during the mycoprotein phase. In addition, an integrative work which again explores the influence of mycoprotein on CVD risk markers and the gut microbiota could help clarify the results and relationships found between microbial genera, blood pressure, and cholesterol.

Turning attention to the theme of meat consumption, isoputreanine in stool was found to be positively associated with the genus *Bacteroides*. This is an organic acid thought to be a potential marker of animal meat consumption and previously shown to be increased in the blood of individuals with increased CRC risk. Future work may consider the relevance of isoputreanine in the gut and CRC, by way of leveraging stool samples from CRC observational models, as well as mechanistic work. The metabolites allysine and P-DMEA in stool were observed to be weak predictors of genotoxicity in our analysis. As

mentioned, to our knowledge, neither compound is an established genotoxin, and may be co-correlates of other unidentified biological genotoxins. Clarifying such genotoxins would be desirable, the ability to identify CRC risk through non-invasive means is a sought-after objective, stool metabolites that can predict a greater genotoxic load in the gut would be step towards attaining this objective.

There were unidentified peaks within the metabolomics dataset. Further analysis might interrogate these unidentified peaks. Identifying these compounds could potentially provide a greater coverage of relationships in the overall data and give a more comprehensive understanding of the explanatory metabolites in the observed correlations between the metabolomics data, faecal genotoxicity, and CVD risk markers.

In conclusion, by integrating rich metabolomic and metagenomic data sets, relationships were uncovered between gut microbial communities, metabolites, gut genotoxicity and risk markers of cardiovascular disease which corroborate with pre-existing data pertaining to the negative influence high meat dietary patterns elicit on gut microbial composition and the metabolic environment within the gut, and how this may compromise both gut and cardiovascular health.

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CHAPTER VII

EXPERIMENTAL CHAPTER IV

The nutritional impact of replacing dietary meat with meat alternatives in the UK: a modelling analysis using nationally representative data.

Abstract

Dietary patterns high in meat compromise both planetary and human health. Meat-alternatives may help facilitate meat reduction, however the nutritional implications of displacing meat with meat-alternatives does not appear to have been evaluated. Here, data from the 9th cycle of the National Diet and Nutrition Survey was used as the basis of models to assess the effect of meat substitution on nutritional intake. I implemented three models; model 1 progressively replaced 25%, 50%, 75%, or 100% of the current meat intake with a weighted mean of meat-alternatives available in the UK market. Model 2 compared different ingredient categories of meat-alternative; vegetable, mycoprotein, a combination of bean and pea, tofu, nut and soy. Model 3 compared fortified versus unfortified meat-alternatives. The models elicited significant shifts in nutrients. Overall, there were increases in carbohydrate, fibre, sugars and sodium, whereas reductions were found for protein, total and saturated fat, iron and B12. The greatest effects were seen for; vegetable-based (+24.63g/day carbohydrates), mycoprotein-based (-6.12g/day total fat), nut-based (-19.79g/day protein, +10.23g/day fibre; -4.80g/day saturated fat, +7.44g/day sugars), soy-based (+495.98mg/day sodium), and tofu-based (+7.63mg/day iron, -2.02μg/day B12). Our results suggest meat-alternatives can be a healthful replacement for meat if chosen correctly. Consumers should seek out meat-alternatives which are low in sodium and sugar, high in fibre, protein and with high micronutrient density, to avoid compromising nutritional intake if reducing their meat intake. Manufacturers and policy makers should consider fortification of meat-alternatives with nutrients such as iron and B12 and focus on reducing sodium and sugar content.

7.1. Introduction

Globally, annual per capita meat consumption has increased by 20 kilograms since 1961 (1). This sector of our food system contributes substantially to greenhouse gas emissions (2), depletes natural resources (3-5), and compromises animal welfare. In the UK, meat can be a valuable source of nutrients, however, overconsumption is associated with an increased risk of cardiovascular disease, type 2 diabetes (6-8), and cancer (9-16). Reducing meat consumption may therefore promote human health, protect animal welfare and benefit the environment.

Changing dietary behaviours to elicit a transition towards alternative protein sources is challenging in part owing to strongly held taste preferences, culinary traditions, and social and cultural norms (17). Meat alternatives, resembling meat in appearance, preparation and eating experience, could therefore play an important role in enabling dietary change (18). Integration of meat alternatives into the diet is subtle and does not require an overhaul of food consumption and meal patterns (19-21). Although meat alternatives have been available for years, their popularity with consumers has increased rapidly in recent times; due in part to producers better simulating the taste, texture and functionality of traditional meat products. There has also been an increase in the direction of marketing towards meat-eating consumers, rather than just vegans and vegetarians (22).

The rapid increase in the consumption of meat alternatives has raised concerns regarding their overall healthfulness and the potential displacement of valuable nutrients. The developing meat alternative market includes an array of food products with variable nutritional quality, some of which might be considered ultra-processed (23). If these foods are to play a role in reducing meat consumption at a population level, it is essential that any population level nutritional benefits and consequences are identified early, and actions taken to mitigate undesirable effects.

Here, I model the potential nutritional implications of meat replacement by using the 9th cycle (years 2016-2017) of the nationally representative UK National Diet and Nutrition Survey rolling program (NDNS RP). I present the nutritional implications of complete and partial meat substitution, using models that either; assume replacement of meat with a range of alternative products, based on current UK purchasing data; or models that assume replacement of meat with specific meat alternative product categories. To our understanding, this is the first study to evaluate the nutritional implications of such dietary replacement scenarios.

7.2. Methods

7.2.1. Study Population

Dietary intake data for the UK population was drawn from the 9th cycle of the NDNS rolling programme. The NDNS rolling programme is a Public Health England and Food Standards Agency funded survey of the food consumption, nutrient intake, and nutritional status of people aged 1.5yr and older living in private households in the UK and is representative of the UK population (24). Detailed descriptions of the NDNS RP survey design and sampling methods can be found elsewhere (25-27). The 2016-2017 survey cycle year used in the present modelling analysis included 1,253 participants aged 1.5-95yr.

7.2.2. Dietary Records

The NDNS RP collected habitual dietary data using four-day estimated food diaries (24-26). Data from food diaries has been aggregated for each respondent to provide publicly available daily averages of self-reported food and nutrient intake. For this analysis, data sets for participants with extreme reported energy intakes (< 500kcal - > 5000kcal) were excluded. Datasets from subjects who self-reported abstaining from meat, or with dietary data devoid of any meat, were also excluded. A common occurrence in the collection of habitual dietary intake data is underreporting of energy intake (28), which the NDNS RP data drawn upon here corroborates. There are different methods to approach underreporting (29); in the present analysis I adjusted nutrients to age and gender specific EARs for energy.

Sub-analyses were performed by stratifying by sex and age group, using the same stratification system as the NDNS RP (male and female; 4-10yr, 11-18yr and 19-64yr, over 65yr). Children in 1.5-3yr subgroups were not included as nutrient requirements are specific for each age in this sub-population. In addition, dietary patterns at this stage in life may not include substantial amounts of solid food such as meat. This left a total of 1,110 respondents for inclusion in the analysis.

7.2.3. Self-reported nutritional intake from meat

The current level of meat consumption in the UK was estimated from the NDNS dataset. Briefly, the NDNS RP reports meat intake in grams per day broken down by type (i.e. “beef, lamb, pork, offal etc”), I allocated these categories into food type groups; ‘red meat; ‘processed red meat’, ‘white meat’, ‘processed white meat’, and ‘seafood’. In this analysis seafood was included with meat as a source of animal flesh. I note that the UK dietary guidelines encourage a population level increase in fish consumption as a source of omega 3 fatty acids and other nutrients, however, most UK vegetarians describe themselves as lacto-ovo-vegetarian or vegan, and only a minority describe themselves as pescatarian.

The allocation and stratification of animal meat within the NDNS dataset is described in **Table 7.1.**

The total nutritional contribution of meat to the UK diet was estimated by combining the intake data with the nutritional composition data from the McCance and Widdowson composition of food tables (30). Briefly, an aggregate quantity of nutrient, per gram, for each of the meat subtypes was multiplied by the intake in grams per day, for each respondent. The totality of these nutrition scores by subtype was then used to estimate the total nutritional value of meat products to the diet for each individual respondent.

Table 7.1. NDNS food groups used to calculate self-reported meat intake

	Meat subtype	NDNS category*	Description of category
Total Meat	Red meat	"beefg"	Amount of beef consumed (g)
		"lambg"	Amount of lamb consumed (g)
		"porkg"	Amount of pork consumed (g)
		"offalg"	Amount of offal consumed (g)
		"liverdishes"	Amount of liver consumed (g)
		"otherredmeatg"	Amount of other red meat consumed (g)
	Processed red meat	"burgersg"	Amount of burger consumed (g)
		"sausagesg"	Amount of sausages consumed (g)
		"baconandham"	Amount of bacon and/or ham consumed (g)
		"burgersandkebabs"	Amount of burger and/or kebab consumed (g)
		"processedredmeatg"	Amount of other processed red meat consumed (g)
	White meat	"poultryg"	Amount of poultry consumed (g)
		"gamebirdsg"	Amount of game consumed (g)
	Processed white meat	"coatedchicken"	Amount of coated chicken products consumed (g)
		"processedpoultryg"	Amount of processed poultry consumed (g)
	Seafood	"whitefishg"	Amount of white fish consumed (g)
		"oilyfishg"	Amount of oily fish consumed (g)
		"cannedtunag"	Amount of canned tuna consumed (g)
		"shellfishg"	Amount of shellfish consumed (g)
		"whitefishcoatedorfried"	Amount of white fish (coated or fried) consumed (g)

*As displayed in NDNS data set.

7.2.4. Nutritional content of meat alternatives

Products were identified through product searches on UK supermarket websites using the key words, 'meat alternatives', 'meat substitutes', 'meat-free', 'plant-based', 'vegan', and 'vegetarian', to ensure a wide capture of meat alternative products. Following data collection, the products that met these criteria were grouped into ingredient categories (**Appendix C, Table 1**).

The nutritional profile of meat alternatives was calculated based on product labels, information from supermarket and manufacturer websites, as well as searching nutrition databases for products. Where nutrient data was not available for a product, manufacturers were contacted to request further details. This data was used to estimate the aggregate nutritional composition per gram for each of the meat alternative ingredient categories, based on the mean nutritional composition of the individual products included in each respective category.

There was incomplete availability of nutritional data across the product categories for many of the micronutrients including iodine, zinc, and selenium. There was available nutritional data for the following nutrients: carbohydrate, protein, total fat, saturated fat, fibre, sugar, sodium, iron and B12. Data on these nutrients were deemed to be of good quality for inclusion in subsequent analysis.

7.2.5. Meat replacement scenarios

Here, I assessed the nutritional implications of a set of scenarios for the widespread replacement of meat with meat alternatives in the UK population (**Table 7.2.**).

<u>Model 1: The effect of meat reduction and replacement with meat alternatives</u>

In the first replacement model, I generated a weighted mean of the nutritional quality of meat alternatives based on the current market share for meat alternative products in the UK; vegetable 23%, mycoprotein 18%, bean 17%, soy 15%, nut 14%, and tofu 13% (31).

I then looked at the effect of progressively reducing the amount of meat in each respondent's diet by 25% (MA-25), 50% (MA-50), 75% (MA-75), and 100% (MA-100), and replacing the removed nutrients with the weighted nutritional score of the meat alternatives on a per weight basis. (**Table 7.2.**).

<u>Model 2: A comparison of the effects of replacing nutrients from meat across specific ingredient categories of meat alternative.</u>

In a second model, I compared the nutritional impact of replacing meat with meat alternatives produced from a particular category of ingredient. Specifically, I replaced the total amount of meat in each individual respondent's diet with vegetable-based (Vegetable), mycoprotein-based (Mycoprotein), a combination of bean and pea-based (Legume), tofu-based (Tofu), nut-based (Nut) and soy-based (Soy) meat alternatives (**Table 7.2.**).

Model 3: substitution with fortified versus unfortified meat alternatives

Within the food products identified, 14% were fortified with either iron, B12, or both. Taking this into account, in a third and final model, I compared the effects of replacing the total amount of meat in each person's diet with either fortified meat alternatives (Fortified) or unfortified meat alternatives (Unfortified) (**Table 7.2.**).

I implemented each replacement strategy by removing the nutrient contribution of meat from each individual respondents' total nutritional intake, followed by replacing this with the nutritional data for the equivalent weight of meat alternatives. I adjusted the final total nutritional intake after substitution to match the energy content of each participant's diet.

7.2.6. Comparators and subjective model corrections

Due to the variable quality of available nutritional data, and the relevance to public health, I have elected in this analysis to focus on the macronutrients, protein, fat and carbohydrate, as well as saturated fat, total sugars and sodium due to public health guidance to limit their intake (32, 33). In addition, fibre was selected as authoritative bodies advise an intake of 30g/day for the adult population (33); which many fail to achieve (34). I hypothesised that fibre would increase in the replacement models as a result of the higher fibre content of meat alternatives compared to meat (35). I also assessed influences on iron and B12, as the intake of these nutrients may be inadequate when transitioning to a dietary pattern devoid of animal based foods (36).

I analysed the nutritional adequacy of the meat replacement models by comparing the nutrient values to the Dietary Reference Values for Food Energy and Nutrients for the UK (DRVs) (32, 33, 37). DRVs comprise a series of estimates of the amount of energy and nutrients needed by different groups of healthy people in the UK population. In accordance with the DRVs, I used the Estimated Average Requirements (EARs) as a comparator for total energy, whereas for protein, sodium, iron and B12, the Reference Nutrient Intakes (RNIs) were used. For fat and carbohydrates (including total sugars, saturated fat, and fibre) I used the UK DRVs which for total fat (35%), saturated fat (11%), and total carbohydrates (50%), are given as a percentage of daily energy intake (32, 33, 37). The current recommendations for protein is to intake 0.75g per kilogram bodyweight (33, 37), as the dataset in the current analysis contains a diverse age range which will also comprise different body types, a protein target of making up the remaining 15% of total energy intake after the contributions of fat (35%) and carbohydrates (50%) was employed instead.
Since the recommendations differ by age and sex, I calculated the average values for nutrients across the population subgroups (male and female; 4-10yr, 11-18yr, 19-64yr, over 65yr). I also incorporated a group that represented the total number of participants (both male and female, ages 4yr and above), and averaged all DRV categories to obtain a population average. This group would act as the representative

of the UK population and used in the main analysis.

7.3. Statistical Analysis

Differences in nutrient intake between the meat replacement models and the current intake were compared using linear models with nutrients as the dependent variable, and the replacement models as the independent variable, using the current intake as the reference. The differences in nutrient intake between all scenarios (current and replacement models) with reference to DRVs were then compared using linear models in the same manner but including the current intake with the replacement models as the independent variable and then using DRVs used as the reference. To assess the nutrient intake between meat replacement models, an Analysis of Variance (ANOVA) was implemented, using an $\alpha = 0.05$, followed by a post hoc Tukey test to determine the significance of differences between models. All analysis was performed using R statistical software (version 4.0.0) (38).

Table 7.2. Meat replacement models implemented in the present modelling analysis

Replacement Scenario Models		Description of model
Progressive (Model 1)	MA-25	Partial replacement substituting 25% of the current self-reported meat intake with a composite of meat alternatives.
	MA-50	Partial replacement substituting 50% of the current self-reported meat intake with a composite of meat alternatives.
	MA-75	Partial replacement substituting 75% of the current self-reported meat intake with a composite of meat alternatives.
	MA-100	Full replacement substituting 100% of the current self-reported meat intake with a composite of meat alternatives.
Ingredient (Model 2)	Vegetable	Full replacement substituting 100% of the current self-reported meat intake with meat alternatives produced predominantly from vegetables.
	Mycoprotein	Full replacement substituting 100% of the current self-reported meat intake with meat alternatives produced predominantly from mycoprotein.
	Legume	Full replacement substituting 100% of the current self-reported meat intake with meat alternatives produced predominantly from bean and pea.
	Tofu	Full replacement substituting 100% of the current self-reported meat intake with meat alternatives produced from tofu.
	Nut	Full replacement substituting 100% of the current self-reported meat intake with meat alternatives produced predominantly from nut.
	Soy	Full replacement substituting 100% of the current self-reported meat intake with meat alternatives produced predominantly from soy.
Fortification (Model 3) *	Fortified	Full replacement substituting 100% of the current self-reported meat intake with meat alternatives that have been fortified with nutrients.
	Unfortified	Full replacement substituting 100% of the current self-reported meat intake with meat alternatives that have not been fortified with nutrients.

*Products identified as fortified if 1) Explicitly stated nutrient fortification had been applied during manufacturing process; 2) Isolated nutrients included in ingredient list. Products not meeting 1) or 2) identified as unfortified.

7.4. Results

7.4.1. Self-reported meat intake

The average self-reported intake of both total meat and meat subgroup for the total population, as well separate population subgroups are shown in **Table 7.3**. For the total population, the self-reported average meat intake was 132.25g/day (95% CI, 128.02-136.48g/day). Processed meat was the largest contributor to total meat intake (35.98%), followed by white meat (27.22), red meat (19.44%), and seafood (13.36%), with processed white meat contributing the lowest (3.99%). Males aged 19-64 years consumed the greatest amount of meat (mean, 177.55g/day; 95% CI, 165.33-189.77g/day), while girls aged 4-10 years consumed the least amount of meat (mean, 98.75g/day; 95% CI, 91.28-106.23g/day).

Table 7.3. Self-reported meat intake (total meat, and meat subtype) stratified by age and gender.

Subgroup	Total meat, g/day	Red meat, g/day	Contribution to total meat, %	Processed meat, g/day	Contribution to total meat, %	White meat, g/day	Contribution to total meat, %	Processed white meat, g/day	Contribution to total meat, %	Seafood, g/day	Contribution to total meat, %
Male, 4 -10yr	106.01 (96.61, 115.42)	14.76 (11.74, 17.77)	13.92 (12.16, 15.39)	47.66 (41.19, 54.13)	44.95 (42.63, 46.89)	26.27 (22.30, 30.24)	24.78 (23.08, 26.20)	6.41 (4.88, 7.95)	6.05 (5.05, 6.89)	10.92 (8.64, 13.20)	10.30 (8.95, 11.43)
Female, 4 -10yr	98.75 (91.28, 106.23)	13.72 (10.92, 16.51)	13.89 (11.97, 15.54)	40.97 (34.84, 47.11)	41.49 (38.17, 44.35)	24.47 (20.88, 28.07)	24.78 (22.87, 26.42)	6.88 (5.26, 8.49)	6.96 (5.77, 7.99)	12.71 (10.20, 15.22)	12.87 (11.18, 14.33)
Male, 11 -18yr	146.71 (134.32, 159.10)	21.4 (16.47, 26.32)	14.59 (12.26, 16.54)	60.37 (50.55, 70.18)	41.15 (37.63, 44.11)	40.79 (33.57, 48.01)	27.8 (24.99, 30.17)	9.14 (5.71, 12.57)	6.23 (4.25, 7.90)	15.01 (10.57, 19.46)	10.23 (7.87, 12.23)
Female, 11 -18yr	131.36 (117.86, 144.87)	19.87 (15.51, 24.23)	15.12 (13.16, 16.72)	50.21 (40.47, 59.94)	38.22 (34.34, 41.38)	39.70 (34.19, 45.21)	30.22 (29.01, 31.21)	9.41 (6.75, 12.07)	7.16 (5.73, 8.33)	12.18 (9.25, 15.10)	9.27 (7.85, 10.42)
Male, 19 -64yr	177.55 (165.33, 189.77)	40.07 (34.21, 45.93)	22.57 (20.69, 24.20)	62.46 (53.53, 71.40)	35.18 (32.38, 37.62)	50.05 (43.67, 56.44)	28.19 (26.41, 29.74)	5.04 (3.34, 6.74)	2.84 (2.02, 3.55)	19.92 (16.09, 23.75)	11.22 (9.73, 12.51)
Female, 19 -64yr	127.84 (119.63, 136.06)	26.33 (22.44, 30.21)	20.59 (18.76, 22.20)	38.94 (33.29, 44.59)	30.46 (27.83, 32.77)	39.41 (34.79, 44.02)	30.83 (29.09, 32.36)	3.31 (2.16, 4.46)	2.59 (1.81, 3.28)	19.86 (16.76, 22.95)	15.53 (14.01, 16.87)
Male, 65yr +	151.01 (133.53, 168.49)	42.22 (33.65, 50.80)	27.96 (25.20, 30.15)	52.64 (40.51, 64.77)	34.86 (30.34, 38.44)	29.01 (19.20, 38.82)	19.21 (14.38, 23.04)	1.18 (0.04, 2.41)	0.78 (0.03, 1.43)	25.95 (20.12, 31.78)	17.18 (15.07, 18.86)
Female, 65yr +	103.97 (94.29, 113.66)	26.61 (20.68, 32.54)	25.60 (21.94, 28.63)	27.29 (21.29, 33.30)	26.25 (22.58, 29.30)	21.98 (16.59, 27.36)	21.14 (17.59, 24.08)	0.80 (0.09, 1.51)	0.77 (0.10, 1.33)	27.29 (21.66, 32.92)	26.25 (22.97, 28.96)
Total Population	132.25 (128.02, 136.48)	25.72 (23.88, 27.55)	19.44 (18.65, 20.19)	47.58 (44.68, 50.49)	35.98 (34.90, 36.99)	36.00 (33.88, 38.12)	27.22 (26.46, 27.93)	5.28 (4.61, 5.95)	3.99 (3.60, 4.36)	17.67 (16.31, 19.04)	13.36 (12.74, 13.95)

Data expressed as means with 95% confidence intervals in parentheses

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7.4.2. Projected changes in nutritional intake

Overall, the implemented meat replacement scenarios elicited many differences in nutritional intake. The current nutrient intake for the total population as well as for each replacement model (MA-25, MA-50, MA-75, MA-100, Vegetable, Mycoprotein, Legume, Tofu, Nut, Soy, Fortified, Unfortified) are included in **Table 7.4.**; while the projected differences for each replacement model in comparison to the current intake are included in **Table 7.5**.

Model 1: The effect of meat reduction and replacement with meat alternatives

In model 1, I explored projected changes in nutritional intake given a graded replacement of meat from replacing 25% of current meat intake, through to a 100% replacement, using weighted composite nutritional values for the meat alternatives based on consumer purchasing data. I observed a linear increase in total carbohydrate consumption with the decrease in meat, reaching an additional 19 grams per day in a 100% meat replacement scenario. This increase in projected carbohydrates was statistically significant in all scenarios except the 25% replacement model (MA-50, P = 0.018; MA-75, P = <0.001; MA-100, P <0.001). Importantly, the projected increased intake of carbohydrates was only associated with a small linear increase in projected sugar intake; this increase did not reach statistical significance until 100% of current meat was replaced (+04.16g/day, *P* = 0.05). In contrast, with decreasing consumption of meat and increasing consumption of meat alternatives there was a graded decrease in projected total protein intake (MA-25, -3.40g/day, P = 0.002; MA-50, -6.99g/day, P = <0.001; MA-75, -10.77g/day, P = <0.001; MA-100, -14.77g/day, P <0.001) . There was also a linear decrease in the intake of fat and importantly, of saturated fat, with decreasing meat and increasing meat alternative intake. The decrease in saturated fat with complete meat substitution was equivalent to 3.96 g per day (P <0.001).

Total fibre intake was increased significantly under each meat replacement scenario. A 100% replacement of meat with meat alternatives was associated with an 8.15g increase in total daily fibre intake (P <0.001)**.** Notably, there was also a linear increase in sodium intake, with a total replacement of meat being associated with a projected 228.03 mg increase in sodium per day (P <0.001). There were non-statistically significant decreases in projected intake of both iron and of vitamin B12. Similar effects were found in sub analyses across each of the population subgroups (**Appendix C, Tables 3-18**).

Model 2: A comparison of the effects of replacing nutrients from meat across specific categories of alternative.

This model is based on a 100% replacement of meat with the mean nutrient values from each of the separate categories of meat alternative. Compared to the current intake, the substitution of meat, with

foods from any of the meat alternative categories, led to a meaningful increase in the projected mean intake of, fibre. The greatest projected increase in total fibre was observed in models replacing meat with nut-based products, (10.23 g day^{-1}), followed closely by substitution with vegetable (8.73 g day^{-1}) or mycoprotein based products (8.71 g day^{-1}). Substitution with tofu-based products had the least impact on projected total fibre intake (+3.14 g day^{-1}).

All product categories were associated with meaningful reductions in projected saturated fat intake. The greatest reduction in saturated fat intake is projected for substitutions with nut-based meat alternatives (-4.8 g day^{-1}, P = <0.001), whilst the greatest reduction in total projected fat intake was associated with substitution for mycoprotein (6.12 g day^{-1}, P = <0.001). Only the replacements with nut-based and tofu-based meat alternatives did not cause significant reductions in projected total fat intake.

Conversely, projected intakes of protein were lower across each meat alternative category. The greatest projected reductions in total protein intake were observed when substituting in products exclusively from the nut (-19.79 g day^{-1}), vegetable (-19.33 g day^{-1}), or legume (-15.43 g day^{-1}) categories. Tofu had the least projected impact on total protein consumption, but it was still associated with a significant reduction in daily intake (-4.87 g day^{-1}). Replacement of meat with meat alternatives was also associated with an increase in total projected sodium consumption except for the nut-based products (-124.30mg day^{-1}). Soy based meat alternatives had the highest sodium content, and replacement with the soy product category raised projected sodium intake by almost 0.5 g day^{-1}.

Regarding micronutrients, the projected intake of vitamin B12 was shown to be statistically significantly reduced in the replacement models with tofu (-2.02ug/day; $P = 0.03$), nut (-1.88ug/day; $P = 0.04$), and soy (-1.76ug/day; $P = 0.05$), although in each case mean projected intake across the population remained in excess of the RNI.
Total iron intake was projected to increase in the substitution model with tofu (+7.63 mg day-1, P <0.001). It was not significantly affected by the other meat replacement scenarios.

In sub-analyses by population group (age and gender) the described influence of these substitution scenarios were replicated in each of the demographics (**Appendix C, Tables 3-18**).

Model 3: substitution with fortified versus unfortified meat alternatives

In this model I compared the impact of selecting iron and B12 fortified, versus unfortified, meat alternatives, assuming a 100% replacement of meat. Fortified products accounted for 14% of the meat alternative products. The projected intake of iron was significantly higher than current intakes when choosing fortified meat alternatives (+3.12mg/day; P <0.001) and would comfortably attain the UK dietary recommendations (Fortified, 16.77mg/day vs 14.8mg/day RNI for girls and women aged 11-50, 8.7mg/day RNI for remaining population). The projected intake of iron was not statistically significantly reduced from current levels of intake in the unfortified model.

The projected intake of vitamin B12 was significantly decreased compared to current intakes in the unfortified model (-1.79ug/day, P = 0.05), however projected mean intake did not fall below the RNI. In the fortified model projected intakes of vitamin B12 were not significantly different from current levels of intake.

Interestingly, fortified meat alternatives were associated with a much smaller projected reduction in total protein from current intakes (-3.09 g day-1 versus -18.19 g day -1 for the unfortified products P = 0.005) however, it was also associated with a greater projected increase in total sodium from current levels of intake (-482 mg day-1 versus +361 mg day -1 for the unfortified products P <0.001). In sub analyses by population group (age and gender) the effect of choosing fortified products described above were replicated across subgroups (**Appendix C, Tables 3-18**).

Table 7.4. Mean nutrient intake of total population (n = 1110) for current and replacement models, with reference to DRVs

			Progressive Model				Ingredient Model						Fortification Model	
Nutrient	**DRV***	**Current**	**MA-25**	**MA-50**	**MA-75**	**MA-100**	**Vegetable**	**Mycoprotein**	**Legume**	**Tofu**	**Nut**	**Soy**	**Fortified**	**Unfortified**
Energy, kcal/day	2150	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate, g/day†	268.80	270.77	275.16	279.78	284.65	289.80	295.39	293.17	290.68	279.77	282.24	285.33	282.01	294.03
Protein, g/day†	80.60	86.67	83.27	79.68	75.90	71.90	67.34	77.86	71.24	81.80	66.88	79.48	83.58	68.49
Fat, g/day	83.60	81.97	81.19	80.38	79.51	78.60	77.58	75.85	78.48	79.60	83.35	77.26	77.08	77.78
Fibre, g/day	30	21.86	23.74	25.71	27.80	30.00	30.59	30.57	28.66	25.00	32.10	28.12	28.94	30.23
Sugars, g/day	90	110.52	111.48	112.49	113.56	114.68	114.94	113.30	113.93	112.66	117.96	112.71	112.20	114.63
Saturated fat, g/day	26.30	30.96	30.05	29.08	28.07	27.00	27.13	27.31	27.23	26.63	26.16	27.27	27.66	27.02
Sodium, mg/day	2400	2413.80	2466.37	2521.74	2580.14	2641.82	2720.90	2726.12	2690.65	2510.20	2289.49	2909.77	2896.42	2774.88
Iron, mg/day	8.7/14.8‡	13.65	13.59	13.52	13.45	13.38	13.17	12.68	13.26	21.28	12.74	13.61	16.77	13.10
B12, ug/day	1.5	7.47	7.07	6.65	6.21	5.74	5.72	5.98	5.77	5.45	5.59	5.71	7.14	5.68

*DRV values correspond to EAR for energy; RNI for Protein, Sodium, Iron, B12; DRV for Carbohydrate, Fat, Fibre, Sugars, Saturated fat.

† Protein target elected as 15% total energy intake and carbohydrate as 50% total energy intake in the current analysis

‡ Iron RNI for females aged 11-50

Colours: Green, meeting DRV. Red, not meeting DRV. Grey, not meeting 15% protein and/or 50% carbohydrate target elected in the current analysis. Blue, not meeting higher RNI for iron.

DRV, Dietary Reference Value; EAR, Estimated Average Requirement; RNI, Reference Nutrient Intake; MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Table 7.5. Projected differences from current intake for meat alternatives across the total population (n = 1110)

Progressive Model

	Current	MA-25	P	MA-50	P	MA-75	P	MA-100	P
Energy, kcal/day	2150	-	-	-	-	-	-	-	-
Carbohydrate, g/day	270.77	+4.39	0.25	+9.01	0.018*	+13.89	<0.001*	+19.04	<0.001*
Protein, g/day	86.67	-3.40	0.002*	-6.99	<0.001*	-10.77	<0.001*	-14.77	<0.001*
Fat, g/day	81.97	-0.78	0.55	-1.60	0.22	-2.46	0.06	-3.37	0.009*
Fibre, g/day	21.86	+1.88	<0.001*	+3.85	<0.001*	+5.93	<0.001*	+8.13	<0.001*
Sugars, g/day	110.52	+0.96	0.66	+1.97	0.36	+3.04	0.16	+4.16	0.05*
Saturated fat, g/day	30.96	-0.91	0.09	-1.88	0.001*	-2.89	<0.001*	-3.96	<0.001*
Sodium, mg/day	2413.80	+52.57	0.21	+107.94	0.01	+166.34	<0.001*	+228.03	<0.001*
Iron, mg/day	13.65	-0.06	0.93	-0.13	0.85	-0.20	0.78	-0.27	0.70
B12, ug/day	7.47	-0.40	0.66	-0.82	0.37	-1.26	0.17	-1.73	0.06

Ingredient Model

	Current	Vegetable	P	Mycoprotein	P	Legume	P	Tofu	P	Nut	P	Soy	P
Energy, kcal/day	2150			-	-	-	-					-	-
Carbohydrate, g/day	270.77	+24.63	<0.001*	+22.40	<0.001*	+19.92	<0.001*	+9.00	0.018*	+11.48	0.003*	+14.56	<0.001*
Protein, g/day	86.67	-19.33	<0.001*	-8.82	<0.001*	-15.43	<0.001*	-4.87	<0.001*	-19.79	<0.001*	-7.19	<0.001*
Fat, g/day	81.97	-4.39	0.001*	-6.12	<0.001*	-3.49	0.007*	-2.37	0.07	+1.38	0.29	-4.71	<0.001*
Fibre, g/day	21.86	+8.73	<0.001*	+8.71	<0.001*	+6.79	<0.001*	+3.14	<0.001*	+10.23	<0.001*	+6.25	<0.001*
Sugars, g/day	110.52	+4.43	0.04*	+2.78	0.20	+3.42	0.11	+2.15	0.32	+7.44	0.001*	+2.20	0.31

Saturated fat, g/day	30.96	-3.83	<0.001*	-3.65	<0.001*	-3.73	<0.001*	-4.33	<0.001*	-4.80	<0.001*	-3.69	<0.001*
Sodium, mg/day	2413.80	+307.10	<0.001*	+312.32	<0.001*	+276.85	<0.001*	+96.40	0.02*	-124.30	0.003*	+495.98	<0.001*
Iron, mg/day	13.65	-0.48	0.49	-0.97	0.16	-0.39	0.57	+7.63	<0.001*	-0.91	0.19	-0.04	0.95
B12, ug/day	7.47	-1.75	0.06	-1.49	0.10	-1.70	0.06	-2.02	0.03*	-1.88	0.04*	-1.76	0.05*

Fortification Model

	Current	**Fortified**	**P**	**Unfortified**	**P**
Energy, kcal/day	2150	-	-	-	-
Carbohydrate, g/day	270.77	+11.24	0.003*	+23.26	<0.001*
Protein, g/day	86.67	-3.09	0.005*	-18.19	<0.001*
Fat, g/day	81.97	-4.90	<0.001*	-4.19	0.001*
Fibre, g/day	21.86	+7.08	<0.001*	+8.37	<0.001*
Sugars, g/day	110.52	+1.68	0.43	+4.12	0.06
Saturated fat, g/day	30.96	-3.30	<0.001*	-3.94	<0.001*
Sodium, mg/day	2413.80	+482.63	<0.001*	+361.09	<0.001*
Iron, mg/day	13.65	+3.12	<0.001*	-0.54	0.43
B12, ug/day	7.47	-0.33	0.72	-1.79	0.05*

Blue indicates significant increase compared to Current. Gold indicates significant decrease compared to Current.
*Differences compared using regression models with significance threshold $P < 0.05$
MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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7.5. Discussion

I have modelled the projected nutritional impact of a shift in the culinary practices of the meat-eating UK population towards favouring meat alternative products and have identified some important nutritional benefits. Notably, our models predict significant increases in fibre intake and significant decreases in both total fat and saturated fat intake as people switch from meat to meat-alternatives. On the other hand, not all projected impacts on nutrient intake would be described as beneficial; there were notable reductions in total protein and vitamin B12, and projected increases in the intake of sodium and of sugars.

The UK dietary guidelines recommend limiting fat to no more than 35% of total energy. This target is generally met by the UK population, but further displacement of fat with fibre may have the potential to reduce total energy intake with consequences for body composition. I observed significant reductions in projected total fat intake across our model scenarios, with the exception of the nut and tofu-based alternatives. The greatest projected reduction in fat was associated with mycoprotein; if I assumed no dietary adjustment for total energy, daily replacement of meat with mycoprotein might be projected to reduce fat intake by the equivalent of 54 kcals per day, a level of reduction more than sufficient to offset weight gain (39).

The UK guidelines set an upper limit of 11% of total energy for saturated fat due to its association with cardiovascular disease (33, 37). Actual intakes of saturated fat remain above this threshold despite long-running public health initiatives. I observed that a 100% replacement of meat with meat-alternatives would take total projected saturated fat intake very close to the 11% of food energy target. This observation was consistent across the categories of meat alternative, but with the greatest projected reductions in saturated fat being observed with the nut-based and tofu-based products categories. Nut and tofu- based meat alternatives may be of further interest as the projected displacement of saturated fat was with mono-unsaturated fatty acids more so than carbohydrate (40-42). Swapping SFA, for MUFAs and PUFAs is considered optimal for heart health (43).

The RNI for protein is greatly exceeded by most UK consumers pursuing an omnivorous, energy balanced diet. However, in our models, the projected reduction in total protein intake from a 100% replacement of meat was equivalent to almost 20 grams per day if alternative foods were drawn exclusively from the vegetable- or nut-based product categories. These losses have the potential to compromise protein status in athletes, in the very young, in the aged and in certain disease states where requirements may be above the RNI. Where protein intake is a concern, I would recommend choosing products from the higher protein, tofu-, soy- and mycoprotein-based meat-alternative categories.

Dietary patterns high in fibre are associated with improved satiety, reduced risk of obesity, improved metabolic health, and reduced risk of diverticular disease, cancer, cardiovascular disease and

hypertension (44, 45); the UK dietary guidelines recommend that adults consume upwards of 30g of AOAC fibre per day (33, 37). Despite consistent public health campaigns over many years, dietary fibre consumption amongst the UK public remains significantly below this recommendation (46). Using a refined NDNS cohort, incorporating meat eaters only, I noted that current mean fibre intake is 21.86g day^{-1}. In our models, under every meat-replacement scenario, projected fibre intake was shown to increase significantly compared to current intake for the total population. Importantly, the models showing a 100% replacement of meat with meat alternatives would increase the mean projected fibre intake, at a population level, to 30 g day^{-1} (**Table 7.4.**). I noted considerable differences in fibre content between, and within, our meat alternative product categories, with the highest projected increase in fibre intake being associated with vegetable, nut, and mycoprotein based products. Not all fibre elicits the same physiological benefits (47), and therefore it is important to note that fibre composition, and functional activity, is better described for soy-based (48) and mycoprotein-based (49) meat alternatives than it is the for the nut, vegetable and tofu categories of product.

Non-milk extrinsic sugar intake in the UK remains well above current guidelines which suggest limiting intake to no higher than 5% of total calories. In our meat eating NDNS cohort, sugar intake was closer to 20% of total calories. A high intake of sugars may be associated with obesity and poor dental health outcomes (50), therefore any intervention that raises sugar consumption must be viewed as problematic. I noted particularly high sugar levels in the nut-based and vegetable-based product categories and in the 100% meat displacement scenarios involving these two product categories, projected sugar intake rose to almost 22% of calories.

Due to its associations with hypertension, kidney disease and some cancers, dietary sodium reduction has been the goal of a multipronged and effective public health campaign over the last two decades (51). Intakes in the UK have decreased by clinically meaningful levels in recent years; in the meat eating NDNS cohort used in this analysis, mean sodium intake was reported as being very close to the current guidelines. Unfortunately, many of the meat alternative products were found to be high in sodium, and in the 100% meat replacement model, total projected sodium intake was increased by 0.23 grams. As with the other nutrients, there was considerable variation in the sodium content of foods from within and across alternative product categories; of note, soy-based meat alternatives were found to be particularly high in sodium, and a scenario with 100% meat displacement for soy-based products was projected to raise sodium intake by almost 0.5 grams day^{-1}. I caution that our displacement models can't fully account for salt added at the table or in the preparation of meat dishes.

Iron intake amongst girls and women in the UK is marginal with the mean intakes for both girls aged 11 to 18 years, and women aged 19 to 64 years below the RNI (56% and 76% of the RNI respectively), and further, 49% of girls aged 11 to 18 years and 25% of women aged 19 to 64 years have iron intakes

below the lower RNI (52). There is also evidence of both iron-deficiency anaemia (as indicated by low haemoglobin levels) and low iron stores (plasma ferritin) in 9% of older girls, 5% of adult women and 2% of older women (52). Replacing meat with meat alternative products did not significantly reduce projected total iron intake; in fact, replacement with either tofu, or with iron-fortified meat alternatives, was associated with a significant increase in iron intake. Therefore, if meat alternatives are integrated into the dietary habits of the UK population, fortification of these foods with iron could facilitate widespread attainment of RNI's.

In the UK population, vitamin B12 deficiency is rarely due to poor dietary intake, but it can be induced by a poorly managed vegan diet (54), and thus B12 intake should be considered in any public health approaches to reducing animal product intake. In our model scenarios I observed slight reductions in B12, approaching statistical significance with a total replacement of meat, and particularly evident when the replacement products were from the nut and tofu product categories. This reduction in projected B12 intake was largely offset in the model with fortified alternatives. In our models, the projected intakes of B12 following meat replacement remained significantly above the RNI, and therefore might be deemed of limited public health consequence. However, I caution that I did not remove milk, dairy, or eggs from our models, and I would expect further reductions in B12 under those scenarios. For individuals pursuing a vegan diet, it remains sensible to choose fortified products and to consider B12 supplementation.

To our knowledge, this is the first study to consider the projected impact of this important, relatively novel, food category at a population level in the UK. The strengths of this work are that I leverage the National Dietary Nutrition Survey dataset; this is a very well developed and informative record of food habits in the United Kingdom. I have also captured a broad picture of the nutritional quality of meat alternative products from within the UK market in 2020.

The limitations of this study are that; I was reliant upon self-reported dietary intake data from the NDNS. The 4 day food record collection tool used in the NDNS can change habitual diet, and the heavy respondent work load may precipitate socioeconomic representation bias in the cohort. I have also made subjective decisions in approaching the analysis; most notably, I chose to keep the energy content of the substitution model's constant by increasing the nutritional intake uniformly across dataset after the substitution. The energy adjustment approach is consisted with previous literature evaluating the impacts of dietary substitutions (55, 56). However, whilst I assume that individuals will maintain energy balance in a meat replacement scenario, but I can't be certain which foods they are likely to adjust their intake of, and this could affect overall projected nutritional intake. I was further limited by the depth of nutritional data and relatively poor characterisation of many of the meat alternatives. Across the product categories, mycoprotein and soy-based products were generally well characterised, but there was a

paucity of B vitamin and mineral data for many of the product categories, which has been noted previously (35). This meant that I was limited in the nutrients that I was able to assess. Data pertaining to the nutritional profile of meat alternatives within the McCance and Widdowson dataset is scarce and there is a need for greater analytical work to be done to update and formulate databases for these foods. If meat alternatives are to be regularly consumed as part of the diet in place of meat, publication of a more comprehensive characterisation their nutritional composition would be welcome.

There are implications arising from this work for consumers, producers, and food policy makers (**Table 7.6.**). For food consumers, it is clear there are many nutritious approaches to reducing meat intake using meat-alternatives. I note, the variable density of different nutrients across meat substitute categories as summarised in **Table 7.7.**, none of these substitutes fully replicates the nutritional composition of meat. If choosing to use meat alternatives, I therefore recommend using a variety of products from across the meat-substitute categories. I also recommend choosing products high in protein, high in fibre, and low in saturated fat and sugar; where possible, I would advise choosing products that are good sources of iron and vitamin B12. Producers of meat alternatives could better serve consumers with stronger nutritional labelling and by focussing on sodium and sugar reduction. Producers might also consider voluntary fortification with iron and B12. Food policy makers might consider regulation around fortification, marketing and labelling to better guide consumer choice.

Reducing meat consumption will be a focus of public health and ecological food policy for the foreseeable future. It is therefore anticipated that meat alternatives will play an increasingly prominent role in the UK food plate. Consumer confidence in the new and dynamic meat alternative market will be an important aspect in facilitating the level of dietary change required to protect planetary and consumer health. Going forward, periodic monitoring of the nutritional quality and healthfulness of meat alternatives will be necessary to ensure these foods compare well on a nutritional playing field, and that nutrient analytical databases are kept up to date with this information. This will then aid better quality research and the compilation of more accurate recommendations that relate to public health policy.

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Table 7.6. Implications and recommendations

i/ Food consumers	Consumers choosing to reduce their meat intake should understand that meat alternative products are a culinary replacement for meat, which differ in their nutritional profile. With the exception of tofu and mycoprotein, I caution that few meat alternative products have been well evaluated for their physiological effects in the consumer. I would therefore strongly recommend selecting meat alternatives based on the level of nutritional characterisation and the availability of high-quality evidence about their healthfulness. It is also important to consider the wider balance of the diet to ensure that the intake of nutrients found in meat are not compromised when reducing intake.
ii/ Producers	Producers of meat alternatives should be encouraged to focus on sodium reduction, deeper nutritional characterisation, and where feasible, investigations evidencing the likely health benefits of their products. Given their market, voluntary product fortification with iron and B12 might also be considered.
iii/ Policy makers	Meat alternatives are intended to displace a nutritionally dense component of the diet, better labelling and nutritional characterisation would be in the interest of the food consumer. Further, in 1960 policy makers in the UK responded to the displacement of butter and animal fats from the diet with legislation instructing the mandatory fortification of margarines with vitamins A and D, there may soon be a need for a discussion about fortification, and or the regulation of nutritional quality and the healthfulness of this category of food; this discussion would be better informed by appropriate dietary intervention studies.

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Table 7.7. Nutritional comparisons across meat substitute product category.

Ingredient Model	Carbohydrate g/day	Protein g/day	Fat g/day	Fibre g/day	Sugars g/day	Saturated fat g/day	Sodium mg/day	Iron mg/day	B12 ug/day
Vegetable	↑	↓	↓	↑	↑	↓	↑	-	-
Mycoprotein	↑	↓	↓	↑	+	↓	↑	-	-
Legume	↑	↓	↓	↑	+	↓	↑	-	-
Tofu	↑	↓	-	↑	+	↓	↑	↑	↓
Nut	↑	↓	+	↑	↑	↓	↓	-	↓
Soy	↑	↓	↓	↑	+	↓	↑	-	↓

+, non-significant increase compared to current intake; -, non-significant decrease from current intake. ↑, significant increase compared to current intake; ↓, significant decrease from current intake. Green indicates best performing model with regards to dietary recommendations for nutrient. Red indicates worst performing model.

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CHAPTER VIII

GENERAL DISCUSSION

I have divided this general discussion into two parts. In the first, I reflect on the value of this project to my development as an aspiring academic/scientist. In the second part, I reflect on the success and failures of the project as a whole; I consider how this work has moved our understanding of meat, mycoprotein and health forward, and I look at where I feel this study field needs to go next.

8.1. Self-reflection

8.1.1. Skills I have attained

I came into this project with minimal experience in the lab; I have developed skills in experimental design, polymerase chain reaction (PCR), faecal DNA extraction, 16S rRNA microbial community analysis via Illumina Sequencing, liquid chromatography–mass spectrometry (LC MS), gas chromatography–mass spectrometry (GC MS), specific gravity normalisation, flame photometry, nitric oxide analyser, Randox Daytona clinical analyser, tissue culture, enzyme-linked immunosorbent assay (ELISA) and microscopy. These skills and the knowledge and understanding of these techniques will without doubt aid in future research endeavours.

I have acquired strong project management skills, predominantly through the design, planning and coordination of the human dietary intervention. This was a tremendous experience where I learnt new skills as well as developed pre-existing ones in the areas of organisation, time management, delegation, and leadership. I believe these skills are transferable to other fields as well as nutrition research and are highly important to have developed and to hone as I continue in my career.

I believe the skills I have established in statistical analysis and bioinformatics are most notable on a personal level. Coming from a non-scientific background, statistical methodologies were quite alien and an area of weakness during my MSc. However, identifying this as a weakness, I spent extra hours reading around the subject into the statistical methodologies and the underpinning mathematical frameworks. I also took the time to learn and develop skills in programming language, and completed all the statistics involved in the project, including complex bioinformatic pipelines and the analysis of large metagenomic and metabolomic datasets.

8.1.2. Research skills I plan to develop further

The process of designing, planning and preparing the human dietary intervention was an aspect of the overall project which I found valuable towards my own self development. There are many skills involved in the implementation of a clinical trial which are not only important in the context of research but are also advantageous across many disciplines due to their transferrable nature. Data analysis was an element of research I was not expecting to acclimatise to, as mentioned previously, it was an area of weakness. However, I became very capable in the process of interpreting complex datasets acquired from metagenomic and metabolomic platforms, and translating this large volume of metrics into tangible results.
An aspect of research which I have a great interest for is dissemination; both written, producing papers to the standard of peer review journals, as well as orally, presenting research findings at conferences and meetings, which I have been able to do on a number of occasions. I believe dissemination is pivotal in research, the aim of research is to make new discoveries and advance knowledge, communicating new findings enables the field to move on and create avenues for future research.

Going forward, I will focus on implementing and honing these skills further as I progress in future research and academic endeavours.

8.2. Reflections on the scientific merit of this thesis

8.2.1. Realisations of thesis objectives

The primary objective of this work was to elucidate the impact of substituting red and processed meat with the meat alternative mycoprotein on markers of gut health. This objective has been realised, in that we now have a better understanding of the implications for gut health of substituting red and processed meat with the meat alternative mycoprotein. The original hypothesis postulated that this dietary substitution would elicit positive influences, grounded on both the high fibre content, as well as type of fibre in mycoprotein; a glucan/chitin complex. In this thesis, I have shown that mycoprotein consumption reduced the genotoxic load in the gut, reduced faecal genotoxin concentrations and increased the abundance of beneficial microbial genera.

Work on this project was interrupted by the coronavirus pandemic, with national lockdowns, facility shutdowns, and government shielding requirements, and some of the originally planned analyses have not yet been completed. These analyses included:

i) Urinary 8-hydroxy-2-deoxyguanosine (8-OHdG). Considered a biomarker of oxidative damage to DNA in the host (1).

ii) Quantified measurement of p-cresol, phenol and indole in stool. These metabolites are predominantly produced from animal protein and have toxic potential in the gut (2).

iii) Plasma micro RNA (miRNA). The area of miRNA study has been building over recent decades, with associations between many disease states, including CRC (3). The measurement of miRNA levels in plasma would be novel with respect to the mechanisms behind the strong associations between meat intake and CRC.

Given more time, I would hope to better quantify a wider array of putative toxins in stool, such as phenol, indole and p-cresol as predictors of the toxic load in the gut. The intervention found reduced faecal water genotoxicity as well as reduced faecal NOCs from mycoprotein consumption relative to both the meat diet and baseline.

In addition, the human intervention study identified significant changes in the gut microbiota, including fluctuations in genera associated with health and disease such as *Roseburia*, *Faecalibacterium*, *Lactobacillus*, *Akkermansia*, *Allistipes* and *Oscillibacter*. 16S rRNA sequencing was employed to characterise these shifts in microbial communities. While this methodology allows a large coverage of microbial populations, it is limited in the depth of coverage (4). The addition of a secondary analysis such as fluorescence in situ hybridization (FISH) or quantitative polymerase chain reaction (qPCR) may have enabled a deeper characterisation of changes down the level of species. With regards to dietary intake data, a single day food record was used, however, a more detailed 3-day record may have developed a better understanding of participants dietary intake (5). Although included as a minor endpoint, a fuller understanding of the volunteers' habitual diet might have enabled some interrogation of the effect of habitual diet on the study outcomes.

I have however been able to deliver some extra science which was not originally planned; notably, I measured a number of secondary endpoints in the human intervention study related to cardiometabolic health. I observed a significant reduction in total blood cholesterol elicited by the mycoprotein diet, which was also observed in low density lipoprotein (LDL) fractions. I also found reductions in both systolic and diastolic blood pressure after the mycoprotein diet. These changes in blood pressure were not linked to salt intake, indeed urinary sodium excretion also increased following the mycoprotein diet, this deserves further investigation. Furthermore, the fourth experimental chapter considered the nutritional implications of replacing meat with meat alternatives in the UK population. This work was not originally planned but arose to mitigate the problems with accessing the lab during a period of national lockdown. To my knowledge, modelling the potential impact of meat alternatives on nutritional intake in the UK had not been reported previously.

8.2.2. Relevance of work for both research and society

Given recommendations to eat less meat (6), in conjunction with an increased availability and uptake of meat alternatives (7), my thesis is timely. The novelty as a food group means there is a dearth of evidence on the health effects of meat alternatives, thus, the present thesis has begun to address these knowledge gaps.

The human intervention showed that mycoprotein consumption reduces the genotoxic load in the gut relative to both a high meat diet, as well as the participants habitual diet (baseline). Whilst the effects of high meat consumption on intestinal genotoxicity are well established, it had not been previously demonstrated that mycoprotein might be independently protective in this context.

The complex fibre fraction of mycoprotein is of particular interest and perhaps underpins the effects of mycoprotein consumption on the gut microbiota *in vivo* – again this had not been previously reported in a dietary intervention context. I reported increases in the abundance of butyrate producing and mucin degrading genera that are associated with improved gut health. As a result of my work, the wider team have begun exploring the utilisation of the various fibre fractions of mycoprotein *in vitro* and the effects on the activity of the microbial community and exploring the relevant Carbohydrate-Active enZYmes (CAZyme) linked pathways.

The human intervention was also the first report on the effects of mycoprotein consumption on the urinary and faecal metabolome. Changes were observed in both microbial and host cell metabolism in response to the different diets. The observed increase in sphingolipid metabolism associated with the meat diet is of particular interest due to the roles of sphingolipid metabolites as signalling molecules in pathways including the regulation of cell cycle kinetics and the DNA damage response (8, 9). To my knowledge, this is a novel finding with respect to mechanisms underlying the relationship between meat consumption and CRC risk, and thus, may warrant further investigation.

Additional questions have been raised about the potential implications of consuming mycoprotein for cardiovascular health. Both total and LDL cholesterol were significantly lower after a mycoprotein diet relative to both a habitual diet and a high meat diet. Although cholesterol reductions have been reported previously for mycoprotein, these were studies which investigated mycoprotein either in an isolated form, a cookie matrix, or as wholefoods under strictly controlled dietary regimens (10-12). In my intervention, controls over the habitual diet of the volunteers during the intervention period were limited to the supplied 240g/day meat and/or mycoprotein. Therefore, it could be argued my study confirms this benefit of mycoprotein in an otherwise free-living population. My intervention also reported non statistically significant, but clinically meaningful reductions of 2.41mmHg and 0.80mmHg in mean systolic and diastolic blood pressure respectively following the mycoprotein diet. Previous dietary intervention studies with mycoprotein have not reported changes in blood pressure. I stress that this

intervention was in metabolically healthy volunteers with normal blood pressure, and I would invite future investigators to consider replicating these studies in more metabolically challenged cohorts.

The changes in the gut microbiota were notable and stimulate questions regarding the interplay between species when degrading mycoprotein. These include reasoning for an increase in *Akkermansia* following the mycoprotein diet when this genus primarily degrades mucins, as well as the role of *Lactobacillus*, a well known probiotic; I believe further study of the fermentative components of the fusarium cell wall will confirm its potential as a prebiotic. Other questions relate to the interplay between *Roseburia*, *Ruminococcus* and *Faecalibacterium*, all understood to be predominant SCFA producers, yet *Faecalibacterium* was reduced relative to *Roseburia* and *Ruminococcus* following the mycoprotein diet. It may be that mycoprotein favours certain polysaccharide degraders over others. The fungal glucan/chitin complex is amongst the most abundant sources of fibre on the planet, which has potential to be utilised in many industrial applications (13). Demonstrating its potential as a prebiotic is of significant commercial interest, particularly to a society under-consuming dietary fibre (14).

Future investigations could complement findings in the present thesis with regards to cohort characteristics and study food quantity. Studies might consider using a larger, mixed gender cohort, to strengthen the findings found in the male cohort recruited. Additionally, large quantities of both foods (240g/day) were prescribed, representing the upper quintile of meat intake in the UK. Importantly, beneficial changes relative to baseline were noted for mycoprotein suggesting that it is a healthy alternative to more regular levels of meat intake. It should, however, need to be considered whether these same benefits are achievable on lower regular consumption of mycoprotein and whether mycoprotein might be beneficial in comparison to a traditional wholefood vegetarian diet.

The modelling analysis found reductions in iron and B12 intake, while increases were found in sugar and salt, if dietary meat was replaced with an assortment of meat alternatives in the UK. Recommendations from that study were geared towards the public, regulators and the food industry, the paper findings suggest public discussion about mandatory fortification and encouragement for the food industry to focus on sugar and salt reduction in the products. I feel this work is socially important, the publication arising from this chapter has already accrued citations.

8.3. Directions for future research

8.3.1. Cardiovascular health

Mycoprotein has previously been submitted for a substantiated health claim under the European Food Safety Authority (EFSA) for maintenance of normal blood LDL-cholesterol concentrations, which was rejected (15). Although cholesterol reduction has now been reported consistently in several dietary intervention studies, more nuanced research could be considered to revisit this claim. The above submission was rejected on grounds that the cause and effect relationship has not been established for mycoprotein beyond what could be expected from the beta-glucan content; which already has a health claim in place for maintenance of normal blood LDL-cholesterol concentrations (16). This is based on oat and barley beta-glucan, which is structurally different from that of the mycoprotein fungal beta-glucan. To address this gap in mechanistic understanding for a health claim, dietary interventions comparing the extracted beta-glucan from mycoprotein with that of oat/barley would confirm whether the structural difference in fibre type elicits differential effects.
EFSA specifies scientific evidence must demonstrate the study group in which the claimed effect is assessed is representative of the target audience, while the dose and pattern of consumption must be attainable as part of a balanced diet (17). Therefore, interventions should involve large cohorts representative of the general population. Additionally, doses prescribed ought to be based on population consumption data. Furthermore, these interventions would require valid control comparisons to generate robust conclusions. This would entail matching intervention foods/diets for fibre and beta-glucan content; previous work observing cholesterol reductions did not have comparable fibre and beta-glucan intake between groups (including the intervention in the present thesis). This would clarify the effects are not a result of increased fibre/beta-glucan intake, but specifically mycoproteins fibre/beta-glucan.
In addition, a systematic review and meta-analysis may now be timely given the growing evidence of effect.

8.3.2. Gut health

Turning attention to gut health, to my knowledge, my study is the first to report gut microbial changes from mycoprotein consumption *in vivo*. Further, my work demonstrated an enrichment in the genus *Lactobacilli* following chronic mycoprotein consumption. On the theme of EFSA health claims, future work could be considered to evidence a 'prebiotic' claim for mycoprotein.

With respect to the *Lactobacilli* increase, to make a prebiotic claim, the microbial strain would be necessary. Although genera can comprise beneficial microorganisms, there can be pathogens within a genus, an example being *Bifidobacterium dentium*, associated with dental caries, in the *Bifidobacterium* genus. Taking this into account, deeper characterisation of the specific species and strains of

Lactobacilli in the study stool samples could be considered via methodology such as shotgun sequencing. In addition, the same could be applied to *in vitro* work in the wider team.

Furthermore, increasing the numbers of any microbial groups, such as *Lactobacilli*, is not established as a beneficial physiological effect, but must be in conjunction with an established effect. Examples include improvements in digestion and absorption of nutrients. Reductions in stool pH and changes in SCFA production are not considered beneficial physiological effects, but may provide evidence on the mechanisms and the biological plausibility of a prebiotic claim (18, 19).

This could be addressed by new investigations underway by the wider team which will be exploring the effects of the fungal cell wall constituents of *Fusarium venanatum* in gut immunity, including immune reactivity and training and the effect of this on inflammation, mediating small intestinal gut barrier integrity, as well as the role of gut microbial fermentation products on the large bowel gut barrier. Subsequently, a human intervention will be implemented, with potential primary outcomes including improvements in gut barrier function as well as suppression of infections (e.g common cold). These endpoints, together with deeper microbial characterisation and related markers (i.e., stool pH, SCFA production) would help move closer to a prebiotic claim for mycoprotein as a food.

8.4. Concluding remarks

The present thesis helps establish mycoprotein as a healthy alternative to meat, it suggests that it may in future be considered as a functional food which can elicit prebiotic influences and benefits for both gut and systemic health. It has begun to address certain gaps in knowledge and has created avenues for compelling research going forward.

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APPENDIX A

Metabolites of variable importance in projection (VIP) within and between study phases

Table 1. VIP faecal metabolites within study phases[1,2]

Mycoprotein		
Metabolite	**VIP**	**Fold Change**
C7 H17 N O2.1	5.54	4.42
C22 H38 N4 O7	5.20	4.15
leucyl-4-hydroxyproline	4.12	3.28
C15 H26 N2 O5	3.75	2.99
C14 H24 N2 O5	3.64	2.91
N-{3-[(4-Acetamidobutyl)amino]propyl-acetamide	2.92	2.33
Acetylcadaverine	2.77	2.21
C12 H25 N3 O2	2.76	2.20
Ile-val	2.63	-2.10
C16 H29 N O3	2.59	2.07
C10 H29 N6 O4 P	2.46	1.96
C25 H36 N8 O4	2.43	1.94
C13 H26 N9 O7 P	2.42	1.93
C20 H42 O11	2.39	1.91
C19 H37 N5 O3 S	2.39	-1.90
EJ4025000	2.36	1.88
C20 H39 N O2 P2	2.33	1.86
C79 H124 N4 O15 P2	2.32	1.85
N-Acetylputrescine	2.29	1.83
C75 H116 N4 O13 P2	2.29	1.83
C24 H44 N4 O4	2.27	1.81
N-[(2Z)-2-Hydroxy-2-octenoyl]glycine	2.24	1.79
C63 H111 N8 O18 P	2.24	1.79
C20 H52 N10 O11	2.21	1.77
L(+)-Ornithine	2.21	1.76
N-Acetylhistamine	2.19	1.75
C77 H120 N4 O14 P2	2.18	1.74
C79 H137 N8 O17 P3	2.18	1.74
C77 H133 N8 O16 P3	2.18	1.74
C35 H42 N8 O4	2.15	-1.71

Table 1 (continued)

C9 H20 N2 O	2.15	1.71
C21 H41 N5	2.15	-1.71
C17 H46 N9 O4 P S	2.09	-1.66
C17 H36 N2 O2	2.06	-1.64
C60 H128 N9 O18 P3 S	2.06	1.64
Thiamine	2.05	1.64
C24 H54 N7 O3 P3	1.97	-1.58
DL-Stachydrine	1.97	1.57
C30 H55 N O7 P2	1.94	1.55
C59 H103 N8 O16 P	1.94	1.54
4-Hydroxyprolylleucine	1.93	-1.54
C13 H28 N2 O2	1.92	-1.54
V557	1.92	1.53
C13 H27 N3 O2	1.92	1.53
C89 H135 N6 O18 P	1.89	1.51
C57 H99 N8 O15 P	1.88	1.50
V354	1.88	1.50
C24 H49 N O13	1.87	1.49
C33 H68 N6 O13	1.87	1.49
C32 H60 N2 O14	1.86	1.48
N-(5-acetamidopentyl)acetamide	1.85	1.47
V498	1.82	1.45
V510	1.81	1.44
Caprolactam.1	1.78	1.42
C18 H37 N4 O5 P	1.77	1.41
C44 H86 N2 O14 P2	1.77	1.41
C19 H50 N9 O4 P S	1.76	-1.41
C8 H16 N2 O2	1.76	1.40
C23 H45 N5 O7	1.76	1.40
8-Azabicyclo[3.2.1]octane-1,2,4,7-tetrol.1	1.75	1.39
C41 H78 N6 O8 P2	1.70	1.35
V490	1.69	1.35
C24 H59 N8 O6 P3	1.66	1.32
C39 H70 N6 O7 P2	1.66	1.32

Table 1 (continued)

C40 H75 N O12 P2	1.65	1.31
C8 H20 N2	1.63	1.30
C16 H28 N2 O5	1.62	-1.30
C11 H24 N2 O2	1.61	-1.28
DL-Carnitine	1.61	1.28
V24	1.60	-1.28
C42 H82 N2 O13 P2	1.60	1.28
C14 H20 N6 O4	1.60	-1.27
C23 H46 N3 O2 P S	1.60	1.27
C38 H71 N O11 P2	1.60	1.27
V580	1.59	1.27
Gamma-Aminobutyric acid (GABA)	1.59	1.27
C22 H45 N O12	1.58	1.26
HMBA	1.57	1.26
C23 H45 N5 O4 S	1.57	-1.25
2-Dimethylamino-5,6-dimethyl-4-hydroxypyrimidine	1.57	1.25
1,3-Dipropylxanthine	1.56	1.25
C30 H59 N O3 P2 S	1.56	-1.24
C15 H32 N2 O2	1.56	-1.24
C16 H27 N3 O7	1.55	-1.24
C36 H71 N O5 P2	1.55	-1.24
C23 H37 N O8	1.54	-1.23
C9 H19 N2 O6 P	1.54	1.23
N-4-hydroxyphenylacetylglutamic acid	1.53	-1.22
C23 H45 N5 O	1.52	-1.21
4-Amino-DL-phenylalanine	1.51	-1.20
Valyl-4-hydroxyproline	1.50	-1.20
C9 H16 N4 O2	1.50	-1.20

Meat

C19 H36 N4 O6	3.07	-1.48
4-Hydroxyprolylleucine	2.57	1.24
C12 H26 N2 O4	2.48	1.20

Table 1 (continued)

C16 H34 N2 O4	2.43	1.17
C24 H43 N O5	2.38	1.15
C87 H157 N8 O15 P3 S2	2.38	1.15
C17 H29 N6 O3 P	2.38	-1.15
C9 H20 N2 O	2.30	1.11
Metalaxyl	2.26	-1.09
N-Butylscopolamine	2.24	-1.08
C7 H12 N4 O2	2.19	1.06
C18 H37 N4 O5 P	2.15	1.04
C77 H120 N4 O14 P2	2.13	1.03
C30 H53 N S	2.12	-1.02
Acetylcadaverine	2.11	1.02
C30 H63 N7 O2	2.10	-1.01
C21 H42 N6 O6	2.09	1.01
C75 H116 N4 O13 P2	2.09	1.01
C7 H12 N2 O2.1	2.08	-1.00
C79 H137 N8 O17 P3	2.07	1.00
Normorphine	2.06	-0.99
tybamate	2.03	0.98
C39 H70 N6 O7 P2	2.02	0.97
C24 H44 N4 O4	2.01	0.97
C54 H73 N4 O10 P S2	1.99	0.96
C31 H65 N7 O2	1.99	-0.96
C60 H128 N9 O18 P3 S	1.98	0.95
3-aza-?-homocholest-4?-en-4-one	1.97	-0.95
C6 H17 N2 O3 P	1.97	0.95
C77 H133 N8 O16 P3	1.97	0.95
CERAMIDE 3	1.96	-0.94
C61 H132 N9 O18 P3 S	1.94	0.94
C89 H135 N6 O18 P	1.94	0.93
C87 H148 N9 O16 P3 S2	1.93	0.93
Armillaramide	1.91	-0.92
V490	1.90	0.92
V538	1.90	0.92

Table 1 (continued)

Ile-val	1.90	0.92
V510	1.89	0.91
C35 H71 N O4	1.89	-0.91
V498	1.89	0.91
Ceramide (d18:1/18:0)	1.88	-0.91
C40 H75 N O12 P2	1.88	0.91
V580	1.88	0.91
C76 H120 N4 O13 P2	1.86	0.90
P-DMEA	1.84	0.89
C43 H75 N8 O4 P	1.83	0.88
V594	1.82	0.88
ile-ala	1.82	0.87
C24 H59 N8 O6 P3	1.81	0.87
C37 H54 N7 O3 P	1.81	0.87
C13 H26 N2 O5	1.79	0.86
12,13-Epoxytrichothec-9-ene-4,7-diol	1.79	-0.86
C35 H75 N7 O P2	1.79	-0.86
C63 H111 N8 O18 P	1.78	0.86
C34 H61 N5 O2 S	1.78	-0.86
C17 H46 N9 O4 P S	1.78	-0.86
3beta-hydroxy-4beta-methyl-5alpha-cholest-7-ene-4alpha-carboxylic acid	1.78	-0.86
C78 H101 N3 O14	1.78	0.86
V354	1.77	0.85
C15 H32 N2 O2	1.76	-0.85
C13 H28 N2 O2	1.76	-0.85
Prolylleucine.1	1.75	0.85
Caprolactam.1	1.75	0.84
C89 H161 N8 O16 P3 S2	1.75	0.84
C23 H45 N5 O	1.74	-0.84
C18 H38 O5	1.73	-0.84
C30 H68 N5 O8 P3	1.72	0.83
4-methylpyridine-3-sulfonic acid	1.72	-0.83
Iprovalicarb.1	1.72	-0.83

Table 1 (continued)

Supinine	1.72	-0.83
C13 H20 N4 O5.1	1.70	-0.82
C35 H42 N8 O4	1.69	-0.82
C27 H44	1.69	-0.82
C38 H71 N7 O4	1.67	-0.81
EJ4025000	1.67	0.80
C45 H90 O18 P2 S3	1.67	0.80
1,3,4-Trihydroxy-8-octadecen-2-yl]octadecanimidic acid	1.66	-0.80
C59 H103 N8 O16 P	1.65	0.80
C88 H181 N10 O16 P3 S5	1.63	0.78
C16-Dihydroceramide	1.63	-0.78
Pheophytin a5	1.62	-0.78
C57 H99 N8 O15 P	1.62	0.78
C42 H75 N O5	1.61	-0.78
V671	1.61	0.78
C38 H71 N O11 P2	1.61	0.78
C80 H128 N4 O15 P2	1.60	0.77
Guanine	1.60	-0.77
V648	1.59	0.77
C17 H36 N2 O2	1.59	-0.77
dinotefuran	1.59	0.76
V448	1.58	0.76
C44 H86 N2 O14 P2	1.56	0.75
C89 H149 N8 O15 P3 S	1.56	0.75
C46 H101 N10 O13 P3	1.56	0.75
C90 H140 N8 O16 P2	1.55	0.75
C10 H16 N6 O2	1.55	0.75
C18 H45 N7 O5	1.55	-0.75
C67 H123 N7 O17 P2 S2	1.55	0.75
C17 H29 N5 O7	1.55	-0.75
leucyl-4-hydroxyproline	1.55	-0.75
C20 H46 N9 O11 P	1.55	-0.74
C8 H16 N2 O	1.53	-0.74
C21 H41 N5 O	1.53	-0.74

Table 1 (continued)

C33 H64 N6 O13	1.53	0.74
C23 H33 N5 O4	1.53	0.74
2,6-Dimethoxyphenol	1.52	-0.73

[1]VIP threshold set at >1.5

[2]Unidentified metabolites presented as chemical formula (i.e., C8 H16 N2 0)

VIP, Variable importance in projection

Table 2. VIP faecal metabolites between study phases[1,2]

Metabolite	VIP	Fold Change (Mycoprotein/Meat)
C22 H38 N4 O7	6.74	4.50
C7 H17 N O2.1	6.39	4.26
C15 H26 N2 O5	5.14	3.43
leucyl-4-hydroxyproline	5.10	3.40
C14 H24 N2 O5	5.01	3.34
C16 H29 N O3	3.87	2.58
4-Hydroxyprolylleucine	3.52	-2.34
C10 H29 N6 O4 P	3.43	2.29
C25 H36 N8 O4	3.33	2.22
N-[(2Z)-2-Hydroxy-2-octenoyl]glycine	3.16	2.11
Ile-val	3.00	-2.00
Asparaginyl-4-hydroxyproline	2.91	-1.94
C8 H16 N2 O.1	2.87	-1.92
C32 H60 N2 O14	2.84	1.90
C13 H26 N9 O7 P	2.82	1.88
C32 H44 N2 O10	2.75	1.83
N~6~,N~6~-Dimethyllysine	2.70	-1.80
dinotefuran	2.65	-1.77
C7 H12 N4 O2	2.57	-1.72
C9 H19 N2 O6 P	2.51	1.67
Isoputreanine	2.42	-1.62
Glycylglutamine	2.41	-1.60
L(+)-Ornithine	2.33	1.55
Gamma-Aminobutyric acid (GABA)	2.26	1.51
C16 H34 N2 O4	2.15	-1.43
C7 H14 N2 O	2.14	-1.43
4-[(3-Acetamidopropyl)amino]butanoic acid	2.14	-1.42
1,3-Dipropylxanthine	2.13	1.42
C12 H26 N2 O4	2.08	-1.39
Butenylcarnitine	2.06	1.37
C10 H15 N O3 S	2.04	1.36
C33 H54 N8 O9 P2	2.02	1.35

Table 2 (continued)

P-DMEA	2.00	-1.34
(2S)-2-Amino-8-hydroxyoctanoic acid.1	1.98	-1.32
C6 H7 N3	1.93	1.29
Sorbic acid	1.90	1.27
Valyl-4-hydroxyproline	1.88	-1.25
Thiamine	1.88	1.25
4-Methyl-5-thiazoleethanol	1.86	1.24
ISOXYL.1	1.85	1.24
Allysine	1.81	-1.21
C10 H18 N4 O3	1.80	-1.20
porfiromycin	1.79	-1.19
C89 H135 N6 O18 P	1.75	1.17
C28 H29 N5 O6	1.75	1.17
C20 H46 N5 O7 P	1.67	-1.11
C9 H18 N4 O	1.65	-1.10
C89 H149 N8 O15 P3 S	1.63	1.09
C10 H16 N6 O2	1.62	-1.08
Homocarnosine	1.58	-1.05
Fusicoccin H	1.57	-1.05
DL-Atenolol	1.54	1.03
V594	1.54	1.03
C16 H27 N3 O7	1.53	-1.02
C35 H71 N O4	1.52	1.01
L-gamma-Glutamyl-L-leucine	1.50	-1.00

[1]VIP threshold set at >1.5.

[2]Unidentified metabolites presented as chemical formula (i.e., C8 H16 N2 0)

Positive fold change indicates greater Mycoprotein, negative fold change indicates greater Meat.

VIP, Variable importance in projection

5354

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Table 3. VIP urinary metabolites within study phases[1,2]

Mycoprotein		
Metabolite	**VIP**	**Fold Change**
balenine.1	5.56	-3.47
C8 H14 N6 O7	5.22	3.26
C11 H18 N2 O6	5.00	3.12
N(alpha)-gamma-L-glutamylhistamine	4.03	-2.52
C17 H31 N O3	3.98	2.49
C10 H21 N5 O3	3.98	-2.48
C9 H15 N3 O	3.59	-2.24
l-Histidinal	3.59	-2.24
C30 H51 N4 O2 P3	3.47	-2.17
L-Anserine	3.13	-1.96
Roxatidine	3.06	-1.91
val-his	3.01	-1.88
C14 H30 N2 O6	2.91	-1.82
C29 H42 N10 O4	2.81	-1.75
C22 H33 N5 O4	2.79	1.74
(5E)-7-methylidene-10-oxo-4-(propan-2-yl)undec-5-enoic acid	2.68	1.67
C7 H13 N3 O	2.66	1.66
gamma-thiomethyl glutamate	2.64	1.65
C34 H44 N8 O2	2.63	-1.64
C14 H16 N6 O6	2.63	1.64
C12 H21 N3 O4 S	2.61	1.63
Cyclamic acid	2.59	1.62
3-[2-[(E)-[3-(2-carboxyethyl)-5-[(4-ethyl-3-methyl-5-oxo-pyrrolidin-2-yl)methyl]-4-methyl-pyrrol-2-ylidene]methyl]-5-[(3-ethyl-4-methyl-5-oxo-pyrrolidin-2-yl)methyl]-4-methyl-1H-pyrrol-3-yl]propanoic acid	2.53	-1.58
C6 H17 N4 O8 P	2.49	1.56
C6 H7 N O2 S	2.41	1.50
2-(6,10-Dimethyl-8-oxospiro[4.5]dec-6-en-2-yl)-2-hydroxypropyl hexopyranoside	2.41	1.50
V489	2.26	-1.41
C10 H19 N5 O2	2.25	-1.41

Table 3 (continued)

C10 H18 N4 O3	2.25	-1.40
C11 H16 N4 O3 S	2.17	1.35
V488	2.15	-1.34
C14 H18 N2 O3 S2	2.07	1.29
C7 H25 N8 O3 P	2.04	1.28
UROBILIN, (-)-	2.02	-1.26
Propionylcarnitine	2.01	-1.25
C13 H20 N2 O5	1.98	-1.24
Acetylagmatine	1.98	-1.23
C5 H4 O7 S	1.97	1.23
Saccharin	1.96	1.22
N-cyclopropylammeline	1.95	-1.22
C10 H25 N6 O9 P S3.1	1.94	-1.21
V359	1.93	1.21
Xanthotoxol	1.93	1.20
C8 H16 N2 O2.1	1.92	-1.20
C10 H27 N4 O3 P3	1.89	1.18
C11 H20 N2 O7 S3	1.88	1.18
Miglitol	1.88	-1.17
(3S,6S)-3-(Hydroxymethyl)-6-{4-[(3-methyl-2-buten-1-yl)oxy]benzyl-2,5-piperazinedione	1.88	-1.17
5-Hydroxydihydro-2,4(1H,3H)-pyrimidinedione	1.86	-1.16
(-)-Galanthamine	1.85	1.15
C3 H2 Cl N2 O17 P3 S5	1.84	-1.15
D-(-)-Quinic acid	1.83	1.14
4-nitrobenzaldehyde N-(5-methyl-3-phenyl-2-thioxo-2,3-dihydro[1,3]thiazolo[4,5-d]pyrimidin-7-yl)hydrazone	1.81	1.13
Succinylacetone	1.79	1.12
C15 H16 N6 O10 S2	1.79	1.12
C20 H35 N O8	1.79	-1.12
lipoic acid	1.79	-1.11
TDIQ	1.78	-1.11
(1R,5S)-8-Thiabicyclo[3.2.1]octan-3-one	1.72	-1.08
C19 H24 O9 S2	1.72	-1.07

Table 3 (continued)

Phthalic acid	1.72	1.07
5-(2,3-Dihydroxy-3-methylbutyl)-4-[(3,3-dimethyl-2-oxiranyl)acetyl]-3,4-dihydroxy-2-(3-methylbutanoyl)-2-cyclopenten-1-one	1.71	1.07
C5 H10 N8 O S2	1.70	1.06
C4 H11 N O S	1.67	-1.04
2-Methyl-4,6-dinitrophenol	1.65	1.03
N-Acetyl-L-cysteine	1.64	1.02
N-[(2Z)-2-Hydroxy-2-octenoyl]glycine	1.64	1.02
Thiamine	1.64	1.02
N-Acetylleucylleucine	1.62	1.01
C14 H20 O13 S	1.61	1.00
Sorbic acid	1.60	1.00
N-Acetyl-S-{[4-(methylsulfinyl)butyl]carbamothioyl-cysteine	1.59	-0.99
C14 H26 N2 O5	1.59	0.99
C10 H18 N8 O3	1.59	0.99
C8 H16 N10 O4	1.59	0.99
Allantoic acid	1.58	-0.98
Levetiracetam	1.55	-0.97
C10 H13 N O5 S.1	1.55	0.97
3-hydroxyoctanoylcarnitine	1.54	-0.96
C12 H18 N4 O10	1.52	0.95
Meat		
C14 H30 N2 O6	6.27	2.78
Miglitol	3.75	1.66
C12 H25 O6 P3	3.44	1.52
C9 H15 N3 O	3.34	1.48
Propionylcarnitine	3.30	1.46
Ethyl-Î²-D-glucuronide	3.28	-1.45
C21 H31 N O9	3.23	1.43
TDIQ	3.21	1.42
Xanthotoxol	3.09	-1.37
diacetylphloroglucinol	3.08	1.36
(1R,3R,6R)-Axomadol	2.98	1.32

Table 3 (continued)

C5 H6 N6 O6 S	2.98	-1.32
Acetyl-Î²-methylcholine	2.93	1.30
9-Nitrooleate	2.90	1.28
Acetylcholine	2.89	1.28
C5 H16 N7 P	2.77	1.23
(3S,6S)-3-(Hydroxymethyl)-6-4-[(3-methyl-2-buten-1-yl)oxy]benzyl-2,5-piperazinedione	2.75	1.22
Acetyl-L-carnitine	2.71	1.20
Tetrahydro-5-oxo-2-furanacetic acid	2.67	1.18
C6 H12 N3 O3 P.1	2.65	-1.17
C10 H25 N6 O9 P S3.1	2.63	1.16
C29 H47 N O8 S	2.49	-1.10
C12 H32 N7 O12 P S4	2.47	1.09
C11 H17 Cl N6 P2 S	2.42	-1.07
C5 H10 N8 O S2	2.38	1.06
Guanadrel	2.36	-1.05
C10 H21 N5 O3	2.28	1.01
Saccharin	2.15	0.95
Butopyronoxyl	2.13	0.94
C8 H18 N5 O6 P S2	2.09	0.93
methoxyresorcinol	2.07	0.92
V1281	2.07	0.92
C22 H35 N O8	2.07	0.92
C4 H8 O4 S	2.05	-0.91
C10 H8 N10 O3	2.05	0.91
C22 H48 N9 O15 P S4	2.04	-0.90
C29 H42 N10 O4	2.01	0.89
C19 H24 O10 S2	1.97	-0.87
C20 H42 N5 O2 P	1.97	-0.87
C13 H22 N9 O P S	1.97	-0.87
4-Ethyl-2-methoxyphenyl hydrogen sulfate	1.96	0.87
Taurine	1.93	0.86
C13 H20 N2 O5	1.91	0.85
C14 H14 N8 O6	1.88	-0.83

Table 3 (continued)

C4 H8 N2 O S	1.86	0.82
C11 H14 N6 O7	1.83	-0.81
9-Decenoylcarnitine	1.83	0.81
balenine.1	1.83	0.81
Mevalonic acid	1.82	-0.81
Carbimazole	1.80	0.80
C34 H44 N8 O2	1.77	0.79
C11 H26 N2 O3	1.77	-0.78
C17 H23 N O7	1.76	0.78
l-Histidinal	1.75	0.78
C22 H31 N O11	1.75	0.78
C11 H20 N2 O7 S3	1.74	0.77
C17 H25 N4 O2 P3 S2	1.73	-0.76
Taurine.1	1.73	0.76
2-Hexenoylcarnitine	1.71	0.76
C12 H14 O12 S	1.68	0.75
Ankorine	1.68	0.74
C8 H7 N O5 S	1.68	-0.74
Gentiopicrin	1.67	-0.74
C12 H20 Cl3 N O11	1.67	-0.74
C11 H34 N9 O3 P	1.64	0.73
Piperine	1.64	0.73
C21 H31 N O9.1	1.64	0.72
C10 H19 N5 O2	1.60	0.71
C16 H31 N P2	1.57	0.70
2-methylbutyrylcarnitine	1.57	0.69
C18 H36 O11	1.56	0.69
C12 H22 N6 O5	1.56	-0.69
C10 H12 N6 O8	1.56	0.69
C13 H22 N4 O2 S	1.56	0.69
Allysine.2	1.54	-0.68
C12 H33 N7 O7	1.52	0.67
C11 H13 N7 O6	1.51	-0.67
(+/-)-Camphoric acid	1.50	0.67

Table 3 (continued)

C10 H25 N6 O9 P S3	1.50	0.67

[1]VIP threshold set at >1.5.

[2]Unidentified metabolites presented as chemical formula (i.e., C8 H16 N2 0)

VIP, Variable importance in projection

Table 4. VIP urinary metabolites between study phases[1,2]

Metabolite	VIP	Fold Change (Mycoprotein/Meat)
balenine.1	5.65	-3.65
C11 H18 N2 O6	4.52	2.92
C9 H15 N3 O	4.11	-2.66
C8 H14 N6 O7	4.08	2.64
C14 H30 N2 O6	3.99	-2.58
C10 H21 N5 O3	3.82	-2.47
C17 H31 N O3	3.76	2.43
l-Histidinal	3.35	-2.16
val-his	3.20	-2.07
N-[(2Z)-2-Hydroxy-2-octenoyl]glycine	2.99	1.94
C12 H25 O6 P3	2.95	-1.90
C13 H20 N2 O5	2.87	-1.85
C7 H13 N3 O	2.85	1.84
Miglitol	2.70	-1.75
C10 H19 N5 O2	2.66	-1.72
C34 H44 N8 O2	2.62	-1.70
C30 H51 N4 O2 P3	2.61	-1.69
C29 H42 N10 O4	2.57	-1.66
C19 H26 O9	2.57	1.66
Propionylcarnitine	2.55	-1.65
gamma-thiomethyl glutamate	2.51	1.62
C10 H4 N3 O3 P	2.51	-1.62
Ibuprofen metabolite B	2.48	1.60
(3S,6S)-3-(Hydroxymethyl)-6-{4-[(3-methyl-2-buten-1-yl)oxy]benzyl-2,5-piperazinedione	2.46	-1.59
TDIQ	2.37	-1.53
2-(6,10-Dimethyl-8-oxospiro[4.5]dec-6-en-2-yl)-2-hydroxypropyl hexopyranoside	2.36	1.52
9-Nitrooleate	2.32	-1.50
N(alpha)-gamma-L-glutamylhistamine	2.26	-1.46
(5E)-7-methylidene-10-oxo-4-(propan-2-yl)undec-5-enoic acid	2.20	1.42

Table 4 (continued)

C22 H33 N5 O4	2.19	1.42
C10 H18 N4 O3	2.19	-1.41
C29 H47 N O8 S	2.12	1.37
Allysine.1	2.11	-1.36
C8 H13 N O4 S.1	2.07	1.34
C10 H13 N O5 S.1	2.05	1.33
Acetyl-Î²-methylcholine	2.03	-1.31
Acetylcholine	2.03	-1.31
Xanthotoxol	2.01	1.30
3-Methylglutarylcarnitine	2.00	-1.29
Mevalonic acid	1.99	1.29
C6 H8 O7 S.1	1.95	-1.26
Sorbic acid	1.92	1.24
Acronycidine	1.92	-1.24
C10 H27 N4 O3 P3	1.90	1.23
C11 H20 N2 O7 S3	1.88	1.21
C21 H31 N O9	1.87	-1.21
5-(2,3-Dihydroxy-3-methylbutyl)-4-[(3,3-dimethyl-2-oxiranyl)acetyl]-3,4-dihydroxy-2-(3-methylbutanoyl)-2-cyclopenten-1-one	1.85	1.20
DL-Î²-Leucine	1.81	-1.17
C8 H12 N2 O5 S	1.81	-1.17
C12 H21 N3 O4 S	1.81	1.17
methoxyresorcinol	1.81	-1.17
C5 H10 N8 O S2	1.78	1.15
C15 H25 N7 O6	1.77	1.15
C10 H25 N6 O9 P S3.1	1.77	-1.14
C16 H34 N O7 P3	1.76	1.14
C14 H20 N2 O8	1.76	-1.14
Taurine	1.75	-1.13
C16 H19 N O6 S.1	1.74	-1.12
Taurine.1	1.74	-1.12
N-[2-Hydroxy-4-(sulfooxy)phenyl]acetamide	1.72	-1.11
C14 H18 N2 O3 S2	1.70	1.10

Table 4 (continued)

C7 H16 N8 O3 S3	1.70	-1.10
C22 H19 N4 O3 P S	1.70	1.10
ibufenac.1	1.70	1.10
2-methoxyacetaminophen sulfate	1.69	-1.10
paracetamol sulfate	1.69	-1.09
Arbusculin A	1.68	1.09
5-Methylangelicin	1.67	-1.08
C6 H17 N4 O8 P	1.67	1.08
C22 H35 N O8	1.67	-1.08
C6 H14 N5 O7 P S	1.65	-1.07
Arsenobetaine	1.65	-1.06
3-[2-[(E)-[3-(2-carboxyethyl)-5-[(4-ethyl-3-methyl-5-oxo-pyrrolidin-2-yl)methyl]-4-methyl-pyrrol-2-ylidene]methyl]-5-[(3-ethyl-4-methyl-5-oxo-pyrrolidin-2-yl)methyl]-4-methyl-1H-pyrrol-3-yl]propanoic acid	1.65	-1.06
N-Acetyl-L-cysteine	1.63	1.05
C24 H26 N6 O6	1.61	1.04
Succinylacetone	1.59	1.03
C5 H4 O7 S	1.56	1.01
L-Anserine	1.55	-1.00
C6 H12 N3 O3 P.1	1.54	1.00
C12 H24 N5 O6 P S	1.54	-0.99
Roxatidine	1.54	-0.99
N-Acetylleucylleucine	1.50	0.97

[1]VIP threshold set at >1.5.

[2]Unidentified metabolites presented as chemical formula (i.e., C8 H16 N2 0)

Positive fold change indicates greater Mycoprotein, negative fold change indicates greater Meat.

VIP, Variable importance in projection

APPENDIX B

Correlation coefficients between metabolites and microbial genera

Table 1. Correlation estimates for relationships between faecal metabolites and gut microbial genera[1].

	Roseburia	Parabacteroides	Bacteroidetes (unclassified)	Acidaminococcaceae (unclassified)	Bifidobacterium	Prevotella	Ruminococcaceae (unclassified)	Proteobacteria (unclassified)	Alphaproteobacteria (unclassified)	Bacteria (unclassified)	Oscillibacter	Pedobacter	Bacteroides
Sorbic acid	0.273	0.256	0.170	0.142	0.130	0.229	0.137	-0.012	-0.021	-0.026	-0.056	-0.212	-0.230
Gamma-Aminobutyric acid (GABA)	0.273	0.255	0.170	0.142	0.129	0.229	0.137	-0.012	-0.021	-0.026	-0.055	-0.211	-0.230
N-[(2Z)-2-Hydroxy-2-octenoyl]glycine	0.276	0.258	0.171	0.144	0.130	0.232	0.139	-0.011	-0.020	-0.025	-0.055	-0.214	-0.233
Butenylcarnitine	0.209	0.174	0.120	0.075	0.062	0.223	0.152	0.136	0.132	0.121	0.116	-0.237	-0.245
L(+)-Ornithine	0.099	0.084	0.058	0.038	0.032	0.101	0.068	0.052	0.050	0.046	0.042	-0.106	-0.110

Table 1 (continued)

leucyl-4-hydroxyproline	0.097	0.083	0.056	0.038	0.032	0.098	0.066	0.049	0.047	0.043	0.038	-0.102	-0.107
Ile-val	0.048	0.044	0.029	0.024	0.022	0.041	0.025	0.001	-0.001	-0.002	-0.007	-0.038	-0.041
Valyl-4-hydroxyproline	0.057	0.055	0.036	0.032	0.029	0.045	0.025	-0.012	-0.014	-0.015	-0.022	-0.039	-0.043
Homocarnosine	-0.035	-0.049	-0.030	-0.044	-0.045	0.007	0.019	0.114	0.117	0.113	0.129	-0.031	-0.024
Glycylglutamine	-0.023	-0.036	-0.021	-0.034	-0.035	0.011	0.018	0.095	0.097	0.094	0.107	-0.030	-0.024
DL-Atenolol	-0.025	-0.028	-0.017	-0.020	-0.019	-0.011	-0.003	0.031	0.032	0.031	0.037	0.004	0.007
dinotefuran	-0.033	-0.036	-0.023	-0.024	-0.024	-0.018	-0.007	0.032	0.033	0.033	0.040	0.010	0.014
4-Hydroxyprolylleucine	-0.014	-0.014	-0.009	-0.008	-0.008	-0.011	-0.006	0.005	0.006	0.006	0.008	0.009	0.010

Table 1 (continued)

(2S)-2-Amino-8-hydroxyoctanoic acid	-0.025	-0.023	-0.015	-0.013	-0.012	-0.020	-0.012	0.002	0.003	0.003	0.006	0.019	0.020
Allysine	-0.005	-0.002	-0.002	0.002	0.002	-0.010	-0.008	-0.018	-0.018	-0.017	-0.018	0.013	0.012
Asparaginyl-4-hydroxyproline	-0.017	-0.011	-0.008	-0.002	0.000	-0.025	-0.019	-0.031	-0.031	-0.029	-0.031	0.030	0.030
P-DMEA	-0.065	-0.050	-0.035	-0.016	-0.012	-0.079	-0.057	-0.072	-0.072	-0.067	-0.068	0.089	0.090
ISOXYL	-0.026	-0.016	-0.012	0.000	0.002	-0.041	-0.032	-0.057	-0.058	-0.054	-0.058	0.051	0.050
porfiromycin	-0.028	-0.016	-0.012	0.002	0.005	-0.047	-0.037	-0.071	-0.072	-0.068	-0.073	0.059	0.058
Thiamine	-0.096	-0.147	-0.087	-0.140	-0.144	0.044	0.075	0.389	0.401	0.385	0.439	-0.124	-0.101
4-Methyl-5-thiazoleethanol	-0.097	-0.148	-0.088	-0.140	-0.144	0.043	0.075	0.389	0.400	0.384	0.439	-0.123	-0.099

Table 1 (continued)

1,3-Dipropylxanthine	-0.099	-0.147	-0.087	-0.136	-0.140	0.035	0.067	0.369	0.380	0.365	0.417	-0.111	-0.088
Isoputreanine	-0.279	-0.242	-0.164	-0.115	-0.099	-0.276	-0.181	-0.117	-0.110	-0.099	-0.084	0.283	0.296
N~6~,N~6~-Dimethyllysine	-0.260	-0.225	-0.153	-0.107	-0.092	-0.257	-0.170	-0.111	-0.105	-0.094	-0.080	0.264	0.277
4-[(3-Acetamidopropyl)amino]butanoic acid	-0.267	-0.233	-0.158	-0.112	-0.096	-0.262	-0.172	-0.107	-0.100	-0.090	-0.074	0.268	0.281

[1]Estimates obtained from sparse partial least squares regression in canonical mode.

Table 2. Correlation estimates for relationships between urinary metabolites and gut microbial genera[1].

	Barnesiella	Akkermansia	Polaromonas	Enterobacteriaceae (unclassified)	Lachnospiraceae (unclassified)	Bifidobacterium	Blautia	Collinsella	Sutterella	Bacteroidetes (unclassified)	Betaproteobacteria (unclassified)	Roseburia	Phascolarctobacterium
N-Acetylleucylleucine	0.232	0.186	0.200	0.183	0.052	0.050	0.359	-0.089	-0.142	-0.088	-0.092	-0.129	-0.155
Allysine	0.199	0.160	0.167	0.152	0.050	0.035	0.300	-0.080	-0.128	-0.093	-0.097	-0.132	-0.157
Mevalonic acid	0.187	0.142	0.186	0.177	0.005	0.093	0.335	-0.045	-0.073	0.046	0.047	0.036	0.034
Arbusculin A	0.129	0.098	0.129	0.122	0.003	0.065	0.232	-0.031	-0.050	0.033	0.034	0.027	0.026
Xanthotoxol	0.334	0.260	0.311	0.291	0.039	0.123	0.560	-0.103	-0.165	-0.016	-0.017	-0.053	-0.072
Sorbic acid	0.291	0.227	0.271	0.254	0.034	0.107	0.489	-0.090	-0.144	-0.014	-0.015	-0.046	-0.063
2-methoxyacetaminophen sulfate	-0.106	-0.112	-0.002	0.020	-0.155	0.164	-0.001	0.135	0.210	0.453	0.469	0.552	0.632

Table 2 (continued)

paracetamol sulfate	-0.111	-0.115	-0.007	0.015	-0.154	0.160	-0.010	0.135	0.211	0.449	0.465	0.548	0.627
N-[2-Hydroxy-4-(sulfooxy) phenyl]acetamide	-0.112	-0.115	-0.008	0.015	-0.154	0.160	-0.011	0.135	0.211	0.449	0.464	0.547	0.626
3-Methylglutarylcarnitine	0.057	0.046	0.050	0.045	0.013	0.013	0.089	-0.022	-0.035	-0.021	-0.022	-0.031	-0.038
Ibuprofen metabolite B	0.057	0.045	0.051	0.047	0.010	0.016	0.092	-0.020	-0.032	-0.013	-0.014	-0.021	-0.026
2DHH	0.045	0.028	0.065	0.066	-0.029	0.066	0.118	0.011	0.016	0.106	0.110	0.122	0.138
5DMD	0.037	0.021	0.060	0.062	-0.032	0.066	0.108	0.015	0.022	0.114	0.118	0.132	0.149

Table 2 (continued)

(5E)-7-methylidene-10-oxo-4-(propan-2-yl)undec-5-enoic acid	0.013	0.004	0.032	0.035	-0.028	0.047	0.059	0.017	0.027	0.094	0.097	0.110	0.125
l-Histidinal	-0.116	-0.101	-0.072	-0.059	-0.067	0.033	-0.128	0.074	0.116	0.173	0.179	0.218	0.252
balenine	-0.038	-0.025	-0.053	-0.054	0.021	-0.051	-0.097	-0.007	-0.009	-0.080	-0.082	-0.091	-0.102
N-Acetyl-L-cysteine	-0.027	-0.021	-0.026	-0.024	-0.002	-0.011	-0.046	0.007	0.012	-0.002	-0.002	0.000	0.001
methoxyresorcinol	-0.038	-0.028	-0.039	-0.037	0.001	-0.022	-0.070	0.008	0.013	-0.016	-0.016	-0.015	-0.016
val-his	-0.028	-0.020	-0.033	-0.033	0.007	-0.025	-0.060	0.002	0.003	-0.030	-0.031	-0.033	-0.037
Roxatidine	-0.039	-0.028	-0.044	-0.043	0.006	-0.030	-0.079	0.004	0.008	-0.032	-0.033	-0.034	-0.037
Taurine	-0.060	-0.046	-0.059	-0.055	-0.004	-0.027	-0.106	0.016	0.026	-0.008	-0.008	-0.004	-0.002

Table 2 (continued)

3CMP	-0.086	-0.068	-0.075	-0.069	-0.017	-0.022	-0.135	0.031	0.050	0.025	0.025	0.038	0.046
9-Nitrooleate	-0.149	-0.117	-0.135	-0.125	-0.024	-0.046	-0.243	0.051	0.081	0.027	0.028	0.048	0.059
TDIQ	-0.179	-0.141	-0.164	-0.153	-0.026	-0.059	-0.295	0.059	0.094	0.024	0.025	0.047	0.060
(3S,6S)-3-(Hydroxymethyl)-6-{4-[(3-methyl-2-buten-1-yl)oxy]benzyl-2,5-piperazinedione	-0.178	-0.140	-0.158	-0.147	-0.031	-0.051	-0.285	0.062	0.099	0.041	0.042	0.067	0.082
Acetylcholine	-0.187	-0.143	-0.181	-0.171	-0.012	-0.083	-0.327	0.050	0.081	-0.023	-0.024	-0.009	-0.004
Acetyl-ß-methylcholine	-0.194	-0.151	-0.178	-0.167	-0.026	-0.067	-0.321	0.062	0.099	0.019	0.020	0.042	0.055
Miglitol	-0.194	-0.151	-0.180	-0.169	-0.023	-0.071	-0.325	0.060	0.097	0.011	0.012	0.033	0.044

Table 2 (continued)

Propionylcarnitine	-0.195	-0.152	-0.181	-0.169	-0.024	-0.071	-0.326	0.061	0.097	0.011	0.012	0.033	0.045

[1]Estimates obtained from sparse partial least squares regression in canonical mode.

Abbreviations: 2DHH, 2-(6,10-Dimethyl-8-oxospiro[4.5]dec-6-en-2-yl)-2-hydroxypropyl hexopyranoside; 5DMD, 5-(2,3-Dihydroxy-3-methylbutyl)-4-[(3,3-dimethyl-2-oxiranyl)acetyl]-3,4-dihydroxy-2-(3-methylbutanoyl)-2-cyclopenten-1-one; 3CMP, 3-[2-[(E)-[3-(2-carboxyethyl)-5-[(4-ethyl-3-methyl-5-oxo-pyrrolidin-2-yl)methyl]-4-methyl-pyrrol-2-ylidene]methyl]-5-[(3-ethyl-4-methyl-5-oxo-pyrrolidin-2-yl)methyl]-4-methyl-1H-pyrrol-3-yl]propanoic acid

APPENDIX C

Supplementary Material

The nutritional impact of replacing dietary meat with meat alternatives in the UK: a modelling analysis using nationally representative data.

Table 1: Meat alternative products included in modelling analysis

Table 2: Mean nutrient intake with 95% confidence intervals for current and replacement models; for total population

Tables 3-4: Population subgroup result tables; males aged 4-10 years

Tables 5-6: Population subgroup result tables; females aged 4-10 years

Tables 7-8: Population subgroup result tables; males aged 11-18 years

Tables 9-10: Population subgroup result tables; females aged 11-18 years

Tables 11-12: Population subgroup result tables; males aged 19-64 years

Tables 13-14: Population subgroup result tables; females aged 19-64 years

Tables 15-16: Population subgroup result tables; males aged 65 years and older

Tables 17-18: Population subgroup result tables; females aged 65 years and older

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Supplementary Table 1.
Meat alternative products included in modelling analysis

	Soy based
Brand/Manufacturer	**Product Name**
Tesco	Meat Free Vegetarian Mince
Tesco	Meat Free Chicken Style Nuggets
Tesco	Meat Free Meat Style
Richmond	Frozen Meat Free Sausages
Richmond	Meat Free Sausages
Fry's Meat Free	Chicken Style Strips
Fry's Meat Free	Chicken Style Burger
Tesco	Vegetarian Bacon Mac & Cheese Melts
Aunt Bessie's	Vegetarian Toad In The Hole
Linda McCartney's	Vegetarian Chicken Bucket
Naked Glory	Vegan Tikka Tenderstrips
Vivera	Veggie Greek Kebab∞
Oumph!	Spiced Kebab
Vivera	Veggie Bacon Pieces∞
Naked Glory	Vegan Quarter Pounders
Naked Glory	Vegan Sausages Pack
Oumph!	Sticky Smokehouse Oumph
Tesco	Plant Chef Herby Bangers
Tesco	Plant Chef Meat Free Balls
Linda McCartney's	Vegetarian Red Onion & Rosemary Sausages
Linda McCartney's	Vegetarian Quarter Pounder Burgers
Linda McCartney's	Vegetarian Chorizo & Red Pepper Sausages
Like Meat	Soya Based Schnitzel
Naked Glory	Mince
Tesco	Plant Chef Breaded Goujons
Linda McCartney's	Mozzarella Burgers
Tesco	Plant Chef Southern Fried Fillets
Tesco	Plant Chef Breaded Meat Free Nuggets
Vivera	Veggie Mince∞
Naked Glory	Quarter Pounders Pack
The Vegetarian Butcher	What The Cluck
Naked Glory	Sausages Pack
Vivera	Plant Chicken Tenders∞
Vivera	Veggie Chicken Pieces∞
The Vegetarian Butcher	Chickened Out Burger∞
The Unbelievable Alt.	Lambless Strips
Tesco	Plant Chef Fish Free Cakes
THIS	Isn't Chicken Salt & Pepper∞
The Unbelievable Alt.	Beefless Strips
Fry's Meat Free	Golden Crispy Fish Style Fillets
Oumph!	Pulled Oumph!
The Vegetarian Butcher	Little Peckers (Nuggets)∞

Vivera	Plant Meatballs∞
The Vegetarian Butcher	Mc2 Burger∞
The Vegetarian Butcher	Little Willies Sausages
Like Meat	Soya Based Nuggets
Linda McCartney’s	Shredded Hoisin Duck
Vivera	Plant Quarter Pounder∞
The Unbelievable Alt.	Mighty Beefless Mince
Tesco	Plant Chef Battered Fish Free Fillets
Linda McCartney’s	BBQ Pulled Pork Burger
Wicked Kitchen	All The Rage Rolls
Fry's Meat Free	Chicken Style Sausage Rolls
Linda McCartney’s	Vegetarian Pork Bao Bun Meal Kit
THIS	Isn't Bacon Rashers∞
Vivera	Shwarma Kebab∞
The Unbelievable Alt.	Chickenless Strips
Vivera	Veggie Burger∞
Sainsbury's	Plant Pioneers Meat Free Mince
Linda McCartney’s	Chilled Vegetarian Meatballs
Linda McCartney’s	Vegetarian Sausages
Fry's Meat Free	Vegan Meat Free Chicken-Style Nuggets
Sainsbury's	Plant Pioneers Meat Free Chicken-Style Pieces
Sainsbury's	Plant Pioneers Meat Free Mini Sausage Rolls
Linda McCartney’s	Vegetarian Fishcake
Sainsbury's	Plant Pioneers Ultimate Plant Burgers
Fry's Meat Free	Vegan Artisan Smoked Hot Dogs
Sainsbury's	Plant Pioneers Vegetarian Chicken Nuggets
Vivera	Plant Steak∞
Gardein	Golden Fishless Fillets
Fry's Meat Free	Vegan Mini Sausage Rolls
Fry's Meat Free	Vegan Artisan Southern-Style Tenders
Gardein	Seven Grain Crispy Tenders
Gardein	Sweet and Sour Porkless Bites
Moving Mountains	Plant-Based Sausage Burgers
Fry's Meat Free	Vegan Twin Sausage Rolls
Squeaky	Satay Kiev
Squeaky	Vegan Nuggets
Asda	Plant Based Vegan Meat Free Chicken Bites
Asda	Plant Based Vegan Meat-Free Chicken Nuggets
Asda	Plant Based Vegan Meat Free Meatballs
Asda	Plant Based Vegan Meat Free Mince
Asda	Plant Based Vegan Quarter Pounders with Cheddar Alternative & Red Onion
Asda	Plant Based 8 Vegan Beef Style Burgers
Oumph!	Vegan Burger
No Chick Meat	Meat Free Strips
Linda McCartney’s	Vegetarian Southern-Style Chicken Fillet Burgers

Nut Based

Brand/Manufacturer	Product Name
Tesco	Plant Chef Nut Cutlets
Tesco	Plant Chef Peanut Burgers

Vegetable Based

Brand/Manufacturer	Product Name
Tesco	Vegetarian Cauliflower Cheese Grills
Tesco	Plant Chef Vegetable Fingers
Tesco	Plant Chef Vegetable Burgers

Birds Eye	Vegetable Fingers
Goodlife	Vegetable Protein Balls With Spinach & Kale
Heck	Ultimate Vegan Burgers
Birds Eye	Green Cuisine Mexican Fajita Style Mix
Strong Roots	Pumpkin Spinach Burger
Strong Roots	Kale & Quinoa Burger
Strong Roots	Spinach Bites
Goodlife	Mighty Non Meaty Sausages
Strong Roots	Broccoli & Purple Carrot Bites
Vivera	Pumpkin Sweet Potato Burgers
Sainsbury's	Sweet Potato, Quinoa & Lentil Burger
Sainsbury's	Vegetable Quarter Pounders
Sainsbury's	Indian Lentil Quarter Pounder
Sainsbury's	Love Your Veg! Vegan Mix Burger
Sainsbury's	Plant Pioneers Chorizo Shroomdogs
Sainsbury's	Plant Pioneers Smoky ‘Jack' Quarter Pounders
Sainsbury's	Plant Pioneers Cumberland Shroomdogs
Heck	Vegan Breakfast Plant Based Sausages
Sainsbury's	Plant Pioneers Cumberland Shroomdogs
Sainsbury's	Plant Pioneers Vegan Steaks∞
Sainsbury's	Plant Pioneers Shroomballs
Sainsbury's	Plant Pioneers Hotnspicy Goujons
Sainsbury's	Love Your Veg! Green Supreme Shroomdogs Sausages
Sainsbury's	Plant Pioneers Caramelised Onion Shroomdogs
Sainsbury's	Love Your Veg! Roasted Red Pepper Shroomdogs
Sainsbury's	Plant Pioneers Fishless Fingers
Moving Mountains	Plant-Based Hot Dogs
Sainsbury's	Plant Pioneers Southern Fried Bites
Sainsbury's	Vegetable Fingers
Moving Mountains	Plant-Based Hot Dogs
Little Roots	Sweet Potato Nuggets
Sainsbury's	Moroccan Vegbab
Goodlife	Supergreen Pesto Burger with Edamame Beans, Kale & Spinach
Rustlers	Moroccan Vegetarian Burger
Heck	Vegfurter
Asda	Plant Based Meat-Free Mince
Asda	Plant Based Meat-Free Burgers
Asda	Plant Based Meat-Free Sausages
Asda	Plant Based Vegan Vegetable Burgers
Asda	Plant Based Vegan Vegetable Fingers
Asda	Plant Based Vegan Nut Cutlets
Asda	Plant Based Vegan Sweet Potato Falafel Burgers
Asda	Plant Based Meat-Free Meatballs
No Bull	Vegan Mushrooms Steaks
Asda	Vegetarian Cauliflower Cheese Crispy Grills

Tofu Based

Brand/Manufacturer	**Product Name**
Cauldron	Organic Tofu
Cauldron	Marinated Tofu
Satono Yukis	Tofu
The Tofoo Co	Smoked Organic Tofu
Yutaka	Tofu
The Tofoo Co	Tempeh
Cauldron	Organic Teriyaki Tofu

The Tofoo Co	Organic Tofu
Clearspring Organic	Organic Tofu
Mori-Nu	Silken Firm Tofu
Plant Power	Original Tempeh Block
Tofurky	Hickory Smoke Flavour Turkey Style Deli Slices
Tofurky	Italian Sausage with Sun-Dried Tomatoes and Basil
The Tofoo Co	The Tofoo Co. Crispy Southern Fried Bites
Tofurky	Plant-Based Deli Slices Smoke Ham Style
Tofurky	Oven Roasted Deli Slices
The Tofoo Co	Crispy Spicy Sweet Chilli Bites
Satonoyuki	Tofu
Ashoka	Palak Paneer
Ashoka	Matar Paneer

Mycoprotein Based

Brand/Manufacturer	**Product Name**
Quorn	Chicken Style Pieces
Quorn	Mince
Quorn	Crispy Nuggets
Quorn	Sausages
Quorn	Cheese Escalopes
Quorn	Southern Fried Bites
Quorn	Cheese & Broccoli Escalopes
Quorn	Chicken Style Burgers
Quorn	Love It Lasagne
Quorn	Tantalising Tikka Masala
Quorn	Family Roast
Quorn	Vegan Smoky Ham Free Slice
Quorn	Roast Style Sliced Fillets
Quorn	Chicken Style Pieces
Quorn	Brilliant Burgers
Quorn	Cocktail Sausages
Quorn	Crispy Fillets
Quorn	Comforting Cottage Pie
Quorn	Picnic Egg
Quorn	Pieces
Quorn	Sausage Rolls
Quorn	Mince
Quorn	Fishless Fillets Salt And Vinegar
Quorn	Bacon Style Rashers
Quorn	Chicken & Bacon Lattice
Quorn	Peppered Steaks
Quorn	Mince
Quorn	Classic Burger
Quorn	Vegan Chicken Free Slices
Quorn	Mozzarella & Pesto Escalope
Quorn	Vegetarian Ham Slices
Quorn	Ultimate Burgers
Quorn	Chilli Bean Bowl
Quorn	Smoky Bacon Style Slices
Quorn	Vegetarian Chicken Slices
Quorn	Biryani Bowl
Quorn	Hot & Spicy Vegan Burger
Quorn	Spiced Chickpea &Lentil Bowl
Quorn	Vegan Pieces

Quorn	Spinach and Red Pepper Slices
Quorn	Sweet Chipotle Goujons
Quorn	Thai Style Wonder Grains
Quorn	Mediterranean Wonder Grains
Quorn	Mexican 3 Bean Wonder Grain
Quorn	Ginsters Vegan Pasty
Quorn	Deli Turkey

Legume Based

Brand/Manufacturer	**Product Name**
Birds Eye	Meat Free Swedish Style Balls
Birds Eye	Meat Free Burgers
Beyond Meat	Beyond Burger Plant Based Burger
Birds Eye	Green Cuisine Meat Free Chicken Pies
Birds Eye	Meat Free Sausages Rolls
Wicked Kitchen	Chorizo Style Bangers
The Meatless Farm	Meat Free Sausages
Beyond Meat	Plant Based Patties
Tesco	Plant Chef Cumberland Style Bangers
Tesco	Plant Chef Meat Free Burgers
Linda McCartney's	Vegetarian Lincolnshire Sausages
The Meatless Farm	Meat Free Burgers
Heck	Meat-Free Vegan Sausages
Birds Eye	Green Cuisine Meat Free Bolognese Mix
Birds Eye	Green Cuisine Meat Free Chilli Mix
Tesco	Plant Chef Meat-Free BBQ Koftas
Wicked Kitchens	Chubby Little Brats
Wicked Kitchens	BBQ Fib Rack
Wicked Kitchens	Jalapeno Griller Patties
Like Meat	Pea Based Smoked Sausage
Beyond Meat	The Beyond Sausage Pack
Wicked Kitchens	Wicked Spiced Amazeballs
Squeaky	Ready To Eat Chicken Style Pieces Thai
Squeaky	Ready To Eat Chicken Style Pieces Tikka
Squeaky	Ready To Eat Chicken Style Pieces BBQ
Linda McCartney's	Vegetarian Lincolnshire Sausages
Sainsbury's	Plant Pioneers Smoky Vacon Rashers∞
Naturli	Pea Based Minced
Squeaky	Ready To Eat Marinated Chicken Style Pieces Kick of Tikka
Squeaky	Ready To Eat Marinated Chicken Style Pieces BBQ
Beanit	Pan-Fry Protein Pieces
Beanit	Pan-Fry Protein Mince
Tesco	Monterey Jack BBQ Bean Burger
Tesco	Plant Chef Spicy Bean Burgers
Strong Roots	Bean Beetroot Burger
Sainsbury's	Spicy Bean Burgers
Asda	Plant Based Vegan Spicy Bean Burgers

∞ Product description indicates fortification with either iron/B12/both iron and B12

Supplementary Table 2.
Mean nutrient intake with 95% confidence intervals for total population (n = 1110) for current and replacement models

Models	Energy, kcal/day	Carbohydrate, g/day	Protein, g/day	Fat, g/day	Fibre, g/day	Sugars, g/day	Saturated fat, g/day	Sodium, mg/day	Iron, mg/day	B12, ug/day
Current	2151 (2111.63, 2190.36)	270.77 (265.61, 275.92)	86.67 (84.96, 88.39)	81.97 (80.14, 83.80)	21.86 (21.35, 22.38)	110.52 (107.55, 113.49)	30.96 (30.18, 31.74)	2413.80 (2359.39, 2468.20)	13.65 (12.70, 14.60)	7.47 (6.20, 8.74)
MA-25	2151 (2111.63, 2190.36)	275.16 (269.96, 280.35)	83.27 (81.63, 84.91)	81.19 (79.37, 83.01)	23.74 (23.21, 24.27)	111.48 (108.50, 114.45)	30.05 (29.27, 30.82)	2466.37 (2411.03, 2521.70)	13.59 (12.63, 14.54)	7.07 (5.80, 8.34)
MA-50	2151 (2111.63, 2190.36)	279.78 (274.54, 285.01)	79.68 (78.11, 81.25)	80.38 (78.57, 82.18)	25.71 (25.16, 26.26)	112.49 (109.51, 115.47)	29.08 (28.32, 29.85)	2521.74 (2465.38, 2578.10)	13.52 (12.57, 14.47)	6.65 (5.38, 7.92)
MA-75	2151 (2111.63, 2190.36)	284.65 (279.37, 289.94)	75.90 (74.40, 77.40)	79.51 (77.71, 81.31)	27.80 (27.22, 28.37)	113.56 (110.57, 116.54)	28.07 (27.31, 28.83)	2580.14 (2522.66, 2637.61)	13.45 (12.50, 14.40)	6.21 (4.94, 7.48)
MA-100	2151 (2111.63, 2190.36)	289.80 (284.46, 295.15)	71.90 (70.47, 73.33)	78.60 (76.81, 80.39)	30.00 (29.38, 30.61)	114.68 (111.69, 117.68)	27.00 (26.24, 27.75)	2641.82 (2583.13, 2700.52)	13.38 (12.43, 14.33)	5.74 (4.48, 7.01)
Vegetable	2151 (2111.63, 2190.36)	295.39 (289.98, 300.81)	67.34 (65.98, 68.70)	77.58 (75.81, 79.36)	30.59 (29.96, 31.22)	114.94 (111.95, 117.94)	27.13 (26.38, 27.89)	2720.90 (2660.58, 2781.22)	13.17 (12.22, 14.12)	5.72 (4.46, 6.99)
Mycoprotein	2151 (2111.63, 2190.36)	293.17 (287.79, 298.56)	77.86 (76.32, 79.39)	75.85 (74.09, 77.60)	30.57 (29.94, 31.20)	113.30 (110.31, 116.28)	27.31 (26.55, 28.06)	2726.12 (2665.69, 2786.55)	12.68 (11.73, 13.63)	5.98 (4.72, 7.25)
Legume	2151 (2111.63, 2190.36)	290.68 (285.33, 296.04)	71.24 (69.82, 72.66)	78.48 (76.69, 80.27)	28.66 (28.06, 29.25)	113.93 (110.95, 116.92)	27.23 (26.48, 27.99)	2690.65 (2630.96, 2750.34)	13.26 (12.31, 14.21)	5.77 (4.50, 7.03)
Tofu	2151 (2111.63, 2190.36)	279.77 (274.53, 285.00)	81.80 (80.19, 83.41)	79.60 (77.81, 81.40)	25.00 (24.46, 25.54)	112.66 (109.68, 115.65)	26.63 (25.88, 27.39)	2510.20 (2454.05, 2566.34)	21.28 (20.29, 22.28)	5.45 (4.18, 6.71)

Nut	2151 (2111.63, 2190.36)	282.24 (276.98, 287.50)	66.88 (65.53, 68.24)	83.35 (81.50, 85.20)	32.10 (31.44, 32.75)	117.96 (114.94, 120.98)	26.16 (25.41, 26.91)	2289.49 (2237.15, 2341.84)	12.74 (11.79, 13.69)	5.59 (4.32, 6.85)
Soy	2151 (2111.63, 2190.36)	285.33 (280.03, 290.62)	79.48 (77.91, 81.05)	77.26 (75.49, 79.04)	28.12 (27.53, 28.70)	112.71 (109.73, 115.70)	27.27 (26.52, 28.03)	2909.77 (2845.33, 2974.21)	13.61 (12.66, 14.56)	5.71 (4.44, 6.97)
Fortified	2151 (2111.63, 2190.36)	282.01 (276.75, 287.26)	83.58 (81.93, 85.23)	77.08 (75.31, 78.85)	28.94 (28.35, 29.54)	112.20 (109.22, 115.18)	27.66 (26.90, 28.42)	2896.42 (2832.28, 2960.56)	16.77 (15.80, 17.73)	7.14 (5.87, 8.41)
Unfortified	2151 (2111.63, 2190.36)	294.03 (288.63, 299.42)	68.49 (67.11, 69.86)	77.78 (76.01, 79.56)	30.23 (29.61, 30.85)	114.63 (111.64, 117.63)	27.02 (26.27, 27.78)	2774.88 (2713.42, 2836.35)	13.10 (12.15, 14.06)	5.68 (4.42, 6.95)

Data presented as means with 95% confidence intervals within parentheses

Supplementary Table 3.
Mean nutrient intake for current and replacement models, with reference to DRVs; males aged 4-10 years (n = 141)

			Progressive Model				Ingredient Model						Fortification Model	
Nutrient	**DRV***	**Current**	**MA-25**	**MA-50**	**MA-75**	**MA-100**	**Vegetable**	**Mycoprotein**	**Legume**	**Tofu**	**Nut**	**Soy**	**Fortified**	**Unfortified**
Energy, kcal/day	1673	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate, g/day†	209.10	229.12	232.64	236.34	240.25	244.38	248.86	247.08	245.09	236.34	238.32	240.79	238.13	247.76
Protein, g/day†	62.70	62.13	59.40	56.53	53.50	50.29	46.64	55.07	49.76	58.23	46.27	56.37	59.65	47.55
Fat, g/day	65.10	62.81	62.19	61.53	60.84	60.11	59.30	57.90	60.01	60.92	63.92	59.04	58.89	59.46
Fibre, g/day	20.00	16.08	17.58	19.16	20.83	22.60	23.07	23.06	21.52	18.59	24.28	21.09	21.75	22.78
Sugars, g/day	90.00	95.11	95.88	96.69	97.55	98.45	98.66	97.34	97.85	96.83	101.08	96.87	96.46	98.41
Saturated fat, g/day	20.50	24.48	23.75	22.98	22.16	21.30	21.41	21.55	21.49	21.01	20.63	21.52	21.83	21.32
Sodium, mg/day	1600	1852.01	1894.15	1938.53	1985.34	2034.78	2098.17	2102.38	2073.93	1929.28	1752.37	2249.58	2238.89	2141.44
Iron, mg/day	7.40	9.35	9.30	9.24	9.19	9.13	8.96	8.57	9.03	15.47	8.62	9.31	11.84	8.91
B12, ug/day	1	4.65	4.33	3.99	3.64	3.26	3.24	3.46	3.28	3.03	3.14	3.23	4.38	3.21

*DRV values correspond to EAR for energy; RNI for Protein, Sodium, Iron, B12; DRV for Carbohydrate, Fat, Fibre, Sugars, Saturated fat.

† Protein target elected as 15% total energy intake and carbohydrate as 50% total energy intake in the current analysis.

Colours: Green, meeting DRV. Red, not meeting DRV. Grey, not meeting 15% protein and/or 50% carbohydrate target elected in the current analysis.

DRV, Dietary Reference Value; EAR, Estimated Average Requirement; RNI, Reference Nutrient Intake; MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 4.
Difference in nutrient intake between replacement models and current intake; males aged 4-10 years (n = 141)

		Progressive Model								Ingredient Model										Fortification Model			
Nutrient	**Current**	**MA-25**	**P**	**MA-50**	**P**	**MA-75**	**P**	**MA-100**	**P**	**Vegetable**	**P**	**Mycoprotein**	**P**	**Legume**	**P**	**Tofu**	**P**	**Nut**	**P**	**Soy**	**P**	**Fortified**	**P**	**Unfortified**	**P**
Energy, kcal/day	1673	-	-	-	-	-	-	-	-			-	-	-	-					-	-	-	-	-	-
Carbohydrate, g/day	229.12	+3.52	0.62	+7.22	0.31	+11.13	0.12	+15.26	0.03*	+19.74	0.01*	+17.96	0.01*	+15.97	0.03*	+7.22	0.31	+9.20	0.20	+11.67	0.10	+9.01	0.21	+18.64	0.01*
Protein, g/day	62.13	-2.73	0.14	-5.60	<0.01*	-8.64	<0.01*	-11.84	<0.01*	-15.50	<0.01*	-7.07	<0.01*	-12.37	<0.01*	-3.90	0.03*	-15.86	<0.01*	-5.77	<0.01*	-2.48	0.18	-14.58	<0.01*
Fat, g/day	62.81	-0.62	0.80	-1.28	0.60	-1.97	0.42	-2.70	0.27	-3.52	0.15	-4.91	0.05*	-2.80	0.25	-1.90	0.44	+1.10	0.65	-3.77	0.12	-3.92	0.11	-3.36	0.17
Fibre, g/day	16.08	+1.50	0.04*	+3.09	<0.01*	+4.76	<0.01*	+6.52	<0.01*	+6.99	<0.01*	+6.98	<0.01*	+5.44	<0.01*	+2.52	<0.01*	+8.20	<0.01*	+5.01	<0.01*	+5.68	<0.01*	+6.71	<0.01*
Sugars, g/day	95.11	+0.77	0.84	+1.58	0.67	+2.43	0.51	+3.34	0.37	+3.55	0.34	+2.23	0.55	+2.74	0.46	+1.72	0.64	+5.96	0.11	+1.76	0.63	+1.35	0.71	+3.30	0.37
Saturated fat, g/day	24.48	-0.73	0.51	-1.50	0.17	-2.32	0.04*	-3.18	<0.01*	-3.07	0.01*	-2.93	0.01*	-2.99	0.01*	-3.47	<0.01*	-3.85	<0.01*	-2.96	0.01*	-2.65	0.02*	-3.16	<0.01*
Sodium, mg/day	1852.01	+42.14	0.57	+86.52	0.25	+133.33	0.08	+182.78	0.01*	+246.16	<0.01*	+250.37	<0.01*	+221.93	<0.01*	+77.27	0.30	-99.64	0.18	+397.57	<0.01*	+386.88	<0.01*	+289.43	<0.01*
Iron, mg/day	9.35	-0.05	0.89	-0.10	0.78	-0.16	0.67	-0.22	0.56	-0.38	0.30	-0.78	0.04*	-0.31	0.40	+6.12	<0.01*	-0.73	0.05*	-0.03	0.93	+2.50	<0.01*	-0.44	0.24
B12, ug/day	4.65	-0.32	0.14	-0.66	<0.01*	-1.01	<0.01*	-1.39	<0.01*	-1.40	<0.01*	-1.19	<0.01*	-1.37	<0.01*	-1.62	<0.01*	-1.51	<0.01*	-1.41	<0.01*	-0.27	0.22	-1.43	<0.01*

Blue indicates significant increase compared to Current. Gold indicates significant decrease compared to Current.
Differences compared using regression models with significance threshold P < 0.05
MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

Supplementary Table 5.
Mean nutrient intake for current and replacement models, with reference to DRVs; females aged 4-10 years (n = 123)

			Progressive Model				Ingredient Model						Fortification Model	
Nutrient	**DRV***	**Current**	**MA-25**	**MA-50**	**MA-75**	**MA-100**	**Vegetable**	**Mycoprotein**	**Legume**	**Tofu**	**Nut**	**Soy**	**Fortified**	**Unfortified**
Energy, kcal/day	1564	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate, g/day†	195.50	212.90	216.17	219.62	223.27	227.11	231.28	229.63	227.77	219.62	221.47	223.77	221.29	230.26
Protein, g/day†	58.60	57.89	55.34	52.67	49.84	46.86	43.45	51.30	46.36	54.25	43.11	52.52	55.58	44.31
Fat, g/day	60.80	59.41	58.83	58.22	57.57	56.89	56.13	54.84	56.80	57.64	60.44	55.90	55.76	56.28
Fibre, g/day	20.00	14.98	16.38	17.85	19.41	21.05	21.49	21.48	20.05	17.32	22.62	19.65	20.26	21.22
Sugars, g/day	90.00	92.86	93.57	94.33	95.13	95.97	96.16	94.93	95.41	94.46	98.41	94.50	94.11	95.93
Saturated fat, g/day	19.10	23.39	22.71	21.99	21.23	20.43	20.53	20.66	20.60	20.16	19.80	20.63	20.92	20.45
Sodium, mg/day	1600	1685.42	1724.67	1766.01	1809.63	1855.68	1914.71	1918.63	1892.14	1757.40	1592.60	2055.74	2045.81	1955.04
Iron, mg/day	7.40	8.44	8.39	8.35	8.29	8.24	8.08	7.72	8.15	14.14	7.76	8.41	10.77	8.04
B12, ug/day	1	4.41	4.11	3.80	3.47	3.12	3.10	3.30	3.14	2.90	3.01	3.09	4.16	3.08

*DRV values correspond to EAR for energy; RNI for Protein, Sodium, Iron, B12; DRV for Carbohydrate, Fat, Fibre, Sugars, Saturated fat.

† Protein target elected as 15% total energy intake and carbohydrate as 50% total energy intake in the current analysis.

Colours: Green, meeting DRV. Red, not meeting DRV. Grey, not meeting 15% protein and/or 50% carbohydrate target elected in the current analysis.

DRV, Dietary Reference Value; EAR, Estimated Average Requirement; RNI, Reference Nutrient Intake; MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 6.
Difference in nutrient intake between replacement models and current intake; females aged 4-10 years (n = 123)

		Progressive Model								Ingredient Model										Fortification Model			
Nutrient	**Current**	**MA-25**	**P**	**MA-50**	**P**	**MA-75**	**P**	**MA-100**	**P**	**Vegetable**	**P**	**Mycoprotein**	**P**	**Legume**	**P**	**Tofu**	**P**	**Nut**	**P**	**Soy**	**P**	**Fortified**	**P**	**Unfortified**	**P**
Energy, kcal/day	1564.00	-	-	-	-	-	-	-	-			-	-	-	-					-	-	-	-	-	-
Carbohydrate, g/day	212.90	+3.28	0.61	+6.73	0.29	+10.37	0.11	+14.22	0.03*	+18.39	<0.01*	+16.73	0.01*	+14.87	0.02*	+6.72	0.29	+8.57	0.18	+10.87	0.09	+8.39	0.19	+17.37	0.01*
Protein, g/day	57.89	-2.54	0.11	-5.22	<0.01*	-8.04	<0.01*	-11.03	<0.01*	-14.44	<0.01*	-6.58	<0.01*	-11.52	<0.01*	-3.64	0.02*	-14.78	<0.01*	-5.37	<0.01*	-2.31	0.15	-13.58	<0.01*
Fat, g/day	59.41	-0.58	0.79	-1.19	0.58	-1.84	0.39	-2.52	0.24	-3.28	0.13	-4.57	0.03*	-2.61	0.23	-1.77	0.41	+1.03	0.63	-3.51	0.10	-3.66	0.09	-3.13	0.15
Fibre, g/day	14.98	+1.40	0.02*	+2.87	<0.01*	+4.43	<0.01*	+6.07	<0.01*	+6.52	<0.01*	+6.50	<0.01*	+5.07	<0.01*	+2.34	<0.01*	+7.64	<0.01*	+4.67	<0.01*	+5.29	<0.01*	+6.25	<0.01*
Sugars, g/day	92.86	+0.72	0.85	+1.47	0.70	+2.27	0.56	+3.11	0.42	+3.31	0.39	+2.07	0.59	+2.55	0.51	+1.60	0.68	+5.56	0.15	+1.64	0.67	+1.26	0.74	+3.07	0.42
Saturated fat, g/day	23.39	-0.68	0.45	-1.40	0.12	-2.16	0.02*	-2.96	<0.01*	-2.86	<0.01*	-2.73	<0.01*	-2.78	<0.01*	-3.23	<0.01*	-3.58	<0.01*	-2.75	<0.01*	-2.46	0.01*	-2.94	<0.01*
Sodium, mg/day	1685.42	+39.25	0.59	+80.59	0.26	+124.21	0.09	+170.26	0.02*	+229.29	<0.01*	+233.21	<0.01*	+206.72	<0.01*	+71.99	0.32	-92.82	0.20	+370.32	<0.01*	+360.39	<0.01*	+269.62	<0.01*
Iron, mg/day	8.44	-0.05	0.89	-0.10	0.78	-0.15	0.66	-0.20	0.55	-0.36	0.29	-0.72	0.03	-0.29	0.39	+5.70	<0.01*	-0.68	0.05*	-0.03	0.93	+2.33	<0.01*	-0.41	0.23
B12, ug/day	4.41	-0.30	0.18	-0.61	0.01*	-0.94	<0.01*	-1.29	<0.01*	-1.31	<0.01*	-1.11	<0.01*	-1.27	<0.01*	-1.51	<0.01*	-1.41	<0.01*	-1.32	<0.01*	-0.25	0.26	-1.34	<0.01*

Blue indicates significant increase compared to Current. Gold indicates significant decrease compared to Current.
Differences compared using regression models with significance threshold P < 0.05
MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

Supplementary Table 7.
Mean nutrient intake for current and replacement models, with reference to DRVs; males aged 11-18 years (n = 112)

			Progressive Model				Ingredient Model						Fortification Model	
Nutrient	**DRV***	**Current**	**MA-25**	**MA-50**	**MA-75**	**MA-100**	**Vegetable**	**Mycoprotein**	**Legume**	**Tofu**	**Nut**	**Soy**	**Fortified**	**Unfortified**
Energy, kcal/day	2680	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate, g/day†	335.00	359.13	364.00	369.13	374.54	380.25	386.45	383.98	381.23	369.12	371.86	375.28	371.60	384.93
Protein, g/day†	100.50	104.21	100.44	96.46	92.26	87.83	82.77	94.43	87.09	98.81	82.26	96.23	100.78	84.04
Fat, g/day	104.20	100.80	99.94	99.03	98.07	97.06	95.94	94.01	96.93	98.18	102.33	95.58	95.37	96.16
Fibre, g/day	30.00	24.05	26.13	28.33	30.64	33.08	33.74	33.71	31.59	27.54	35.41	30.99	31.91	33.34
Sugars, g/day	90.00	137.19	138.26	139.38	140.56	141.81	142.10	140.27	140.98	139.57	145.45	139.63	139.06	141.76
Saturated fat, g/day	32.80	38.69	37.68	36.61	35.48	34.30	34.44	34.64	34.56	33.89	33.37	34.60	35.03	34.32
Sodium, mg/day	2400	2969.08	3027.40	3088.82	3153.61	3222.03	3309.76	3315.57	3276.21	3076.02	2831.19	3519.28	3504.49	3369.66
Iron, mg/day	8.70	14.42	14.35	14.28	14.20	14.12	13.89	13.35	13.99	22.89	13.42	14.38	17.88	13.82
B12, ug/day	1.5	6.93	6.49	6.02	5.53	5.01	4.99	5.28	5.04	4.69	4.84	4.97	6.56	4.94

*DRV values correspond to EAR for energy; RNI for Protein, Sodium, Iron, B12; DRV for Carbohydrate, Fat, Fibre, Sugars, Saturated fat.

† Protein target elected as 15% total energy intake and carbohydrate as 50% total energy intake in the current analysis.

Colours: Green, meeting DRV. Red, not meeting DRV. Grey, not meeting 15% protein and/or 50% carbohydrate target elected in the current analysis.

DRV, Dietary Reference Value; EAR, Estimated Average Requirement; RNI, Reference Nutrient Intake; MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 8.
Difference in nutrient intake between replacement models and current intake; males aged 11-18 years (n = 112)

		Progressive Model								Ingredient Model										Fortification Model			
Nutrient	**Current**	**MA-25**	**P**	**MA-50**	**P**	**MA-75**	**P**	**MA-100**	**P**	**Vegetable**	**P**	**Mycoprotein**	**P**	**Legume**	**P**	**Tofu**	**P**	**Nut**	**P**	**Soy**	**P**	**Fortified**	**P**	**Unfortified**	**P**
Energy, kcal/day	2680.00	-	-	-	-	-	-	-	-			-	-	-	-					-	-	-	-	-	-
Carbohydrate, g/day	359.13	+4.87	0.69	+10.00	0.42	+15.41	0.21	+21.12	0.09	+27.32	0.03*	+24.85	0.04*	+22.10	0.07	+9.99	0.42	+12.73	0.30	+16.15	0.19	+12.47	0.31	+25.80	0.04*
Protein, g/day	104.21	-3.78	0.34	-7.76	0.05*	-11.95	<0.01*	-16.38	<0.01*	-21.45	<0.01*	-9.78	0.01*	-17.12	<0.01*	-5.40	0.17	-21.95	<0.01*	-7.98	0.04*	-3.43	0.39	-20.18	<0.01*
Fat, g/day	100.80	-0.86	0.84	-1.77	0.68	-2.73	0.53	-3.74	0.39	-4.87	0.26	-6.79	0.12	-3.87	0.37	-2.62	0.54	+1.53	0.72	-5.22	0.23	-5.43	0.21	-4.64	0.28
Fibre, g/day	24.05	+2.08	0.07	+4.27	<0.01*	+6.58	<0.01*	+9.02	<0.01*	+9.68	<0.01*	+9.66	<0.01*	+7.54	<0.01*	+3.48	<0.01*	+11.35	<0.01*	+6.94	<0.01*	+7.86	<0.01*	+9.28	<0.01*
Sugars, g/day	137.19	+1.06	0.89	+2.19	0.78	+3.37	0.67	+4.62	0.56	+4.91	0.53	+3.08	0.69	+3.79	0.63	+2.38	0.76	+8.25	0.29	+2.44	0.76	+1.87	0.81	+4.57	0.56
Saturated fat, g/day	38.69	-1.01	0.63	-2.08	0.32	-3.21	0.12	-4.40	0.04*	-4.25	0.04*	-4.05	0.05*	-4.14	0.05*	-4.80	0.02*	-5.32	0.01*	-4.09	0.05*	-3.66	0.08	-4.37	0.04*
Sodium, mg/day	2969.08	+58.32	0.66	+119.74	0.37	+184.53	0.16	+252.95	0.06	+3400.68	0.01*	+346.49	0.01*	+307.13	0.02*	+106.94	0.42	-137.89	0.30	+550.20	<0.01*	+535.41	<0.01*	+400.58	<0.01*
Iron, mg/day	14.42	-0.07	0.91	-0.14	0.81	-0.22	0.72	-0.30	0.62	-0.53	0.38	-1.07	0.07	-0.43	0.47	+8.47	<0.01*	-1.01	0.09	-0.04	0.94	+3.46	<0.01*	-0.60	0.32
B12, ug/day	6.93	-0.44	0.31	-0.91	0.04*	-1.40	<0.01*	-1.92	<0.01*	-1.94	<0.01*	-1.65	<0.01*	-1.89	<0.01*	-2.24	<0.01*	-2.09	<0.01*	-1.96	<0.01*	-0.37	0.40	-1.99	<0.01*

Blue indicates significant increase compared to Current. Gold indicates significant decrease compared to Current.
Differences compared using regression models with significance threshold P < 0.05
MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 9.
Mean nutrient intake for current and replacement models, with reference to DRVs; females aged 11-18 years (n = 115)

			Progressive Model				Ingredient Model						Fortification Model	
Nutrient	**DRV***	**Current**	**MA-25**	**MA-50**	**MA-75**	**MA-100**	**Vegetable**	**Mycoprotein**	**Legume**	**Tofu**	**Nut**	**Soy**	**Fortified**	**Unfortified**
Energy, kcal/day	2304	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate, g/day†	287.90	304.27	308.63	313.22	318.07	323.18	328.73	326.53	324.06	313.21	315.67	318.74	315.43	327.38
Protein, g/day†	86.40	89.78	86.40	82.84	79.08	75.11	70.58	81.02	74.45	84.94	70.12	82.64	86.71	71.72
Fat, g/day	89.60	88.01	87.24	86.42	85.57	84.66	83.65	81.93	84.54	85.66	89.38	83.33	83.15	83.85
Fibre, g/day	30.00	21.90	23.77	25.73	27.80	29.98	30.57	30.55	28.65	25.02	32.07	28.11	28.94	30.21
Sugars, g/day	90.00	116.35	117.31	118.31	119.37	120.49	120.75	119.11	119.75	118.49	123.74	118.53	118.03	120.44
Saturated fat, g/day	28.20	31.66	30.75	29.79	28.78	27.72	27.85	28.03	27.95	27.36	26.89	27.99	28.38	27.74
Sodium, mg/day	2400	2601.91	2654.14	2709.14	2767.14	2828.41	2906.96	2912.17	2876.93	2697.68	2478.45	3094.57	3081.31	2960.59
Iron, mg/day	14.80	14.72	14.66	14.59	14.52	14.45	14.24	13.76	14.33	22.30	13.82	14.68	17.82	14.18
B12, ug/day	1.5	5.19	4.79	4.38	3.94	3.47	3.45	3.71	3.50	3.18	3.32	3.44	4.86	3.41

*DRV values correspond to EAR for energy; RNI for Protein, Sodium, Iron, B12; DRV for Carbohydrate, Fat, Fibre, Sugars, Saturated fat.

† Protein target elected as 15% total energy intake and carbohydrate as 50% total energy intake in the current analysis.

Colours: Green, meeting DRV. Red, not meeting DRV. Grey, not meeting 15% protein and/or 50% carbohydrate target elected in the current analysis.

DRV, Dietary Reference Value; EAR, Estimated Average Requirement; RNI, Reference Nutrient Intake; MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 10.
Difference in nutrient intake between replacement models and current intake; females aged 11-18 years (n = 115)

		Progressive Model								Ingredient Model										Fortification Model			
Nutrient	**Current**	**MA-25**	**P**	**MA-50**	**P**	**MA-75**	**P**	**MA-100**	**P**	**Vegetable**	**P**	**Myco protein**	**P**	**Legume**	**P**	**Tofu**	**P**	**Nut**	**P**	**Soy**	**P**	**Fortified**	**P**	**Unfortified**	**P**
Energy, kcal/day	2304.00	-	-	-	-	-	-	-	-			-	-	-	-					-	-	-	-	-	-
Carbohydrate, g/day	304.27	+4.36	0.73	+8.95	0.48	+13.79	0.28	+18.91	0.14	+24.46	0.05*	+22.25	0.08	+19.79	0.12	+8.94	0.48	+11.40	0.37	+14.46	0.26	+11.16	0.38	+23.10	0.07
Protein, g/day	89.78	-3.38	0.27	-6.94	0.02*	-10.70	<0.01*	-14.67	<0.01*	-19.20	<0.01*	-8.76	<0.01*	-15.33	<0.01*	-4.84	0.12	-19.66	<0.01*	-7.14	0.02*	-3.07	0.32	-18.06	<0.01*
Fat, g/day	88.01	-0.77	0.85	-1.58	0.70	-2.44	0.56	-3.35	0.42	-4.36	0.29	-6.08	0.14	-3.47	0.40	-2.35	0.57	+1.37	0.74	-4.67	0.26	-4.86	0.24	-4.16	0.32
Fibre, g/day	21.90	+1.86	0.07	+3.82	<0.01*	+5.89	<0.01*	+8.08	<0.01*	+8.67	<0.01*	+8.65	<0.01*	+6.75	<0.01*	+3.12	<0.01*	+10.17	<0.01*	+6.21	<0.01*	+7.03	<0.01*	+8.31	<0.01*
Sugars, g/day	116.35	+0.95	0.90	+1.96	0.79	+3.02	0.69	+4.14	0.58	+4.40	0.56	+2.76	0.71	+3.39	0.65	+2.13	0.78	+7.39	0.32	+2.18	0.77	+1.67	0.82	+4.09	0.58
Saturated fat, g/day	31.66	-0.91	0.61	-1.86	0.29	-2.87	0.11	-3.94	0.03*	-3.80	0.03*	-3.63	0.04*	-3.70	0.04*	-4.30	0.02*	-4.77	0.01*	-3.66	0.04*	-3.28	0.07	-3.91	0.03*
Sodium, mg/day	2601.91	+52.23	0.68	+107.23	0.40	+165.23	0.20	+226.49	0.08	+305.05	0.02*	+310.25	0.02*	+275.02	0.03*	+95.76	0.45	-123.47	0.33	+492.65	<0.01*	+479.39	<0.01*	+358.67	0.01*
Iron, mg/day	14.72	-0.06	0.99	-0.13	0.97	-0.19	0.96	-0.27	0.94	-0.47	0.90	-0.96	0.80	-0.39	0.92	+7.58	0.05*	-0.90	0.81	-0.04	0.99	3.10	0.41	-0.54	0.89
B12, ug/day	5.19	-0.40	0.18	-0.81	0.01*	-1.25	<0.01*	-1.72	<0.01*	-1.74	<0.01*	-1.48	<0.01*	-1.69	<0.01*	-2.01	<0.01*	-1.87	<0.01*	-1.75	<0.01*	-0.33	0.26	-1.78	<0.01*

Blue indicates significant increase compared to Current. Gold indicates significant decrease compared to Current.
Differences compared using regression models with significance threshold P < 0.05
MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 11.
Mean nutrient intake for current and replacement models, with reference to DRVs; males aged 19-64 years (n = 198)

			Progressive Model				Ingredient Model						Fortification Model	
Nutrient	**DRV***	**Current**	**MA-25**	**MA-50**	**MA-75**	**MA-100**	**Vegetable**	**Mycoprotein**	**Legume**	**Tofu**	**Nut**	**Soy**	**Fortified**	**Unfortified**
Energy, kcal/day	2662	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate, g/day†	332.80	317.53	323.43	329.63	336.18	343.09	350.59	347.61	344.27	329.62	332.94	337.08	332.62	348.76
Protein, g/day†	99.80	112.20	107.63	102.81	97.73	92.37	86.24	100.36	91.48	105.66	85.63	102.54	108.04	87.78
Fat, g/day	103.50	99.68	98.64	97.54	96.38	95.16	93.79	91.46	95.00	96.51	101.53	93.37	93.11	94.06
Fibre, g/day	30.00	27.25	29.77	32.42	35.22	38.17	38.97	38.94	36.37	31.47	40.99	35.65	36.76	38.49
Sugars, g/day	90.00	126.83	128.12	129.47	130.91	132.42	132.77	130.56	131.42	129.71	136.82	129.78	129.09	132.35
Saturated fat, g/day	32.50	36.64	35.41	34.12	32.75	31.31	31.50	31.73	31.63	30.83	30.20	31.69	32.21	31.35
Sodium, mg/day	2400	3114.72	3185.31	3259.64	3338.03	3420.85	3526.99	3534.03	3486.41	3244.14	2947.85	3780.55	3762.67	3599.48
Iron, mg/day	8.70	16.05	15.96	15.88	15.78	15.69	15.41	14.75	15.52	26.30	14.83	15.99	20.23	15.32
B12, ug/day	1.5	8.98	8.45	7.88	7.29	6.66	6.63	6.99	6.69	6.27	6.45	6.61	8.54	6.58

*DRV values correspond to EAR for energy; RNI for Protein, Sodium, Iron, B12; DRV for Carbohydrate, Fat, Fibre, Sugars, Saturated fat.

† Protein target elected as 15% total energy intake and carbohydrate as 50% total energy intake in the current analysis.

Colours: Green, meeting DRV. Red, not meeting DRV. Grey, not meeting 15% protein and/or 50% carbohydrate target elected in the current analysis.

DRV, Dietary Reference Value; EAR, Estimated Average Requirement; RNI, Reference Nutrient Intake; MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 12.
Difference in nutrient intake between replacement models and current intake; males aged 19-64 years (n = 198)

		Progressive Model								Ingredient Model										Fortification Model			
Nutrient	**Current**	**MA-25**	**P**	**MA-50**	**P**	**MA-75**	**P**	**MA-100**	**P**	**Vegetable**	**P**	**Myco protein**	**P**	**Legume**	**P**	**Tofu**	**P**	**Nut**	**P**	**Soy**	**P**	**Fortified**	**P**	**Unfortified**	**P**
Energy, kcal/day	2662.00	-	-	-	-	-	-	-	-			-	-	-	-					-	-	-	-	-	-
Carbohydrate, g/day	317.53	+5.89	0.60	+12.10	0.29	+18.64	0.10	+25.56	0.02*	+33.06	<0.01*	+30.08	0.01*	+26.74	0.02*	+12.09	0.29	+15.41	0.18	+19.55	0.09	+15.09	0.19	+31.23	0.01*
Protein, g/day	112.20	-4.57	0.11	-9.39	<0.01*	-14.46	<0.01*	-19.83	<0.01*	-25.96	<0.01*	-11.83	<0.01*	-20.72	<0.01*	-6.54	0.02*	-26.57	<0.01*	-9.66	<0.01*	-4.15	0.14	-24.42	<0.01*
Fat, g/day	99.68	-1.04	0.77	-2.14	0.54	-3.30	0.35	-4.52	0.20	-5.89	0.10	-8.22	0.02*	-4.69	0.18	-3.18	0.37	+1.85	0.60	-6.32	0.07	-6.57	0.06	-5.62	0.11
Fibre, g/day	27.25	+2.52	0.04*	+5.17	<0.01*	+7.97	<0.01*	+10.92	<0.01*	+11.71	<0.01*	+11.69	<0.01*	+9.12	<0.01*	+4.21	<0.01*	+13.74	<0.01*	+8.39	<0.01*	+9.51	<0.01*	+11.23	<0.01*
Sugars, g/day	126.83	+1.29	0.84	+2.65	0.68	+4.08	0.53	+5.59	0.39	+5.94	0.36	+3.73	0.57	+4.59	0.48	+2.88	0.66	+9.99	0.12	+2.95	0.65	+2.26	0.73	+5.53	0.40
Saturated fat, g/day	36.64	-1.23	0.42	-2.52	0.10	-3.88	0.01*	-5.32	<0.01*	-5.14	<0.01*	-4.90	<0.01*	-5.01	<0.01*	-5.81	<0.01*	-6.44	<0.01*	-4.95	<0.01*	-4.43	<0.01*	-5.29	<0.01*
Sodium, mg/day	3114.72	+70.58	0.60	+144.91	0.28	+223.31	0.10	+306.13	0.02*	+412.26	<0.01*	+419.30	<0.01*	+371.68	0.01*	+129.42	0.34	-166.87	0.21	+665.82	<0.01*	+647.95	<0.01*	+484.75	<0.01*
Iron, mg/day	16.05	-0.08	0.90	-0.17	0.79	-0.26	0.69	-0.36	0.58	-0.64	0.33	-1.30	0.05*	-0.52	0.42	+10.25	<0.01*	-1.22	0.06	-0.05	0.93	+4.19	<0.01*	-0.73	0.26
B12, ug/day	8.98	-0.54	0.65	-1.10	0.35	-1.69	0.15	-2.32	0.05*	-2.35	0.05*	-2.00	0.09	-2.29	0.05*	-2.72	0.02*	-2.53	0.03*	-2.37	0.04*	-0.44	0.71	-2.40	0.04*

Blue indicates significant increase compared to Current. Gold indicates significant decrease compared to Current.
Differences compared using regression models with significance threshold P < 0.05
MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 13.
Mean nutrient intake for current and replacement models, with reference to DRVs; females aged 19-64 years (n = 257)

			Progressive Model				Ingredient Model						Fortification Model	
Nutrient	**DRV***	**Current**	**MA-25**	**MA-50**	**MA-75**	**MA-100**	**Vegetable**	**Mycoprotein**	**Legume**	**Tofu**	**Nut**	**Soy**	**Fortified**	**Unfortified**
Energy, kcal/day	2127	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate, g/day†	265.90	257.49	261.74	266.21	270.92	275.90	281.30	279.15	276.75	266.20	268.59	271.57	268.36	279.98
Protein, g/day†	79.80	90.18	86.89	83.42	79.76	75.90	71.49	81.66	75.26	85.47	71.05	83.23	87.19	72.60
Fat, g/day	82.70	81.92	81.17	80.38	79.54	78.66	77.68	76.00	78.54	79.63	83.25	77.37	77.19	77.87
Fibre, g/day	30.00	23.50	25.32	27.23	29.24	31.37	31.94	31.92	30.07	26.54	33.40	29.55	30.35	31.59
Sugars, g/day	90.00	104.22	105.15	106.12	107.15	108.24	108.50	106.90	107.52	106.29	111.41	106.34	105.85	108.20
Saturated fat, g/day	26.00	30.33	29.45	28.52	27.54	26.50	26.63	26.80	26.73	26.15	25.70	26.77	27.14	26.53
Sodium, mg/day	2400	2416.49	2467.30	2520.83	2577.28	2636.91	2713.34	2718.42	2684.12	2509.67	2296.33	2895.91	2883.03	2765.54
Iron, mg/day	14.80	15.93	15.87	15.80	15.74	15.67	15.47	14.99	15.55	23.31	15.05	15.89	18.94	15.40
B12, ug/day	1.5	10.85	10.46	10.06	9.63	9.18	9.16	9.41	9.20	8.89	9.03	9.14	10.53	9.12

*DRV values correspond to EAR for energy; RNI for Protein, Sodium, Iron, B12; DRV for Carbohydrate, Fat, Fibre, Sugars, Saturated fat.

† Protein target elected as 15% total energy intake and carbohydrate as 50% total energy intake in the current analysis.

Colours: Green, meeting DRV. Red, not meeting DRV. Grey, not meeting 15% protein and/or 50% carbohydrate target elected in the current analysis.

DRV, Dietary Reference Value; EAR, Estimated Average Requirement; RNI, Reference Nutrient Intake; MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 14.
Difference in nutrient intake between replacement models and current intake; females aged 19-64 years (n = 257)

		Progressive Model								Ingredient Model										Fortification Model			
Nutrient	**Current**	**MA-25**	**P**	**MA-50**	**P**	**MA-75**	**P**	**MA-100**	**P**	**Vegetable**	**P**	**Myco protein**	**P**	**Legume**	**P**	**Tofu**	**P**	**Nut**	**P**	**Soy**	**P**	**Fortified**	**P**	**Unfortified**	**P**
Energy, kcal/day	2127.00	-	-	-	-	-	-	-	-			-	-	-	-					-	-	-	-	-	-
Carbohydrate, g/day	257.49	+4.24	0.59	+8.71	0.27	+13.42	0.09	+18.40	0.02*	+23.81	<0.01*	+21.66	0.01*	+19.25	0.02*	+8.70	0.27	+11.09	0.16	+14.08	0.08	+10.86	0.17	+22.48	<0.01*
Protein, g/day	90.18	-3.29	0.14	-6.76	<0.01*	-10.41	<0.01*	-14.28	<0.01*	-18.69	<0.01*	-8.52	<0.01*	-14.92	<0.01*	-4.71	0.04*	-19.13	<0.01*	-6.95	<0.01*	-2.99	0.18	-17.58	<0.01*
Fat, g/day	81.92	-0.75	0.79	-1.54	0.59	-2.38	0.41	-3.26	0.25	-4.24	0.14	-5.92	0.04*	-3.37	0.24	-2.29	0.42	+1.33	0.64	-4.55	0.11	-4.73	0.10	-4.05	0.16
Fibre, g/day	23.50	+1.81	0.05*	+3.72	<0.01*	+5.74	<0.01*	+7.86	<0.01*	+8.44	<0.01*	+8.42	<0.01*	+6.57	<0.01*	+3.03	<0.01*	+9.89	<0.01*	+6.04	<0.01*	+6.85	<0.01*	+8.09	<0.01*
Sugars, g/day	104.22	+0.93	0.84	+1.91	0.69	+2.94	0.53	+4.03	0.39	+4.28	0.36	+2.68	0.57	+3.30	0.48	+2.08	0.66	+7.19	0.13	+2.12	0.65	+1.63	0.73	+3.98	0.40
Saturated fat, g/day	30.33	-0.88	0.46	-1.81	0.13	-2.80	0.02*	-3.83	<0.01*	-3.70	<0.01*	-3.53	<0.01*	-3.60	<0.01*	-4.18	<0.01*	-4.64	<0.01*	-3.57	<0.01*	-3.19	0.01*	-3.81	<0.01*
Sodium, mg/day	2416.49	+50.81	0.51	+104.34	0.18	+160.79	0.04*	+220.43	<0.01*	+296.85	<0.01*	+301.93	<0.01*	+267.64	<0.01*	+93.18	0.23	-120.15	0.12	+479.42	<0.01*	+466.54	<0.01*	+349.05	<0.01*
Iron, mg/day	15.93	-0.06	0.98	-0.12	0.95	-0.19	0.92	-0.26	0.90	-0.46	0.82	-0.94	0.64	-0.38	0.85	+7.38	<0.01*	-0.88	0.66	-0.04	0.98	+3.01	0.13	-0.53	0.79
B12, ug/day	10.85	-0.39	0.92	-0.79	0.84	-1.22	0.76	-1.67	0.67	-1.69	0.67	-1.44	0.72	-1.65	0.68	-1.96	0.62	-1.82	0.65	-1.71	0.67	-0.32	0.94	-1.73	0.66

Blue indicates significant increase compared to Current. Gold indicates significant decrease compared to Current.
Differences compared using regression models with significance threshold P < 0.05
MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 15.
Mean nutrient intake for current and replacement models, with reference to DRVs; males aged ≤ 65 years (n = 66)

			Progressive Model				Ingredient Model						Fortification Model	
Nutrient	**DRV***	**Current**	**MA-25**	**MA-50**	**MA-75**	**MA-100**	**Vegetable**	**Mycoprotein**	**Legume**	**Tofu**	**Nut**	**Soy**	**Fortified**	**Unfortified**
Energy, kcal/day	2318	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate, g/day	289.80	263.86	268.87	274.15	279.72	285.60	291.98	289.44	286.60	274.14	276.97	280.49	276.69	290.42
Protein, g/day	86.90	94.26	90.37	86.27	81.96	77.39	72.18	84.19	76.63	88.70	71.66	86.04	90.72	73.49
Fat, g/day	90.10	89.56	88.68	87.74	86.76	85.72	84.56	82.57	85.58	86.86	91.14	84.19	83.98	84.78
Fibre, g/day	30.00	24.15	26.29	28.55	30.93	33.44	34.12	34.10	31.91	27.74	35.84	31.29	32.24	33.71
Sugars, g/day	90.00	115.14	116.23	117.39	118.61	119.89	120.19	118.31	119.04	117.59	123.64	117.65	117.06	119.84
Saturated fat, g/day	28.30	33.45	32.41	31.31	30.15	28.93	29.08	29.28	29.20	28.51	27.98	29.24	29.69	28.96
Sodium, mg/day	2400	2460.51	2520.54	2583.76	2650.44	2720.88	2811.16	2817.13	2776.64	2570.59	2318.58	3026.83	3011.61	2872.80
Iron, mg/day	8.70	15.10	15.03	14.95	14.87	14.79	14.55	13.99	14.65	23.81	14.06	15.05	18.66	14.48
B12, ug/day	1.5	8.41	7.95	7.47	6.97	6.44	6.41	6.71	6.46	6.10	6.26	6.39	8.03	6.37

*DRV values correspond to EAR for energy; RNI for Protein, Sodium, Iron, B12; DRV for Carbohydrate, Fat, Fibre, Sugars, Saturated fat.

† Protein target elected as 15% total energy intake and carbohydrate as 50% total energy intake in the current analysis.

Colours: Green, meeting DRV. Red, not meeting DRV. Grey, not meeting 15% protein and/or 50% carbohydrate target elected in the current analysis.

DRV, Dietary Reference Value; EAR, Estimated Average Requirement; RNI, Reference Nutrient Intake; MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 16.
Difference in nutrient intake between replacement models and current intake; males aged ≤ 65 years (n = 66)

		Progressive Model								Ingredient Model										Fortification Model			
Nutrient	**Current**	**MA-25**	**P**	**MA-50**	**P**	**MA-75**	**P**	**MA-100**	**P**	**Vegetable**	**P**	**Myco protein**	**P**	**Legume**	**P**	**Tofu**	**P**	**Nut**	**P**	**Soy**	**P**	**Fortified**	**P**	**Unfortified**	**P**
Energy, kcal/day	2318.00	-	-	-	-	-	-	-	-			-	-	-	-					-	-	-	-	-	-
Carbohydrate, g/day	263.86	+5.01	0.66	+10.29	0.37	+15.86	0.17	+21.74	0.06	+28.12	0.01*	+25.58	0.03*	+22.74	0.05*	+10.28	0.37	+9.20	0.20	+16.63	0.15	+12.83	0.26	+26.56	0.02*
Protein, g/day	94.26	-3.89	0.26	-7.98	0.02*	-12.30	<0.01*	-16.86	<0.01*	-22.08	<0.01*	-10.07	<0.01*	-17.62	<0.01*	-5.56	0.11	-22.60	<0.01*	-8.21	0.02*	-3.53	0.31	-20.77	<0.01*
Fat, g/day	89.56	-0.89	0.88	-1.82	0.75	-2.81	0.62	-3.85	0.50	-5.01	0.38	-6.99	0.22	-3.99	0.48	-2.70	0.63	+1.57	0.78	-5.37	0.34	-5.59	0.32	-4.78	0.40
Fibre, g/day	24.15	+2.14	0.16	+4.40	<0.01*	+6.77	<0.01*	+9.29	<0.01*	+9.96	<0.01*	+9.94	<0.01*	+7.76	<0.01*	+3.58	0.02*	+11.69	<0.01*	+7.14	<0.01*	+8.09	<0.01*	+9.55	<0.01*
Sugars, g/day	115.14	+1.10	0.89	+2.25	0.78	+3.47	0.67	+4.75	0.56	+5.05	0.54	+3.17	0.70	+3.90	0.63	+2.45	0.77	+8.50	0.30	+2.51	0.76	+1.92	0.81	+4.70	0.57
Saturated fat, g/day	33.45	-1.04	0.66	-2.14	0.37	-3.30	0.17	-4.53	0.06	-4.37	0.07	-4.17	0.08	-4.26	0.07	-4.94	0.04*	-5.48	0.02*	-4.21	0.08	-3.77	0.11	-4.50	0.06
Sodium, mg/day	2460.51	+60.03	0.65	+123.25	0.35	+189.93	0.15	+260.37	0.05*	+356.65	0.01*	+356.62	0.01*	+316.13	0.02*	+110.08	0.41	-141.93	0.28	+566.32	<0.01*	+551.10	<0.01*	+412.29	<0.01*
Iron, mg/day	15.10	-0.07	0.97	-0.15	0.95	-0.22	0.92	-0.31	0.89	-0.55	0.80	-1.11	0.61	-0.45	0.83	+8.72	<0.01*	-1.04	0.63	-0.05	0.98	+3.56	0.10	-0.62	0.77
B12, ug/day	8.41	-0.46	0.56	-0.93	0.23	-1.44	0.06	-1.97	0.01*	-2.00	0.01*	-1.70	0.03*	-1.95	0.01*	-2.31	<0.01*	-2.15	0.01*	-2.01	0.01*	-0.38	0.63	-2.04	0.01*

Blue indicates significant increase compared to Current. Gold indicates significant decrease compared to Current.
Differences compared using regression models with significance threshold P < 0.05
MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 17.
Mean nutrient intake for current and replacement models, with reference to DRVs; females aged ≤ 65 years (n = 98)

			Progressive Model				Ingredient Model						Fortification Model	
Nutrient	**DRV***	**Current**	**MA-25**	**MA-50**	**MA-75**	**MA-100**	**Vegetable**	**Mycoprotein**	**Legume**	**Tofu**	**Nut**	**Soy**	**Fortified**	**Unfortified**
Energy, kcal/day	1876	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydrate, g/day†	234.50	226.87	230.32	233.95	237.78	241.83	246.23	244.48	242.53	233.94	235.89	238.31	235.70	245.15
Protein , g/day†	70.40	76.98	74.30	71.48	68.51	65.37	61.78	70.05	64.84	73.15	61.42	71.32	74.54	62.68
Fat, g/day	73.00	74.96	74.35	73.71	73.03	72.31	71.51	70.15	72.21	73.10	76.04	71.26	71.11	71.67
Fibre, g/day	30.00	21.05	22.53	24.08	25.72	27.45	27.91	27.90	26.39	23.52	29.10	25.97	26.62	27.63
Sugars, g/day	90.00	100.74	101.50	102.29	103.13	104.02	104.22	102.93	103.43	102.43	106.59	102.47	102.07	103.98
Saturated fat, g/day	22.90	30.43	29.71	28.96	28.16	27.32	27.42	27.56	27.50	27.03	26.66	27.53	27.84	27.34
Sodium, mg/day	2400	2038.91	2080.24	2123.78	2169.69	2218.18	2280.33	2284.47	2256.57	2114.70	1941.19	2428.82	2418.35	2322.78
Iron, mg/day	8.70	13.97	13.93	13.87	13.82	13.76	13.60	13.21	13.67	19.98	13.26	13.94	16.43	13.55
B12, ug/day	1.5	6.72	6.41	6.08	5.73	5.36	5.34	5.55	5.38	5.13	5.24	5.33	6.46	5.31

*DRV values correspond to EAR for energy; RNI for Protein, Sodium, Iron, B12; DRV for Carbohydrate, Fat, Fibre, Sugars, Saturated fat.

† Protein target elected as 15% total energy intake and carbohydrate as 50% total energy intake in the current analysis.

Colours: Green, meeting DRV. Red, not meeting DRV. Grey, not meeting 15% protein and/or 50% carbohydrate target elected in the current analysis.

DRV, Dietary Reference Value; EAR, Estimated Average Requirement; RNI, Reference Nutrient Intake; MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

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Supplementary Table 18.
Difference in nutrient intake between replacement models and current intake; females aged ≤ 65 years (n = 98)

		Progressive Model								Ingredient Model										Fortification Model			
Nutrient	**Current**	**MA-25**	**P**	**MA-50**	**P**	**MA-75**	**P**	**MA-100**	**P**	**Vegetable**	**P**	**Mycoprotein**	**P**	**Legume**	**P**	**Tofu**	**P**	**Nut**	**P**	**Soy**	**P**	**Fortified**	**P**	**Unfortified**	**P**
Energy, kcal/day	1876.00	-	-	-	-	-	-	-	-			-	-	-	-					-	-	-	-	-	-
Carbohydrate, g/day	226.87	+3.45	0.69	+7.09	0.42	+10.92	0.21	+14.97	0.09	+19.36	0.03*	+17.61	0.05*	+15.66	0.07	+7.08	0.42	+9.02	0.30	+11.45	0.19	+8.84	0.31	+18.29	0.04*
Protein, g/day	76.98	-2.68	0.31	-5.50	0.04*	-8.47	<0.01*	-11.61	<0.01*	-15.20	<0.01*	-6.93	0.01*	-12.13	<0.01*	-3.83	0.15	-15.56	<0.01*	-5.65	0.03*	-2.43	0.36	-14.30	<0.01*
Fat, g/day	74.96	-0.61	0.86	-1.25	0.71	-1.97	0.42	-2.65	0.44	-3.45	0.31	-4.81	0.16	-2.74	0.42	-1.86	0.59	+1.08	0.75	-3.70	0.28	-3.85	0.26	-3.29	0.34
Fibre, g/day	21.05	+1.47	0.22	+3.03	0.01*	+4.66	<0.01*	+6.39	<0.01*	+6.86	<0.01*	+6.85	<0.01*	+5.34	<0.01*	+2.47	0.04*	+8.05	<0.01*	+4.92	<0.01*	+5.57	<0.01*	+6.58	<0.01*
Sugars, g/day	100.74	+0.75	0.90	+1.55	0.79	+2.39	0.69	+3.27	0.58	+3.48	0.56	+2.18	0.71	+2.69	0.65	+1.72	0.64	+5.85	0.32	+1.73	0.77	+1.32	0.82	+3.24	0.59
Saturated fat, g/day	30.43	-0.72	0.65	-1.48	0.35	-2.27	0.15	-3.18	<0.01*	-3.01	0.06	-2.87	0.07	-2.93	0.07	-3.40	0.03*	-3.77	0.02*	-2.90	0.07	-2.59	0.10	-3.10	0.05*
Sodium, mg/day	2038.91	+41.33	0.63	+84.87	0.33	+130.78	0.13	+179.27	0.04*	+241.42	0.01*	+245.56	<0.01*	+217.66	0.01*	+75.80	0.38	-97.72	0.26	+389.91	<0.01*	+379.44	<0.01*	+283.87	<0.01*
Iron, mg/day	13.97	-0.05	0.99	-0.10	0.98	-0.15	0.97	-0.21	0.96	-0.38	0.92	-0.76	0.84	-0.31	0.94	+6.00	0.12	-0.71	0.85	-0.03	0.99	+2.45	0.52	-0.43	0.91
B12, ug/day	6.72	-0.31	0.77	-0.64	0.54	-0.99	0.35	-1.36	0.20	-1.38	0.20	-1.17	0.27	-1.34	0.21	-1.59	0.13	-1.48	0.16	-1.39	0.19	-0.26	0.81	-1.41	0.19

Blue indicates significant increase compared to Current. Gold indicates significant decrease compared to Current.
Differences compared using regression models with significance threshold P < 0.05
MA-25, Replacement model substituting 25% of self-reported meat intake with composite of meat alternatives; MA-50, Replacement model substituting 50% of self-reported meat intake with composite of meat alternatives; MA-75, Replacement model substituting 75% of self-reported meat intake with composite of meat alternatives; MA-100, Replacement model substituting 100% of self-reported meat intake with composite of meat alternatives.

5470

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