Investigating the effects of mycoprotein on glycaemic control and appetite in South Asian and white European adults with Type 2 Diabetes

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La feina mal feta no té futur, la feina ben feta no té fronteres.

Mama (2001)

ABSTRACT

Type 2 diabetes (T2D) is a disease with a high prevalence in south Asian countries. Diet is the cornerstone strategy for the management of T2D. South Asians may have increased postprandial blood glucose responses following the same dietary challenge compared to white Europeans. Dietary fibre and protein play a role in regulating blood glucose and appetite via a myriad of mechanisms such as gut microbiota fermentation to short-chain fatty acids. Mycoprotein is a food ingredient high in both fibre and protein which has a positive effect on blood glucose and appetite in humans. Likewise, guar gum is a fibre-rich ingredient that decreases blood glucose and appetite in people with T2D. The aim of this thesis is to determine the effect of mycoprotein on glycaemia and energy intake in humans and the mechanisms underpinning these. In **Study 1**, the associations between mycoprotein-based food consumers and non-consumers with non-communicable disease markers such as blood glucose, energy intake and diet quality are studied in the UK free-living population using the National Diet and Nutrition Survey. The results showed that there is a positive association between mycoprotein consumers and healthy diet scores, fibre, fasting glucose and glycated haemoglobin. In **Study 2**, a systematic review of the effects of mycoprotein on glycaemic control and appetite in humans is performed. The results showed that the acute effects of mycoprotein on glycaemia are unclear, there is a consistent decrease in insulinaemia and energy intake in healthy humans. In **Study 3**, the acute effect of mycoprotein and guar gum in glycaemic control and appetite in people with T2D of South Asian and European ethnicity is investigated via a randomised controlled trial. The results showed that mycoprotein decreases postprandial blood glucose and that enriching chapati with guar gum induces a decreased blood glucose response. South Asians, however, had an increased postprandial glucose response compared to white Europeans. In **Study 4**, the effects of mycoprotein fermentation by the healthy gut microbiota is investigated using *in vitro* batch fermentation cultures. The results on breath hydrogen, pH, SCFAs, and bacterial communities, showed that mycoprotein may not be fermented.

DECLARATION OF CONTRIBUTORS

The work of this thesis is original. Most of the work in this thesis was performed by the author. Everything else has been appropriately cited. All collaboration and assistance are detailed below.

All the clinical studies were performed with the help of the research nurses and administration staff at the NIHR/Wellcome Trust Imperial Clinical Research Facility. The medical support was covered by Dr Martina Tashkova.

Chapter 2: The data used in Chapter 2 was collected by the National Diet and Nutrition Survey-Rolling Programme (NDNS-RP) and conducted by the NatCen Social Research working with the Medical Research Council Epidemiological Unit. The dietary data were coded by the MRC team. The Public Health England and the UK Food Standards Agency jointly fund the UK NDNS-RP. The analysis of the associations of interest was conducted by myself Anna Cherta-Murillo. The study was published in the British Journal of Nutrition but permission to use the published material in this thesis from the copyright holder was sought and granted. The proof of this permission for the right to publish can be found in Appendix 28.

Chapter 3: The study was published in the British Journal of Nutrition but permission to use the published material in this thesis from the copyright holder was sought and granted. The proof of this permission for the right to publish can be found in Appendix 28.

Chapter 5: The test foods were produced by NPD Ltd and funded by Quorn Foods Ltd. The human study visits were conducted with the assistance of Miss Kexin Zhou and Dr Martina Tashkova. Insulin and gut hormone radioimmunoassays were performed with the assistance of Dr Edward Chambers, Dr. Georgia Becker, Professor Kevin Murphy, Jamie Frampton, Claire Ho, Ana Claudia Oliveira, Mingzhu Cai, Shilpa Tejpal, Jen Pugh.

Chapter 6: The *in vitro* batch cultures were performed in collaboration with the Department of Hepatology in St Marys Hospital, under the supervision of Dr Julie McDonald and with the help of Jesús Miguens Blanco, Dr Nathan Danckert who helped with the stool samples 16S rRNA gene sequencing analysis, Dr Lauren Roberts, who helped with the *in vitro* batch culture fermentation experiment.

The GC-MS/MS experiment and analysis of the cultures were done in collaboration with the Department of Metabolism, Digestion and Reproduction under the supervision of Maria Valdivia-Garcia, who ran the experiment and provided guidance with the analysis.

CHALLENGES

This thesis was framed under very constrained circumstances such as the COVID-19 pandemic outbreak. Because of it, recruitment, study visits for the human study as well as any bench laboratory work were stopped for 6 months from March 2020 to September 2020. Without the impact of the COVID-19 pandemic, a long-term human study was planned to be conducted.

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ACHIEVEMENTS

Publications

Corrado, M., **Cherta-Murillo, A.,** Chambers, E. S., Wood, A. J., Plummer, A., Lovegrove, A., & Hazard, B. A. (2020). Effect of semolina pudding prepared from starch branching enzyme IIa and b mutant wheat on glycaemic response *in vitro* and in vivo: a randomised controlled pilot study. *Food & Function*, *11*(1), 617-627.

Cherta-Murillo, A., Lett, A. M., Frampton, J., Chambers, E. S., Finnigan, T. J., & Frost, G. S. (2020). Effects of mycoprotein on glycaemic control and energy intake in humans: a systematic review. *British Journal of Nutrition*, *123*(12), 1321-1332.

Cherta-Murillo, A., Frost, G. S. (2021). The association of mycoprotein-based foods consumption with diet quality, energy intake and non-communicable diseases' risk in the UK adult population using the National Diet and Nutrition Survey (NDNS) years 2008/09-2016/17: A cross-sectional study. *British Journal of Nutrition*, *123*(12), 1321-1332.

Cherta-Murillo, A., Alaraj-Alshehhi, S., Pugh, J , Hajjar, D., Chambers, E., Frost, GS. (2021) The effect of short-chain fatty acids on glycemic control in humans: A systematic review and meta-analysis. *American Clinical Journal of Nutrition. [Accepted on 7th of April 2022]*

Cherta-Murillo, A. Danckert, N, Valdivia-Garcia, M, Chambers, E, Roberts, L, Miguens-Blanco, J, Seone-Castro, R, McDonald, J, Marchesi, J, Frost, GS. Gut microbiota fermentation profiles of pre-digested mycoprotein using *in vitro* batch culture: A pilot study *[Manuscript under preparation]*

Cherta-Murillo, A. Zhou, K., Oliveira, AC., Chambers, ES., Frost, GS. The effect of mycoprotein and guar gum on blood glucose, appetite and energy intake in people with Type 2 Diabetes of south Asian and white European ethnicities. *[Manuscript under preparation]*

Posts in the Media

"Is Mycoprotein an ideal food for managing blood sugar levels in Type 2 Diabetes?". Imperial College London Medicine Blog, June 2019. Available from: https://blogs.imperial.ac.uk/imperial-medicine/author/ac6717/. Date of access: October 2021.

"Mycoprotein: a potential beneficial food for people with Type 2 Diabetes". Imperial College London Felix Newspaper, May 2019. Available from: https://old.felixonline.co.uk/articles/2019-05-17-mycoprotein-a-potential-beneficial-food-for-people-with-type-2-diabetes/. Date of access: October 2021.

Academic Experience

Supervised and mentored 2 MRs students (Imperial College London, 2018-2019,2019-2020).

Tutored 3rd year Medical Students on the Endocrinology intercalated BSc, (Imperial College London, 2019 and 2020).

Taughted on an endocrinology practical lesson for disadvantaged youth and assisted the Imperial College London Summer School in 2018 and 2019.

Awards

Travel Grant for a 3-day Symposium European Endocrinology in St. Odile, Strasbourg 2018.

Short-listed 3-minute thesis competition ICL, Summer competition, 2020.

Conferences and Presentations

Poster presentation in research day Imperial NIHR, 2019.

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ABBREVIATIONS

ADA American Diabetes Association

ADP Adenosine Diphosphate

AGRP Agouti-Related Protein

ALT Alanine Transferases

ANOVA Analysis of Variance

AOAC Association of Official Agricultural Chemists

ARC Arcuate Nucleus of The Hypothalamus

AT Adipose Tissue

ATP Adenosine Triphosphate

AUC Area Under the Curve

BCAAs Branched Chain Amino Acids

BIA Bioelectrical Impedance Analysis

BMI Body Mass Index

CCK Cholecystokinin

CHO Carbohydrate

CI Confidence Interval

CNS Central Nervous System

CONSORT Consolidated Standards of Reporting Trials

CRP C-Reactive Protein

CV Coefficient of Variability

DASH Dietary Approaches to Stop Hypertension

DPP4i Dipeptidyl Peptidase-4 Inhibitor

DRA Dietary Recommended Allowance

EE Energy Expenditure

EFSA European Food Safety Authority

FAO Food Agriculture Organisation

FAO Food Agriculture Organisation

FBG Fasting Blood Glucose

FDA Food and Drug Agency

FFAR Free Fatty Acid Receptor

G Grams

GC-MS/MS Gas Chromatography – Mass Spectrometry

GCPR G-Coupled Protein

GE Gastric Emptying

GIP Gastric Inhibitory Peptide or Glucose-Dependent Insulinotropic Polypeptide

GIT Gastrointestinal Tract

GLP-1 Glucagon-Like Peptide 1

HbA1c Glycated Haemoglobin A1c

HDI Healthy Diet Index

HDL-c High Density Lipoprotein Cholesterol

IAUC Incremental Area Under the Curve

IR Insulin Resistance

IRS Insulin Receptor Substrate

ISI Insulin Sensititivy Index

INSR Insulin Receptor

kDa Kilodalton

LC-MS/MS Liquid Chromatography with Tandem Mass Spectrometry

LDL-c Low Density Lipoprotein Cholesterol

LMM Linear Mixed Model

Min Minute

MMTT Mixed Meal Tolerance Test

MSSP Macronutrient Self-Selection Paradigm

mTOR Mammalian Target of Rapamicin

n.d Non-Detectable

n Sample Size Per Group

N Total Study Sample Size

NCDs Non-Communicable Diseases

NDNS National Diet and Nutrition Survey

NPY Neuropeptide Tyrosine

NSP Non-Soluble Polysaccharide

OGTT Oral Glucose Tolerance Test

OR Odds Ratio

Pa Pascal

PBS Phosphate Bovine Serum

PDCAAS Protein Digestibility-Corrected Aminoacid Score

POMC/CART Proopiomelanocortin and Cocaine- And Amphetamine-Regulated Transcript

PPM Parts per Million

PRISMA Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PUFA Polyunsaturated Fatty Acids

PYY Peptide Tyrosine Tyrosine

RCF Relative Centrifuge Force

RCTs Randomised Clinical Trials

RIA Radioimmunoassay

ROS Reactive Oxygen Species

RP Rolling Programme

SCFAs Short Chain Fatty Acids

SD Standard Deviation

SEM Standard Error Of The Mean

SFA Saturated Fatty Acids

SGLT2i Sodium Glucose Transporter-2 Inhibitor

SV Structural Variants

T2D Type 2 Diabetes

TAGs Triacylglycerides

UEM Universal eating monitor

VAS Visual Analogue Scale

WHO World Health Organisation

CHAPTER 1: Introduction

1.1 Type 2 Diabetes

1.1.1 Definition, prevalence, burden, causes

Diabetes is an endocrine and metabolic disorder characterised by chronically increased blood glucose (hyperglycaemia). Most diabetes cases (~90%) are type 2 diabetes (T2D); formerly known as non-insulin-dependent or adult-onset diabetes, which occurs when there is a deficiency in insulin hormonal secretion and/or the body's ability to sense insulin (DeFronzo *et al*., 2015). The global age-standardised prevalence of T2D has continued to increase. Global cases of diabetes rose from 108 million to 150 million between 1980 and 2000 (Cho *et al*., 2018, King *et al*., 1998). As of 2019, approximately 420 million adults are currently diagnosed with T2D (IDF, 2019); which is predicted to escalate to 693 million by 2045 (Cho *et al*., 2018). The leading countries with the greatest number of cases are China (89.5 million, the 6.45% of the Chinese population), India (67.8 million, 5% of the Indian population) and the United States of America (USA) (30.7 million, the 9.4% of the population in the USA) (as of 2017) (Lin *et al*., 2020).

T2D entails an important global public burden involving human health and economic losses, all having a colossal impact worldwide. On the human side, T2D-related hyperglycaemia, if not well managed, can increase the risk of developing long-term complications such as blindness, kidney failure and limb amputations (Skyler *et al*., 2012). Additionally, T2D increases the risk of heart attacks and strokes by 2 to 3-fold (Yang *et al*., 2019, ERF Collaboration, 2010). These complications impair quality of life and contribute to the increased risk of mortality, compared to healthy subjects (Skyler *et al*., 2012). In 2015, it was estimated that 5 million deaths were attributed to diabetes (of which 90% of cases are T2D) in adults aged 20-79 years, representing 6.8% of global mortality, and one of the 10 main causes of death worldwide (Roglic *et al*., 2010). Of importance, 87% of deaths related to diabetes occur in low- and middle-income countries (Diabetes Atlas, 2019). On the economic side, the global cost of diabetes is almost $1.25 trillion and is set to double by 2030. Per year, the global cost of treating and managing T2D is calculated to be of $825 billion, with the largest cost being in China, the US, and India (Bommer *et al*., 2018). Particularly, in the UK, T2D costs the NHS £7.5 billion a year (Stedman *et al*., 2019).

Lowering such large figures often comes with the scientific understanding of its cause. However, the aetiology of T2D is poorly understood and it is thought that a complex interplay of genetic, epigenetic and lifestyle factors within a socio-cultural environment are involved in its development (Zheng *et al*., 2018). The major driving factors are: increased body mass index (BMI) (kg/m^2) (representing the single strongest factor (Ganz *et al*., 2014)), sedentary lifestyle and unhealthy diets –particularly, overconsumption of energy. Data from a meta-

analysis suggests that lifestyle modifications can prevent T2D (Schellenberg *et al*., 2013). However, other factors comprise age, family history of T2D, high blood pressure, high-density lipoprotein (HDL) or triacylglycerol (TAG) and ethnicity also contribute to increased T2D risk.

1.1.2 South Asian and white European differences in T2D

Ethnicity is important for understanding disparities in health, in particular, for the condition of T2D, ethnic differences in its diagnosis, postprandial metabolism and its potential causes have been observed. Ethnicity has been defined as “a complex multidimensional construct reflecting the confluence of biological factors and geographical origins, culture, economic, political and legal factors” (Williams *et al*., 1997). The largest ethnic minority group in the UK are the south Asian, forming 5.3% of the population in England and Wales (Office for National Statistics, 2011). In this section, the T2D ethnic differences between white Europeans and south Asians will be reviewed.

1.1.2.1 Prevalence and Diagnosis

A white European can be defined as a native of Europe with light complexion suggesting white European ancestry (Bhopal *et al*., 1998). On the other hand, south Asian ethnicity is attributed to people with ancestors born in India, Pakistan, Nepal, Bhutan, Bangladesh, and Sri Lanka (Naganathan *et al*., 2015). Currently, south Asians have approximately a 23% prevalence of T2D, which is the highest prevalence compared to white Europeans (6%), African Americans (18%), Latinos (17%), Chinese Americans (13%) (Kanaya et al., 2014), and Middle-East countries (14.6%) (Farmanfarma *et al*., 2020). In this thesis, a focus will be placed on the migrant south Asian. Migrant south Asians from rural to urban regions have increased T2D prevalence. This is exacerbated when south Asians migrate to high-income countries (Figure 1) (Sattar *et al*., 2015). Their risk of developing T2D is 2 to 4 times higher than compared to white Europeans (Sattar *et al*., 2015). This may be due to an alteration of lifestyle such as reduced physical activity, greater intake of energy-dense foods, language and cultural barriers in a health-care setting (Misra *et al*., 2018).

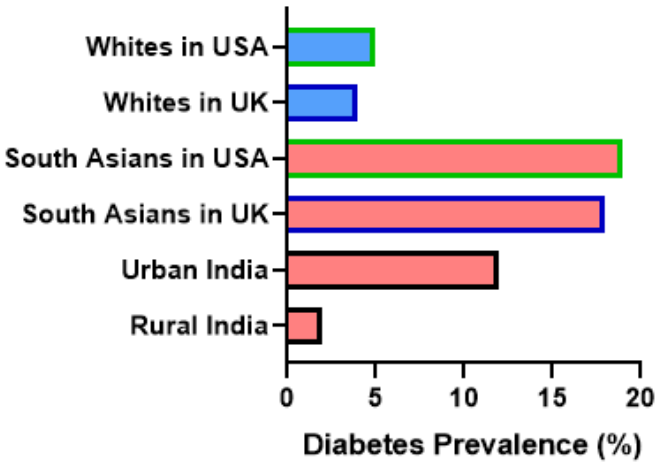

Figure 1. Diabetes prevalence in south Asians and white Europeans by country and area of residence. The prevalence of T2D in urban India is almost 5 times higher than in rural India, phenomena which have been mirrored in Pakistan, Bangladesh and Nepal. Adapted from Hall *et al*., 2008.

In addition, T2D onset and complications are fundamentally different between ethnicities. South Asians are diagnosed with T2D 5 to 10 years earlier (Mather *et al*., 1985), occurring at a lower BMI (Lear *et al*., 2007, Lee *et al*., 2011) and with a greater degree of β-cell dysfunction, insulin resistance (the body's incapacity to sense insulin despite it being produced) and ectopic fat deposition, compared to white European counterparts (Hills *et al*., 2018). Furthermore, south Asians have a worse glycaemic response to the same dietary challenge (Bakker *et al*., 2014, Henry *et al*., 2008) and glycaemic management (Rawshani *et al*., 2015), and therefore a rapid progression to diabetic complications relative to white European counterparts (Kirk *et al*., 2005, Misra *et al*., 2018).

1.1.2.2 Causes

The basis of the ethnic differences in T2D prevalence, diagnosis and management is not well-understood.

1.1.2.2.1 Thrifty gene/phenotype hypothesis

The thrifty phenotype hypothesis was postulated by Barker and Hales in 1992 to explain T2D aetiology. This hypothesis states that poor foetal and post-natal nutrition (over and undernutrition) can increase the risk for future type 2 diabetes (Hales et al., 1992). This hypothesis was tested using a cohort of 468 grown-up 64-year-old men in Hertfordshire and still living there at the time whose birth weight was known at year 1. They underwent an OGTT and had their insulin-related profile measured. The study showed that there was a strong correlation between reduced birth growth and impaired glucose tolerance (Hales et al., 1991). It was suggested that the foetal undernutrition leads to impaired pancreatic beta-cell function and a thrifty genotype (which is likely to store energy). In which, a scenario of continued poor nutrition in adult life, poor beta-cell function would not confer a disadvantage, but the most likely scenario in westernised countries (i.e. the obesogenic environment) is

characterised by overnutrition in which poor beta-cell function would result in a disadvantage and likelihood to develop T2D.

It is plausible that impaired beta-cell function, thrifty genotype and increased T2D risk observed in south Asians is caused from low birth weights in this group as a study conducted in the START and FAMILY birth cohorts in Canada including 401 south Asian and 389 white European pregnant women showed that south Asian newborns had a lower birthweight and greater skinfold thickness than white European babies (Anand et al., 2016). This was also shown to be influenced by maternal body fat and glucose. This brings up the importance of maternal nutrition and body weight in the development of T2D in their offspring.

1.1.2.2.2 South Asian phenotype

It is possible that foetal nutrition may have led to epigenetic changes in the foetal genome favouring a nutritional thrifty phenotype (Hills et al., 2018) such as the so-called "South Asian phenotype" observed in adults with T2D and newborn south Asian babies (Unnikrishnan *et al*., 2014, Sletner et al., 2013). This phenotype is mainly characterised by a high body fat and low lean mass percentage, within a healthy BMI (i.e. <25 kg/m^2 as defined by the WHO based on data from the Seven Countries Study, these being mostly western: the Netherlands, Greece, USA, Finland, Italy, Yugoslavia and Japan). The metabolic profile of this phenotype includes insulin resistance, hyperglycaemia, low HDL-cholesterol and high TAGs (Banerji *et al*., 1999). Particularly, body fat disposition differs amongst ethnicities. Compared to white Europeans, south Asians have a higher waist-to-hip ratio (indicating central obesity), subscapular skinfold thickness as well as body, subcutaneous and intra-abdominal visceral fat (including liver fat) (Hills *et al*., 2018). Visceral fat and truncal fat (central fat) correlates with insulin action in south Asians (Banerji *et al*., 1999, Sandeep *et al*., 2010, Chandalia *et al*., 2007). Furthermore, south Asians have shown to have a lower cardiorespiratory fitness (respiratory and circulatory capacity to provide oxygen to the mitochondria in the skeletal muscle) compared to BMI-matched white Europeans (Hall *et al*., 2010), which explains 68% of the ethnic difference in insulin resistance (Ghouri *et al*., 2013). Altogether, these metabolic features may predispose this subgroup to early insulin resistance, leading to compensatory hyperinsulinaemia. For instance, higher insulin levels have been found in the umbilical cord plasma levels of Indian newborns compared to white European newborns (Yajnik *et al*., 2004), suggesting an early development of insulin resistance. Overall, these observations suggest that south Asians exhibit earlier β-cell insufficiency and/or failure, compared to white Europeans, especially when exposed to "urbanised" western lifestyle characterised by sedentariness and high-calorie intake (Sattar *et al*., 2015).

1.1.2.2.3 South Asian metabolic and body composition and its impact on glycaemic control

South Asians have been shown to have a "thin-fat phenotype" (anthropometrically thin but metabolically obese), in which for a normal BMI (according to western criteria in between 18.5-25 kg/m^2), they have a greater percentage of body fat and low percentage of muscle mass, and this is evident even from infancy (Yajnik et al., 2003). The south Asian subgroup, they present a higher waist-to-hip ratio (indicating central obesity), subscapular skinfold thickness as well as body, subcutaneous and intra-abdominal visceral fat (including liver fat) (Hills *et al*., 2018). Visceral fat and truncal fat (central fat) correlates with insulin action in south Asians (Banerji *et al*., 1999, Sandeep *et al*., 2010, Chandalia *et al*., 2007). In addition, visceral and abdominal obesity is also related to glucose intolerance as adiposity is related with macrophage's infiltration and pro-inflammatory cytokines which are involved in insulin resistance pathogenesis (Moritai et al., 2020, Apovian et al., 2008). This has been shown in a study in which south Asians had almost double the amount of the pro-inflammatory C-reactive protein levels which correlated with their central obesity (Forouhi et al., 2001). Altogether, leads to a state of insulin resistance and compensatory hyperginsulinemia from an early age in the south Asian group which could explain the impairment in glucose clearance observed in clinical trials compared to white Europeans (Bakker et al., 2014)..

Furthermore, south Asians have lower brown adipose tissue volumes activity and resting energy expenditure than matched white healthy white Europeans (Bakker et al., 2014) as studied in cold-induced 18F-fluorodeoxyglucose positron emission tomography computed tomography scanning. This is coupled with a higher endocannabinoid tone in this ethnic group, which plays a role in appetite regulation and energy expenditure which may lead to a disadvantageous metabolic phenotype in later life (Kantae et al., 2017). Overactivation of the endocannabinoid system is associated with obesity and low brown adipose tissue. A low brown adipose tissue would make south Asian susceptible to a reduced glucose clearance, since brown adipose tissue is an organ that dissipates triglycerides and glucose towards heat.

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1.1.2.2 Dietary intake

As stated earlier, T2D's aetiology, amongst other genetic and epigenetic factors, is strongly determined by lifestyle factors, particularly, dietary intake. For example, high intake of simple carbohydrates may lead to hyperinsulinaemia, whereas fat intake is associated with insulin resistance (Riccardi *et al*., 2004). Saturated fatty acid intake has also been shown to be an independent predictor of fasting and postprandial insulin concentrations (Parker *et al*., 1993).

Data from 7-day weighted diary reports indicated that the diet of south Asians living in London, are typically lower in energy and, total fat but higher in starch, polyunsaturated fatty acids (PUFA) and dietary fibre intake (Sevak *et al*., 1994) compared to Europeans. More recent data from the UK National Diet Nutrition Survey (NDNS, year 9) also supports this finding (Table 1), suggesting that south Asians may follow a more favourable diet pattern compared to Europeans.

Table 1. Comparison of the intake of nutrients in people with Asian/British Asian and white European ethnicities from the UK for years 2008/09 to 2016/17.

	Asians or British Asians[1] (n=186)	UK white Europeans[1] (n=5083)
Total energy (kcal)	1740.22 (66.32)	1827.59 (10.14)
Carbohydrates (% totE)	51.36 (0.72)	45.21 (0.13)*
Total fat (% totE)	31.75 (0.62)	33.44 (0.11)*
SFA (% totE)	10.34 (0.25)	12.56 (0.06)
CN3PUFA (% totE)	0.98 (0.03)	0.97 (0.00)
CN6PUFA (% totE)	5.78 (0.19)	4.67 (0.02)*
Trans-fat (% totE)	0.44 (0.02)	0.55 (0.00)*
Protein (% totE)	16 (0.35)	16.7 (0.07)
Fibre (g/day)	21.41 (1.2)	18.49 (0.14)*
Alcohol (g/day)	3.23 (1.26)	13.48 (0.48)*

G, grams; PUFA, polyunsaturaded fatty acids; SFA, saturated fatty acids; totE, total energy; n, sample size. Values expressed as survey-adjusted means and standard error of means. T-test of independent groups. % totE, percentages of total energy.
[1]Data calculated using UK adults from NDNS database year 1 (2008/09) to year 9 (2016/17).
*A two-sided P-value was considered to be significant if ≤0.05

Nevertheless, information from Table 2 should be interpreted with caution due to the unbalanced group sizes (n=186 *vs.* n=5083), and the fact that the Asian group may also include non-south Asians (e.g. Chinese). Furthermore, there is a possibility that both British Asians and migrant Asians are included in the same group when they may have different eating patterns. Furthermore, some evidence suggests that migrant Asians undergo "acculturation" which consists of adopting elements of their recipient countries' diet resulting in a reduction in fibre and increased intake of energy, fat, meat and highly processed foods (Holmboe-Ottesen *et al*., 2012, Raj *et al*., 1999).

However, despite these data suggesting that the macronutrient profile intake of migrant south Asians seems more favourable compared to white-Europeans, studies have reported that the south Asian have practices such as religious Ramadan fasting or Jainism intermittent fasting, re-heating of oils promoting trans fats intake, unhealthy choice of oils, traditional fondness for sugar which can increase the risk for T2D development and poor management (Misra *et al*., 2018).

Interestingly, a study administrating a 5-day dietary challenge mimicking western-diets (high fat, high-calorie diet) showed that healthy south Asians exhibited increased insulin resistance compared to matched-white European counterparts (Bakker *et al*., 2014) independent of changes in skeletal muscle metabolism. This study suggests that divergent metabolic responses to the same western diets may contribute to the ethnic inequalities in T2D development and progression.

1.1.2.3 Management

Despite T2D being considered a chronic disease, there have been cases in which T2D has been remitted permanently or for a finite amount of time. This has been mainly achieved via bariatric surgery (Buchwald *et al*., 2004) and weight loss induced by low-energy diets (825-850 kcal/day) (Lean *et al*., 2018), whose success mainly relies on the sustained and substantial body weight loss they induce. Nevertheless, these approaches are not suitable and/or safe for most people with T2D as a series of requirements need to be met to be eligible (e.g. BMI ≥35) for bariatric surgery and low-energy diets can have adverse effects such as dizziness, headache and constipation.

The main cornerstone for T2D management is lifestyle modifications involving a healthy balanced diet and increase in physical activity – along with structured education and drug therapy -. Evidence-based nutrition guidelines for T2D encourage a sustained weight loss of at least 5% in overweight people by reducing energy intake and increasing energy expenditure (Diabetes UK, 2018). More specifically, eating carbohydrate from fruit, vegetables and whole grains and pulses, low-fat dairy products, oily fish and limiting the amount of food that contains saturated fats and trans fatty is advised (NICE, 2015). Some examples of diets including these nutritional features are the Mediterranean diet or the New Nordic diet; however, adopting these diets may not be suitable for all cultures such as the South Asian.

Expert health care commissions (Diabetes UK and NICE) have highlighted the importance of tailoring T2D care according to ethnicity and culture. Nevertheless, a systematic review and meta-analysis investigating the effect of culturally specific interventions in South Asian people with T2D in the UK, compared to standard educations, showed no differential effect on glycaemic control; although there was no specific mention on the inclusion of cultural-specific dietary education (Hawthorne *et al*., 2009). General recommendations for this subgroup include increasing monounsaturated fatty acids, changing from white to brown rice, increasing the consumption of low glycaemic index foods and dietary protein as well as switching to healthier cooking oils and cooking methods such as not using re-heated oils which can affect trans fatty acid transformation (Misra *et al*., 2018). However, there is a widespread poor adherence in this community to following guidelines, primary care

appointments or testing, coupled with poor awareness and cultural barriers (Misra *et al*., 2018). Considering that early provision of basic treatments is key in this subgroup and that sociocultural nuances must be accounted for to address this, there is a need to develop ethnic-specific food that can help decrease postprandial blood glucose, along with first-line drug therapy and lifestyle modification.

1.2 Blood glucose homeostasis

Maintaining normal blood glucose levels is achieved via a homeostatic process which is tightly regulated mainly via two hormones: insulin and glucagon. T2D is related to a malfunctioning of this homeostatic process. In the following sections, both hormones and other factors which are important at modulating blood glucose will be discussed in the context of healthy and T2D physiology, with a special focus on insulin.

1.2.1 Blood glucose

Blood glucose homeostasis is the maintenance of blood glucose concentration (glycaemia) at a steady-level (euglycaemia). Euglycaemia in the healthy can range from 3.9 to 10 mmol/L in the situations of fasting or 2 hours postprandial levels (Bode *et al*., 2005). This is a vital physiological phenomenon critical for the correct functioning of the body in humans as glucose constitutes the major energy substrate for many tissues in the body- and essentially for the brain. Euglycaemia is the result of the balance between glucose appearance and glucose clearance in the systemic circulation. In the fed status, glucose is provided from the intestinal absorption of monosaccharides via sodium glucose transporters and GLUT transporters. During the fasted status, blood glucose is derived from the catabolic processes in some tissues such as glycogenolysis (e.g. hydrolysis of glycogen in the muscle), gluconeogenesis (formation of glucose *di novo* in the liver). On the other hand, glucose clearance depends on kidney clearance, tissue's storage and utilisation (Gerich *et al*., 2000).

Blood glucose and T2D

In T2D, euglycaemia is not maintained and it moves towards high blood glucose levels (hyperglycaemia). In individuals with T2D, glycaemia reaches ≥7.0 mmol/l at fasting and ≥11.1 mmol/l at 2 hours postprandially (fed a 75 g of an oral glucose load) reaches (American Association for Diabetes, 2020). Diabetes-specific microvascular damage may lead to blindness, renal failure, and nerve damage (Kitada *et al*., 2010). Also, diabetes accelerates atherosclerosis leading to increased risk of myocardial infarction, stroke, and limb amputation. It is hypothesised that hyperglycaemia mediates vascular damage through four main molecular mechanisms. These are: increased polyol pathway flux driving retinopathy; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux (Brownlee *et al*., 2001).

1.2.2 Pancreas and its role in glucose homeostasis

The pancreas is an endocrine and exocrine gland that regulates both macronutrient digestion and energy homeostasis. The exocrine or acinar cells secrete pancreatic juice with digestive enzymes (e.g. amylase, lipase, trypsinogen) into the pancreatic duct to the gallbladder. On the other hand, the endocrine cells constitute the 1-2% of the entire pancreas and they are clustered in the islets of Langerhans. These cells secrete different hormones into the bloodstream, being the most abundant (in 65-80%) the β-cells that secrete insulin and c-peptide. The other cells are called alpha (secreting glucagon), gamma (secreting pancreatic polypeptide – involved in glycogen storage in the liver), delta cells (secreting somatostatin – inhibits the secretion of insulin and glucagon), and epsilon cells (secreting ghrelin - an orexigenic hormone) (Röder *et al*., 2016). In this thesis a focus will be placed in insulin and glucagon.

1.2.3 Insulin and glucagon – the primary glucoregulatory hormones

Euglycaemia is achieved by the balance of the action of the pancreatic hormones insulin and glucagon. Insulin is a 51-amino acid polypeptide hormone produced by the pancreatic β-cells of the islets of Langerhans (Banting *et al*., 1922). The main function of insulin is to decrease glycaemia by facilitating glucose transport into insulin-sensitive cells (e.g. myocytes, adipocytes, hepatocytes) and stimulating the storage of energy via anabolic effects in these cells (e.g. glycogenesis, lipogenesis). In addition, insulin also inhibits glucagon secretion. The secretion of insulin is primarily modulated by hyperglycaemia, but also by other factors such as incretin hormones (e.g. glucagon-like peptide 1, GLP-1) or nutrients (e.g. fatty acids and amino acids) (Charles *et al*., 1982, Yaney *et al*., 2003).

1.2.3.1 Insulin secretion

In healthy individuals, insulin secretion occurs in the pancreatic β-cell and is mainly promoted by the uptake of glucose (>5.5 mM) (via glucose transporter 2, GLUT2), which is then hydrolysed yielding adenosine triphosphate (ATP) as a by-product. An increase in the ATP to adenosine diphosphate (ADP) ratio triggers the closure of ATP-sensitive K^+ channels, depolarising the β-cell membrane. This depolarisation leads to the opening of voltage-dependent Ca^{+2} channels, thereby increasing intracellular Ca^{+2} concentrations (Figure 2). Increased Ca^{+2} levels lead to the exocytosis of insulin granules outside the β-cell into the portal vein, where part of it will be extracted by the liver, and then found in the systemic circulation (Cantley *et al*., 2015).

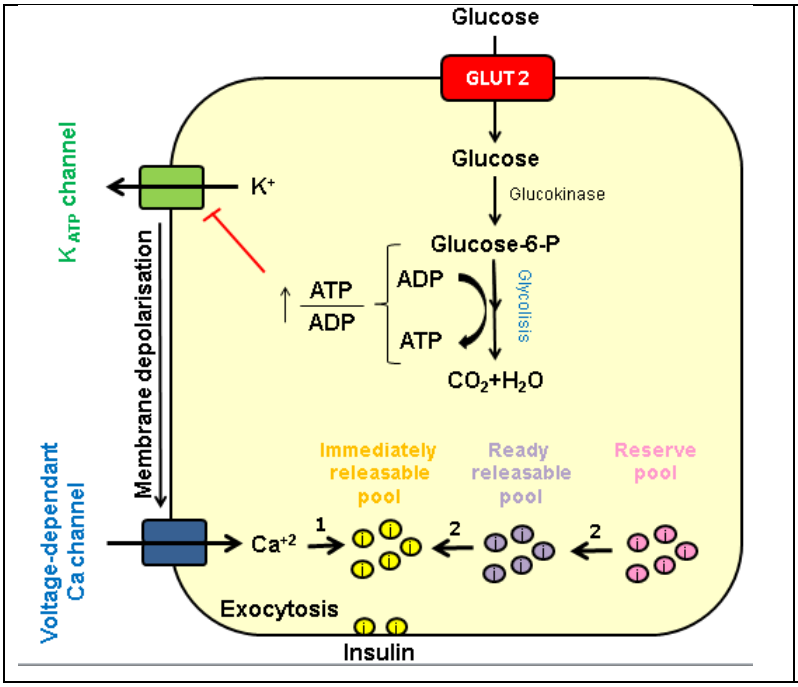

Figure 2. Schematic representation of the mechanism of insulin secretion in a pancreatic β-cell. In brief, glucose is uptaken by GLUT2 and phosphorilated to Glucose-6-phosphate by the glucokinase enzyme which then undergoes glycolysis. Increased ATP/ADP ratio inhibits K^+-ATP channel causing membrane depolarisation which stimulates the opening of Ca^{+2} channels. Intracellular Ca^{+2} levels increase the exocytosis of insulin immediately releasable pool. Figure adapted from Cantley *et al*., 2015.

Insulin secretion and T2D

In healthy individuals, insulin secretion pattern following a glucose bolus is biphasic. This pattern consists of a sharp insulin rise and a decrease within the first 10 min of stimulus (first phase), followed by a "slow-ramp" during the following 20 min (second phase) (Curry *et al*., 1968). T2D is associated by a loss of the first-phase insulin secretion pattern, as well as a reduction in the second-phase secretion (Seino *et al*., 2011). Some authors attributed this to a β-cell secretory defect due to a loss in the releasable pools of insulin (responsible for the first and second insulin phase), complete defect in the exocytosis of insulin or disturbance in the actin-filament network which plays a role in the insulin secretory granules release.

Additionally, research shows that T2D is also associated with β-cell mass loss, with >15-year old diagnosed T2D patients showing a >50% reduction, compared to patients without T2D (Rahier *et al*., 2008).

1.2.3.1 Insulin signal transduction pathway

In the context of a healthy physiology, once insulin is secreted into the circulation from the pancreas, insulin is sensed by target tissues with insulin receptors (INSR) such as the liver, skeletal muscle and adipose. Briefly, INSR consists of a heterotetramer of 2 α-subunits and 2 β-subunits linked by a disulphide bond. When insulin binds to the α-subunit of the INSR, it leads to a cascade of intracellular molecular processes that promote an anabolic response in the target cell. Briefly, insulin binding to α-subunit of the INSR activates the tyrosine kinase activity of the β-subunit. This triggers the auto-phosphorilation of the β-subunit leading to the further phosphorylation and activation of insulin receptor substrate (IRS) proteins and of phosphatidylinositol (PI) 3-kinase (Saini *et al*., 2010) (Figure 4). This leads to the activation

of Akt which will trigger several processes, such as GLUT4 translocation to the membrane to facilitate glucose uptake in the target cell or to activate mammalian target of rapamycin (mTOR), a transcriptional factor involved in upregulating anabolic genes (e.g. fatty acid and cholesterol biosynthesis) (Saxton *et al.*, 2017).

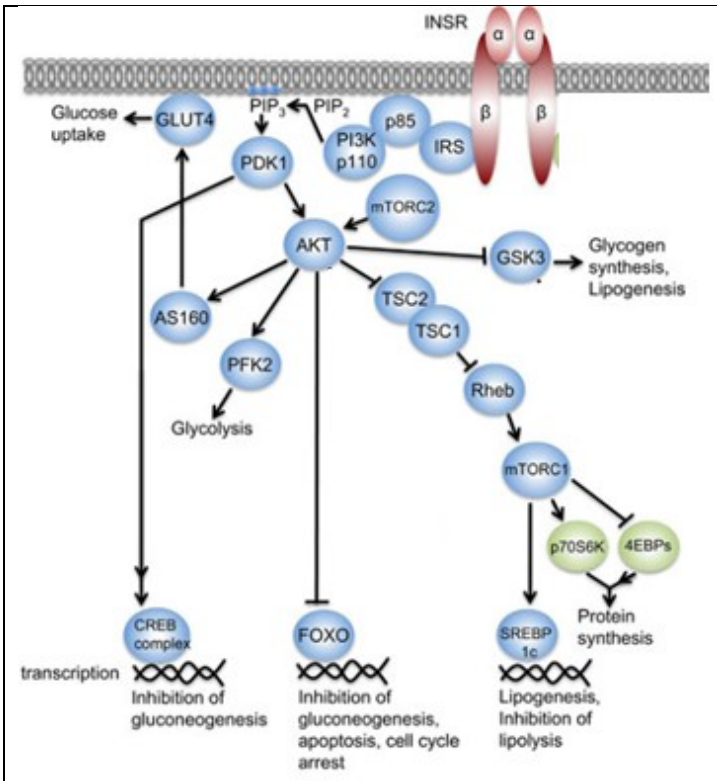

Figure 3. Schematic representation of insulin signal transduction pathway in insulin-senstive cells. Insulin binding to INSR causes its autophosphorilation, activating IRS, which then activates PI3K and AKT, triggering the translocation of GLUT4 to the membrane and other anabolic effects such as activation of mTOR promoting expression of anabolic enzymes. Illustration reproduced is an adaptation courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com). AKT, serine/threonine-specific protein kinase; FOXO, forkhead box protein O1; GLUT4, glucose transporter 4; GSK3, glycogen synthase kinases (GSK3); INSR, insulin receptor; IRS, insulin receptor substrate; mTORC, mammalian target of rapamycin; PDK, pyruvate dehydrogenase kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PFK, phosphofructokinase; PI3K, phosphatidylinositol 3-kinase; PIP, phosphatidylinositol phosphate; SREBP, sterol regulatory element-binding protein.

Insulin signalling and T2D

T2D is also characterised by a deficiency in the insulin transduction signalling pathway leading to IR (the inability of insulin to be sensed by its receptor) in the insulin-sensitive tissues. IR may lead to a deregulation of metabolic processes in such a way that anabolic routes are not regulated, whereas catabolic routes will not be inhibited explaining hyperglycaemia characteristic in T2D. For example, the muscle is the major site tissue for glucose disposal in the body. The major fate for myocellular glucose is glycogen synthesis. However, glycogen synthesis is markedly impaired in people with T2D by 50%. Studies in humans, revealed a malfunction in GLUT4 translocation as well as decreased surface IRe and insulin receptor kinases activities (Petersen *et al.*, 2018).

Causal factors for insulin resistance could be the role of unhealthy diets or obesity at inducing inflammation processes affecting molecular aspects of insulin signal transduction

pathway (Muoio *et al*., 2008). For example, processed red meat high-intake poses a relative risk for T2D of 1.51 (1.25-1.83) (Petersen *et al*., 2018). Higher animal protein intake may result in higher branch chain amino acids (BCAAs) intake. Supplementation of BCAAs has been shown to induce IR in regular chow-fed rats (Newgard *et al*., 2009). One of the mechanisms proposed is that BCAA activates mTOR providing negative feedback to insulin signal at the level of insulin receptor substrate-1.

1.2.3.3 Glucagon

Glucagon is a 29-amino acid peptidic hormone produced by the pancreatic α-cells in the islets of Langerhans (Kimball *et al*., 1923). Glucagon derives from the preproglucagon peptide, encoded by the preproglucagon gene, also expressed in CNS and intestinal L-cells. Post-translational cleavage yields different mature peptides including glucagon, glucagon-like peptide 1 (GLP-1) and GLP-2 (Mojsov *et al*., 1986). The main trigger for glucagon release is insulin-induced hypoglycaemia (<3.9mmol/L) (Nadal *et al*., 1999), although mixed nutrient meals, amino acid and fatty acids administration and autonomic nervous system also trigger a rise in glucagon. Pancreatic α-cells can sense a drop in blood glucose and release glucagon- even when insulin is exerting an inhibitory effect on these cells- (Walker *et al*., 2011). The main function of glucagon is to increase blood glucose through its effect mainly on the liver stimulating glycogenolysis and gluconeogenesis, converting the liver into a glucose production organ, while inhibiting glycogenesis and glycolysis. In addition, glucagon is also involved in providing gluconeogenic substrates to the liver by promoting uptake of amino acids in the liver and mediating lipolysis in the adipocytes to mobilise glycerol to the liver. In general, glucagon catabolic effects ensure an appropriate supply of glucose to the body and the brain.

Glucagon and T2D

It is well established that in T2D a loss of insulin is paraleled by an increase in glucagon at high glucose levels, which further excarcerbates hyperglycaemia (e.g. by increasing hepatic glucose output). This could be due to a lack of insulin-inhibitory effect on glucagon production or defects in α-cells. On the other hand, T2D is also related to a lack of glucagon response to low blood glucose levels, increasing the risk of hypoglycaemic episodes in T2D, particularly, on late-stage T2D (Quesada *et al*., 2008).

1.2.4 Other mechanisms that regulate blood glucose

As discussed earlier, blood glucose levels are tightly regulated mainly by insulin and glucagon which lower and increase blood glucose levels, respectively, to maintain normal glucose homeostasis. While blood glucose elevation is a redundant system (adrenaline and cortisol being also able to directly exert this effect) (Klausner *et al*., 1967, Sherwin *et al*.,

1984), direct decrease of blood glucose is exclusively attributed to one sole hormone: insulin. Nevertheless, some hormones can stimulate the secretion of insulin (namely incretins) as well as physiological processes that can decrease blood glucose (e.g. gastric emptying). Altogether, they importantly contribute to regulate blood glucose, and will be reviewed in this section.

1.2.4.1 Gastric emptying

Gastric emptying is a process occurring in the stomach in which liquid and solid food is emptied into the duodenum via gastric muscular contractions. The stomach is a muscular digestive organ responsible for the mechanical (gronding) and chemical (acid-peptidic) digestion of food, which is divided into 2 functional sections: the fundus (proximal stomach) and the antrum (distal and terminal stomach). The fundus is mainly responsible for storing food (although it also mills food) and the antrum for mixing and breaking down solid chyme into <2.0 mm food pieces (chymous-like consistency), which are then emptied into the duodenum through the pylorus (gastric emptying). This process is coordinated by antropyloric propulsive pressure waves, pyloric-sphincter relaxation and duodenal pressure waves. Once in the duodenum, the chyme stimulates several neural and hormonal reflexes (Section 1.2.4.2) that decelerate the emptying (Verhoeckx *et al*., 2015). Since gastric emptying controls the flow of nutrients to be further digested and absorbed (including carbohydrates) into the blood, it plays an important role in appetite regulation and meal-related glycaemia. Gastric emptying accounts for approximately 35% of the variance in the postprandial blood glucose excursion as well as for the overall area under the curve of postprandial glycaemia (Horowitz *et al*., 1985, Jones *et al*., 1996).

Gastric emptying and T2D

Cross-sectional studies have reported that 30-50% of patients with diabetes have gastroparesis (delayed gastric emptying), which is commonly associated with late-stage diabetes consequence of neuropathy, although this is more probable for T1D than T2D (Marathe *et al*., 2020). It has been reported in the literature that in early T2D, hyperglycaemia plays a role at reducing gastric emptying in both healthy and people with T2D (without neuropathy) exposed to induced-hyperglycaemia, when compared to induced-normoglycaemia (Schvarcz *et al*., 1997). This is in line with other studies (Matsumoto *et al*., 2007, Bharucha *et al*., 2015). Interestingly, one study found opposite findings, in which T2D individuals with a good glycaemic management showed a relative faster gastric emptying compared to BMI- and age-matched healthy controls in a large cross-sectional study (Watson *et al*., 2019). The authors attribute this discrepancy in the results to their use of people with T2D who have good glycaemic management (i.e. only diet or metformin), andare early diagnosed individuals, relative to the other studies.

1.2.4.2 Gut hormones

There is evidence supporting the physiological role of some gut hormones such as GLP-1 in modulating glycaemia, accounting for a 65% of postprandial insulin release in response to oral glucose in healthy people (Nauck *et al*., 1986). GLP-1 postprandial responses have been shown to correlate with insulin secretion (Nauck *et al*., 1993).

GLP-1

GLP-1 is a peptidic hormone present in many forms, the most common being GLP-1(7-36NH_2). Active forms are rapidly degraded by dipeptidyl peptidase-4 (DPP-4), an enzyme naturally present in endothelial cells, liver and blood, rendering GLP-1 to a half-life of approximately 1-2 min *in vivo*. GLP-1 is secreted mainly from the enteroendocrine L-cells in the proximal gut and distal gut (where most L-cells reside) stimulated by nutrient sensing through its chemoreceptors, although it is also produced by neurones from the nucleus tractus solitarius. The secretion pattern of GLP-1 is unclear, with most studies showing it to be monophasic (Vollmer *et al*., 2008, Toft-Nielsen *et al*., 2001, Vilsboll *et al*., 2001) and some others biphasic (Ahren *et al*., 2001, Rask *et al*., 2001). In general, a quick rise in GLP-1 is observed 5-15 min following oral glucose tolerance test (OGTT) or mixed meal tolerance test (MMTT) reaching maximum values at 40-60min. Considering that GLP-1 is secreted by L-cells which are mainly found in the distal gut, such quick initial GLP-1 response (5-15 min) could arise from an indirect neural or hormonal mechanism, since nutrients take longer than 15 min to reach the distal gut (Patricia *et al*., 1991).

GLP-1 secretion is mainly stimulated by carbohydrate intake although oral protein and fat stimuli have a slower onset and sustained response compared to carbohydrate. Mixed nutrient meals show a biphasic secretion pattern, with secondary peaks evident at 60-120 min. GLP-1 receptors are expressed in the gastrointestinal tract (GIT), pancreas, heart, abdominal vagal afferents and brain affecting physiologically to these tissues via G-protein-coupled receptor binding (Steinert *et al*., 2017). GLP-1 has been associated to multiple biological roles compiled in Table 2.

Table 2. GLP-1 biological sites of action and effects in tissues involved in glucose homeostasis

Site of action	Effect
Pancreas	• Stimulates glucose-dependant insulin release • Stimulates insulin gene transcription • Stimulates islet cell growth and neogenesis
GI tract	• Inhibits gastric emptying • Decreases gastric motility and small intesine movement via direct effects on gastric smooth muscle • Inhibits postprandial acid secretion

Source: MacDonald *et al*., 2002.

One of the most important effects of GLP-1 in the pancreas is insulin release stimulation (insulinotropic or incretin hormone) therefore reducing blood glucose levels. This effect is thought to be possible due to the inhibition of K_{ATP} channels in the β-cell by GLP-1, resulting in membrane depolarisation (Holz *et al*., 1993). Furthermore, evidence from rodents studies suggest that GLP-1 can also stimulate proinsulin gene promoter activity (Skoglun *et al*., 2000,Fehmann *et al*., 1992). GLP-1 also seems to be a glucose-dependant inhibitor of glucagon release accounting for 22% of inhibition at fasting and 80% at the postprandial state (Muskiet *et al*., 2017).

Furthermore, GLP-1 also exerts important effects on the GIT motility and digestive secretion, termed as the "ileal brake" phenomenon, described as the negative feedback mechanism in the GIT in which digestion is slowed down to optimise digestion and absorption upon meal consumption (Van Citters *et al*., 1999). Delay of gastric emptying, digestive acid secretion and gastric motility results in an overall decrease in nutrient absorption from the GIT, including decreased postprandial glucose fluctuations, which reduces the necessity for a postprandial insulin response, therefore modulating glucose homeostasis.

GLP-1 and T2D

Since T2D may be due to an insulin secretory problem and GLP-1 represents an important incretin, it is logical to think that T2D may have reduced GLP-1 responses. Furthermore, if T2D is characterised by a loss of β-cell mass, particularly, at the end-stages of T2D, the response to GLP-1 may also be limited. However, a meta-analysis of 22 clinical studies (Calanna *et al*., 2013) showed that patients with T2D do not have lower GLP-1 responses compared to matched-healthy counterparts following the same OGTT or MMTT. Furthermore, a study using exenedin (9-39), a GLP-1 receptor antagonist used to block the action of GLP-1, resulted in no differences in endogenous postprandial insulin secretion in T2D patients with good glycaemic management compared to matched healthy controls (Salehi *et al*., 2010).

Other hormones

Amylin, also referred as islet amyloid polypeptide (IAPP), is composed of 37 aminoacids and it is secreted from the pancreatic beta-cells, co-secreted with insulin, upon consumption of carbohydrate and fat. Studies show that amylin plays a role in glucose homeostasis by suppressing glucagon release from the pancreatic alpha-cells, therefore preventing hepatic gluconeogenesis, it also decreases gastric emptying which can also contribute to decreasing the postprandial blood glucose response (Pillay *et al*., 2013).

Pancreatic polypeptide (PP), is a hormone secreted by the endocrine F cells in the pancreatic islets in response to food intake via vagal cholinergic nerve. PP inhibits gastric

emptying of solid foods in humans, therefore delaying the postprandial glucose response to foods and insulin secretion (Schmidt *et al*., 2005).

1.2.4.3 Chrononutrition

An important factor for blood glucose control is the time of day at which the food is consumed. This is linked to the circadian rhythm and how it interacts with nutrients eaten to influence metabolism, in a phenomenon called chrononutrition (Henry *et al*., 2020). Circadian rhythms in mammals (e.g. sleep-wake cycle, hormone secretion such as insulin and cortisol) are tissue-specific 24-h cycles regulated by endogenous molecular oscillators. Circadian rhythm influence and regulate the expression and/or activity of enzymes involved in glucose homeostasis. Glucose tolerance typically peaks during day-light hours and reduces to night-dark hours when fasting usually occurs.

This was elegantly shown in a human study including healthy elderly Chinese who had continuous glucose monitoring and showed that the intake of either low or high-GI meals induced a significantly increased postprandial blood glucose when consumed at dinner time (8pm) compared to breakfast time (8 am), independently of the GI amount, with low-GI inducing a higher PBG response compared to the high-GI (Haldar *et al*., 2019). This effect is also observed in young non-diabetic individuals (Leung *et al*., 2019 and Gibbs *et al*., 2014). This knowledge has important public health implication in dietary planning as some ethnic groups, such as the Indian, consume 32% of their daily calories between 7 pm and 11 pm, with the median dinner time being at 10 pm (Gupta *et al*., 2017). Macronutrient changes such as increasing protein and fat content at dinner time is a simple strategy to improve overall daily postprandial blood glucose control.

1.2.4.4 Food components

Phytochemicals

Spices which are used to flavour and preserve food and are particularly prominently used within the south Asian community (ref) have been shown to reduce postprandial blood glucose in clinical trials due to their phytochemical content. Cinnamon is the most frequently used spice and recognised as safe by the Food and Drugs Agency (FDA). There are many species of cinnamon, but the Chinese type *Cinnamomum cassia* is the one with the strongest hypoglycaemic effects. Cinnamon has some phytochemicals such as cinnamonaldehyde, A and B-type procyanidins. These phytochemicals have been shown to upregulate the expression of GLUT4, be an insulin mimetic, and improve insulin sensitivity, respectively, collectively making cinnamon the strongest hypoglycaemic spice compared to other spices and herbs extracts, with its effects being 20-fold times higher (Bi *et al*., 2017).

Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) have been shown to bing to G-protein coupled receptor (GPCR) 40 expressed in the pancreatic beta-cell and GPCR120 in the enteroendocrine L-cell inducing a direct effect on insulin and glucagon like peptide 1 (GLP-1) secretion respectively, modulating postprandial insulin levels. Furthermore, the coupling with GPCR120 also promotes the upregulation of GLUT4, promoting glucose disposal as well as the reduction of inflammation which in the long term is associated with increased insulin sensitivity. Because of these pleiotropic roles of PUFA it is considered that they could modulate blood glucose in humans.

However, a meta-analysis has shown not have beneficial effects on blood glucose response in type 2 diabetes (Wu et al., 2012). However, one study (Li et al., 2015) showed conflicting results according to patient's ethnicity, in which white Europeans exhibit no improvement, while Asian type 2 diabetics may have increased insulin sensitivity in response to omega-3 PUFAs supplementation with doses ranging from 0.42-5.2g/day. Nevertheless, higher doses of PUFA above 5.2g/day were not shown to have this effect (Coelho et al., 2017).

Coelho, O. G. L., da Silva, B. P., Rocha, D. M. U. P., Lopes, L. L., & Alfenas, R. D. C. G. (2017). Polyunsaturated fatty acids and type 2 diabetes: impact on the glycemic control mechanism. *Critical reviews in food science and nutrition*, *57*(17), 3614-3619.

1.3 The regulation of energy homeostasis

As demonstrated in the previous section (1.2.2.1), unhealthy foods/diets and obesity likely play a role in the pathophysiology of T2D. Furthermore, foods high in carbohydrate further exacerbate glycaemic responses in those with T2D. Appetite is the drive for food intake, and it is linked with both excess energy intake and obesity which contribute to the pathophysiology of T2D, and it is linked with unhealthy food consumption which contributes to the poor management of glycaemic control. Therefore, understanding how appetite is regulated in the body, and how this is influenced by macronutrient intake, is important in the context of T2D. Appetite is a physiological process which is homeostatically regulated in humans. It can be regulated by the levels of adiposity in the body or by the food we eat. In regard to the latter, appetite regulation mainly consists of three main elements: the central nervous system, the peripheral gut hormones from the GIT, and the abdominal viscera. In the following sections, a focus will be placed on the central and the peripheral regulation of appetite in humans and the role of specific macronutrients on appetite.

1.3.1 Central regulation of appetite

The central nervous system (CNS) is the major modulator of energy homeostasis, adjusting both energy intake and energy expenditure (EE) based on, nutritional and hormonal cues. The central nervous system receives information about the nutritional status of the individual via the vagus nerve and spinal cord (Morton *et al*., 2006). These messages are being integrated by multiple regions within the central nervous system, with the hypothalamus regarded as the most important site.

The hypothalamus is a complex and integrated neural network formed by nuclei including the arcuate nucleus (ARC), which plays a role in energy homeostasis. The ARC is situated in an area with an incomplete blood-brain barrier, allowing it to sense circulating metabolic and endocrine signals (Broadwell *et al*., 1976). The ARC also receives neural signals from the nucleus solitary tract in the brainstem, which in its turn received signals from the GIT via the vagus nerve regarding nutritional status. These signals in the ARC stimulate two distinct neural populations (orexigenic and anorexigenic) within the ARC which co-express two types of neuropeptides (Table 3).

Table 3. Neurones, expressed neuropeptides and actions on appetite and energy expenditure.

Neurones	Neuropeptides co-expressed	Action on appetite	Action on EE	Ref
Orexigenic	Agouti-related peptide (AgRP)	Increase	Decrease	Broberger *et al*., 1998
	Neuropeptide Y (NPY)			
Anorexigenic	Cocaine- and amphetamine-regulated transcript (CART)	Decrease	Increase	Elias *et al*., 1998
	Pro-opiomelanocortin (POMC)			

EE, energy expenditure.

These neurones communicate with other hypothalamic and CNS regions to modulate energy homeostasis (Bagnol *et al*., 1999). AgRP/NPY are appetite-stimulant neuropeptides and decrease energy expenditure, whereas POMC/CART are appetite-suppressant neuropeptides which stimulate energy expenditure. In addition, NPY/AgRP neurones directly inhibit the activity of POMC/CART neurones through the neurotransmitter γ-aminobutyric acid.

1.3.2 Peripheral regulation of appetite

In light of the above, the CNS receives information about the nutritional status of the individual via the vagus nerve and spinal cord (Morton *et al*., 2006). This information can come directly from nutrients that are able to cross the brain blood barrier (such as acetate) (Frost *et al*., 2014) or from hormones secreted by the gut after sensing nutrients (Chambers *et al*., 2015). Gut hormones controlling appetite can be orexigenic (e.g. ghrelin) or anorexigenic (e.g. GLP-1 and peptide tyrosine tyrosine (PYY)) depending on whether they stimulate or supress the orexigenic or anorexigenic neurones. They can be secreted by the enteroendocrine cells found along the GIT, in which they hold nutrient-sensing mechanisms driving these hormone release responses.

1.3.2.1 Orexigenic hormones

Ghrelin

Ghrelin is a 28-amino acid hormone released by enteroendocrine cells in oxyntic glands in the stomach. Ghrelin can also be secreted by other regions including along the GIT and the arcuate nucleus of the hypothalamus. Ghrelin is found in blood as desacyl-ghrelin (90%) and acyl-ghrelin (10%), being the latter form the active form of the hormone (Delporte *et al*., 2013). Its secretion regulators are thought to be cephalic (olfactory, visual, gustatory food cues) and intestinal driven, with gastric ghrelin secreting cells also expressing several nutrient-sensing receptors which may stimulate ghrelin secretion. The ghrelin receptor is named growth hormone-secretagogue receptor-1A and it is expressed peripherally and centrally. Acyl-ghrelin can act on the brain by crossing the blood-brain barrier. Ghrelin is the only known factor to increase appetite in the circulation. However, other functions have been attributed to ghrelin such as glycaemic control by accelerating gastric emptying, inhibiting insulin secretion and stimulating glucagon secretion (Steinert *et al*., 2017).

Ghrelin and T2D

Low ghrelin levels have been associated with insulin resistance and obesity in overweight people (Ukkola *et al*., 2011) and with abdominal adiposity, hyperinsulineamia, insulin resistance in people with T2D (Katsuki eit al., 2004). Mutations in the ghrelin 51Gln gene, which is responsible for changing the molecular structure of the mature form of the hormone (reducing its levels), has been shown to increase the T2D risk (Pöykkö *et al*., 2003), which could be related to body weight gain.

1.3.2.2 Anorexigenic hormones

GLP-1

GLP-1 characteristics were reviewed previously in Section 1.2.4.2 as an incretin hormone; however, it can also act as an anorexigenic hormone. Central administration of GLP-1

inhibits food intake in rodents, whereas peripheral administration inhibits appetite in animals and humans (Drucker *et al*., 2006). Also, acute administration induces satiety and decreases energy intake in humans (Shah *et al*., 2014).

GLP-1 and T2D

GLP-1 levels in people with T2D compared to healthy individuals do not appear to show any different following nutrient stimulation (Nauck *et al*., 2011). Potential determinants of GLP-1 secretion following OGTT/MMTT were found to be age, glucagon and non-esterified fatty acids. Furthermore, polymorphisms in GLP-1 receptors are associated with BMI (De Luis *et al*., 2015).

Peptide tyrosine tyrosine (PYY)

Peptide tyrosine tyrosine (PYY) is a 36-amino acid hormone whose bioactive form is PYY_{3-36} which has a high affinity for the G protein-coupled receptor subtype Y2 named NPY2R. This receptor is expressed throughout the body, including the brain, GIT and vagal afferents. PYY is synthesised and secreted by enteroendocrine L-cells, found abundantly in the lower intestine (terminal ileum, colon and rectum) (Murphy *et al*., 2004). PYY secretion is induced by lipid intake more than protein intake, with CHO intake stimuli being less clear (Adrian *et al*., 1985). Plasma levels of PYY increase within 15-30 min of meal ingestion, reaching maximum values at 60-90 min, and are suppressed by fasting (Steinert *et al*., 2017). PYY secretion has an early and late phase. Early secretion is regulated by a neuroendocrine gut hormone named cholecystokinin (CCK) (Pilichievicz *et al*., 2007). It has also been suggested that PYY as well as GLP-1 have a second phase secretion wave until the food has reached the distal GIT, in which L-cells are found in abundance.

A central biological role associated with PYY is the ileal brake as it inhibits CCK, gastric acid and pancreatic secretion as well as gastric emptying and motility in the stomach (via a direct effect). PYY also decreases meal-related glycaemia and decreases food intake by acting centrally, and in a less established manner, via vagal afferent nerves in the lamina propria (Steinert *et al*., 2017).

PYY and T2D

Compared to BMI-matched healthy individuals, patients with T2D have low circulating PYY levels following a meal despite, displaying elevated fasting PYY levels (English *et al*., 2004). Similarly, healthy patients with first-degree relatives with T2D show a decrease PYY secretion compared to controls with no family history for T2D (Viardot *et al*., 2008, Boey *et al*., 2006). Furthermore, certain variants in the PYY gene modestly correlated with T2D (Torekov *et al*., 2005).

Other hormones

Amylin or IAPP, a hormone co-secreted in a fixed molecular ratio with insulin by the pancreatic beta-cells following the consumption of carbohydrate and fat. Like with insulin, fasting plasma levels of amylin are low and they increase with meal or glucose intake proportionally to body fat and meal size without producing taste aversion. IAPP has been shown to decrease gastric emptying, modulate appetite (Pillay *et al*., 2013), inhibit pancreatic glucagon and gastric enzyme secretion. Amylin has been shown to act synergistically to reduce meal size with CCK. Amylin acts directly at the area postrema of the hindbrain to reduce energy intake, and amplifies and or facilitates other satiety signals such as the ones elicited by CCK. IAPP is inhibited by somatostatin secreted by the pancreatic delta-cells (Woods *et al*., 2006).

Pancreatic polypeptide (PP) is a hormone secreted by the endocrine F-cells in the pancreatic islets in response to food intake. The secretion of PP is proportional to the caloric load eaten. Trans-genic mice who over-express PP are hypophagic and lean. In humans, PP inhibits gastric emptying of solid foods, which can also contribute to modulating appetite (Woods *et al*., 2006). This was shown in a study in which a 90-min infusion of PP at 10 pmol/kg/min 2 h prior an *ad libitum* meal led to decreased appetite and food intake (Schmidt *et al*., 2005).

1.3.3 Nutrient sensing of enteroendocrine cells

The intestinal cells that secrete hormones are called enteroendocrine cells, and they do so upon the specific stimulation of nutrients in the luminal content via receptors. For example, free fatty-acids are sensed in the luminal space by the enteroendocrine L- cells (Psichas *et al*., 2015) via free fatty acid receptors (FFARs)-2 and -3, belonging to the G-protein coupled receptors (GPCRs) family. Its stimulation leads to intracellular signals, which, although not fully understood, are responsible for the release of hormones such as GLP-1 and PYY (Chambers *et al*., 2015) into the blood that induce the aforementioned GIT functions related to appetite regulation (Efeyan *et al*., 2015). Similar mechanisms exist for amino acids, sensed via a variety of receptors including the calcium-sensing receptor.

1.4 Role of specific nutrients on blood glucose and appetite regulation

In the previous sections, I have discussed blood glucose and appetite regulation in the context of healthy and T2D individuals and how nutrition plays an important modulatory role in such regulations. In particular, dietary fibre and protein play an important role in blood glucose homeostasis and are generally considered the most satiating of the macronutrients. In the following sections, the effects of dietary fibre and protein on glycaemic control and appetite regulation will be discussed, as well as the mechanisms underpinning these effects.

1.4.1 Dietary fibre

Dietary fibre is defined as “carbohydrate polymers with 10 or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans” by the CODEX Alimentarius Commission (Codex Committee, 2009). This definition also specifies that dietary fibre must be originated from: (1) naturally occurring in the food as consumed*, (2) food raw material by physical, enzymatic or chemical means or (3) synthetic polymers, in which (2,3) have shown to have a benefit in human physiology according to the scientific evidence accepted by competent authorities. Of interest, CAC also notes that dietary fibre derived from a plant source may contain lignin and/or other compounds typical of cell walls.

Epidemiological and clinical evidence highlight the beneficial effects of dietary fibre consumption in humans to many aspects of health (Newby *et al*., 2007, Liu *et al*., 2003, Aune *et al*., 2017). For example, dietary fibre such as β-glucan intake promotes blood cholesterol reduction, maintenance, or achievement of normal body weight, increase satiety and decrease postprandial blood glucose (European Food and Safety Authority (EFSA), 2011). Given the well-recognised health benefits following dietary fibre consumption, in the UK, the daily recommendation of dietary fibre (AOAC) intake for adults is 30g a day as advised by the UK Scientific Advisory Committee on Nutrition. However, as of June 2020, this recommendation is not met in the population, as the mean intakes reported are of 19.05g a day, with only 8.5% of the population sampled meeting the recommendations (NDNS years 7 and 8, 2020).

Dietary fibre can be generally classed according to 3 characteristics: composition, solubility and fermentability, although not all fibres have these characteristics defined. Depending on the main chemical component forming its structure, fibres can be non-starch polysaccharides (NSP) or oligosaccharides, carbohydrate analogues, lignin, and non-plant origin fibres. Depending on their capacity or not to dissolve in water (solubility), dietary fibres could be either soluble or insoluble. Within soluble fibres, there are 2 distinctions: viscous or non-viscous depending on their resistance to deformation. Moreover, dietary fibres, as they are not hydrolysed by the endogenous enzymes in the small intestine of humans, they may be susceptible to different degrees of fermentability by the gut microbiota within the small and large intestine. Table 4 shows the types of dietary fibres by composition and their properties.

Table 4. Examples of dietary fibres and their classification according to composition and their properties.

Fibre Constituents	Principal groupings	Fibre name	Water Solubility	Viscosity	Ferment-ability
	Cellulose	Cellulose	No	No	Low

NSP & oligo-saccharides	Hemicellulose	β-glucan	Yes	Yes	High
	Polyfructose	Inulin	Yes	No	High
	Gums	Galactomannan (guar gum)	Yes	Yes	High
	Mucilages	Psyllium	Yes	Yes	Low
Carbohydrate analogues	Pectins	Pectin	Yes	Yes	High
	Resistant starches	Wheat dextrin	Yes	No	Low
	Enzymatic synthesis	Fructooligosaccharide	Yes	No	High
Lignin	Lignin	Lignin	No	No	Low
Animal or fungal origin	N-acetylglucosa-mine	Chitin	No	No	Unknown Low

NSP, non-starchy polysaccharides. Table adapted from Tungland *et al*., 2002, Slavin *et al*., 2009 and amplified with Slavin *et al*., 2009, Mensink *et al*., 2015, Wanders *et al*., 2011, Chutkan *et al*., 2012.

These biological and physico-chemical properties of dietary fibre are involved in the physiological effects in humans following dietary fibre intake. In particular, the effects on blood glucose and appetite homeostasis of the main soluble groups of fibres will be covered in section 1.4.1.1 and 1.4.1.2.

1.4.1.1 Effects of dietary fibre on blood glucose homeostasis

Acute effects

In healthy subjects, the acute effect of soluble viscous fibre such as barley β-glucan (5 g with 75 g of available carbohydrate), reduced both glucose and insulin incremental area under the curve (iAUC) by ~25% and ~50%, respectively compared to only glucose (Behall *et al*., 2006). Several studies conducted on soluble non-viscous fibres such as resistant maltodextrin were meta-analysed (Livesey *et al*., 2009) showing that 5-8 g (with 350 g of available carbohydrate), attenuated postprandial glycaemic response by ~20% when placed into drinks and by ~10% when placed into food. Insulin response was also reduced by ~25% with 5-10 g of resistant maltodextrin co-ingested with 50-130 g of available CHO. However, prior to this, the EFSA issued a non-favourable opinion on the cause-effect relationship between resistant maltodextrin and reduction of postprandial glycaemia (EFSA, 2011). Insoluble fibres such as 13 g of wheat fibre (70% cellulose) (with 50 g available carbohydrate), modestly reduced both glucose and insulin post-prandial responses at specific time-points compared to white bread (Weickert *et al*., 2005).

In people with T2D, the effect of soluble-viscous fibre such as psyllium, showed a decreased postprandial glucose by ~14% (and ~12% for insulin) at breakfast and by ~31% at lunch, relative to placebo (Pastors *et al*., 1991). In addition, non-viscous fibres such as dextrin showed a modest decrease in mean postprandial blood glucose by ~10% when added to a cake, compared to a control cake (Argyri *et al*., 2013). Insoluble fibre such as soy or lupin

(12.5 g of fibre with 50 g of available carbohydrate), decreased postprandial glucose and increased insulin response at specific time-points, compared to only 50 g of glucose (Dove *et al*., 2011).

Chronic effects

In healthy individuals, the chronic effects of the soluble viscous fibre β-glucan are studied in 5 meta-analyses, of which only two studies (doses of ~5.5 and 6 g/day during 7.5 and 6 weeks-long interventions, respectively) showed a decrease in fasting blood glucose (of ~2.3 and 44.6 mg/dL, respectively) and in fasting insulin of ~6.3 pmol in two studies (Andrade *et al*., 2015, Tiwari *et al*., 2011, He *et al*., 2016, Zou *et al*., 2015, Bae *et al*., 2014). Furthermore, one meta-analysis on another soluble fibre namely psyllium (10g/day x 9.5 weeks) showed a tendency to decrease fasting blood glucose by ~1.6 mg/dL. The effects of chronic supplementation of soluble non-viscous fibre such as fructooligosaccharide (20g/day x 4 weeks) resulted in no change in fasting blood glucose (FBG), but a decrease in hepatic glucose production without changes in insulin stimulation of glucose uptake as measured with hyperinsulinemic clamps (Luo *et al*., 1996). The chronic effect of insoluble fibres such as wheat bran (20g/day x 7 weeks) decreased postprandial blood glucose and increased insulin postprandial levels following a meal intake, being the greatest effect 24 days after the intervention compared to 10 days or 45 days (Villaume *et al*., 1984).

In those with T2D, two types of soluble fibre [β-glucan (~3 g) and psyllium (~12 g)], were meta-analysed independently (Shen *et al*., 2016, Gibb *et al*., 2015). A supplementation of β-glucan for ~4.5 weeks-long decreased FBG by ~8 mg/dL. Psyllium supplementation for ~8.5 weeks induced the greatest reduction by ~37.0 mg/dL. Consistent with this,a a more recent meta-analysis has reported this effect to be of ~31.0 mg/dL (Xiao *et al*., 2020). The effects on HbA1c resulted in a drop of ~0.21% for β-glucan, whereas a greater reduction was observed for psyllium of about ~0.95%. Soluble non-viscous fibres such as resistant dextrin (10g/day x 8 weeks) did not show any effect on FBG or glycated haemoglobin A1c (HbA1c), but it reduced fasting insulin by ~22.8% compared to maltodextrin (Farhangi *et al*., 2018). These beneficial effects in glycaemic control are not reported for insoluble fibres such as wheat bran in people with T2D following a 3-month intervention, being the reason for this unclear (Jenkins *et al*., 2002). Table 5 summarises the effects of dietary fibre following acute and chronic interventions on blood glucose control in healthy and T2D subjects.

Table 5. Summary of the magnitude of the effect of different types of fibre on blood glucose levels in healthy and people with T2D, following acute and chronic interventions.

	Acute			**Chronic**		
	Soluble viscous	**Soluble non viscous**	**Insoluble**	**Soluble viscous**	**Soluble non viscous**	**Insoluble**

Healthy	β-glucan ↓↓↓	Resistant maltodextrin ↓↓↓	Wheat fibre ↓	β -glucan ↓↓↓ Psyllum -	Fructo-oligo-saccharide -	Wheat bran ↓↓
T2D	Psyllum ↓↓↓	Dextrin ↓	Soy/lupin ↓↓↓	B-glucan ↓ Psyllum ↓↓↓	Resistant dextrin -	Wheat bran -
-, no effect; ↓, modest reducing effect ≤10%); ↓↓ moderate reducing effect (≥10.1≤20%); ↓↓↓ considerable reducing effect (≥20.1%)						

1.4.4.2 Mechanisms of blood glucose homeostasis

The mechanisms underpinning the effects of dietary fibre on glucose homeostasis mainly depend on biological and physico-chemical properties of such.

Soluble viscous fibres possess water-soluble gel-forming properties forming a viscous solution delaying gastric emptying (Holt *et al*., 1979) and consequently delaying carbohydrate digestion and absorption. In line with this, some authors have reported correlations between reduced blood glucose levels and reduced gastric emptying rates following the consumption of soluble fibre (Torsdottir *et al*., 1991, Jenkins *et al*., 1978, Edwards *et al*., 1987).

Other potential mechanisms behind the effect of dietary fibre on blood glucose might be the increased viscosities of digesta and its effect at reducing the contact between co-ingested carbohydrate and the absorptive epithelium, further contributing to the delay of carbohydrate absorption (Edwards *et al*., 1988, Dhital *et al*., 2014) as well as modestly diminishing starch hydrolysis *in vitro* (Dhital *et al*., 2014).

These diverse physiological processes during digestion may lead to the observed dual and consequent decrease in blood glucose and insulin levels following soluble fibre ingestion.

On the other hand, the mechanisms underpinning the effect of insoluble fibre on glycaemia could be due to an increase in gastrointestinal solids accelerating gastric emptying and intestinal transit (Dove *et al*., 2011, Villaume *et al*., 1984). This could explain the increased rise in insulin secretion observed compared with insoluble fibres compared to soluble fibres, resulting to a stimulation of glucose uptake from cells and therefore reduced blood glucose levels (Morgan *et al*., 1990). Also, it has been shown that some insoluble fibres including oat bran and wheat bran can alter digestive enzymes by decreasing their activity (Dunaif *et al*., 1981) or trigger GIP secretion (Weickert *et al*., 2005). It is of importance noting that some insoluble fibre interventions (Dove *et al*., 2011) are administrated in combination with protein, which may explain the greater glucose-dependent insulinotropic polypeptide as well as insulin responses (Weickert *et al*., 2005) consequently decreasing blood glucose acutely,

compared to fibre alone. These observations provide insights into the mechanisms underpinning the acute effects of dietary fibre on blood glucose. However, dietary fibre has also been reported to exert an effect on blood glucose in the long term. A possible explanation for this could be the phenomenon known as the “second-meal effect” (Jenkins *et al*., 1982) in which malabsorbed carbohydrate leads to improved glycaemic responses at a subsequent meal. This may be credited to the ability of fibres to be fermented to by specific resident gut microbiota. As a result, short chain fatty acids (SCFAs) are produced which are sensed via FFAR-2 and -3 expressed in the enteroendocrine L-cells (Psichas *et al*., 2015), stimulating incretin secretion (e.g. GLP-1) (Chambers *et al*., 2015). Furthermore, SCFAs have been shown to appear in portal circulation in molar ratios of 60:20:20 (for acetate, propionate and butyrate, respectively) (Cummings *et al*., 1987) as well as in systemic circulation in humans (Frampton *et al*., 2020), suggesting they may act on tissues with FFARs receptors such as liver, adipose tissue or muscle, which are glucose-disposal tissues (Thorburn *et al*., 1993, Venter *et al*., 1990). An example of this was observed in an early study showing that carbohydrate fermentation (as measured with breath hydrogen) decreased hepatic gluconeogenesis by 30% (Thorburn *et al*., 1993). More recent evidence showed that targeted delivery of propionate in the colon improved insulin sensitivity in humans (as measured by Matsuda index) (Chambers *et al*., 2019), and was in line with *in vitro* cell work suggesting that propionate inhibited pancreatic β-cell apoptosis (Pingitore *et al*., 2017).

1.4.1.3 Effects of dietary fibre on appetite regulation and energy intake

Acute effects

The acute effects of fibre on appetite outcomes in healthy individuals were studied in a systematic review (Wanders *et al*., 2011) where 58 studies showed that there was a ~10% reduction of appetite feelings following fibre consumption. This outcome was mainly driven by the intake of pectins, most glucans and soluble fibres. This finding was confirmed with a reduction of acute energy intake reported on 26 other studies in which dextrin, β-glucan-rich, pectin and resistant starch intake showed the greatest effect.

In individuals with T2D, only one study was found to investigate the acute effects of soluble fibre on appetite, in which pumpernickel rye bread (19.2g of fibre) showed no effect on appetite compared to other breads (3.5-7.5g of fibre) (Breen *et al*., 2013).

Chronic effects

38 other studies on the chronic effects of dietary fibre on appetite and energy intake in healthy subjects were meta-analysed and it was concluded that there was an overall average reduction of ~238.85 kcal/day (Wanders *et al*., 2011) and a possibly consequent reduction in body weight of ~0.4%. Nevertheless, the types of dietary fibre that showed a

greater effect at supressing food and energy intake were arabinoxylan-rich fractions (insoluble), fructans and mannans (soluble).

No studies were found to investigate the effects of chronic dietary fibre intake on appetite/energy intake in people with T2D.

1.4.1.4 Mechanisms of dietary fibre on appetite regulation

Dietary fibre has been shown to reduce appetite and subsequent energy intake via various mechanisms depending on the type of fibre. Soluble viscous fibres can increase exposure time in the oral cavity that may trigger sensory-mediated appetite regulatory mechanisms (Wanders *et al.*, 2014). Moreover, viscous fibres can retain water molecules promoting stomach distension sensed by the mechano-receptors, which in its turn may promote fullness signals via the afferent vagus (Browning *et al*., 2017). Furthermore, their water-holding capacity may also delay gastric emptying (Zhu *et al*., 2013) and prolongate nutrient absorption in the small intestine resulting in nutrient-sensing stimulatory effects on anorexogenic hormones release (Juvonen *et al*., 2009). This has been confirmed by correlations between gastric emptying and appetite feelings (Bergmann *et al*., 1992). For insoluble fibres, it has been observed increases in cholecystokinin levels following its intake, which may have contributed to the appetite-suppressant effects (Samra *et al*., 2007).

The longer-term effects of fibre on appetite could be owed to the “ileal and colonic brake” (Layer *et al*., 1990, Nightingale *et al*., 1996). This phenomenon is a feedback inhibitory mechanism in which energy content found at the end of the GIT signals the end of digestion via neuropeptide release (e.g. GLP-1 and PYY) which slows down gastric emptying. It has been observed that CHO in the distal small intestine can increase and prolong GLP-1 secretion for 2-4 hours (Qualman *et al*., 1995, Seifarth *et al*., 1998). In addition, some fibres can be fermented by the gut microbiota resident in the ileum and colon producing SCFAs, which in its turn stimulate the release of the anorexigenic hormones GLP-1 and PYY from the enteroendocrine L-cells via FFAR-2 and -3 receptors. In addition, studies have been shown that SCFAs such as acetate and butyrate can cross the blood brain barrier and may affect appetite directly in the central nervous system (Li *et al*., 2018, Frost *et al*., 2014).

1.4.2 Dietary protein

Dietary protein is a polypeptidic molecule composed of amino acids, naturally found in foods, most predominantly in animal-derived foods. Proteins provide 4 kcal/g and unlike dietary fibres, are considered a macronutrient, constituting the building blocks for growth and synthesis of structural proteins, enzymes, etc. Furthermore, aside from its nutritious role, dietary protein has been shown to positively affect blood glucose levels and to regulate appetite. In the UK, the daily recommendation of dietary protein intake for adults is

approximately 50g/day (Public Health of England, 2016). A sample of the UK adult population (n=648) has reported an average intake of 72g/day, with 82% of people meeting the recommendations (NDNS, 2020).

1.4.2.1 Effects on glucose homeostasis

Acute effects

In both healthy individuals and patients with T2D, protein ingestion induces a reduction in glucose and a rise in plasma insulin levels, compared to carbohydrate consumption alone (Nuttal *et al*., 1984, van Loon *et al*., 2003). The type of protein and factors such as digestibility and amino acid composition can have a differential effect on insulin release. A comprehensive study in healthy people using several amino acids (1 mmol/kg lean body mass), co-ingested with 25 g of glucose showed a similar effect, in which in general, blood glucose was reduced, insulin secretion modestly increased, and glucagon reduced. This could be interpreted because amino acids stimulated insulin which promoted glucose disposal. Both the rise in insulin secretion and the decrease in blood glucose and may have had a role at subsequently reducing any stimulation on glucagon secretion (Gannon *et al*., 1988).

Chronic effects

Interestingly, even though it seems that protein has a clear acute effect on glycaemia, long-term interventions do not show to be as efficient. A high-protein diet (27% kcal as protein), compared to a high-carbohydrate diet, did not change HbA1c, fasting glucose or insulin sensitivity parameters after 8 weeks of intervention (Sargrad *et al*., 2005). In line with this finding, supplementing the amino acid leucine (7.5g/day) for 6 months did not change glycaemic control in people with T2D compared to placebo (Leenders *et al*., 2011). The reasons as to why this occurred are not clear although authors suggest that the dietary intake of participants was already optimal for protein intake and leucine, with further increases not resulting in any improvement. Nevertheless, it is noteworthy that this study was performed in elderly people in which aging is associated with loss of skeletal muscle which may explain the lack of any effect following leucine supplementation.

1.4.2.2 Mechanisms of protein on blood glucose homeostasis

Some mechanisms have been reported for the acute effects of protein on blood glucose regulation. These include delaying gastric emptying therefore decreasing intestinal delivery and absorption of glucose or by indirectly stimulating incretin hormones such as GLP-1 and glucose-dependant insulinotropic peptide (GIP) which promoted insulin secretion and subsequent blood glucose regulation (Ma *et al*., 2009). Furthermore, some amino acids have been also shown to directly stimulate pancreatic β-cells insulin secretion, function, and mass. Studies have shown that BCAAs acutely stimulates insulin secretion and activate

intracellular signalling molecules involved in insulin transducing signalling pathway responsible for β-cell mass and function stimulation (Cheng *et al*., 2016). In addition, glutamate has also been shown to potentiate insulin secretion (Javed *et al*., 2019). Interestingly, a recent study has highlighted another mechanism by which protein exposure in the upper gut regulates glycaemia attributing the upper gut sensory receptor named peptide transporter 1 as the mediator by which hepatic glucose output decreases and glucose tolerance increases in rodents (Dranse *et al*., 2018).

1.4.2.3 Effects of protein on appetite regulation

Dietary protein is widely reported to be the most satiating macronutrient (Velhorst *et al*., 2008) (de Castro *et al*., 1987). Amongst body weight-reducing diets, high-protein diets have become popular as they have shown to be effective at preventing body weight regain (Layman *et al*., 2003, Skov *et al*., 1999, Lejeune *et al*., 2005, Westerterp *et al*., 2004). A possible explanation for this is their role at reducing appetite and energy intake.

In healthy subjects, whey protein (forming 20% of total protein in bovine milk) reduced appetite long term, although it did not show an effect the short-term compared to CHO, as reported in a meta-analysis including 8 studies (Mollahosseini *et al*., 2017). Interestingly, in a study which combined proteins (bovine milk) compared to proteins alone (casein and whey), no differences were found in subjective appetite, although milk decreased energy intake by ~9% compared to both casein and whey alone (Lorenzen *et al*., 2012). When compared to low protein preloads, higher protein preloads are more powerful at increasing fullness feelings (Dhillon *et al*., 2016, Wycherley *et al*., 2012). However, when chronically administered, low protein doses showed a greater effect on appetite than higher-protein, suggesting an adaptive response to the appetite-supressing potential of protein (Long *et al*., 2000).

For patients with T2D, fewer studies have been found, of which all are chronic interventions. Results indicate that replacing a so-called “diabetes diet” (20% of energy as protein) for a Palaeolithic diet (25% of energy as protein) for 3 months induced an increase in perceived satiety per calorie (Jonsson *et al*., 2013). However, it is important noting that the Palaeolithic di*et al*so had increased amounts of fruits and vegetables (therefore dietary fibre), which may have contributed to increasing one’s perception of satiety. Another 14-day interventional study of low-carbohydrate, high-protein, and fat diets in obese with T2D, showed a decrease in appetite and energy intake to ~2200 kcal/day (similar to normal-weight individuals) (Boden *et al*., 2005).

1.4.2.4 Mechanisms of protein on appetite regulation and energy intake

The mechanisms underpinning the effects of dietary protein on appetite regulation are not well understood, although several hypotheses have been proposed (Mellinkoff *et al*., 1956, Westerp-Platenga *et al*., 1997, Westerp-Plantenga *et al*., 1999, Ravn *et al*., 2013, Veldorst 2012). In the mid-1950s, a moderate but significant relationship (r~0.5) between amino acid serum levels and fluctuations in appetite was identified, which was termed the aminostatic hypothesis (Mellinkoff *et al*., 1956). Interestingly, similar relationships although weaker, were found for glucose in a phenomenon denominated glucostatic hypothesis (Mayer *et al*., 1953) (Mellinkoff *et al*., 1956). At the end of the 20th century, Westerp-Plantenga and team proposed the hypothesis that the magnitude rate at which dietary protein is metabolised is related to the perception of satiety (Westerp-Platenga *et al*., 1997). However, subsequent work did not find any relationship between protein metabolism and appetite over 24 hours (Westerp-Plantenga *et al*., 1999). Furthermore, since protein intake causes a higher degree of diet-induced thermogenesis (DIT) compared to other macronutrients (van Baak *et al*.,2004), it was thought that DIT caused increased oxygen consumption and body temperature that lead to a feeling of satiety (Westerp-Platenga *et al*., 1999). However, no association between satiety, composite appetite score and DIT has been reported (Ravn *et al*., 2013). High-protein diets are widely acknowledged to increase gluconeogenesis in humans (Veldhorst *et al*., 2009), increasing glycaemia, and consequently may inhibit meal initiation- based on the glucostatic hypothesis. However, this relationship was later failed to be proved in humans (Veldorst *et al*., 2012).

A later review on the topic (Bendtsen *et al*., 2013), suggested that factors such as amino acid composition, rate of absorption, protein/food texture all may play a role in the appetite-supressing effects of protein. Recently, findings were reported for the effects of dietary proteins consumption on gut anorexigenic hormone release, which appear to have a greater effect on the release of CCK, GLP-1 and PYY compared to other macronutrients (Raben *et al*., 2003, Giezenaar *et al*., 2018). This could explain the acute effects of protein on appetite and energy intake. However, the effects of protein on ghrelin secretion stimulation are inconsistent (Erdmann *et al*., 2003, Foster *et al*., 2008, Greenman *et al*., 2004), although this could be explained by the different food formats used between studies. It has been demonstrated that ileal protein infusions in humans activate the so-called ileal brake –which reduces food intake and satiety-, explaining the effects of protein on appetite and energy intake at the long term, although differences in these effects exist for diverse proteins and amino acids (Maljaars *et al*., 2008).

1.5 Guar gum and the effects of blood glucose and appetite

Soluble fibres have been consistently shown to greatly reduce postprandial blood glucose and appetite in humans. In particular, the effect of the soluble fibre guar gum on glycaemia and appetite has a long track record in human studies since 1980. In addition, guar gum allows to be incorporated into staple foods such as bread dough almost unnoticeably to taste, which makes it widely accepted as a supplement for foods. In this section, its origin, properties, the magnitude of its effects on glycaemia and appetite as well as its potential mechanisms underpinning these effects, will be reviewed in detail.

1.5.1 Guar gum, origin, properties and uses

Guar gum is derived from the processed endosperm of a plant member of Leguminosae family named *Cyamopsis tetragonoloba*. Guar gum is mainly composed of a linear long backbone composed of β-1,4-mannose and α-1,6-galactose. Guar gum molecular structure can comprise from 50 to $80x10^5$ galactomannan units, having one of the highest molecular weights (50-8000kDa) of all naturally occurring water soluble molecules. The most characteristic property of guar gum is its hydration capacity, giving highly viscous solutions. When in water, guar gum forms a colloidal suspension, whose viscosity starts to develop after 10-15 min (Holt *et al*., 1979). The viscosity of 1% of guar gum is 2700 mPa/s. Guar gum is classed as a non-newtonian fluid, meaning that its viscosity is not constant, but changes when different forces are applied (e.g. temperature changes, shearing force, pressure). Other factors such as pH, degree of processing, concentration, and molecular weight are also important for guar gum's viscosity (Berthold *et al*., 2008). As a result of its thickening property, guar gum has been used for both non-food (e.g. as paper, printing pastes, finishing operations, water-blocking agent in dynamites) and as a food additive in foods such as gravies, ice creams, sauces, cake mixes and cheese spreads.

Guar gum has also an important biological property which is that is practically not digested in the human small intestine, and therefore is considered as dietary fibre. However, it is considerably fermented by human gut microbiota mainly by Bifidobacterium and Clostridium genres, with the subsequent production of SCFAs (Hartemink *et al*., 1999).

1.5.2 Effects of guar gum on blood glucose in T2D

Acute effects

For individuals with T2D, doses ranging from 7.6-16 g of guar gum incorporated into bread co-ingested with carbohydrate loads reduced glucose incremental area under the curve (iAUC) by 26-51% compared to controls (Jenkins *et al*., 1976, Jenkins *et al*., 1980, Gatenby *et al*., 1996). The strongest effect was observed for guar gum incorporated into crispbreads (Jenkins *et al*., 1980). However, crispbreads are generally high in fibre which may have

confounded the sole effect of guar gum. Similarly, another study (Jenkins *et al*., 1976) also used 10 g of pectin in addition to guar gum which may have confounded the specific effect of guar gum. Other forms of guar gum administration such as dissolved into water (and co-ingested with carbohydrate) showed a decrease in glucose iAUC of 18-32% for doses ranging from 5 to 9 g (Russo *et al*., 2003, Carvalho *et al*., 2017). Of these studies, one did not use pure guar gum (de Caravalho *et al*., 2017), but a mixture of 60% guar gum and 40% inulin, potentially confounding the results.

Chronic effects

Several studies have been found to investigate the effects of chronic guar gum supplementation on blood glucose in individuals with T2D who were body-weight stable. Guar gum administration of doses ranging from 5-26 g a day in the form of diet supplements or within food products (e.g. crispbreads, bread) for 5 days to 12 weeks induced a reduction of FBG by 5-16%, compared to controls (Jenkins *et al*., 1978, Aro *et al*., 1981, Fuessl *et al*., 1987, Peterson *et al*., 1987, Niemi *et al*., 1988, Lalor *et al*., 1990). The strongest effect was observed with 15g of guar gum a day (as 3 doses of 5 g granules at mealtimes), for 12 weeks, achieving a reduction in FBG of 16%, compared to placebo (Lalor *et al*., 1980). In contrast to this, one study with the same dosing and time of administration reported no changes in FBG in patients with T2D and poor glycaemic management compared to microcrystalline cellulose, although an effect was seen at 6 weeks (Niemi *et al*., 1988). It is possible that poor compliance after 6 weeks may have driven this change as the authors implemented no objective measure of adherence. Consistent reductions in glucose iAUC of 10-50% were observed following 4-24 week-long interventions of guar gum doses ranging from 5 to 31.7 g a day in form of tablets, sprinkled in food or high-carbohydrate bar. The strongest reduction (50%) was achieved with 5g/day of sprinkled guar gum in food for 4 weeks, compared to wheat bran (Fuessl *et al*., 1987). Furthermore, glycated haemoglobin A1c was also reduced by 4.30% with 5 g of guar gum/day (sprinkled over food) for 4 weeks (Fuessl *et al*., 1987). Interestingly, a cohort of patients with T2D with good glycaemic management from the UK Prospective Study who achieved near-normal FBG, did not show any change in FBG, HbA1c or glucose iAUC (Holman *et al*., 1987) following a 8-week long intervention of 15 g of guar gum/day, suggesting no additional effect of guar gum at decreasing blood glucose levels when these patients have a good glycaemic management.

1.5.2.1 Mechanisms of guar gum on blood glucose

The mechanistic basis underlying guar gum's blood glucose lowering effects do not appear to be due to an increase in insulin secretion or in insulinotropic hormones such as GIP (Jenkins *et al*., 1980, Jenkins *et al*., 1978, Taylor *et al*., 1977). It is therefore possible that the

effects of guar gum on glucose homeostasis are related to its gelling-forming properties, increasing viscosity of digesta, and consequently promoting a physical interaction with different digestive elements (Jenkins *et al*., 1978). It is known that guar gum delays gastric emptying, which has been shown to be correlated with blood glucose levels (Daumerie *et al*., 1982, Torsdottir *et al*., 1989, Russo *et al*., 2003). Furthermore, guar gum was shown to slow the passage rate of digesta through the small intestine by ~25% and to increase the viscosity of ileal digesta by ~70% (Owusu-Asiedu *et al*., 2006). Guar gum's viscosity may also reduce the permeability of compounds passing to the epithelial layer(Jenkins *et al*. 1978; Leeds *et al*., 1979; Taylor *et al*., 1977). This means that glucose diffusion and the accessibility of digestive enzymes such as α-amylase may be decreased, delaying CHO digestion and absorption (Dall'Alba *et al*., 2013), translating to a lower postprandial blood glucose. Furthermore, *in vitro* studies have shown that galactomannan directly and non-competitively inhibits α-amylase by the formation of a complex galactomannan-amylase with a Ki (inhibitory constant value, K_i) of 3.3 μM. (Slaughter *et al*., 2002). This inhibition is not a consequence of impaired diffusion of enzyme, increased viscosity or decrease in available water. Other studies have shown how guar gum in comparison to other gums (i.e. acacia, tragacanth) have the lowest IC50 for α-amylase with only 28.4 μg/mL of guar gum concentration needed to inhibit the enzyme activity by 50% (Singh et al., 2020).

While the ling-term effects of guar gum on glycaemia could be due to the cumulative effect of postprandial blood glucose reduction resulting from the above-mentioned acute mechanisms, other mechanisms could also be involved. One chronic interventional study observed decreased insulin to c-peptide ratio (Groop *et al*., 1993). Considering that both peptides are secreted in an equimolar ratio, this suggests that guar gum may promote hepatic insulin extraction and possibly inhibit hepatic gluconeogenesis, ameliorating glycaemia. Furthermore, guar gum has been shown to be fermentable *in vitro* by human faecal bacteria (Khan *et al*., 2005, Yang *et al*., 2013) leading to the production of SCFAs. SCFAs can trigger incretin secretion from enteroendocrine L-cells as well as direct effect on glucose-disposal tissues (Pingitore *et al*., 2016, Ximenes *et al*., 2007). Although no studies were found to measure plasmatic SCFAs in humans following guar gum ingestion, one study using partially hydrolysed guar gum (which has reduced viscosity compared to guar gum), did not show changes in days 4, 5, 6 post-ingestion of compared to a diet without fibre (Alam *et al*., 1998). This could have been due to a loss of this effect with partial hydrolysation compared to pure guar gum.

1.5.3 Effects of guar gum on appetite and energy intake

Research studies investigating the effects of guar gum on appetite and energy intake have mainly been conducted on healthy populations showing a lowering effect (Rao *et al*., 2016,

Salleh *et al*., 2019). Only two studies have been found to include patients with T2D, one (Chow *et al*., 2007) consisting of a controlled RCT testing the acute effect of a snack bar containing 5.7 g of guar gum, showing a suppression in appetite feelings by approximately 20% compared to a control bar. However, it is important noting that the food test product used (bar) also included 3.4 g of other types of fibre. The second study consisted of a chronic intervention with whey preload (17 g) in combination with guar gum (5 g) twice-a-day, over a 12-week period, showing no effects on energy intake assessed by 3-day food diary compared to placebo (Watson *et al*., 2019). This evinces the current gap in knowledge in regard to the effects of guar gum interventions alone, acutely and chronically, specifically on subjective appetite feelings and energy intake in people with T2D. Nevertheless, other studies including T2D individuals have focused on body weight as a primary outcome, which can be used to make differences about changes in energy balance. Overall, these studies show that interventions from 4 to 18 weeks of doses ranging from 7. 5 g to 15 g/day did not show a reduction in body weight compared to controls (Uusutipa *et al*., 1984 and 1989). This suggests that guar gum may not have an effect on energy intake and appetite feelings in the long-term.

1.5.3.1 Mechanisms of the effects of guar gum on appetite and energy intake

The acute appetite and energy intake suppressing effects of guar gum may be mainly explained by its increased viscosity (Marciani *et al*., 2001) exerting physical actions on different elements of digestion along the GIT. Viscosity is likely to increase bolus transit time and mastication in the oral cavity (Matsuo *et al*., 2013). It is recognised that increasing oral processing time increases the timespan for satiety signals to act (Hogenkamp *et al*., 2013). However, for guar gum, oral exposure time was studied by video observation showing no time differences compared to a macronutrient-matched control (Wanders *et al*., 2013). At the level of the stomach, guar gum has been shown to delay gastric emptying in most studies (Blackburn *et al*., 1983, Torsdottir *et al*., 1989, Russo *et al*., 2003), with some studies demonstrating a correlation between appetite feelings and gastric emptying (French *et al*., 1994) which may explain the acute effects of guar gum on appetite feelings. At the level of the proximal intestine, viscosity generally results in a prolonged exposure of nutrients to the absorptive epithelium and the nutrient-sensing mechanisms. In line with this, studies have shown CCK (Heini *et al*., 1998 and Meier *et al*.,1993) and GLP-1 (Adam *et al*., 2005) acute release following guar gum intake, which are two anorectic hormones that act centrally via afferent nerves, suggesting that nutrient-sensing mechanisms may have promoted its secretion in the enteroendocrine cells.

A potential long-lasting effect of guar gum on appetite and energy intake could be due to its potential of being fermented by resident gut microbiota and subsequent production of SCFAs (Khan *et al*., 2005, Yang *et al*., 2013), which via nutrient-sensing mechanisms, stimulate the release of anoretic gut hormones (Rao *et al*., 2016). Furthermore, it has been observed how colonic acetate can cross the blood brain barrier and affect the metabolism (activates Acetyl-CoA carboxylase) and expression patterns of regulatory neuropeptides that modulate appetite (Frost *et al*., 2014). Differences in resident gut microbiota between healthy and people with T2D have been described (Ahmad *et al*., 2019) with losses in bacterial diversity and butyrate-producing species being characteristic for T2D patients (Qin *et al*., 2012). This could potentially explain the loss of an effect in appetite and energy intake following guar gum's chronic intervention in T2D patients (Watson *et al*., 2019).

1.6 Mycoprotein

Foods high in both fibre and protein are of interest for the management of T2D since these macronutrients play a role in glycaemic control and appetite regulation. Importantly, a recent Lancet Commission (2019) called EAT - which surged following the concerns raised at the Lancet Commission on Global Syndemic obesity, undernutrition and climate change-, put together worldwide experts with the aim of developing healthy diets that can feed the growing global population using sustainable food production systems (Commissions from the Lancet journals, 2019). Altogether, this suggests that sustainable healthy high-fibre and protein-foods which are palatable and acceptable to the consumer are possibly the way forward to improve people's health. In this context, mycoprotein is an ingredient originated from fungi (naturally rich in fibre) that is concentrated achieving increased content of protein and it is produced in a sustainable way. Mycoprotein is shaped into foods that resemblance in appearance and taste to meat-counterparts. Mycoprotein represents a good food option which fits within the current planetary and human health demands. In the following section, I will provide an overview of the origin, history and reach of mycoprotein, accompanied by a detailed review of its nutritional profile. Even though the nutritional composition of a food is a classical measure to determine if a food is potentially healthy, recently, scientists have also placed a special focus on how these nutritional components are structurally arranged in food (food matrix and structure). Therefore, I will also review mycoprotein's food matrix and structure as well as its derived physico-chemical and biological properties, and how this may relate to any potential effects on glycaemia and appetite.

1.6.1 Mycoprotein, a unique ingredient

Mycoprotein ("myco", from the Greek word "fungus") is an ingredient of fungal nature which is rich in dietary fibre (6 g/100 g wet weight) and a source dietary protein (>12% of energy) (Quorn's website). Mycoprotein is made of the biomass of a filamentous fungus named

Fusarium venenatum A3/5 (PTA-2684), from the Ascomycota phyla, obtained through continuous fermentation of a carbohydrate substrate (Finnigan *et al.*, 2017). Mycoprotein can be processed into foods that resemble meat in shape and flavour, and are first launched in 1985, are sold under the brand name of Quorn™. Quorn foods became the first commercially available vegetarian meat alternative resembling meat (Ronald Bergers' website). Figure 4 shows two typical Quorn products available in the market (i.e. pieces and mince).

Quorn pieces	Quorn mince
Marketed package	
	
Frozen raw product	
	
Cooked product in a meal	
	

Figure 4. Pictures of marketed package, frozen raw and cooked Quorn products of pieces and mince.
Sources: https://www.quorn.co.uk/products/chicken-style-pieces (accessed: January 2022),

http://www.veganoo.net/2015/11/review-quorn-vegan-meat-free-pieces.html (accessed: January 2022), https://www.quorn.co.uk/products/meat-free-mince (accessed: January 2022), https://topfoodinfo.com/can-you-freeze-quorn-mince/ (accessed: January 2022).

Quorn foods are currently commercially available in the UK, Europe, North America, Oceania and Asia. Mycoprotein is regarded as a healthy product, exerting benefits in lipid profile, appetite and energy intake as well as glycaemia and insulinaemia (Turnbull *et al.*, 1993, Turnbull *et al.*, 1995).

1.6.2 Discovery

The fungus *F. venenatum* was discovered in the 1960s in Buckinghamshire (UK) by the former group of companies mainly devoted to flour-based foods, named Rank Hovis McDougall (RHM) group. Lord J. Arthur Rank was the chair of RHM at the time, and it is believed that his drive to search for alternatives to animal protein rose from the fear of global protein shortage and a subsequent undernutrition, which was already seen in some areas of the world at that time (Finnigan *et al.*, 2017). The strategy that RHM employed was to seek for a naturally occurring microorganism able to produce edible high-quality protein from fermentable glucose derived from starch within a flour surplus. 3000 soil samples around the UK, and later the world, were seeded with flour and analysed for microorganism growth able to ferment carbohydrate into protein. The screening of soil samples lasted 3 years until a fungal species named *F. venenatum* (ATCC PTA-2684, formerly identified as *F. graminearum* A3/5) (O'Donnell *et al.*, 1998; Yoder *et al.*, 1998) was selected as the best fungus able to ferment carbohydrate into protein, which was found in the field of Marlow, Buckinghamshire (UK). Since the discovery of *F.venenatum* in 1967, it took approximately 20 years of research and development before it could be sold for human consumption (Finnigan *et al.*, 2017).

1.6.3 Production

Mycoprotein is currently mass-produced in Stokesley, North Yorkshire (UK) using a 150,000 litre pressure-cycle reactor (named "fermenter"; Figure 4) which meets the required conditions that allow axenically (one-species culture) continuous fermentation by *F.venenatum*. These conditions are temperature (28-30°C), a pH of 6.0, dissolved oxygen, sterility, and an excess of growth medium (Trinci *et al.*, 1991). The growth medium is mainly composed of glucose as a source of carbon, and ammonia as a source of nitrogen and water. A few milligrams of a batch culture of *F. venenatum* are inoculated into the fermenter which is in a continuous flow process (Trinci *et al.*, 1994), allowing a constant growth rate of 0.17-0.20 μ/h, enabling the production of 2 tons of biomass/h (Wiebe *et al.*, 2004). This process is in circulation for a maximum of 6 weeks, before highly branched forms of mycoprotein overtake the culture, when the desired mycoprotein biomass is generated and

harvested. During this 6-week interval, regular testing for mycotoxins and potentially harmful substances is carried out every 6 h.

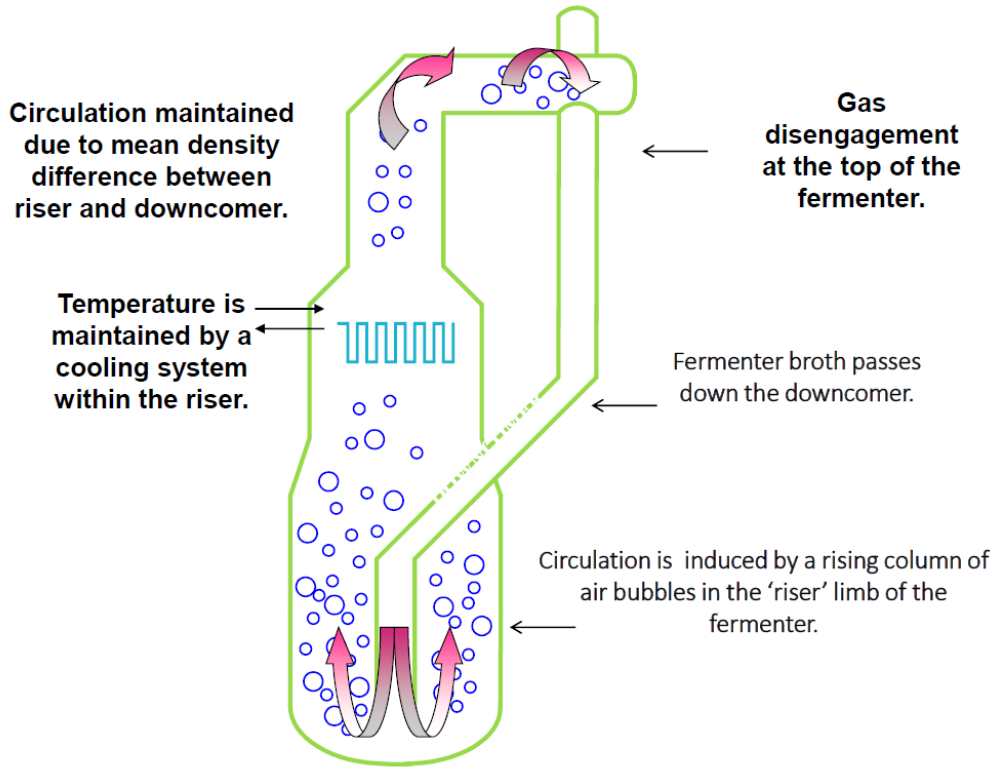

Figure 4. Schematic representation of the fermenter and the continuous flow process of *F. venenatum* fermentation. Source: Finnigan et al., 2017

As a result, fungal biomass is produced and harvested to make raw mycoprotein (paste). Mycoprotein biomass is subject to heat exchange and pasteurisation which activates intracellular RNAses to reduce RNA content and therefore meet food safety standards (Finnigan *et al.*, 2017). Following RNA treatment, mycoprotein biomass is heated to 90ºC and centrifuged to concentrate the biomass of fungal filaments or hyphae (mycelia) yielding a solid content greater than >20% (w/v) (Wiebe *et al.*, 2001). At this point, a paste of mycoprotein (raw mycoprotein) is generated. Raw mycoprotein is edible and typically consumed in research studies in the form of a soup or drink.

Nevertheless, raw mycoprotein is further processed to obtain commercially available Quorn™ food. This processing involves mixing with other ingredients, shaping, steaming, chilling and freezing. Addition of a protein binder, typically egg albumen or potato protein for vegan formats, combined with freezing are two key processes thought to be responsible for developing the meat-alike texture in the finished product. This may be regarded as a fibre-gel composite. In some products, cations, typically calcium are added to promote interhyphal

crosslinking whereas freezing may form controlled growth of ice crystals effectively pushing filamentous hyphae together to create fibrous bundles emulating the texture of whole muscle found in meaty products (Figure 9) (Finnigan *et al*., 2017).

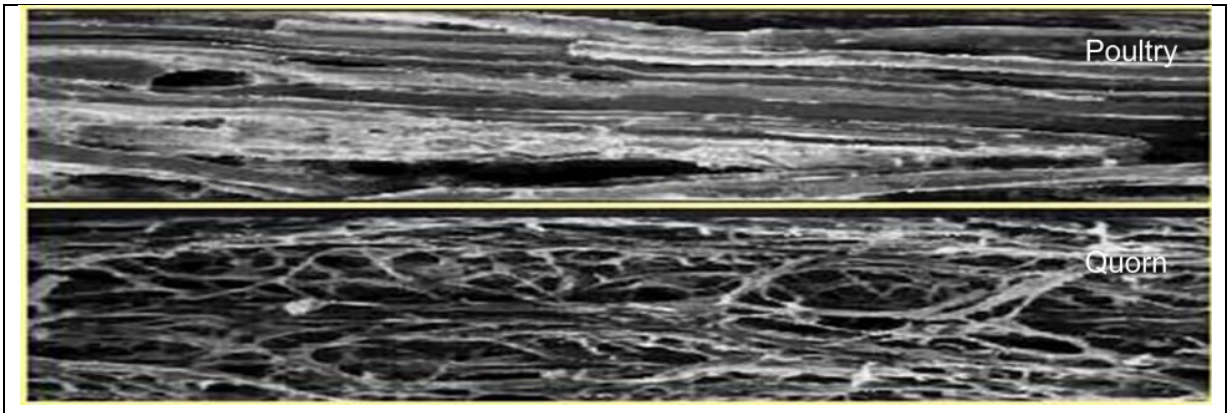

Figure 5. Microscopic view of poultry tissue (above) and Quorn (below). A similar pattern in filament distribution can be observed, probably responsible for the meat-alike texture properties of Quorn. Adapted from Finnigan *et al*., 2017

It is important to note that mycoprotein production is regarded as more sustainable when compared to the production of protein from animal livestock, as much reduced levels of CO_2 emission, land and water resources are used to produce the same amount of mycoprotein (Finnigan *et al*., 2017).

1.6.4 Mycoprotein on the market

Since its discovery, approximately 20 years of research and development followed to ensure an efficient way of producing mycoprotein as well as to establish its safety for human consumption (Finnigan *et al*., 2019). In 1983, mycoprotein obtained sale approval from the Ministry of Agriculture, Fisheries and Food in the UK. In 1985, mycoprotein was first sold as the main ingredient of a savoury pie in the UK under the name of Quorn™ in Sainsbury's supermarkets (Wiebe *et al*., 2002). At present, Quorn™ foods are consumed in 17 countries (Finnigan *et al*., 2019) with almost 7 billion servings consumed worldwide since launch. Quorn™ is mainly sold in North Europe, but it is available in Asia (Philippines, Singapore), Australia, New Zealand, and the US.

1.6.5 Composition

Mycoprotein consists of approximately 75% water and 25% dry matter. Table 6 shows the nutritional composition of raw mycoprotein (dry weight), Quorn (wet weight) in comparison with lean chicken breast.

Table 6. Nutritional composition of mycoprotein, Quorn and chicken breast per 100g.

Per 100g	**Mycoprotein (dry weight)[1]**	**Quorn (wet weight)[2]**	**Chicken breast, meat only (wet weight)[3]**

Energy (kcal)	340	85	160
Protein (g)	44	11	28.4
Carbohydrate (g) **Of which sugars (g)**	36 0	9 0	0 0
Fat (g) **Of which saturated (g)**	12 2.8	3 0.7	5.2 1.5
Fibre (AOAC) (g)	24	6	0.9
AOAC, Association of Analytical Chemists; G, gram; kcal, kilocalories. Source[1]: Finnigan *et al*., 2017. Source[2]: Quorn, website. Source[3]: Sainsbury's website.			

Mycoprotein-containing foods (Quorn)'s nutrition profile, allow some nutrition claims to be made (according to EFSA, Regulation EU No 1047/2012) such as: low in fat (<3 g/100 g in solids), low in saturated fat (<1.5 g/100 g in solids), low in sugar (<5 g/100 g in solids), high in fibre (>6g/100g). Mycoprotein is also a source of riboflavin, folate, vitamin B12, phosphorous, zinc, choline and manganese. It is also low in sodium and is free from cholesterol. Mycoprotein-containing foods are regarded as foods to belong as part of a healthy diet (Derbyshire *et al*., 2019) and marketing since 1994 has revolved around this claim.

1.6.5.1 Dietary fibre content

The fibre content of mycoprotein (24 g/100 g dry weight) is attributed mainly to the cell wall (25% of cell's dry weight) and is mainly composed of β-glucan (mainly insoluble) and chitin (in a ratio 2:1). β-glucans are non-starch polysaccharides sharing a common backbone of β(1,3)-linked D-glucose units. Nevertheless, they can differ in the glycosidic linkages and branching structure depending on the origin. For example, plant β-glucans (e.g. oats, barley) are characterised by the linear β(1,3),(1,4)-linked D-glucose units. However, fungal β-glucans are characterised by the presence of β(1,6)-linked branches coming from a β(1,3) backbone (Du *et al*., 2019). The different linkages define the molecular structure (helix or coil conformation) influencing molecular weight, which in its turn determines properties like gel, viscosity and texture of β-glucans (Du *et al*., 2019). These properties are important as they are responsible for some human physiological effects attributed to β-glucans (e.g. slowed gastric emptying rate due to increased viscosity properties). Plant β-glucans are known to be water-soluble, whereas fungal β-glucans can exist in both soluble and particulate forms (Yadomae *et al*., 2000). In fungi, β-glucans form 66% of total fibre. Fungal β-glucans are typically polymers covalently bound to chitin via hydrogen bonds, forming a network within the β-glucan matrix which results in a strong cell wall that is insoluble (Sietsma *et al*., 1981, Rosenberger *et al*.,1976).

Chitin (from the Greek word "covering") is also an important component of the fungal cell wall, making up the remaining 33% of the total fungal fibre. Fungal chitin is made of long-

chains of the β-(1-4) polymer N-acetylglucosamine which is largely insoluble (Sietsma *et al*., 1981). Chitin is found in nature either as a dietary source (exoskeleton of crustaceans, insects, and of course fungi) or in pathogenic parasites. Humans have been shown to express exochitinases in the stomach (mammalian chitinase, AMCase) and duodenum (chitotriosidase) which hydrolyses chitin to N-acetyl-glucosamine which can be easily metabolised by N-acetylglucosamine kinase (an enzyme present in all human tissues) (Gindzienski *et al*., 1974). Interestingly, the gene for chitotriosidase is maintained by 99% of individuals from Africa, whereas for individuals from Western countries it is 55-65% (Malaguarnera *et al*., 2003). This suggests that it is possible that by an environment of higher socio-economic status -including a reduction in dietary chitin and/or parasitic infections- may have selected the observed gene redundancy in Westerns. Furthermore, even though presence of AMCase has been detected in 80% of Western healthy individuals, its activity values ranged broadly from 0.21 to 36.27 nmol/ml/h (Paoletti *et al*., 2007). Since the amount of activity needed to digest dietary chitin is unknown, it is currently uncertain whether western humans can effectively digest chitin. Another study found in human faeces presence of the commensal *Clostridium paraputrificum* which has endochinase activity, suggesting possible fermentation of chitin in the human gut microbiota (Simunek *et al*., 2002).

1.6.5.2 Dietary protein content

The dietary protein content of mycoprotein (44 g/100 g dry weight) is attributed mainly to structural proteins such as the cytoskeleton or microtubules of the fungal cell (42-50% of cell's dry weight), which are proteins that allow movement, shape and expansion to the cell. Despite being a source of non-animal derived protein, it is logical to question whether its protein quality compares to that of animal-derived protein. Protein quality can be assessed using the protein digestibility-corrected amino acid score (PDCAAS) (FDA), which captures both human digestibility and distribution of the 9 essential amino acids. Mycoprotein's PDCAAS was determined to be 0.996 (Finnigan *et al*., 2017), similarly to animal-derived protein sources such as whole milk, chicken breast and eggs (PDCAAS of 1). Furthermore, recent studies on protein bioavailability and muscle anabolism in humans have shown mycoprotein to be insulinotropic (stimulate insulin release) and to stimulate both resting and post-exercise muscle protein synthesis to a greater extent than leucine-matched milk protein, in resistance-trained young men (Dunlop *et al*., 2017, Monteyne *et al*., 2020). Altogether, these data suggests that mycoprotein is a high-quality protein source.

1.6.6 Food Structure

In addition to the healthy nutritional profile of mycoprotein, it is very important to consider that its nutritional components are found in a unique whole food matrix and structure. Food

matrix could be defined as the three-dimensional assembly that describes the entirety of the food form. On the other hand, food structure could be defined as the arrangement of food constituents at multiple spatial scales, including macro- (>1 mm) and microstructures (<1 mm) (Cai *et al*., 2021). A recent meta-analysis showed how food structure factors such as high amylose:amylopectin ratio, less gelatinated starch, retrograded starch, and larger particle size had a significant effect at reducing blood glucose levels since they affect the digestibility of the carbohydrate content of foods (Cai et al., 2021). The dry matter of mycoprotein consists of fungal mycelium (a thread-like fungal mass with branches). Each fungal thread or filament is named hyphae (from the Greek word “web”) which in its turn, is composed of single fungal cells encapsulated by a cell wall and separated by septum. In Figure 6 it is shown mycoprotein structures at different length scales from a macroscopic view to a microscopic view.

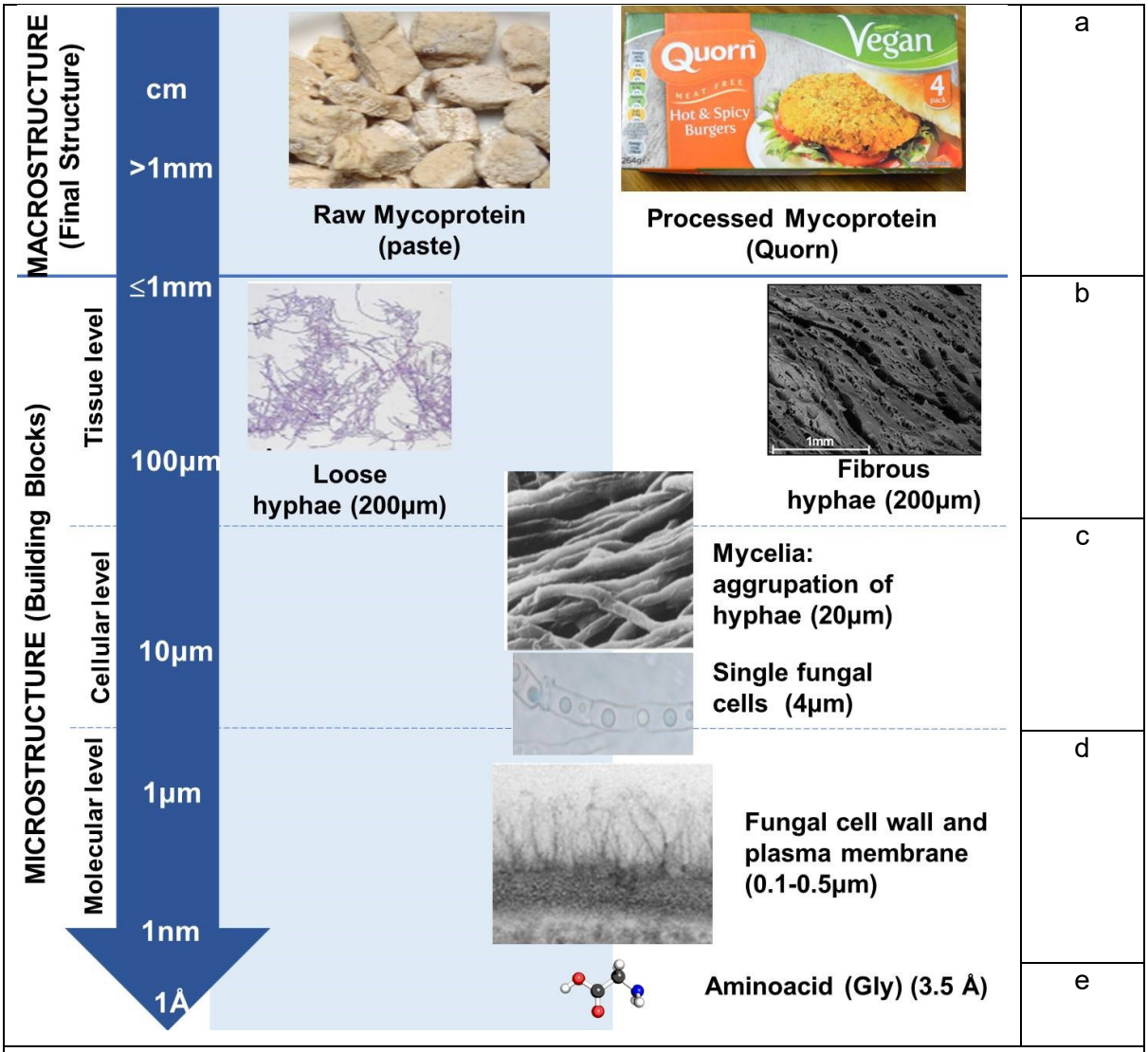

Figure 6. Mycoprotein structures at different lengh scales from a macroscopic view (top) to a microscopic view (bottom). a) Mycoprotein macrostructure can exist in two forms (raw mycoprotein) or processed mycoprotein. Subsequent levels of microscopic detail reveal the fungal tissue level which is formed by b) mycaelia which is an aggregation of hyphae (filaments). These can be loose or fibrous (after processing with ice crystals and acetate addition). The hyphae are formed by a cellular level of c) single fungal cells lined up contiguously and separated by a septum. A fungal cell is defined by macromolecular structures such as a d) cell wall which delimits the cell and it is formed of mannose, chitin and β-glucan. Inside the cell where are other e) molecular structures such as basic amino acids, peptides, etc. A, angstrom; Gly, glycine; µm, micometre. Picture adapted using images from Miller *et al*., 2001, Trinci *et al*., 1992 and Ene *et al*., 2015.

A plasmatic membrane is encapsulating the fungal cells which consists of a phospholipidic bilayer with no intralipid cholesterol (unlike animal cells). The cell wall is composed of mannose, β-glucan and chitin (Figure 7). β-glucan and chitin compose the dietary fibre content of mycoprotein-containing foods whereas the intracellular structural proteins compose the protein content.

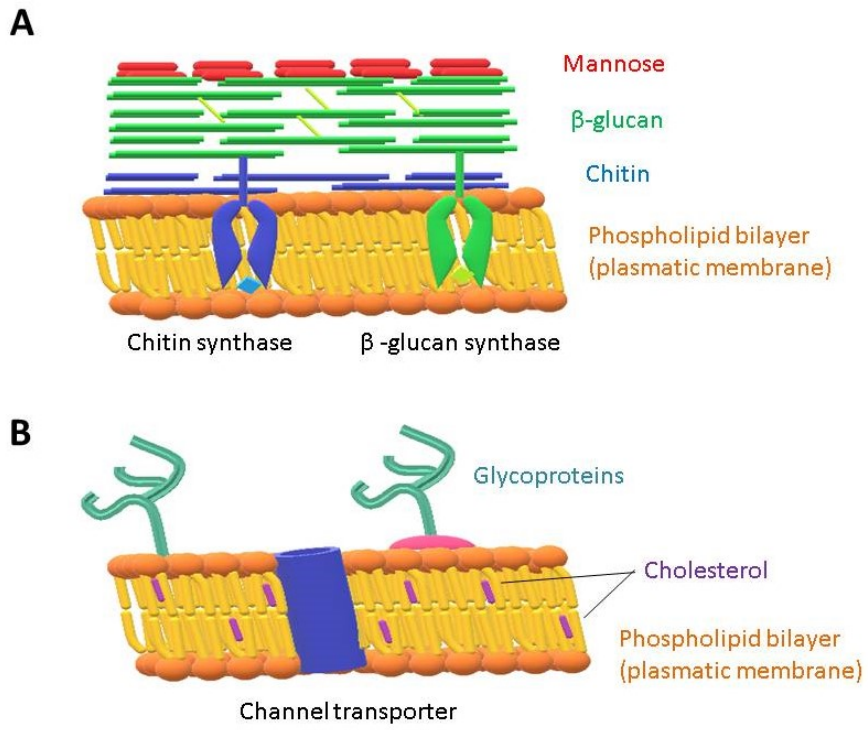

Figure 7. Schematic representation of the extracellular matrices and plasmatic membranes elements of a fungal (A) and animal (B) cell. The main difference between the fungal and animal extracellular matrices is the presence of fibre, the fungal cell (A) consisting of layers (from distance to proximity to the plasmatic membrane) of mannose, β-glucan, chitin compared to the animal cell (B) which is mainly composed of glycoproteins. This constitutes the main difference responsible for the presence of dietary fibre in mycoprotein-based foods compared to animal-derived proteins. Source: Cherta-Murillo *et al*., 2020 and Cherta-Murillo unpublished.

1.6.7 Physico-chemical and Biological Properties

Mycoprotein has been reported to exert diverse effects on some aspects of human physiology which are thought to be due to its composition and unique food structure conferring specific physico-chemical and biological properties. For example, longer hyphae filament lengths decrease glucose diffusion by 57% relative to shorter hyphae filaments *in vitro* (Marks *et al*., 2004a). This suggests that mycoprotein hyphae plays a role at reducing gastric emptying rate (therefore modulating glycaemia and appetite) in humans in comparison to the short filament, which was later confirmed with the paracetamol method. This effect was independent of GLP-1 secretion and was accompanied by no changes in blood glucose or insulin (Marks *et al*., 2004b). The mechanism underpinning this finding could be due to a particle size effect as it has been shown in a study using the gold-standard measure for gastric emptying (scintigraphy) that fine and coarse brain (two different particle sizes of bran) have a different effect on gastric physiology (Vincent *et al*., 1995). Furthermore, the fibre fraction of mycoprotein may play a role in gastric emptying when compared to animal-derived protein sources, since plant-based β-glucans are well-known to

decrease gastric emptying via increased viscosity in the gastric lumen (Kristensen *et al*., 2011). However, little is known about the effect of fungal β-glucans.

Human studies have tested the effect of solid mycoprotein compared to macronutrient matched-chicken, and showed no changes in gastric emptying or in GLP-1 levels (Bottin *et al*., 2016). Nevertheless, the findings of this study should be treated with caution since the method used for assessing gastric emptying (paracetamol) is not regarded as the gold-standard method for measuring gastric emptying in solid food (^{13}C-octanoid acid breath test), possibly confounding the results. An important biological property of mycoprotein is its effect on digestive enzyme activity. A recent *in vitro* study has shown that digestive enzymes such as α-amylase can be physically entrapped by the fungal cell walls delaying starch hydrolysis (Colosimo *et al*., 2020a). Furthermore, another study by the same author suggested that proteases can diffuse through the fungal cell wall due to its porosity and permeability and therefore modulate protein bioavailability (Colosimo *et al*., 2020b). This suggests that mycoprotein's own digestion and any potential co-ingested food digestion may be affected, subsequently affecting postprandial levels of nutrients such as glycaemia. Altogether, this data suggests that mycoprotein may play a role in physiological aspects like glycaemia and appetite through mechanisms such as delaying gastric emptying and digestive enzyme activity modulation.

Another relevant biological property of mycoprotein is its fermentability. Most β-glucans are considered to be non-digestible carbohydrates and are fermented in various degrees by the resident gut microbiota (Chan *et al*., 2009) producing SCFAs. For chitin, some bacterial species have been found to express chitinases and to be able to utilise chitin as a substrate such as *Clostridium putrefacis* (Chan *et al*., 2009). To date, only one study has investigated mycoprotein fermentation (Harris *et al*., 2019). Using an *in vitro* batch culture, the fermentation of whole mycoprotein showed a total SCFAs production which was non-significantly different to the control (blank). However, isolated mycoprotein fibre led to the significant production of total SCFAs of 61 mmol/L compared to control and similarly to other fermentable fibres, and with acetate being the largest SCFAs produced. Nevertheless, some flaws have been identified from this study, such as the small sample size (n=3) used as well as the lack of pre-digested mycoprotein use, which would be a more representative substrate of *in vivo* digestion.

1.7 Thesis Hypothesis

I hypothesised that acute mycoprotein in combination with guar gum intake would have a positive effect on glycaemic control and suppress appetite in people with T2D and that these effects may be mediated by gut hormones. I hypothesised also that there may be differential

effects between ethnicities. Furthermore, I hypothesised that mycoprotein may be fermented by gut microbiota with the consequent production of SCFAs.

1.8 Thesis Aims and Rationale

The aims of this thesis are:

1) To determine the associations of mycoprotein-based foods consumers and metabolic parameters such as blood glucose, diet quality and appetite in the free-living population.
2) To determine the effect of mycoprotein intake on glycaemia and appetite in humans by performing a systematic review and meta-analysis of the available peer-reviewed evidence.
3) To determine the acute effect of mycoprotein in combination with guar gum on glycaemic control and appetite in south Asian and European people with T2D by performing a randomised controlled trial.
4) To investigate the effect of pre-digested mycoprotein on gut microbiota fermentation and SCFA production using *in vitro* batch cultures as a mechanism underpinning the potential chronic effects of mycoprotein supplementation on glycaemic and appetite outcomes in healthy humans.

The rationale behind these purposes are:

1) Mycoprotein-based foods have been consumed for decades in the UK. This is coupled with a recent increase in people adopting flexitarian trends. Mycoprotein-based foods consumed as meat replacements therefore may represent an increase in fibre intake in the diet. Fibre intake has been associated with reduced risk of mortality and CVD-related markers such as high blood glucose, and to decrease appetite. Therefore, it needs to be understood whether mycoprotein-based food consumers in the free-living population have a better blood glucose profile, reduced energy intake and healthy diets.
2) *In vitro* and mechanistic human studies suggest that mycoprotein intake has an effect on gastric emptying, digestive enzyme activity such as α-amylase and gut microbiota fermentation, all of which constitute mechanisms underpinning postprandial metabolic responses such as glycaemia and appetite. Therefore, a systematic review of the literature is needed to assess what is the pooled effect of mycoprotein on glycaemic control and appetite in humans. This will identify the pits and falls present in the current literature and subsequently rationally design research studies to address the unanswered questions on the effects of mycoprotein on glycaemia and appetite.

3) There are no human studies that investigate the effect of mycoprotein in combination with guar gum on glycaemic control and appetite in white European and south Asian people with T2D. Developing foods/ingredients that can reduce glycaemic response of carbohydrate-rich staple foods, are ethnic-specific and are acceptable to the consumer are needed. Furthermore, studying the effects of mycoprotein on south Asians with T2D is of importance as there is scarce information on the effects of food on glycaemia in this subgroup, and this suggests that they have a worse metabolic response to a same food/diet compared to European counterparts. Understanding south Asian metabolic behaviour in response to a food could help develop foods that ameliorate their glycaemic control.
4) There is only one study (Harris *et al*., 2019) that have investigated *in vitro* gut microbiota fermentation of mycoprotein, however, the sample size used was very small (n=3) and mycoprotein used was not pre-digested which would be a more realistic approach. Understanding whether pre-digested mycoprotein is fermented by gut microbiota, as well as what are the bacterial changes and the SCFAs profile from mycoprotein fermentation, could help establishing a mechanistic link between mycoprotein and its reported effects on health such as glycaemic control and appetite.

1.9 Thesis Objectives

The objectives of this thesis are:

- To determine the associations between mycoprotein-based foods consumers and non-consumers with non-communicable disease markers such as blood glucose, energy intake and diet quality in UK free-living population using the National Diet and Nutrition Survey (NDNS).
- To systematically review and meta-analyse all evidence available in peer-reviewed journals on the effects of mycoprotein on glycaemic control and appetite in humans.
- To determine the acute effect of mycoprotein and guar gum in glycaemic control and appetite in people with T2D of south Asian and European ethnicity.
- To investigate the potential of mycoprotein in being fermented by the gut microbiota from healthy donors using static *in vitro* batch cultures.

1.10 Bibliography of Chapter 1

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CHAPTER 2: The associations between being a mycoprotein-based food consumer and metabolic markers and energy intake: a cross-sectional study using the National Diet and Nutrition Survey (NDNS) years 2008/09-2016/17

2.1 Introduction

2.1.1 Non-communicable diseases and metabolic markers

Non-communicable diseases (NCDs) are diseases of long duration (chronic) which can arise from the interrelation of genetic, physiological, environmental, and behavioural factors (WHO, 2018). NCDs are the leading cause of mortality worldwide, being responsible for 7 out of 10 deaths (Center for Disease Control and Prevention). Examples of NCDs are cancers, cardiovascular disease and T2D. Metabolic changes such as raised blood pressure, blood glucose and cholesterol as well as being overweight/obese are associated with increased risk of NCDs, all of which are modifiable risk factors mainly via lifestyle interventions (Global Burden Disease, 2016). The main lifestyle modification to decrease the risk of developing NCDs is through diet. For example, dietary protein has been shown to modulate blood glucose response by stimulating insulin release via yet unclear mechanisms (Gannon *et al*., 2010), and to decrease energy intake associated to increased satiety signals (Paddon-Jones *et al*., 2008) and increased energy expenditure (Tremblay *et al*., 2007).

2.1.2 Associations between diet and metabolic markers

High-red and processed meat intake is positively associated with diabetes, cardiovascular disease, weight gain, total mortality. Inversely, the substitution of meat-protein for plant-protein was associated with lower mortality (Song *et al*., 2016, Godfray *et al*., 2018). Interestingly, the amount and amino acid have a differential effect on glucose homeostasis. For example, the branched amino acid leucine is linked with impaired peripheral glucose uptake (Tremblay *et al*., 2007), and high-protein diets are linked to increased risk of insulin resistance and insulin levels in rodent studies (Tremblay *et al*., 2007).

Currently, industrialised nations mainly rely on animal-based protein, which not only have been linked to poor health outcomes but also to high-environmental impacts through fossil fuel usage, animal methane production, effluent waste, water, and land consumption (Swinburn *et al*., 2019). Therefore, it is imperative to switch towards sustainable meat alternatives with a healthy nutrition value as well as to understand its effects on health.

On the other hand, dietary fibre intake (mainly from plant sources which have a lower environmental impact compared to meat and dairy (Davis *et al*., 2010)) has been shown to decrease blood glucose via delayed gastric emptying and gut microbiota fermentation (Gill *et al*., 2020). A systematic review and meta-analysis has shown that high-fibre diets have been linked to an improvement in glycaemia, body weight management and reduction of premature mortality (Reynolds et al., 2019 and 2020), for most types of fibres (high-fibre diets, whole grains and low glycaemic index foods) and for which such effects are greater with higher doses of fibre. Furthermore, fibre also has a positive effect at lowering total

cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), which are important factors in atherogenesis and high blood pressure, which are risk factors for NCDs. The effects of fibre on lipid profile are possibly brought about via mechanisms such as decreased insulin secretion of hepatic cholesterol synthesis, prevention of bile salt reabsorption and increased SCFAs production (Gunness *et al*., 2010). Furthermore, dietary fibre intake influences body weight possibly because its presence in food is linked to reduced energy density of foods, and delays gastric emptying, subsequently decreasing appetite, energy intake and ultimately modulating body weight (Wanders *et al*., 2011).

2.1.3 Mycoprotein and metabolic markers

Mycoprotein is a fungal-based ingredient which represents a sustainable meat alternative, with a heathy nutritional profile. The nutrition profile is characterised by being high in bothdietary fibre (66% fungal β-glucan and 33% chitin) and protein, and low in energy. The protein content of mycoprotein has been shown to be as bioavailable as whole milk, chicken and eggs, and to stimulate muscle anabolism to similarly to leucine-matched milk protein (Edwards et al., 2010, Monteyne *et al*., 2020, Dunlop *et al*., 2017).

The effects of mycoprotein on health show that it increases insulin sensitivity and decreases energy intake in healthy lean and overweight individuals compared to matched animal-derived protein (Cherta-Murillo *et al*., 2020). In addition, replacing meat for mycoprotein for 7 days has resulted in a mean reduction of blood lipid profiles such as LDL-C and TC in a free-living setting (Coelho *et al*., 2020). This effect was consistent with the evidence showed in *in vitro* and *in vivo* short-term studies (Turnbull *et al*., 1992, Colosimo *et al*., 2020a). Although the mechanisms underpinning these effects are poorly understood, it is possible that the high-fibre and protein content of mycoprotein along with its unique food structure (hyphae) may act as a mechanism underpinning the metabolic effects by acting in the upper and lower gastrointestinal tract. *In vitro* evidence suggests that the hyphal structure can trap digestive enzymes such as amylase and bile salts during digestion, consequently decreasing starch hydrolysis and lipolysis, and hindering the digestion, absorption and appearance in blood of carbohydrates and lipids (Colosimo *et al*., 2020a and b). Other mechanisms underpinning may involve gut microbiota fermentation of mycoprotein undigested parts of and SCFAs production, as observed in one *in vitro* (Harris *et al*., 2019). SCFAs have a role at modulating glucose metabolism and appetite central regulation (Byrne *et al*., 2015).

So far, the evidence available on mycoprotein comes from acute and short-term (7 days) well-controlled *in vivo* and *in vitro* studies, which do not represent a free-living setting. Furthermore, they are not representative of the mycoprotein health effects experienced by

those consumers who have a regular intake of mycoprotein. Therefore, there is a need to investigate the effect of being a regular consumer of mycoprotein-based foods on NCDs markers such as glycaemic control, energy intake, blood lipid profile, healthy diet indices and anthropometric parameters.

2.2 Purpose of the Study

2.2.1 Hypothesis

I hypothesised that being a mycoprotein-based foods consumer is associated with a better diet quality, NCDs' metabolic markers and reduced energy intake.

2.2.2 Aims

The overall aim of this exploratory study was to investigate the association between being a mycoprotein-based food consumer and diet quality, energy and nutrient intake as well as NCDs' risk factors using data from a nationally representative cohort to provide insight into the impact of mycoprotein consumption on NCDs' risk in a free-living setting. For this, a cross-sectional analysis was performed and reported in line with the STROBE-Nut checklist (Lachat *et al.,* 2016).

2.2.3 Outcomes Measures

The co-primary outcome measures are:

1. NCDs' risk markers in relation to glycaemic control (fasting glucose, HbA1c)
2. Energy intake

The co-secondary outcome measures are:

1. Other NCDs' risk markers such as BMI and blood lipids profile
2. Diet quality (Dietary approaches to stop hypertension score (DASH) and Healthy diet index (HDI))

2.3 Methods

2.3.1 The National Diet and Nutrition Survey Rolling Programme (NDNS-RP)

The National Diet and Nutrition Survey Rolling Programme (NDNS-RP) is a long-term initiative from the UK government that started in 2008, consisting of a survey scheme to assess nutrient intake and health outcomes of free-living population households in the UK (England, Scotland, Wales and Northern Ireland) that are ≥1.5 years old. The aim of the NDNS-RP is to monitor the nutritional intake of the UK population, to provide the foundation from which government policies will be based on and inform the Food Standars Agency to

inform risk assessments of chemicals in food. In these surveys, people from the UK were randomly selected and invited to complete a self-reported 4 days food diary (including 2 weekend days). Participants also had an interview about dietary habits, socio-demographic background and lifestyle every year since 2008. An example of the interview for year 5 is available from: http://doc.ukdataservice.ac.uk/doc/6533/mrdoc/pdf/6533_ndns_yr5_interviewing_documents.pdf. Some of them accepted to undergo a nurse visit to have anthropometric measurements undertaken such as blood pressure and BMI, and to provide overnight fasting blood (for healthy subjects without diabetes, otherwise non-fasting samples were obtained) and urine samples. The NDNS-RP was conducted by the NatCen Social research working with the MRC Epidemiological Unit. The Public Health England and the UK Food Standards Agency jointly fund the UK NDNS-RP.

In the present study the publicly available raw data from the databases of years 2008 to 2017 were used in this thesis to inform the present study and analysed for associations by myself Anna Cherta-Murillo.

2.3.2 Food diary

2.3.2.1 Food diary collection

Food diary data were collected from self-reported 4-day food diaries (including 2 weekend days). Participants were instructed to keep a record of their food intake both inside and outside home. More details on how the food diary data were collected available from: https://dapa-toolkit.mrc.ac.uk/instrument/242 (accessed: February 2022).

2.3.2.2 Food diary data treatment

Food diary data were coded by trained researchers and entered into the Medical Research Council human nutrition research dietary assessment system called “Diet In Nutrients Out” (MRC, 2013). Food composition from the Department of Health’s NDNS Nutrient Databank was used to calculate macro- and micronutrient intake. Composite items such as sandwiches and home-made meals were coded as individual foods. Further details on the NDNS-RP methodology are described elsewhere (Bates *et al*., 2014). In this study, the mean of the 4-consecutive days of reported dietary data were calculated and used for the analysis.

2.3.2.3 Food diary quality control

Coded food diary data underwent two quality controls by researchers independent to the coders:

1. Portion sizes were checked against the maximum and minimum portion sizes for each subsidiary food group in a random subsample (10% of total diary sample) to identify missing data fields.
2. Extreme values were checked against the food diary entries to identify extreme high or low nutrient intakes (outside the 2.5 and 97.5 percentiles for their age and sex group as based on previous NDNS data).

2.3.2.4 Mycoprotein-based foods treatment

Participants were classed as mycoprotein consumers when consuming >1% kcal of daily average energy intake from mycoprotein, while the remaining (<1% of average energy intake from mycoprotein) were classed as non-mycoprotein consumers. Other variables in relation to mycoprotein intake were created: Daily average of mycoprotein-based foods energy (kcal) and weight (g) intake. Mycoprotein-based foods labelled with the name “Quorn” included: dippers, goujons, burgers, mycoprotein and pieces, quarter pounder grilled, sausage, mini eggs, sliced meats for all years (1-9); in addition to vegetarian sausage roll, pie, bacon style strips, cottage pie for most years (1-8), as well as breadcrumbs including nuggets, stir fried, lasagne for years 1-4.

2.3.3 Participants

All participants who were adults (≥18 years old) were selected and included in the analysis. This yielded a total of N=5507 of adult participants who partook in years 1-9 of the NDNS-RP (2008-2017). The NDNS-RP was conducted according to the Declaration of Helsinki guidelines and ethical approval was obtained by the local Research Ethics Committees of the areas covered by the survey. All participants gave informed consent.

2.3.4 NCDs’ risk markers

The NCDs’ risk markers that were included for analysis were BMI, systolic and diastolic pressure (mmHg), TC (mmol/l), triacylglycerides (mmol/l), HDL-C (mmol/l), low density lipoprotein LDL-C (mmol/l), TC to HDL ratio, C-reactive protein (CRP) (mg/l), FBG (mmol/l) and HbA1c (%). Protocol for blood sample collection, processing, analysis and quality controls are described elsewhere (Huijbregts *et al*., 1997).

2.3.5 Diet quality assessment

The quality of the diet was assessed by calculating the healthy diet indicator (HDI) (Stefler *et al*., 2014) and the dietary approaches to stop hypertension (DASH) score (Sacks *et al*., 1995).

2.3.5.1 Healthy Diet Indicator

HDI was developed in 1997 based on the WHO dietary recommendations established in 1990 (i.e. maintain a healthy body weight, eat variety of foods, have a diet low in fat, saturated fats, cholesterol, sugars, alcohol and eat salt in moderation) (Dietary Guidelines for Americans, 1990) which were aimed at preventing the development of chronic diseases. HDI has been associated negatively with overall and NCDs' mortality (Berentzen *et al*., 2013). HDI is calculated by assigning a score of one if the score targets for the following 9 nutrients and foods intake a day are met. These are: saturated fatty acids (0-10% of total energy), CN-3 PUFA (1-2% of total energy), CN-6 PUFA (5-8% of total energy), trans fatty acids (<1% of total energy), protein (10-15% of total energy), mono- and disaccharides (0-10% of total energy), fruit/vegetables (>400 g), cholesterol (0-300 mg), non-starchy polysaccharides (>20 g).

2.3.5.2 Dietary Approaches to Stop Hypertension

DASH was established in 1995 by the DASH Collaborative Research Group in the USA with the initial aim to reduce blood pressure (Sacks *et al*., 1995). Adherence to a DASH diet has shown benefits at reducing cardiovascular risk, low-density lipoprotein and cholesterol levels as well as components of metabolic syndrome (Mellen *et al*., 2008). DASH score is calculated by assigning a score of one if the score targets a day for the following 9 nutrients are met. These are: total fat (27% of total energy), saturated fat (6% of total energy), protein (18% of total energy), fibre (31g), cholesterol (150 mg), calcium (1240 mg), magnesium (500 mg), potassium (4700 mg) and sodium (2400 mg). The maximum score is a 9 but DASH scores ≥ 4.5 are considered to be DASH concordant (Mellen *et al*., 2008). Cholesterol intake data were missing in the NDNS-RP database, therefore this component was omitted from its calculation.

2.3.6 Statistical analysis

Normality of the data were checked visually from the normal residual distributions Q-Q plots. If not normal, a squared root transformation of skewed variables was performed. Normally distributed data are presented as mean and standard error of the means (SE) and non-normally distributed data are presented as medians and interquartile range (IQR) for demographic variables, and as survey-weighted means and standard error of the means (SE) for nutrients, diet quality scores and NCDs' risk factors for both non- and consumers of mycoprotein-based foods. Not all participants had measures on blood, anthropometric and blood pressure parameters therefore the associations were calculated for only those

individuals with no missing data for those parameters. To assess the association between being a mycoprotein consumer and demographic variables a survey-weighted general linear model (GLM) was used (Lumley et al., 2015). The GLM was adjusted for potential co-founders such as age, sex, body mass index (BMI), ethnicity, region of residency, socio-economic and smoking status, and alcohol, energy density (kcal/g food), total energy and non-mycoprotein fibre intakes, and HDI. Three models were conducted as following:Model 1: Survey-adjusted generalised linear model with predictors: age, sex and BMI. Model 2: Survey-adjusted generalised linear model with predictors: age, sex, BMI, ethnicity, region of residency, socio-economic and smoking status. Model 3: Survey-adjusted generalised linear model with predictors: age, sex, BMI, ethnicity, region of residency, socio-economic and smoking status, alcohol, fibre, energy density and energy intake was used for all measurements.For the association with BMI, alcohol, fibre, energy intake and energy density the above adjusted model was not including the covariates BMI, alcohol, fibre, energy intake and energy density, respectively. Collinearity of variables was checked using variance inflation factor, values less than 10 were considered not colinear. Survey weight factors for individuals (wti_), nurses (wtn_) and blood measurements (wtb_) were used for each specific survey year to control for any potential selection bias in the associations with nutrient intake, diet quality and body weight (wti_ used), blood pressure (wtn_ used) and blood markers (wtb_ used). Pre-processing of the data were performed using R Studio 1.3.1056 using the tidyverse, tidyr, reshape2 and dplyr packages and statistical analysis was performed using the survey and svydiags package. A two-sided p-value of ≤0.05 was considered as statistically significant. A sensitivity analysis to compare the group with participants with full dataset and those with missing data for blood, BMI and blood pressure was done using a t-test considering unequal variance and one-way ANOVA (Appendix 0).

Missing data for blood pressure, BMI and blood analytes were omitted and only available data were included in the analysis.

2.4 Results

2.4.1 Demographic characteristics

The 3.44% of the adult cohort were identified as mycoprotein consumers, of which 28.27% are vegetarian, 0.5% are vegan and 71.73% are omnivores. On the other side, the remaining 96.56% of the adult cohort was classed as non-mycoprotein consumers, a 1.42% of these being vegetarian, 0.15% vegan and 98.41% omnivores. Mycoprotein consumers were 7 years (median) younger than non-mycoprotein consumers. There were more 24% more women than men, compared to the non-mycoprotein consumers for which the difference was 16%. The predominant ethnicity in the consumer group was white in a 95%, whereas for non-consumers it was of 92%, and most of the population was from the England region in

the consumer (67%) and non-consumer (59%) groups. Mycoprotein consumers had a 6.30-6.20% more high and lower managerial professional occupations compared to non-consumers. This group also had a 9% fewer proportion of smokers compared to the non-consumers. The survey-adjusted generalised linear model showed that mycoprotein consumption in adults was significantly associated with age ($p<0.001$) and smoking status ($p=0.02$). The demographic characteristics for both consumer groups, as well as the associations between mycoprotein consumer group and demographic characteristics are shown in Table 7.

Table 7. Characteristics of mycoprotein-based foods consumers compared to non-consumers in the UK adult population (>18 years old) based on NDNS years 2008/09-2016/17 (N=5507)

Only adults		**Mycoprotein-based foods consumers n=191**	**Non-mycoprotein consumers n=5316**	**P-value**
Quorn kcal/day	Median	127.4	-	
	IQR	151.37	-	
Quorn (g/day)	Median	112.48		
	IQR	132.88	-	
Total energy from Quorn (%)	Median	7.84	-	
	IQR	8.91	-	
Age	Median	42	49	<0.001
	IQR	20	28	
Sex (%)	Male	38	42	0.24
	Female	62	58	
Ethnicity (%)	White	95	92	0.42
	Mixed ethnic	1	0.8	
	Black or Black British	1	2.1	
	Asian or Asian British	2.6	3.4	
	Any other group	0.5	1.3	
Region (%)	England	67	59	0.18
	Scotland	12	14	
	Wales	15	14	
	Northern Ireland	3.1	5.4	
Socio-economic statues (%)	Higher managerial and professional occupations	22	15.7	0.12
	Lower managerial and professional occupations	30	23.8	
	Intermediate occupations	12	9.9	
	Small employers and own account workers	8.4	10.7	

	Lower supervisory and technical occupations	7.3	9	
	Semi-routine occupations	9.4	14.4	
	Routine occupations	6.2	12	
	Never worked	0.5	0.3	
	Others	3.7	0.04	
Smoking status (%)	Current smoker	13	22	0.02
	Ex-regular smoker	26	25	
	Never regular smoker	61	53	
IQR, interquartile range; DASH, dietary approaches to stop hypertension; HDI, healthy diet index. Survey-adjusted generalised linear model was used to investigate the association between being a mycoprotein consumer and demographic variables. No variables were used to adjust the model. P-value ≤0.05 considered to be a significant association.				

2.4.2 Nutrient intake and diet quality

The models 1-3 of the associations between being a mycoprotein consumer with nutrient intake and diet quality are shown in Table 8. In model 3, when adjusted for all the co-founders (age, sex, BMI, ethnicity, region of residence, socio economic and smoking status, alcohol, energy intake, energy density and non-mycoprotein fibre intake), mycoprotein consumers were statistical significantly associated with slightly increased food energy (kcal) (+0.22, p<0.001) and total energy (kcal) (+3.09, p<0.001), but reduced energy density (food kcal/gram) (-0.08, p<0.04). Furthermore, positive statistical significantly associations between mycoprotein consumers and specific nutrients were found for poly unsaturated fatty acids CN6 (CN6-PUFA) (+0.57%, p<0.04), CN3-PUFA (+0.10%, p<0.04), starch (+1.97%, p<0.04), fibre (g) (+3.17, p<0.001). There were statistical significantly associations between mycoprotein consumers and reduced intake of total sugars (-1.75%, p<0.001), non-milk extrinsic sugars (-3.20%, p<0.001), free sugars (-2.83%, p<0.001) and alcohol intake (g) (-1.35, p<0.001). For healthy diet scores, mycoprotein consumers were statistically significantly associated with increased HDI (+0.19, p<0.001) and DASH score (+0.26, p<0.001).

Table 8. Association between mycoprotein consumption and nutrients in UK adult population cohort from the NDNS 2008/09-2016/17 (N=5507)

	Model 1		Model 2		Model 3	
Nutrients (percentage of total energy, kcal)	**Beta**	**P-value**	**Beta**	**P-value**	**Beta**	**P-value**
Food energy (kcal)	38.38	0.46	18.61	0.72	0.22	<0.001
Energy (kcal)	25.31	0.65	-3.58	0.95	3.09	<0.001
Energy density (food kcal/food grams)	-0.04	0.02	-0.03	0.08	-0.08	0.04
Protein	-1.31	<0.001	-1.27	<0.001	-0.01	0.75
Fat	0.23	0.69	0.40	0.49	0.76	0.19
Saturated fatty acids	0.19	0.60	0.18	0.62	0.04	0.89
CMUFA	-0.34	0.18	-0.23	0.37	-0.12	0.63
CN6PUFA	0.25	0.12	0.31	0.05	0.57	<0.001
CN3PUFA	0.06	0.10	0.06	0.11	0.10	0.01
Trans fatty acids	0.03	0.27	0.03	0.27	0.04	0.35
Carbohydrate	1.73	0.01	1.96	<0.001	0.21	0.73
Total Sugars	0.24	0.64	-0.02	0.97	-1.75	<0.001
Starch	1.48	0.02	1.98	<0.001	1.97	<0.001
Non milk extrinsic sugars	-0.56	0.33	-0.66	0.23	-3.20	<0.001
Free Sugars	-0.38	0.47	-0.41	0.42	-2.83	<0.001
Alcohol (g)	-1.87	0.30	-3.17	0.04	-1.35	<0.001
Fibre (g)	5.41	<0.001	4.96	<0.001	3.17	<0.001
HDI	0.57	<0.001	0.57	<0.001	0.19	<0.001
DASH score	0.42	<0.001	0.38	<0.001	0.26	<0.001

Model 1: Survey-adjusted generalised linear model with predictors: age, sex and BMI.
Model 2: Survey-adjusted generalised linear model with predictors: age, sex, BMI, ethnicity, region of residency, socio-economic and smoking status.
Model 3: Survey-adjusted generalised linear model with predictors: age, sex, BMI, ethnicity, region of residency, socio-economic and smoking status, alcohol, fibre, energy density and energy intake was used for all measurements except for energy density (where energy density as a predictor was omitted), energy (where energy intake as a predictor was omitted), alcohol (where alcohol as a predictor was omitted) and fibre (where fibre as a predictor was omitted).
P-value <0.05 was considered to be a statistically significant association. CMUFA, monounsaturated fatty acids; CN6PUFA, CN6 polyunsaturated fatty acids; CN3PUFA, CN3 polyunsaturated fatty acids; HDI, healthy diet indicator; DASH, dietary approaches to stop hypertension score; SE, standard error of the mean.

2.4.3 NCDs' risk markers

The models 1-3 of the associations between being a mycoprotein consumer with NCDs's risk markers are shown in Table 9. In model 3 -adjusted for all the co-founders (age, sex, BMI, ethnicity, region of residence, socio economic and smoking status, alcohol, energy intake, energy density and non-mycoprotein fibre intake)-, the results showed that being a mycoprotein consumer was almost significantly associated with a lower BMI (-0.80 kg/m^2, p=0.051). Mycoprotein consumers were statistical significantly associated with lower FBG (-0.31 mmol/L, p<0.01) and HbA1c (-0.06%, p=0.01). There was no statistically significant association between being a mycoprotein consumer and the rest of NCDs' markers.

Table 9. Association between mycoprotein consumption and NCDs' risk markers NCDs' risk markers						
	Model 1		Model 2		Model 3	
NCDs' risk marker	**Beta**	**P-value**	**Beta**	**P-value**	**Beta**	**P-value**
BMI (kg/m^2)	-0.92	0.02	-0.87	0.051	-0.80	0.051
SBP (mmHg)	-0.52	0.77	-0.39	0.82	-0.36	0.84
DBP (mmHg)	-0.48	0.70	-0.13	0.91	-0.16	0.89
TC (mmol/L)	0.11	0.33	0.11	0.37	0.09	0.48
TAG (mmol/L)	0.05	0.53	0.05	0.51	0.01	0.94
HDL-C (mmol/L)	0.03	0.43	0.02	0.59	0.02	0.72
LDL-C (mmol/L)	0.06	0.58	0.06	0.57	0.06	0.61
TC:HDL-C	-0.06	0.68	-0.02	0.90	-0.04	0.76
CRP (mg/L)	-0.15	0.78	0.13	0.87	-0.05	0.95
Fasting blood glucose (mmol/L)	-0.31	<0.001	-0.32	<0.001	-0.31	<0.001
HbA1c (%)	-0.16	<0.001	-0.14	<0.001	-0.15	0.01

Model 1: Survey-adjusted generalised linear model with predictors: age, sex and BMI.
Model 2: Survey-adjusted generalised linear model with predictors: age, sex, BMI, ethnicity, region of residency, socio-economic and smoking status.
Model 3: Survey-adjusted generalised linear model with predictors: age, sex, BMI, ethnicity, region of residency, socio-economic and smoking status, alcohol, fibre, energy density and energy intake.
Due to missing values for anthropometric and blood sample values, the sample sizes for each measurement respectively are 176, 119, 119, 94, 94, 94, 94, 94, 95, 83, 90 for consumers and 5069, 3446, 3446, 2843, 2835, 2843, 2812, 2843, 2843, 2716, 2754 for non-consumers.
For BMI, BMI as a predictor was omitted in all models. P-value <0.05 was considered to be a statistically significant association. BMI, body mass index; SBP, systolic blood pressure, DBP, diastolic blood pressure; TC, total cholesterol; TAG, triacylglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; CRP, C-reactive protein; HbA1c, glycated haemoglobin A1c; SE, standard error of the mean.

2.5 Discussion

In the present study it has been investigated for the first time the association of being a mycoprotein-based food consumer with demographics, nutritional profiles as well as with NCDs's risk markers, showing that being a mycoprotein consumer was significantly positively associated with HDI, DASH score, fibre, PUFA, total and food energy intake, but negatively associated with energy density, sugars, and alcohol intake, as well as lower FBG and HbA1c. It was showed that those who were classed as mycoprotein consumer were the 3.44% of the surveyed adult participants and had an average of 147 g/day of mycoprotein, equivalent to 2 servings of mycoprotein. This represented an average of 7.84% of their total energy intake.

2.5.1 Associations between being a mycoprotein consumer and nutritional profile and diet quality

Being a mycoprotein consumer was associated negatively with energy density intake. This is in disagreement with a macronutrient self-selection paradigm study showing that overweight pre-menopausal females increased their intake for high energy density foods such as high

fat/high sugar and low fat/high sugar foods, following acute mycoprotein intake, compared to tofu and similarly to chicken (Williamson *et al*., 2006). However, the conditions of these studies are not comparable since the acute interventional study was performed in a controlled settings such as a metabolic chamber and this may have influenced the participant's choices would have had in a free-living setting. Mycoprotein consumers obtained on average 22% more dietary fibre than the non-consumer group, which is a macronutrient with increased effects on satiation. However, it is possible that overall, mycoprotein-consumers did not consume enough amounts of satiating macronutrients (such as protein), explaining their increased total energy intake. The positive association of being a mycoprotein consumer with energy intake are unexpected since mycoprotein is rich in two macronutrients (dietary protein and fibre) which are satiating (Williamson *et al*., 2006, Bottin *et al*., 2016, Swindell *et al*., 2018). Clinical acute studies have consistently shown an effect between mycoprotein intake and decreases in appetite subjective feelings, energy intake post-24 hours and at *ad libitum* (Bottin *et al*., 2016, Swindell *et al*., 2018). A high energy balance (measured as BMI), determined by a higher energy intake relative to energy expenditure, is an important determinant of NCDs' risk. In this regard, it was showed that BMI was almost significantly negatively associated with this consumer group, suggesting that the mycoprotein group may be more physically active, despite an association with higher energy intake. However, levels of physical activity were not possible to assess as a standard measurement for such this parameter was missing for some survey years.

Being a mycoprotein consumer was positively associated with HDI and DASH score, suggesting that mycoprotein consumers in general have a healthier choice of foods, including foods and nutrients aimed at lowering hypertension, which is a NCD's risk marker. In particular, the nutrient intake mycoprotein consumers were associated with were with higher starch. In this database it was not possible to distinguish between resistant starch and non-resistant starch. Resistant starch constitutes the fraction of starch that resists digestion, and it is a source of dietary fibre. Resistant starch intake is positively linked to improved NCDs' risk factors such as glycaemia, insulinaemia, bowel health, blood lipid profile, and increased satiety and reduced energy intake (Nugent *et al*., 2005). In addition, mycoprotein consumers were associated with were with higher CN3- and CN6-PUFA intake. PUFA intake has been consistently shown to have favourable effects in glycaemic control, insulin resistance and insulin secretion (Imamura *et al*., 2016), and to be linked to amelioration of cardiometabolic health (Monnard *et al*., 2021).

Being a mycoprotein consumer was significantly associated with a 3.71g increase in fibre intake and it was estimated that mycoprotein intake contributed to the 29% of the total dietary fibre intake. Considering the daily recommended intake for dietary fibre of 30g/day,

mycoprotein consumers were estimated to eat 23 g of fibre/day on average, being closer to the Scientific Advisory Committee on Nutrition recommendations (Janelle *et al*., 1995), compared to the non-consumers who had an average fibre intake of 17 g/day. This suggests that adding mycoprotein into one's diet could possibly increase one's dietary fibre intake to aid meeting the current dietary fibre intake daily recommendations.

2.5.2 Associations between being a mycoprotein consumer and NCDs's risk factors

A recent systematic review and meta-analysis of epidemiological studies and RCTs with people with T2D showed that dietary fibre intake improved glycaemic control, blood lipids, body weight, and inflammation, as well as a reduction in premature mortality, which are all linked to NCD's risk prevention (Reynolds *et al*., 2020). In line with this, in the present study it is shown how mycoprotein consumers were associated with lower FBG. However, experts question the validity of FBG as a clinical marker for NCDs' risks (Cavalot *et al*., 2006). Instead, other glycaemic measures such as oral glucose tolerance test or Hb1Ac have been shown to be better at predicting NCDs' risk (Hill *et al*., 1986). In the NDNS database, oral glucose tolerance test data were not available, instead HbA1c was, and it was shown to be strongly negatively associated with being a mycoprotein consumer, when adjusted for all cofounders, including healthy diet. This is in line with acute interventions feeding mycoprotein in healthy humans that have shown that while there is not a significant effect on postprandial blood glucose following mycoprotein intake, a reduced postprandial insulin is evident compared to macronutrient matched-chicken or soy (Bottin *et al*., 2016, Turnbull *et al*., 1995). Furthermore, analysis have shown a significant increase in insulin sensitivity following doses ranging 44-132 g of mince mycoprotein in overweight and obese humans (Bottin *et al*., 2016). However, these effects were not observed following a 7-day chronic administration of mycoprotein where meat and fish was replaced for mycoprotein in healthy participants (Coelho *et al*., 2020). Despite the associations of being a mycoprotein food consumer with lower FBG and HbA1c and a tendency towards a lower BMI, there was no association with lower levels of triacyl glycerides or LDL-C as it could be expected given the healthy profile of mycoprotein consumers. This lack of significance could be due to the small sample size for these parameters coupled with the variance of these blood markers. Although, the current evidence from human trials is small and heterogenous, it is suspected that the mechanisms behind the potential effects of mycoprotein on blood glucose are its fibre content and food structure. Fibre intake is related to the second meal effect in which postprandial blood glucose responses following posterior meal intake are blunted due to the fibre intake (Thornburn et al., 1993). Furthermore, *in vitro* studies have shown that mycoprotein hyphal food structure inhibited starch hydrolysis (Colosimo et al., 2020). Moreover, *in vitro* studies using fluorescent microscopy, suggest that the mycoprotein

physically entraps the amylase enzyme within the hyphal structure, suggesting that during digestion mycoprotein may delay carbohydrate digestion and absorption, reducing postprandial blood glucose responses (Colosimo *et al*., 2020).

Mycoprotein-based foods are an appealing alternative to help swapping animal-based to plant-based protein since qualities such as meat-alike taste and texture as well as protein bioavailability are not compromised. Furthermore, as previously shown, being a mycoprotein consumer is positively associated with higher diet quality scores, and lower NCD's risk factors whichcould be possibly related to a healthy consumer bias. This was confirmed when the demographic profile of mycoprotein consumers was explored, in which there were more likely to be women, white, younger, living in England, belonging to a higher socio-economic status, have greater dietary quality scores and less likely to be current smokers relative to non-consumers. In this study there was an attempt to control for factors which are determinant of a healthy lifestyle, nevertheless, it is possible that other unknown confounders were not controlled for as well as the physical activity factor which was not available.Since most of the mycoprotein group was mainly representative of white, young, women with high professional occupations, there was a small representation of other subgroups which may be at greater risk of NCDs (e.g. males, older individuals, Black, Asian or minority ethnicities). Considering the positive associations between mycoprotein intake and NCDs' risk markers, this underrepresented subgroup could benefit from including mycoprotein-based foods into their diets. The reasons behind the smaller representation of other demographic subgroups within the mycoprotein consumer group should be explored, although factors such as economic power, and education could be involved. It has been reported that the differences in diet quality in the different socioeconomical classes is related to a higher cost of healthy diets (Darmon *et al*., 2008). Non-white ethnicities and lower socioeconomical classes spend on average less pence per person per week (ppw) in meat and vegetables compared to white and higher socioeconomical statuses (Family Food datasets 2017-2019, UK Government). In general, mycoprotein-based products have matching costs (£5.67-£6.67/kg) to their animal-derived protein equivalents (£3.30-£8/kg) (Sainsbury's website). 7 portions a week (75 g each a day) of mycoprotein-based foods would approximately cost 297 pence per week (ppw), which is within the average meat expenditure of non-white communities (299-303 ppw). This suggests that the cost of mycoprotein products in the UK may not be the reason why non-white ethnicities are not consuming mycoprotein-based foods in comparison to white communities. Therefore, understanding their lack of mycoprotein-based foods consumption and finding ways to promote mycoprotein-based food within non-white, men and older demographic subgroups

may potentially promote healthy dietary intakes and decrease NCDs' risk at a population level.

2.6 Limitations

The epidemiological study is a cross-sectional study, meaning that data are studied at a specific timepoint. This limits the ability to infer cause-and-effect relationships. For this reason, longitudinal studies, which include measurements of the same individual over time, are needed to capture the real effect of chronic exposure to mycoprotein-based foods in each individual. Furthermore, NDNS is a survey-database in which participants self-reported their dietary intake for 4 days. Despite, the food was weighed, participants were not trained to do such task and self-reported energy intake is regarded by the scientific community as a high-risk biased methodology to report (Lichtman *et al.*,1992, Schoeller *et al.*, 1990 and 1995). Instead, objective food intake measures should be employed but at the time of writing this thesis, no objective method has been yet developed and validated against the gold standard.

In this study, it was not possible to adjust for physical activity levels because the NDNS database lacked a standard measurement for it across the survey years. Physical activity has been linked to glycaemic control, metabolic parameters and energy intake in a wide range of studies (Frampton *et al.*, 2021). Using other databases such as the AIRWAVE or NHANES database with a standard measure for physical activity was not possible because they did not capture specifically Quorn branded products. Importantly, the NDNS database was not complete as some blood markers were missing in almost 50% of the participants for both consumer and non-consumer group. This is an inevitable and intrinsic consequence of the nature of mass-databases, and it may have been due to either a refusal by the participants to have the nurse visit or due to issues during the laboratory analysis. To assess whether the missing data may have led to biased results, a post-hoc analysis was performed and showed some statistically significant differences in nutrient intakes between participants with and without missing data. Therefore, it is possible that should the full dataset for all parameters had been available, the real associations of being a mycoprotein-based food consumer on blood parameters and blood pressure may differ from the results obtained in this study. A way to address the missing data issue would have been using multiple imputation. However, this option was discarded as imputing data for such high number of missing participants from the available participants' data may have led to a decreased group variance, increasing the chances of detecting statistical differences between groups, this leading to biased results.

Furthermore, the demographic profile observed for mycoprotein consumers was more likely to be women, white, younger, living in England, belong to a higher socio-economic status, have greater dietary quality scores and less likely to be current smokers relative to non-consumers. This may have led to a demographic unrepresentativeness of other population groups such as men, black, and of lower socio-economic status which may have represented a limitation. Therefore, care should be taken when extrapolating these results in the real-world population.

2.7 Conclusion

In conclusion, mycoprotein consumers (3.44% of the sample population) were likely to be women, white, younger, living in England, belong to a higher socio-economic status, and less likely to be current smokers relative to non-consumers. Higher HDI, DASH score, fibre, total and food energy intake, but lower energy density, sugars, alcohol intake, as well as FBG and HbA1c was associated with being a mycoprotein consumer. This suggests that mycoprotein-based foods may have a positive effect at reducing NCDs's risk, in particular, those related with glycaemia. It would be interesting to find ways to promote the consumption of mycoprotein-based foods consumption within demographic groups at risk of NCDs's such as people T2D or of minority ethnicities such as Black African or south Asian. Further cross-sectional studies using other databases in other countries are encouraged to contrast these findings. Furthermore, longitudinal studies are also needed to understand the impact of mycoprotein-based foods in the long-term NCDs's risk of people over time.

2.8 Bibliography of Chapter 2

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CHAPTER 3: The effects of mycoprotein on glycaemic control and appetite in humans: A Systematic Review

3.1 Introduction

Non-communicable diseases (NCD) are responsible for 41 million deaths annually, which represents the 71% of the global deaths (WHO, 2018). Two of the most important metabolic risk factors for NCD are elevated blood glucose (hyperglycaemia) and being overweight or obese (Lim *et al*., 2012). According to WHO, hyperglycaemia is defined as blood glucose levels ≥7.8 mmol/L (considered as impaired glucose tolerance) or ≥11.1 mmol/L (considered as diabetes) 180 min after the consumption of a 75 g glucose load (WHO, 2006). Whereas on the other hand, being overweight or obese results from a positive energy balance where energy intake exceeds energy expenditure and it is diagnosed using body mass index (BMI, kg/m^2) of which values ≥25-29.9 or ≥30 kg/m^2 are categorised as "overweight" or "obese" under white European standards- respectively (Bray *et al*., 1978). The cause of the appearance for hyperglycaemia and BMI >25 is multi-factorial, with lifestyle (e.g. diet and exercise) being a significant contributing factor to both (Murea *et al*., 2012).

These two risk factors are modifiable and in particular dietary interventions are a cornerstone strategy for the prevention and management of both hyperglycaemia and increased body weight (Ajala *et al*., 2013). As explained in Chapter 1, high-fibre diets have been shown to improve glycaemic control and body weight for many types of fibre and diabetes, and that moving from a low to moderate or high intake had greater improvements (Reynolds et al., 2019). Evidence has shown that dietary fibre modulates glucose and energy homeostasis via a myriad of mechanisms. In terms of glucose homeostasis, soluble fibres can delay gastric emptying, glucose absorption in the small intestine and starch degradation as well as can stimulate the secretion of hormones such as glucagon-like peptide 1 (GLP-1) which act as an incretin (insulin-secreting hormones) and promotes pancreatic β-cell growth (Chutkan *et al*., 2012). On the other hand, the other type of fibres, the insoluble fibres modulate glycaemia by interacting with host digestive enzymes to attenuate digestible polysaccharide hydrolysis and consequently dampen the glycaemic response (Dhital *et al*., 2015). Furthermore, dietary fibres can be fermented by the gut microbiota when short chain fatty acids (SCFAs) namely acetate, butyrate, and propionate (Tarini *et al*., 2010) are produced as by-products. These can stimulate incretin secretion by the enteroendocrine L-cells (Wong *et al*., 2006) or affect directly metabolically active tissues or tissues involved in insulin secretion such as pancreatic beta-cells (Pingitore et al., 2017).

In relation to energy homeostasis, dietary fibre has been shown to reduce energy intake via increasing satiety feelings. This has been achieved via various mechanisms (Wanders *et al*., 2011) such as increased bulk and viscosity (Slavin *et al*., 2007), increased gastric distension (De Graaf *et al*., 2004) and decreased gastric emptying (Bergmann *et al*., 1992), stimulation of the release of the anorexigenic hormones GLP-1 and peptide tyrosine tyrosine (PYY) following fibre ingestion (e.g. via gut microbiota-derived SCFA production (Byrne *et al*., 2017)). Overall, these processes result in a decrease in appetite and energy intake which can ultimately influence body weight loss.

Evidence has shown that dietary protein modulates glucose homeostasis (Nuttal *et al*., 1984, Tremblay et al., 2007), and that short-term studies feeding dietary proteins showed that these stimulate insulin secretion in both healthy and diabetic humans. The mechanisms by which protein modulates glycaemia not yet fully defined but may involve effects on incretin secretion [e.g. GLP-1 and gastric inhibitory peptide (GIP) (Ma *et al*., 2009)], beta-cell insulin secretagogue effects of amino acids (Fajans et al., 1967), effects on glucagon secretion, or the modulation of insulin receptor mRNA expression (Tremblay et al., 2007). Moreover, dietary protein can increase satiety and suppress energy intake, possibly via its effect on gluconeogenesis (Mithieux *et al*., 2005), diet-induced thermogenesis (Rampone *et al*., 1991) and/or release of anorexigenic hormones such as PYY from the gastrointestinal tract (Belza *et al*., 2013, Batterham et al., 2006). Therefore, foods containing high amounts of both dietary fibre and protein represent an interesting dietary strategy with the potential to modulate blood glucose levels, appetite and consequently energy intake to a greater extent than foods that are either high in fibre or protein.

As introduced in Chapter 1, mycoprotein is a food ingredient high in both dietary fibre (6g/100g wet weight - composed of 1/3 chitin and 2/3 β-glucan) and non-animal derived protein (11g/100g wet weight). Mycoprotein has a unique food structure consisting of hyphae which are essentially fungal filaments made of fungal beta-glucan and chitin surrounding the intracellular contents. Food structure plays a key role in the digestibility of food and the delivery of nutrients. For example, for carrot, an intact cell wall and chromoplast are barriers to carotenoid bioaccessibility during *in vitro* and *in vivo* digestion, and that cell wall rupture before digestion modulates carotene bioaccessibility in the upper gut (Ogawa *et al*., 2018). Another example is observed with whole natural and roasted, and chopped almonds led to less metabolizable energy than almond butter (Gebauer *et al*., 2016). The modulation of

release of nutrients affects host physiology and postprandial responses. This was shown in a recent meta-analysis showed how food structure factors such as high amylose:amylopectin ratio, less gelatinated starch, retrograded starch, and larger particle size had a significant effect on reducing blood glucose levels since they affect the digestibility of the carbohydrate content of foods (Cai et al., 2021).

Of importance, mycoprotein has a wide reach in the population as it is commercially available in 17 countries across the globe and it is of increasing popularity within the vegetarian collectives as a meat-replacement (Finnigan *et al*., 2017). For this reason, mycoprotein represents a significant source of dietary fibre and non-animal derived protein for numerous people worldwide. As such, and as mycoprotein consumption continues to increase, it is of imperative to understand its role in health outcomes such as glycaemia and energy intake which are risk factors for NCD's that are responsible for such elevated number of deaths. Randomised control trials (RCTs) investigating the effects of mycoprotein on blood glucose and energy intake in humans have been conducted since mycoprotein was first sold in the market under the name of Quorn. However, to the best of our knowledge, such evidence has not been systematically reviewed, appraised and synthesised to provide a consensus on the role of mycoprotein in glycaemic control and energy intake modulation.

3.2 Purpose of the Study

3.2.1 Hypothesis

I hypothesised that mycoprotein intake has a positive effect on glycaemic control and decreases energy intake in humans.

3.2.2 Aims

The overall aim of this exploratory study was to systematically review the RCTs investigating the effects of mycoprotein on glycaemic control and energy intake in humans as well as to explore the mechanisms underpinning these effects.

3.2.3 Outcomes Measures

The co-primary outcome measures are:

1. Postprandial blood glucose (area under the curve)
2. Postprandial insulin (area under the curve)

The co-secondary outcome measures are:

1. Subjective appetite feelings
2. Energy intake

3.3 Methods

A systematic review was conducted including only peer-reviewed papers published up to the 3rd of November 2019. The systematic review was conducted, in adherence to the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines (Moher *et al*., 2009). The protocol for this review has been registered in PROSPERO 2018 (CRD42018114566) (Supplementary Table S1) and is available from: https://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=114566.

3.3.1 Eligibility Criteria

Table 10 describes the PICOS (patients, intervention, comparator, outcome, study design) criteria used to establish study eligibility and focus the research question.

Table 10. PICOS criteria for inclusion and exclusion of studies.

Criteria	**Inclusion**	**Exclusion**
Participants	Humans	Animals
Intervention	Studies assessing mycoprotein intake in any form.	Not mycoprotein or mycoprotein mixed with other sources of fibre.
Comparator		No comparators were excluded
Outcome	Studies that contain a quantifiable measure of glucose, insulin appetite and/or energy intake.	Studies, which do not contain a quantifiable measure of the outcomes of interest.
Study Design	Study designs which generate empirical data from RCTs. Only results analysed statistically will be included.	Reviews, conference abstracts, dissertation abstracts, lectures, information pieces, and *corrigendums* were not included. Studies were limited to English language up to 3rd of November 2019.

3.3.2 Search Strategy

The search strategy was performed in the following databases: PubMed, EMBASE, Web of Science and Google Scholar database. The search strategy for Pubmed included the following key search terms: *(mycoprotein[All Fields] OR myco-protein[All Fields] OR fungi-derived protein[All Fields] OR quorn[All Fields]) AND (glucose[MeSH Terms] OR A1c[All Fields] OR glycated-haemoglobin[All Fields] OR hba1c [All Fields] OR haemoglobin a [All Fields] OR dysglycemia [All Fields] OR dysglycaemia [All Fields] OR glycaemia[All Fields] OR glycemia [All Fields] OR glycaemic[All Fields] OR glycemic[All Fields] OR insulin*[All Fields] OR insulin [All Fields] OR fasting [All Fields] OR postprandial [All Fields] OR homeostatic model assessment [All Fields] OR oral glucose tolerance test [All Fields] OR oral glucose tolerance test [All Fields] OR appetite [All Fields] OR energy intake [All Fields] OR food intake [All Fields] OR food behaviour [All Fields] OR food behavior [All Fields] OR eating behaviour [All Fields] OR eating behavior [All Fields] OR hunger [All Fields] OR food consumption [All Fields]).* Other search strategies are found in the Appendix 1.

In addition, a manual search of papers and previous reviews was performed, to identify additional relevant articles. The search was conducted within the English language and the cut-off date was of 3rd November 2019.

3.3.3 Study Selection

The title and abstract of the articles identified by the search strategy were screened by 2 reviewers. Those eligible according to PICOS, underwent a post-screening, where full text was retrieved, and study eligibility assessed against PICOS criteria. All unclear eligibility of papers was resolved by consulting a third party. Duplicates were removed.

3.3.4 Data Extraction and Management

Eligible articles were assigned to subject categories (glycaemic control or energy intake/appetite), depending on the outcome of the study and the following data were extracted from the papers and summarised in tables for each outcome.

- Reference - Describes the main author and year of publication of the study.
- Participants – Describes the characteristics of the population (sample size (N), gender, age, BMI, healthy and smoker status).
- Study Information – Describes the study design (randomised, controlled, blinded, crossover), the test food (form, quantity of mycoprotein, control type and amount of carbohydrate given with the meal) and the protocol of the study.

- Outcome – Describes the result of the outcome of interest in the study. For energy intake and appetite, the methods of assessment are described in each.

3.3.5 Risk-of-Bias Assessment

Studies were assessed for risk of bias by 2 independent reviewers following the Cochrane Risk of Bias Assessment Tool. Studies were assessed for risk of bias depending on their subject category. The 5 methodological features assessed were selection, performance, detection, attrition and reporting bias. Studies were classified as “high risk” if they contained methodological flaws that may have influenced the results, “low risk” if the flaw was not deemed to have affected the results and “unclear risk” if not enough information was provided to perform a judgement. Disagreements in the classification were resolved by consulting a third party.

3.3.6 Data Analysis

Demographic data and outcome values were reported as mean ± standard deviation (SD). Glycaemic control was defined as blood glucose and insulin area under the curve (AUC) or incremental AUC (iAUC). In addition, for 1 study (Dunlop *et al*., 2017), mean glucose levels (mmol/L) were reported at fasting (0 hours), early (0-2 hours) and late (2-4 hours) postprandial stages. Energy intake was defined as acute energy intake (≤5h post-ingestion) which included *ad libitum* meal and as short-term energy intake (>5h post-ingestion) which included food diaries. Energy intake at *ad libitum* was assessed via buffet meal, macronutrient self-selection paradigm (MSSP) and universal eating monitor (UEM) and energy intake post-visit was assessed using self-reported food diaries. Appetite was measured via subjective appetite feelings scoring (mm) using a visual analogue scale (VAS). When possible, all outcomes were calculated as the percentage of change compared to control.

A statistical meta-analysis of the results was not possible due to the heterogeneity in the reported glycaemic outcomes and the insufficient power of studies assessing the energy intake outcomes measurement. Therefore, for all outcomes, a systematic, narrative approach was conducted according to the Cochrane handbook for systematic review of interventions (Higgins and Green, 2008). Results were synthesised according to outcome measure (glycaemic control or appetite/energy intake).

3.3 Results

3.3.1 Overview of Studies Identified

The search strategy yielded a total of 23 potentially relevant articles. Of which, 11 duplicates were identified and removed. Of the remaining 12 potentially relevant articles, 6 were excluded post-abstract-screening as they were a review (n=1), an EFSA report (n=1), a conference paper (n=2), an *in vitro* study (n=1), and had an irrelevant outcome measure (n=1). A total of 6 potentially relevant full-text articles were therefore assessed for eligibility, during which 1 was excluded for not meeting the PICOS criteria on study design as it was not a RCT. A total of 5 articles were eligible for inclusion and therefore included in the final synthesis (See Figure 8). All studies were acute interventions (up to 24 hours).

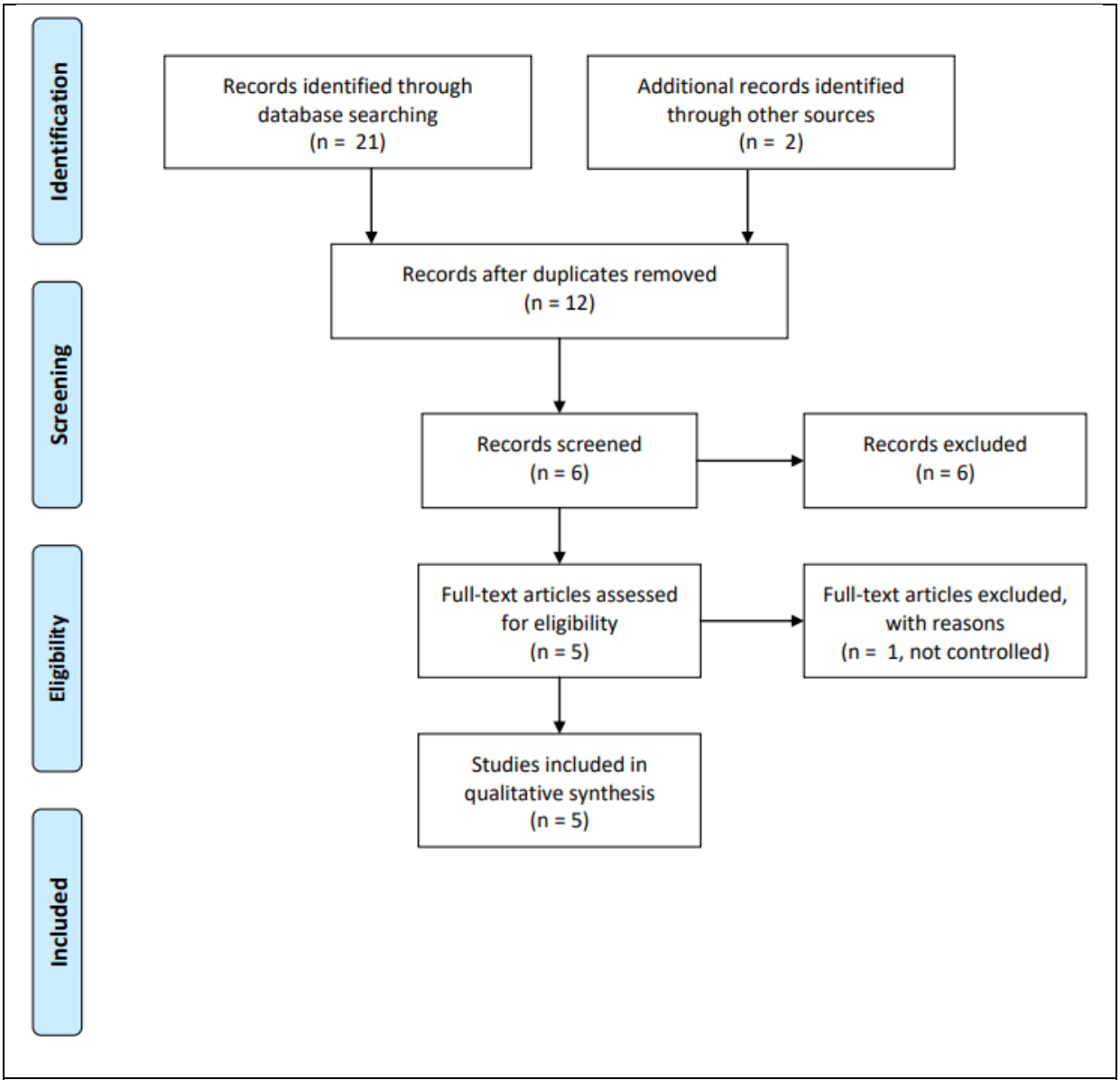

Figure 8. PRISMA Flow Diagram of references identified and evaluated. PRISMA, preferred reporting items for systematic reviews and meta-analyses.

3.3.2 Risk-of-bias

Individual studies were analysed for risk of bias and classed as high, low, or unclear risk of bias for the following 5 methodological features depending on the subject category (See Table 11).

Table 11. Risk of bias. Quality assessment of the included studies.

Study	Random sequence generation	Allocation concealment	Method of blinding	Blinding of outcome assess-ment	Handling of drop-outs	Selective reporting
Type of bias	Selection	Selection	Performance	Detection	Attrition	Reporting
Glycaemic control						
Turnbull (1995)	Unclear	Unclear	Low	Unclear	High	Unclear
Bottin (2016)	Low	Unclear	Low	Unclear	High	Unclear
Dunlop (2017)	Unclear	Unclear	Low	Unclear	High	Unclear
Energy intake/Appetite						
Turnbull (1993)	Unclear	Unclear	High	Low	High	Unclear
Williamson (2006)	Unclear	Unclear	Unclear	High	High	Unclear
Bottin (2016)	Low	Unclear	Low	Unclear	High	Unclear
Dunlop (2017)	Unclear	Unclear	Low	Unclear	High	Unclear

For studies assessing energy intake/appetite, all studies (75%) were judged as having an unclear risk for selection bias and 25% of studies were judged as having a low risk of selection bias (random sequence generation). 100% of the studies had an unclear risk of bias for allocation concealment (selection bias) and selective reporting (reporting bias). 100% of the studies had a high risk of attrition bias (handling of drop-outs). For performance bias (method of blinding), a low (50%), high (25%) and unclear (25%) risk was given, whereas for detection bias (blinding of outcome assessment), an unclear (50%), high (25%) and low (25%) risk was given.

For studies assessing glycaemic control, for the methodological features of random sequence generation (selection bias), the majority of the studies (66%) were judged as having an unclear risk of bias whereas the remaining 33% was given a low risk of bias. For the allocation concealment (selection bias), all studies (100%) had an unclear risk of bias. For the method of blinding (performance bias), 100% of the studies had a low risk of bias. For the blinding of the outcome assessment (detection bias), 100% of the studies had an unclear risk of bias. For the attrition bias, all studies (100%) had a high risk of bias. Selective reporting bias was unclear for all studies (100%).

3.3.3 Studies on Glycaemic Control

Table 12 shows the effect of mycoprotein on glycaemic control. In total, 3 randomised controlled and crossover trials assessed glycaemic control. 2 studies (Bottin *et al*., 2016, Dunlop *et al*., 2017) were conducted in healthy [no chronic disease such as type 2 diabetes (T2D)] overweight (BMI, 25-29.9 kg/m^2) and obese (BMI ≥30 kg/m^2) adults and 1 study (Turnbull *et al*., 1995) with healthy lean (BMI, 18.5-24.9 kg/m^2) adults. 2 studies were conducted using mycoprotein as liquid (Turnbull *et al*., 1995, Dunlop *et al*., 2017) whereas 1 used mycoprotein as solid (Bottin *et al*., 2016). The amounts of mycoprotein ranged from 20 g to 132 g. The controls used were chicken (Bottin *et al*., 2016), soy-based protein (Turnbull *et al*., 1995) and milk protein (Dunlop *et al*., 2017) drink. All 3 studies assessed insulin AUC and 2 studies assessed glucose AUC (Turnbull *et al*., 1995, Bottin *et al*., 2016). 1 study assessed mean glucose levels at fasting, early (0-2 hours) and late (2-4 hours) postprandial state (Dunlop *et al*., 2017).

Table 12. Summary of the acute randomised trials on glycaemic control.						
Refs.	**Participants**		**Study information**			**Outcome**
	N (M/F)	**Characteristics (Age / BMI / health and smoking status)**	**Study design**	**Test Food (Form / Quantity of mycoprotein / Control type / CHO)**	**Protocol**	**Glycaemic and Insulinaemic response**
Turnbull *et al*., 1995	19 (4/15)	22.8 ± 3.55 y, 22.74 ± 2.59 kg/m^2, healthy, not reported	RCT, single-blinded, cross-over	Drink / 20g dry weight / energy and macronutrient-matched (except fibre) soy-based protein drink / 75g CHO	Participant fasted overnight. Blood sampling at fasting, 30, 60, 120 min relative to test food.	**Glucose AUC$_{0\text{-}60\ min}$**: 9% decrease with mycoprotein (p<0.03). **Glucose AUC$_{0\text{-}120\ min}$**: NSSD. **Insulin AUC$_{0\text{-}60\ min}$**: 20% decrease with mycoprotein (p<0.01). **Insulin AUC$_{0\text{-}120\ min}$**: 13% decrease with mycoprotein (p<0.05).
Bottin *et al*., 2016	36 (19/17)	33 ± 14 y, 28.1 ± 2.3 kg/m^2, healthy, non-smokers	RCT, single-blinded, cross-over	Solid / 44g, 88g, 132g wet weight / energy and macronutrient-matched (except fibre) chicken (22,44,66g) / 25-30g CHO	Participant had a standard evening meal, 12h overnight fast, asked to refrain from alcohol and avoid any strenous exercise 24h prior study visit. Test meal followed by an *ad libitum* meal at 180 min. Blood sampling at -15, 0, 15, 30, 45, 60, 90, 120, 150, 180 min relative to test food.	**Glucose iAUC$_{0\text{-}180\ min}$:** NSSD with 44, 88 and 132g of mycoprotein *vs.* matched controls. **Insulin iAUC$_{0\text{-}180\ min}$:** 9% (p<0.01), 12% (p<0.01) and 21% (p<0.01) decrease with 44, 88 and 132g of mycoprotein, respectively, *vs.* matched controls.
Dunlop *et al*., 2017	12 (12/0)	28 ± 6.92 y, 26 ± 3.46 kg/m^2, healthy,	RCT, single-blinded,	Drink / 20g (mass-matched), 40g (protein-matched), 60g	Participant fasted. Abstained from physical activity and	**Mean Glucose (mmol/L) (0-120 min):** NSSD with any test drink *vs.* fasting levels. **Mean Glucose (mmol/L) (120-240 min):** 5% decrease with 20g

		non-smokers	cross-over	and 80g wet weight / Not energy or other macronutrient-matched milk-protein (20g) / protein:CHO ratio of 23 (in 20g of milk) and of 4.5 (in 20, 40, 60 and 80g of mycoprotein).	alcohol 48h prior to the study visit. Blood sampling at -5, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240 min relative to test food.	of milk and 4% reduction with 20g and 40g of mycoprotein *vs.* fasting levels ($p<0.05$). 2% decrease with 20g of milk, 20g, 40g, and 60g of mycoprotein *vs.* glucose (0-120 min) ($p<0.05$). **Insulin $AUC_{0\text{-}240\ min}$:** 53% decrease with 20g of mycoprotein *vs.* mass-matched milk (20g) ($p<0.05$). NSSD with 40g of mycoprotein *vs.* protein-matched milk (20g). 70%, 77% and 80% increase with 40g, 60g, 80g of mycoprotein *vs.* 20g mycoprotein ($p<0.05$). NSSD with 40g *vs.* 60g and 80g and 60g *vs.* 80g of mycoprotein.
Glucose and insulin AUC are expressed as a percentage change (%) from control food. Age and BMI expressed as mean ± SD. AUC, area under the curve; BMI, body mass index; CHO, carbohydrate; F, female; g, grams; iAUC, incremental AUC; kilogram/square metre; M, male; min, minutes; N, sample size; NSSD, no statistically significant difference; p, p-value; RCT, randomised controlled trial; refs, references; y, years; kg/m^2, *vs.*, versus.						

3.3.4 Studies on Energy Intake and Appetite

Table 13 shows the effect of mycoprotein on energy intake and appetite. In total, 4 randomised controlled and crossover trials assessed energy intake and appetite. All studies were conducted in healthy humans of which 3 studies (Bottin *et al*., 2016, Williamson *et al*., 2006, Dunlop *et al*., 2017) included adults with a BMI ≥25 kg/m^2 and 1 study (Turnbull *et al*., 1993) with a BMI <25 kg/m^2. 3 studies were conducted using solid mycoprotein (Turnbull *et al*., 1993, Bottin *et al*., 2016, Williamson *et al*., 2006) and 1 as liquid (Dunlop *et al*., 2017). The amounts used ranged from 44g to 132g of mycoprotein. The controls used were chicken in 2 studies (Bottin *et al*., 2016, Turnbull *et al*., 1993) , milk-protein in 1 study (Dunlop *et al*., 2017) and both chicken and tofu in 1 study (Williamson *et al*., 2006). 2 studies (Bottin *et al*., 2016, Williamson *et al*., 2006) measured acute energy intake (<5h post-ingestion) via *ad libitum* intake, of which 1 study used UEM and MSSP (Williamson *et al*., 2006) and 1 study using a buffet meal (Bottin *et al*., 2016). 2 studies measured energy intake post-24 hours using 3-day food diaries (Bottin *et al*., 2016, Turnbull *et al*., 1993). All 4 studies explored appetite feelings using subjective appetite scoring via VAS rating some of the following items: appetite, fullness, prospective food intake, hunger and desire to eat (Bottin *et al*., 2016, Turnbull *et al*., 1993, Dunlop *et al*., 2017, Turnbull *et al*., 1993). All studies assessed feelings of nausea and an eating behaviour questionnaire performed to participants in 3 studies (Bottin *et al*., 2016, Turnbull *et al*., 1993, Turnbull *et al*., 1993).

Table 13. Summary of the acute randomised trials on energy intake and appetite.

Refs.	Participants		Study information			Outcomes			
	N (M/F)	Characteristics (Age, BMI, health and smoking status)	Study design	Test Food (Form / Quantity of Mycoprotein / Control type / CHO)	Protocol	Energy intake assessment method	Effect on energy intake	Appetite assessment method	Effect on appetite subjective feelings
Turnbull *et al*., 1993	13 (0/13)	24.8 ± 7.9 y, 22.25 ± 2.50 kg/m^2, healthy, 1 smoker	RCT, cross-over, not blinded	Solid / 130 g wet weight / energy and macronutrient-matched (except from fibre) chicken / 77-80g CHO	Participant fasted from midnight. No breakfast was given. Test food served at lunchtime.	Weighted food diary using digital food balances recorded for 3 days (the day before, on the day, day after study visit)	24% decrease on the day ($p<0.01$) and by 16% on the day after ($p<0.05$) with mycoprotein.	VAS (100 mm) right after the meal and at 60, 120 and 180 min.	Decrease by 25% in the desire to eat at 180 min ($p<0.05$) and by 39% in prospective consumption feeling at 180 min ($p<0.01$) with mycoprotein. NSSD in hunger or fullness feelings with mycoprotein.
Williamson *et al*., 2006	42 (0/42)	30.14 ± 9.59 y, 27.42 ± 1.62 kg/m^2, healthy, non-smokers	RCT, cross-over,*	Solid / 44.3g wet weight/ energy and macronutrient-matched chicken (except fibre) and tofu / 63.6-65.3g	Participant fasted overnight for 12h and deprived of exercise and alcohol 24h prior study visit. Standard	*Ad libitum* meal at lunch (20 min post-test food ingestion) using UEM	12% decrease intake of total grams of food, energy and all macronutrient at *ad libitum*, *vs.*chicken, ($p<0.05$), and similarly to tofu.	VAS (100 mm) before and after the 3 meals and at 30, 60, 90, 120, 180, 240 min after lunch.	SSD in time effect. NSSD in treatment effect. SSD in interaction time x treatment for hunger. NSSD for subjective appetite AUC.

				CHO	breakfast served in the study visit. 4h later a 220g pasta meal with test food is served. 20 min later an *ad libitum* meal of sandwiches is served and ask to consume as much as wished. 4.5h later an *ad libitum* dinner is served using MSSP.		Mycoprotein did not affect the duration of food intake.		
						Ad libitum meal at dinner (4.5h post-test food ingestion) using MSSP	NSSD in energy intake with mycoprotein, chicken or tofu (p=0.33). 18% (+102 kcal) increase in high fat/high sugar foods with mycoprotein *vs.* tofu (p<0.01), and similarly to chicken (-20 kcal) (p<0.01).		
Bottin *et al*., 2016	36 (19/17)	33 ± 14 y, 28.1 ± 2.3 kg/m^2, healthy, non-smokers	RCT single-blinded, crossover	Solid / 44g, 88g,132 g wet weight / energy and macronutrient-matched (except fibre)	Participant had a standard evening meal, 12h overnight fast, asked to refrain from alcohol and	*Ad libitum* meal served at 180 min	8% (-48 kcal) decrease in energy intake with 132g of mycoprotein *vs.* matched chicken (p≤0.01). NSSD with 44 or 88g of	VAS (100 mm) at -15, 0, 15, 30, 45, 60, 90, 120, 150, 180 min relative to test food.	22% increase in fullness score at 150 and 180 min with 132g of mycoprotein (p≤0.05). NSSD in fullness with 44, 88g of mycoprotein.

				chicken (22,44,66 g) / 25-30 g CHO	avoid any strenuous exercise 24h prior study visit. Test food followed by an *ad libitum* meal at 180 min.		mycoprotein *vs.* matched chicken.		NSSD in hunger, desire to eat and prospective food intake scores with 44, 88, 132 g of mycoprotein.
						3-day food diary (results reported for energy intake over 24h overall)	13% (-249 kcal) decrease in energy intake with 44g (p=0.047) and 132g (almost SDD, p=0.083). NSSD with 88g of mycoprotein.		
Dunlop *et al*., 2017	12 (12/0)	28 ± 6.92 y, 26 ± 3.46kg/m^2, healthy, non-smokers	RCT single-blinded, cross-over	Drink / 20 g (mass-matched), 40 g (protein-matched), 60 g and 80 g wet weight / Not energy or other macronutrient-matched milk-protein (20 g) / protein:CHO ratio of 23 (in 20g of milk) and of 4.5 (in 20, 40, 60 and 80 g of mycoprotein).	Participant fasted. Abstained from physical activity and alcohol 48h prior the study visit. Test food served.	N/A	N/A	VAS (100 mm) at -15, 30, 60, 90, 120, 150, 180, 210, 240 min relative to test food.	From 0-120 min, decrease of appetite with 20 g of milk and 20, 40, 60 and 80g of mycoprotein (P<0.05) compared to baseline levels. From 120-240 min, increase of appetite with 20 g of milk, 20, 40 and 60 g of mycopotein (P<0.05) compared to baseline levels. NSSD in appetite with 80 g of mycoprotein compared to baseline from 120-240 min. Statistical analysis against milk-protein

									and other doses of mycoprotein N/A.

VAS score is expressed as mm. Energy intake at *ad libitum* and post-24h is expressed as percentage (%) change in kcals from control food. Age and BMI expressed as mean ± SD. *, In Williamson *et al*., 2006 blinding is not mentioned, although authors report that meals were prepared to be as equal as possible in palatability and appearance. BMI, body mass index; CHO, carbohydrate; F, female; g, grams; kilogram/squared metre; M, male; min, minutes; MSSP, macronutrient self selection paradigm; N/A, not/available; NSSD, no statistically significant difference; N, sample size; p, p-value; RCT, randomised controlled trial; refs, references; SSD, statistically significantly different; UEM, universal eating monitor; y, years; kg/m^2, VAS, visual analogue scale; *vs.*, versus.

3.5 Discussion

In the present study it has been systematically reviewed for the first time the evidence available in peer reviewed journals about the effects of mycoprotein on glycaemic control, appetite and energy intake in humans.

3.5.1 Summary of Findings

- There was an unclear effect of acute mycoprotein on blood glucose levels, but there was a consistent effect of mycoprotein at decreasing insulin levels.
- Acute mycoprotein intake led to a decrease in energy intake at an *ad libitum* meal and post-24 hours in healthy lean, overweight, and obese humans.
- The mechanisms underpinning these effects such as gut hormonal response and gastric emptying remain unclear.

3.5.2 Detailed Discussion

In this systematic review, 3 studies were identified to report the the effects of mycoprotein on glycaemia and insulinaemia, of which 1 was performed in healthy lean adults (Turnbull *et al*., 1995) and 2 in healthy overweight and obese (Bottin *et al*., 2016, Dunlop *et al*., 2017). On the outcome of appetite/energy intake 4 studies were identified to report the acute effects of mycoprotein on energy intake in healthy lean, overweight, and obese humans, with 2 studies analysing *ad libitum* meal (Bottin *et al*., 2016, Williamson *et al*., 2006), *2* post-24 hours energy intake (Bottin *et al*., 2016, Turnbull *et al*., 1993) and 1, energy intake during the day (Turnbull *et al*., 1993).

3.5.2.1 Effect of Mycoprotein on Glycaemic Control

In line with the hypothesis, the studies showed that acute mycoprotein had a positive effect on blood glucose since it increased insulin sensitivity, although the evidence is small in number of studies and heterogenous.

3.5.2.1.1 Glycaemia

Blood glucose AUC showed to be reduced in the first phase (0-60 min) but not in the second phase (60-120 min) in healthy lean adults, following the intake of 20 g of mycoprotein, compared to a combination of milk and soy protein drink (Turnbull *et al*., 1995). A similar experiment on healthy and overweight adults showed no differences in blood glucose $iAUC_{0\text{-}180}$ following the intake of mycoprotein at any dose (44, 88, 132 g) compared to matched-chicken for energy and macronutrient (Bottin *et al*., 2016). These similar effect on blood

glucose is interesting as both studies are conducted in different population characteristics (lean *vs.* overweight and obese) with different doses of mycoprotein. When different doses of mycoprotein are compared over time, it is observed that mean glucose values are not changed for in the first phase (0-120 min) relative to baseline levels, although they are decreased in the second phase (120-240 min) relative to the first phase (0-120 min) with 20, 40 and 60 g of mycoprotein (Dunlop *et al*., 2017).

3.5.2.1.2 Insulinaemia

Insulin AUC was decreased with 20g mycoprotein at both phases (0-60 and 60-120 min) in healthy lean individuals compared to a drink composed of soy and milk protein (Turnbull *et al*., 1995). A similar effect in $iAUC_{0\text{-}180}$ was shown in overweight and obese individuals when 44, 88 and 132 g of mycoprotein were compared to matched-chicken (Bottin *et al*., 2016). These findings, coupled to the observations on blood glucose suggest that mycoprotein may be increasing insulin sensitivity, which was suggested by Bottin and colleagues reporting an increased Matsuda index (which correlates with an $r=0.73$, $p<0.01$ with hyperinsulinaemic clamp which informs about peripheral insulin sensitivity) (Matsuda *et al*., 1999) and reduced insulinogenic index compared to matched-chicken (Bottin *et al*., 2016). This is of particular importance as overweight and obese people are at great risk of developing NCDs, and in such pathophysiology factors such as hyperglycaemia and hyperinsulinemia are involved, as it is the case of diseases such as T2D (Zheng *et al*., 2018) and CVD (Coutinho *et al*., 1999).

The effect of different doses (20, 40, 60 and 80 g) of mycoprotein on insulin $AUC_{0\text{-}240}$ was studied and it was showed that 40g induced a similar insulin $AUC_{0\text{-}240}$ response to protein-matched milk (20 g) suggesting that both protein sources have similar effects on insulinaemia when matched for protein content. 40, 60 and 80 g of mycoprotein induced a proportionally but non-significant increase in insulin $AUC_{0\text{-}240}$ which was significantly different compared to 20 g of mycoprotein. This is likely as the protein insulinotropic effect is proportional with the dose (Gunnerud *et al*., 2013). Interestingly, a saturation effect is observed with doses of 40, 60 and 80 g of mycoprotein intake. It is hypothesised that this effect may be influenced by a similar digestion pattern since a similar total postprandial plasma total amino acid concentration was observed following the intake of 60 and 80 g of mycoprotein during 45 to 120 min (Dunlop *et al*., 2017). This probably was influenced by the hyphae and a similar fibre content (5 g of difference from one to the other).

3.5.2.1.3 Possible mechanisms underlying the effect of mycoprotein on glycaemic control

The mechanisms involved in the effects of mycoprotein on blood glucose and insulin response may be due to a combination of different factors (e.g. digestive, endocrine), acting independently or together, and potentially influenced by the unique food matrix and nutritional profile high in both fibre and protein content.

It is possible that the mycoprotein food structure (particle size and fibre content) may have slowed gastric emptying reducing glucose absorption in the gut lumen, therefore decreasing postprandial blood glucose levels. However, the fibre content of mycoprotein has been described to be largely insoluble (Turnbull *et al*., 1998), and in theory insoluble fibre are unlikely to increase the viscosity in the stomach, influencing gastric emptying. Nevertheless, an in vitro study has shown that chitin can be converted to its soluble form namely chitosan via alkaline deacetylation, although evidence in vivo is lacking (Turnbull *et al*., 1998). Even though mycoprotein has not shown to have an effect on gastric emptying when compared to energy and macronutrient matched chicken in 1 study (Bottin *et al*., 2016), the methodology (paracetamol) used in the study was inadequate to measure gastric emptying on solid foods. This is because paracetamol follows a liquid-phase gastric emptying (Wagner *et al*., 1964), and liquid and solid phases empty at different rates and in patterns (Horowitz *et al*., 1985), therefore a better methodology should be employed to measure gastric emptying in solid mycoprotein such as ^{13}C-octanoid acid test. Other mechanisms such as the secretion of hormones (e.g.GLP, PYY) which are also involved in gastric emptying via activation of the ileal brake has been shown to be no different in mycoprotein relative to macronutrient-matched chicken (Bottin *et al*., 2016), although this study was not powered to detect differences in gut hormone release.

Protein content may have also been involved in the effects of mycoprotein on blood insulin. A slightly low insulinotropic amino acid profile coupled to a decreased amino acid availability in mycoprotein compared to protein-matched milk (Dunlop et al., 2017) may have explained a reduced insulin output (Bottin *et al*., 2016).

In summary, the mechanisms underpinning the blood glucose and insulin response of mycoprotein are unclear. Mechanisms such as gastric emptying and incretin and gastric emptying-regulatory gut hormone release (e.g. cholecystokinin, GIP) deserve to be further investigated with adequate methodologies.

3.5.2.2 Effect of Mycoprotein on Energy Intake

In line with the hypothesis, a single dose of mycoprotein reduced energy intake on the day and the following day in healthy lean individuals and at *ad libitum* on the following day in healthy overweight and obese population. Nevertheless, the evidence is small in number of studies and heterogenous.

3.5.2.2.1 Acute energy intake

Ad libitum energy intake was measured in healthy lean, overweight and obese individuals showing an overall reduction. In healthy lean individuals, *ad libitum* energy intake was reduced by 236 kcal following 130 g of mycoprotein after 180 min compared to matched-chicken, possibly influenced by the decreased subjective feeling of desire to eat and prospective consumption observed following mycoprotein intake (Turnbull *et al*., 1993).

In healthy overweight and obese individuals, a 166 kcal reduction in *ad libitum* energy intake was achieved 20 min following 44.3 g of mycoprotein ingestion compared to matched-chicken and similarly to matched-tofu (Williamson *et al*., 2006), this effect being independent of any change in subjective appetite. Another study (Bottin *et al*., 2016) tested 44g and 88g of mycoprotein showing no effect on energy intake 180 min after, possibly related to the increased energy requirements displayed by this population (overweight and obese). Interestingly, food intake using the macronutrient self-selection paradigm (MSSP) was also assessed and showed that mycoprotein promoted an increased intake of high fat/high sugar foods compared to tofu and similarly to chicken without inducing an increase in energy intake during a second *ad libitum* meal (4.5 h after mycoprotein ingestion) (Williamson *et al*., 2006). However, in this study it is not clear on whether mycoprotein would have resulted in an increase on overall energy intake during that day given the increased intake in high calorie foods on the previous meal. When a higher dose of mycoprotein (132 g) was tested, a 67 kcal reduction is observed 180 min following mycoprotein intake compared to matched-chicken possibly influenced by the increased subjective feeling of fullness reported after mycoprotein intake (Bottin *et al*., 2016). The differences in the *ad libitum* energy intake reduction observed between studies could be related to the difference in the timeframes compared (e.g. the time between protein ingestion and presentation of the *ad libitum* meal) and the different types of population tested (lean *vs.* overweight and obese displaying different energy demands). However, the small number of studies and the heterogeneous methods employed prevents any definitive conclusions from being made.

3.5.2.2.2 Short-term energy intake

Short-term (12-24h post ingestion) energy intake was assessed in both healthy lean (Turnbull *et al*., 1993) and overweight and obese population (Bottin *et al*., 2016) showing an overall decrease in energy intake. Healthy lean people reduced their energy intake by 25% on the day and by 16.5% on the following day with 130 g of mycoprotein relative to matched-chicken (Turnbull *et al*., 1993). On the other hand, healthy overweight and obese people showed a slightly less decrease in energy intake (by 10%) on the following day with the same dose and comparator (Bottin *et al*., 2016). This difference in energy intake could be in relation to energy demands depending on BMI or a misreporting in the food diaries from participants. Interestingly, despite mycoprotein reduced energy intake on the day, it did not induce a compensatory overeating on the next day. It has been reported that a reduction of energy intake of 100 kcal per day can contribute to weight maintenance (Hill *et al*., 2003), however, it is not clear on whether mycoprotein could possess long-term effects on energy intake reduction, which warrants further research. Interestingly, it has been shown that 60, 80 g and 132 g of mycoprotein increased resting energy expenditure following mycoprotein ingestion (Dunlop *et al*., 2017, Bottin *et al*., 2016). It is possible that diet-induced thermogenesis may be a contributor in this effect. The potential thermic effect of mycoprotein, coupled to the effect on reduction energy intake represent 2 important attributes of mycoprotein-containing foods which could facilitate weight maintenance and possibly induce weight loss, which deserve further investigation.

3.5.2.2.3 Possible mechanisms underlying the effect of mycoprotein on energy intake

Different mechanisms, acting separately or together may have been involved in the effects on energy intake.

The decrease in energy intake at subsequent meals may have been triggered by decreased subjective appetite feelings by mycoprotein (Turnbull *et al*., 1993, Bottin *et al*., 2016, Dunlop *et al*., 2017) probably due to the action of appetite-regulating gut hormones such as GLP-1 and PYY, subsequently reducing energy intake. Nevertheless, mycoprotein showed no differences in these gut hormone concentrations compared to macronutrient matched-chicken (Bottin *et al*., 2016), although the role other appetite-regulating gut hormones such as cholecystokinin and ghrelin have not been yet investigated. Metabolomics data also indicated that mycoprotein intake was related with increases in the presence of molecules which are related to appetite (Bottin *et al*., 2016). Furthermore, while a slowed down gastric

emptying and/or transit time can also modulate appetite feelings, evidence for this is weak as 1 study showed no effect on gastric emptying using an inadequate method (paracetamol) for measuring gastric emptying in solid mycoprotein (Bottin *et al*., 2016) and there is no published data on the effect of mycoprotein on gut transit time. In support of this, the presence of insoluble fibre within mycoprotein (of which certain types can increase gut transit time (Burkitt *et al*., 1972)), as well as data showing a steady plasma amino acid appearance following mycoprotein intake (Dunlop *et al*., 2017) (compared to an acute appearance with protein-matched milk protein), suggests that mycoprotein may decrease the rate of gastric emptying. Further research is needed to elucidate this.

The effects of mycoprotein on short-term (>5h post-ingestion) energy reduction could be in part due to fibre fermentation by the resident gut microbiota and the subsequent production of the SCFAs acetate, butyrate and propionate. A recent study using *in vitro* batch culture fermentation with human faeces showed that isolated mycoprotein fibre increased SCFAs production by almost 6 times compared to control, similarly to other fermentable fibres such as laminarin, rhamnose, and oligofructose (Harris *et al*., 2019). Studies have shown that propionate stimulates the increase of GLP-1 and PYY production and decreases acute energy intake in overweight humans (Chambers *et al*., 2015) and that acetate and butyrate can cross the blood brain barrier and may affect appetite directly in the central nervous system (Li *et al*., 2018, Frost *et al*., 2014). Therefore, it is plausible that the one-time intake of mycoprotein may have stimulated gut microbiota fermentation of the unfermentable fractions of mycoprotein with the production of SCFA that may have affected appetite feelings therefore explaining the decreased energy intake post-visit >5 hours post-intake.

3.6 Limitations

In the systematic review the evidence found from the systematic search was limited in number of studies (n=3, for glycaemic outcomes and n=4, for energy intake outcomes). Furthermore, these had diverse ways of reporting the glycaemic outcomes (e.g. mean glucose at specific time frames and iAUC) and energy intakes (post-20 min and post-180 min) which could not be pooled together for a meta-analysis as they are not physiologically comparable. Meta-analysing the results is important to improve precision of estimates of the overall effect and to settle controversies arising from conflicting studies. This highlights the need for further research on the acute effects of mycoprotein on glycaemia and energy intake in a diverse range of populations (healthy, metabolically compromised and with T2D)

using physiologically relevant and similar ways to report the outcome so meta-analyses are possible. Chronic studies are important to understand the health impact of a long-term exposure to mycoprotein as they provide a realistic context within a free-living setting. Such studies should be high-quality, meaning that they should abide by PROSPERO protocols, follow CONSORT reporting guidelines to avoid risk-of-bias, and have an adequate control of outcome co-founders. Furthermore, high-quality studies also mean employing gold-standard methodologies for glucose management assessment such as euglycaemic-hyperinsulinaemic clamps and energy intake such as automatic re-fillable *ad libitum* meals in a controlled environment (Wanskin *et al*., 2005) and free-living energy intake reporting using objective measures, which are still under-development at the time of writing this thesis. Only with high-quality studies it is possible to generate high-quality and trustable data from meta-analysis.

Equally as important, uncovering the mechanisms relating to mycoprotein-induced decreases in subjective appetite, acute and short-term energy intake and any potential effect glycaemic control is key to inform re-development of mycoprotein-containing foods or the use of different mycoprotein variants with characteristics that can optimise health outcomes in the population. So far, data are scarce and inconsistent regarding the mechanisms underpinning mycoprotein's effects, and studies investigating gut hormone release, gastric emptying, gut microbiota fermentation following mycoprotein intake are greatly required.

3.7 Conclusions

Mycoprotein is a food ingredient of growing popularity within the vegetarian community in many countries across the globe. Its nutritional profile rich in both dietary fibre and protein make it an ingredient with potential to affect positively glycaemia and appetite. In this systematic review it has been shown that the acute intake of mycoprotein has an unclear effect on glycaemia, but likely decreases insulinaemia and energy intake (at an *ad libitum* meal and post-24 hours) in healthy lean and overweight and obese humans acutely. The mechanisms underpinning these effects are not fully understood and future research should uncover them. In addition, there is a need to conduct well-controlled chronic investigations to translate the acute effects in a real-world setting.

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CHAPTER 4: Experimental Methods for the human and *in vitro* batch fermentation study

4.1 Analysis of Blood samples

4.1.1 Blood samples collection, processing, and storage

Blood samples were collected from the human study to analyse glucose, insulin and gut hormones. They were collected using BD Vacuntainers® blood tubes (BD, USA). A total of 8 mL of whole blood were drawn from the cannula and 2 mL were poured into each of the 4 blood tubes (Table 14, column 1). Each blood tube was spray-coated with additives that preserved the analyte of interest (Table 14, column 3). In addition, the heparin blood tube for the collection of gut hormones (GLP-1 and PYY) was pre-treated on the morning of the study visit with aprotinin (Trasylol, Nordic Pharma, Norway) (20µL/ 1mL whole blood).

Table 14. Blood tubes type, specimen and analytes used for the human study

BD Vacuntainer® type (Spray-coated additives) + extra additive	Blood specimen	Analytes
Fluoride (glycolytic inhibitor)	Plasma	Glucose
SST™ (Silica/Gel for serum separation)	Serum	Insulin
Heparin (Lithium Heparin) + Aprotinin	Plasma	GLP-1 and PYY

GLP-1, glucagon-like protein 1; PYY, peptide tyrosine tyrosine; SST, serum separator tubes.

Immediately after blood collection, all tubes except SST™, were immediately placed on wet ice to paralyse the metabolism of blood cells with potential degradation of the analytes of interest. Blood tubes were promptly centrifuged for 10 min at 4°C and 2500 relative centrifugal force (RCF) to obtain 1 mL of plasma (Table 14, column 2). The resultant plasma was aliquoted (2 x 500 µL) into 1 mL microcentrifuge tubes. SST™ was left at room temperature for approximately 10 min to allow the blood sample to clot prior to being centrifuged under the aforementioned conditions. The resultant serum was aliquoted (2 x 500 µL) into 1 mL microcentrifuge tubes. Aliquoted samples were immediately stored at -80°C until further analysis.

4.1.2 Colorimetry Assay

A colorimetry assay is an assay in which a substrate is catalysed by an enzyme into a coloured product, that can be quantified by measuring the absorbance of a specific wavelength. This allows the indirect concentration quantification of the substrate.

4.2.2.1 Glucose Analysis

Plasma samples from the human study were analysed for glucose using a commercially available enzymatic colorimetric assay kit (Glucose GOD/PAP GL 364, RANDOX Laboratories Ltd, UK). The principle of this kit is that it contains glucose oxidase (GOD), an enzyme responsible for the oxidative catalysis of glucose, oxygen and water present in the serum sample into gluconic acid and oxide peroxide. The latter, in presence of 4-aminophenazone and phenol (both present in the assay kit), are catalysed by peroxidase (PAP) (also present in the assay kit). This reaction yields quinoneimine, a chromogen (pink coloured dye) that can be measured at 504 nm (Figure 9).

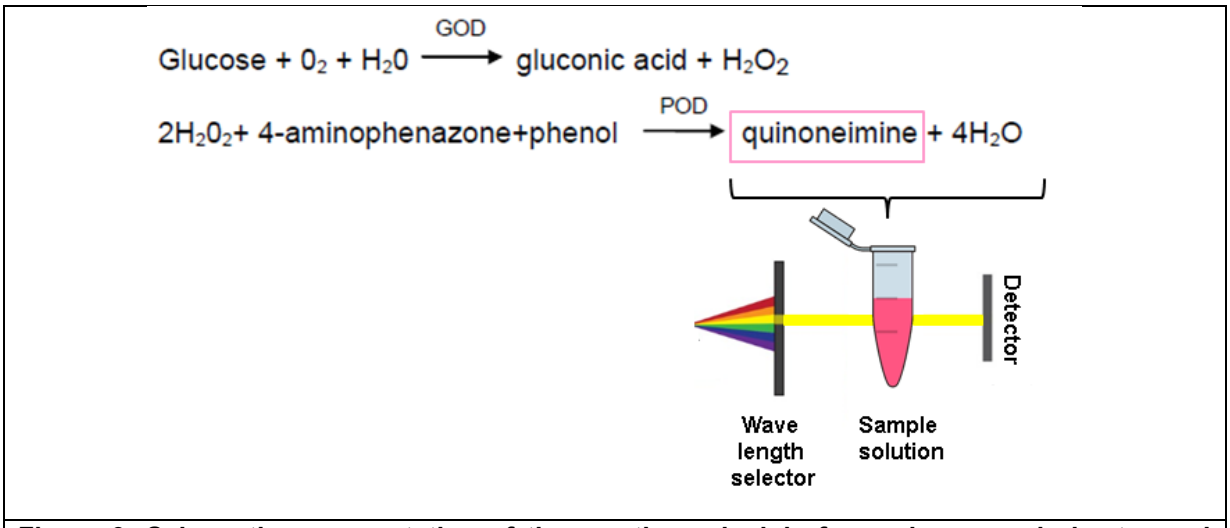

Figure 9. Schematic representation of the reaction principle for a glucose colorimetry and enzymatic assay. GOD, glucose oxidase; POP or PAP, peroxidase.

Briefly, 20 µL of serum samples were aliquoted in duplicates into microcentrifuge tubes followed by the addition of 1000 µL of a previously mixed reagent (phosphate buffer + enzymes GOD-PAP + enzymatic substrates). Quadruplicates of blanks (no serum sample) and calibrator (also known as the standard, with a known concentration of glucose ~5.55 mmol/L) were also included. Next, following vortex, samples were incubated at 37°C for 10 min. Following, 200 µL of the mixture was transferred into a 96-well plate and absorbance (A) of a 504 nm wavelength was measured in the standard (known concentration of glucose) and the samples, against the blanks using a spectrophotometer (Elx808, BioTek, BioTek Instruments, Inc, USA) at 37°C. The glucose concentrations (mmol/L) were calculated using the following formula on Microsoft Excel.

$$\text{Glucose concentration } (\frac{\text{mmol}}{\text{L}}) = \frac{\text{A Sample x Standard concentration (mmol/L)}}{\text{A Standard}}$$

The manufacturer reported coefficient of variance (CV, %) was not reported. Samples were assayed in duplicate to increase the accuracy of the results. 32 assays were performed. The intra-assay CV for the 32 assays were: 3.9, 3.7, 5.9, 3.6, 4.4, 4.6, 5.1, 3.7, 2, 3.4, 3.1, 3.2, 5, 2.1, 3.4, 4.8,2.4,3.1,2.2, 2.2, 2.2, 1.7 1.4, 1.8 2.1, 1.2, 4.3, 6.2, 5.8 4.8, 4.8, 5.6%. The average inter-assay coefficient of variability (CV) was 3.55%. A CV <10% was accepted.

These assays were performed in the Department of Metabolism, Digestion and Reproduction, Commonwealth Building, Hammersmith Campus, Imperial College London.

4.1.3 Radioimmunoassay

A radioimmunoassay (RIA) is an *in vitro* technique that employs both radiation and antibodies used to determine the concentration of specific hormones. The principle behind it is that a known concentration of radioactively labelled hormone competes with the same unlabelled hormone (of interest; unknown concentration) for a specific binding site of an antibody, for which its concentration is known. After separating the antibody-bound from the free fraction, the amount of radiolabelled hormone can be assessed by counting the radioactivity of one of the fractions (Figure 10). To extrapolate the concentration of the unlabelled hormone from this data, a standard curve is generated with serial dilutions of unlabelled hormone of known concentration (standards).

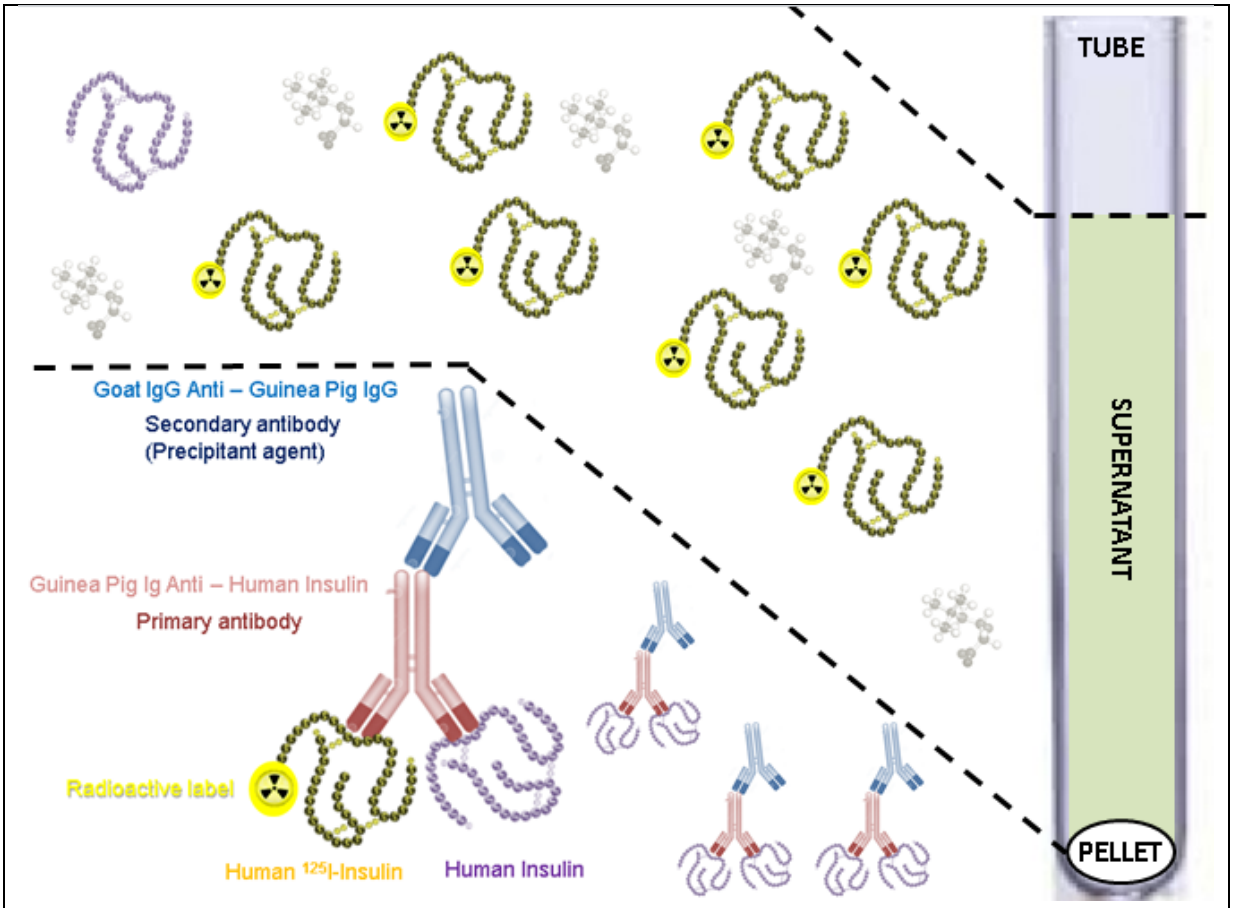

Figure 10. Graphic representation of the mechanism behind the RIA assay, in this case, for determination of serum insulin. In short, after incubation of the serum sample with a known concentration of radio-labelled insulin, the primary antibody and the secondary antibody (acting as a precipitating agent), competition of labelled and unlabelled insulins for binding sites of the antibody occurs. In this case, the more amount of unlabelled insulin found the serum sample, the more radio-labelled insulin will be displaced from binding to the primary antibody, and therefore more radioactivity will be found in the supernatant. Ig, immunoglobulin (or antibody). ^{125}I, iodine-125-labelled; Ig, immunoglobulin.

RIA is a very sensitive and specific assay and was used in this research project to measure the concentration of insulin, GLP-1 and PYY.

These assays were performed in the Department of Metabolism, Digestion and Reproduction, Commonwealth Building, Hammersmith Campus, Imperial College London.

4.1.3.1 Insulin Analysis

Serum samples from the human study were analysed for insulin concentrations using a quantitative commercially available assay kit named Millipore Human Insulin Specific RIA Kit (HI-14K, Millipore Corporation, Billerica, USA), according to the manufacturer's protocol, but using half volume.

In short, on day 1, 50µL of serum sample was added into a borosilicate glass tube with 50 µL of phosphosaline buffer. Next, 50µL of hydrated radio-labelled I^{125}-human insulin and 50 µL of human insulin antibody was added to all tubes (except total count and non-specific binding tubes), and vortexed. The assay was performed in a total volume of 200 µL and incubated for 20 hours at room temperature before the separation of the free from antibody-bound. On day 2, 500 µL of precipitating reagent (secondary antibody) was added to all tubes (except total count tubes). Tubes were vortexed and incubated for 20 min at 4˚C. Following, tubes were centrifuged for 30 min, at 2500 RCF and at 4˚C. Immediately, the supernatant was discarded, and pellets were counted using a gamma counter (LB2111 Multy Crystal Gamma Counter, Berthhold Technologies, Bad Wildbad, Germany) for 1 minute. Insulin concentration was extrapolated from the standard curve according to manufacturer's indications, using GraphPad Prism version 9 (Graphpad Software, San Diego, CA, USA).

One standard curve per sample 100 tubes was used, with the known concentration of human insulin ranging from 3.125 uU/mL to 100 uU/mL. Two quality controls in duplicates were used per assay. Samples were analysed in duplicates.

This assay did not cross-react with proinsulin. The limit of detection was 2.715 µU/mL. Samples were assayed in duplicate to increase the accuracy of the results. The manufacturer reported CV was 3.2% (within sample) and 3.8% (between sample). All samples were analysed in 4 insulin assays. The intra-assay CVs of the present assay were 6.4,6.04,4.16 and 3.53%. The inter-assay CV was 5.03%. A CV < 10% was accepted (Morgan and Lazarow, 1963, Thorell, 1973, Deshpande, 2012).

4.1.3.2 GLP-1 analysis

Plasma samples from the human study were analysed for GLP-1 concentrations using a specific and sensitive in-house RIA radioimmuno-assays, as previously described (Kreymann *et al*., 1987). The radiolabelled I^{125}-GLP-1 was prepared by Professor Kevin Murphy using the iodogen method (Wood *et al*., 1981) and purified by high pressure liquid chromatography (HPLC). The assay was performed in 350 µl of 0.06 M phosphate buffer (pH=7.34) containing 0.3% BSA (1ml/100ml) and Tween 20 diluted in 1:10 (Sigma, Poole, UK) (200 µl/100ml). Briefly, on day 1, 50 µL of plasma sample was added to the borosilicate

glass tube containing 150 µL of buffer. Next, 50 µL of label and 50 µL rabbit anti-GLP-1 primary antibody were added. The standard curve was prepared by adding 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100 µL of GLP-1 at a concentration of 0.083 pmol/mL. Tubes were vortexed and incubated for 4 days at 4°C. On day 5, 250 µL of dextrin-coated charcoal (Merck) was added to each tube and samples were immediately centrifuged at 1318 RCF for 30 minutes at 4°C. After centrifugation, the supernatant, which contained the antibody-bound labelled GLP-1, was separated from the charcoal pellet using Pasteur glass pipettes and added to new tubes. The final step included the counting using gamma scintillation counter (LB2111 Multy Crystal Gamma Counter, Berthhold Technologies, Bad Wildbad, Germany) for 3 minutes. GLP-1 peptide concentrations in each sample were calculated by using a non-linear two-phase decay plot.

For GLP-1, the assay uses a polyclonal antibody which detects the $GLP\text{-}1_{1\text{-}36}$ amide, $GLP\text{-}1_{7\text{-}36}$ amide and $GLP\text{-}1_{9\text{-}36}$ amide forms of the hormone. This antibody does not cross-react with other known pancreatic or gastrointestinal hormones. The limit of detection was 7.5 pmol/L. Samples were assayed in duplicate to increase the accuracy of the results. The samples were analysed in 5 separate assays. The intra-assay CV was 5.12, 4.75, 4.71, 4.81 and 4.09%. The inter-assay CV was 5.03%. A CV <10% was accepted.

4.1.3.3 PYY Analysis

Plasma samples from the human study were analysed for PYY concentrations using a specific and sensitive in-house RIA (Kreymann *et al*., 1987). The assay measured the hormone fragment, $PYY_{3\text{-}36}$, and the full-length hormone, $PYY_{1\text{-}36}$, both of which are biologically active. The radiolabelled $I^{125}\text{-}PYY_{3\text{-}36}$ was prepared by Professor Kevin Murphy using the iodogen method (Wood *et al*., 1981) and purified by high pressure liquid chromatography (HPLC). The assay was performed in 350 µL of 0.06 M phosphate buffer (pH=7.4) containing 0.3% BSA (1ml/100ml) and Tween 20 diluted in 1:10 (Sigma, Poole, UK) (200 µl/100ml). Briefly, on day 1, 100 µL of plasma sample was added to the borosilicate glass tube containing 200 µL of buffer. Next, 50 µL of label and 50 µL rabbit anti-$PYY_{3\text{-}36}$ primary antibody were added. The standard curve was prepared by adding 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100 µL of $PYY_{3\text{-}36}$ at a concentration of 1 pmol/ml. Plasma was added to the standard curve to account for plasma matrix effects. Tubes were vortexed and incubated for 3 days at 4°C. On day 4, 250 µL of dextrin-coated charcoal (Merck) was added to each tube and samples were immediately centrifuged at 1318 RCF for 30 minutes at 4°C.

After centrifugation, the supernatant, which contained the bound PYY_{3-36}, was separated from the charcoal pellet using Pasteur glass pipettes and added to new tubes. The final step included the counting using gamma scintillation counter (LB2111 Multy Crystal Gamma Counter, Berthhold Technologies, Bad Wildbad, Germany) for 3 minutes. PYY concentrations in each sample were calculated by using a non-linear two-phase decay plot.

The antibody cross-reacted fully with the biologically active circulating forms of PYY (PYY_{1-36} and PYY_{3-36}) but not with pancreatic polypeptide or other known gastro-intestinal hormones. The detection limit of the assay was 7.5 pmol/L. Samples were assayed in duplicate to increase the accuracy of the results. The samples were analysed in 5 separate assays. The intra-assay CV were 9.67, 7.52, 5.81, 4.52 and 5.28%. The inter-assay CV was 5.07%. A CV <10% was accepted.

4.2 Analysis of Energy intake and appetite

4.2.1 Visual Analogue Scales (VAS)

Visual analogue scales (VAS) were used to assess subjective appetite feelings (Flint *et al*., 2000) at specific time points (-15, -5, 15, 30, 45, 60, 90, 120, 180 relative to test meal intake) during the study visit. VAS has been shown to be a reproducible way to measure subjective appetite feelings in studies using single meals which do not seem to be influenced by standardised diet prior the study visit.

During the study visit, smell from other foods in the immediate nearby areas was controlled and avoided as they could trigger hormone release (insulin and gut hormone) and influence their appetite feelings (Morquecho-Campos *et al*., 2020). In addition, watching, talking or reading about foods was not encouraged during the study visit to avoid any further appetite queues.

The VAS consists of a set of questions to assess appetite feelings which were:

- Hunger (“How hungry do you feel right now?”)
- Fullness (“How full do you feel right now?”)
- Thirst (“How thirsty do you feel right now?”)
- Desire to eat (“How strong is your desire to eat?”)
- Nausea (“How sick do you feel right now?”)

- Prospective food intake (“How strong is your appetite for a meal?”)
- Appetite for a snack (“How strong is your appetite for a snack?”)
- Appetite for savoury (“How strong is your appetite for something savoury?”)
- Appetite for sweet (“How strong is your appetite for something sweet?”)

Volunteers were asked to answer to these questions by tracing a clean and vertical line across a 100 mm scale ranging from “not at all” (right extreme) and “extremely” (left extreme).

Answers were obtained by measuring the distance between the left extreme and the vertical line traced at each time point for each VAS question. Time points -15 and -5 were averaged to obtain a baseline (timepoint 0) value. In addition, a composite appetite score (CAS) was calculated by averaging hunger, desire-to-eat, prospective food intake and 100-fullness (Stubbs *et al*., 2000).

4.2.2 *Ad libitum* energy intake

A meal served at *ad libitum* was used to measure *ad libitum* energy intake in the human study. *Ad libitum* is a Latin expression for “at one’s pleasure” or “as you desire”. Hence *ad libitum* energy intake refers to the intake of energy when this is offered in abundance, without restriction.

The *ad libitum* meal consisted of a bowl filled with homogenous ~3 kg of boiled white pasta with tomato sauce and vegetable oil (approximately 2500 kcal). Pasta was cooked as per manufacturer’s instructions in a standard way each time to avoid differences in water content in the pasta that may influence energy intake. The *ad libitum* meal is a reproducible method for measuring spontaneous energy intake which is recommended to be used along a VAS for appetite (Gregersen *et al*., 2008).

At 180 min following the test meal intake, volunteers were served the *ad libitum* meal freshly cooked. Each time, they were specifically asked to consume until they felt “comfortably full” and to take as much time as needed. To avoid potential cofounders in their intake, participants were specifically asked to avoid entertainments (such as use of phone, tablets, music or reading) during their intake. In addition, volunteers were left in an isolated area (curtains delimiting their area were pulled to create a division between them and the rest of the unit) to prevent social interaction during food intake. External food smells and noises were kept to minimum to avoid interfering with the experiment. Volunteers were told that if

not strictly necessary, drinking water was advised at the end of the experiment to avoid them filling up on water.

The bowl of food was measured before and after serving using the same weighing scale to calculate the difference of grams of food remaining (amount of food eaten). This data were collected to then calculate the energy intake using the manufacturer's food label for the energy values per 100g (note: "cooked" kcal/100g for pasta was used).

4.2.3 Food diaries analysis

Volunteers were asked to complete a 4-day food diary analysis to assess their usual dietary intake (baseline) and a 2-day food diary post-study visit to assess the effect of the test meals on their dietary intake post-visit in a free-living setting.

Volunteers were asked to fill in the diaries with the unweighted food they ate on specific days with as much detail as possible reporting measuring units or equivalents (such as grams, pint, cup, teaspoon), ingredients, method of cooking and brands of food. Instructions and examples of how to report with detail were provided to volunteers.

The food diaries were analysed for energy and macronutrients using DietPlan 6 (Foresfield Software Ltd, West Sussex, UK) according to a diary coding protocol developed within the Section for Nutrition Research by a qualified group of dieticians, to assure analysis quality. Food portion sizes were estimated using Food Standards Agency Portion sizes references (FSA, 2002). Food weights were estimated using Carbs & Cals (Chris Cheyette and Yello Balolia, 2013).

4.2.4 Assessment of under-reporting

Assessing dietary intake using unweighted food diaries has many limitations. Firstly, the use of household measures to estimate quantities may lead to imprecision both from the volunteer and the researcher conducting the analysis. Weighted food diaries are a better measure than unweighted as they lead to less variance (Tod *et al*., 1983). Secondly, T2DM is associated with overweight and obese phenotypes, and this population may have a tendency to intentionally under-report (food eaten but not reported) (Goris *et al*., 2000, Hill *et al*., 2001). Under-reporting can also result from an unintentional behaviour (food intake reduced or types of food avoided, motivated by their participation in a research study) (Macdiarmid and Blundell, 1998). In addition, because 2-day food diaries were asked to be

filled on 6 times, it is possible that a lack of interest/motivation in participant may have led to underreporting.

4.3 Statistical Analysis

Data for the above outcomes was checked for normality using the quantile-quantile (Q-Q) plot using GraphPad Prism version 9.0.0 (GraphPad Software, CA, USA). For non-parametric data, data were log-transformed prior to carrying out parametric statistical tests. Data are presented as means ± standard error of the mean (SEM), unless otherwise stated. Missing timepoints data were interpolated with the mean value of the following and previous timepoint. For last missing timepoints (at 180 min) the average of the 180 min group was taken. For missing study visits, the average of the food group was taken. For blood analytes and VAS, the average of two separate baseline measurements (-15 and -5 min relative to meal intake) were used for the 0 min measurement. Baseline values were compared using a one-way analysis of variance (ANOVA). If baseline values were significantly different between groups, results are presented as change from baseline. Incremental area under the curve (AUC) were calculated using the trapezoidal rule using GraphPad Prism version 9.0.0 (GraphPad Software, CA, USA). Because some participants were vegetarian (n=3) and did not have the two chicken meals, there were missing samples for these conditions, therefore a linear mixed model (LMM) was used as it handles well in a situation of missing data. LMM was used to analyse the effect of glucose, insulin and VAS $iAUC_{0\text{-}180}$, *ad libitum* and post-visit energy intake with fixed effects for type of chapati (with or without guar gum), protein type (soy, chicken or mycoprotein) and ethnicity (white European or south Asian), and their interaction, and random effects for participant. To perform pairwise comparisons, Bonferroni test were performed. A two-tailed P-value≤0.05 was considered statistically significant. Statistical analyses were performed using STATA version 16.0 (Stata Corp, Texas, USA). Graphs were prepared using GraphPad Prism version 9.

4.4 Analysis of *in vitro* Batch Culture Samples

4.4.1 Microbial Compositional Analysis

Compositional analysis was performed in the Dept. Gastroenterology and Hepatology, Queen Elizabeth Mother Queen Wing, St. Mary's Hospital Campus, Imperial College London.

4.4.1.1 DNA extraction

DNA extraction is a technique in which the molecules of DNA are isolated via a multi-step process of lysis and precipitation of cellular components.

Faecal slurry form Study 2 were collected at 0 hours and 24 hours following fermentation in the *in vitro* batch cultures and stored at –80°C for between 6-9 months before processing. The microbial community DNA was extracted with PowerLyzer PowerSoil DNA Isolation Kits (Mo Bio, Carlsbad, CA, USA). The manufacturers protocol was followed with minor alterations to the bead beating process, whereby samples were beaten for 3 min at speed 8 in a Bullet Blender Storm (Chembio Ltd, St. Albans, UK). All samples were analysed in a single batch. DNA concentration was measured using Invitrogen Qubit fluorometric quantification machine (Qubit 2.0, ThermoFisher Scientific, USA) using a Qubit dsDNA broad range buffer and reagent (1:200) (Q32850, ThermoFisher Scientific, USA). This technique has been used before for similar nature studies (Chambers *et al*., 2019)

4.4.1.2 Bacterial composition profiling using 16S rRNA gene sequencing

The basic principle of profiling bacterial composition is that bacterial cells have an intracellular organelle called ribosome composed of a small ribosomal subunit named 16S rRNA, which is unique to the Bacteria Kingdom. The gene that codes for the 16S rRNA is highly preserved in the evolution of bacteria. This allows bacterial phylogeny to be identified.

The remaining analysis was performed by Dr. Nathan Danckert. Sample libraries were prepared following Illumina's 16S Metagenomic Sequencing Library Preparation Protocol with the following alterations. First, the index PCR reactions were cleaned up and normalized using the SequalPrep Normalization Plate Kit (Life Technologies, Paisley, UK). In addition, sample libraries were quantified using the Qubit fluorometry (as described above) and NEBNext Library Quant Kit for Illumina (New England Biolabs, Hitchin, UK). Sequencing was performed on an Illumina MiSeq platform (Illumina Inc., Saffron Walden, UK) using the MiSeq Reagent Kit v3 (Illumina) using paired-end 300 bp chemistry. The resulting sequencing data were processed following the DADA2 pipeline as previously described (Callahan *et al*., 2016). The SILVA bacterial database version 132 was used to classify the sequence variants. Phangorn was used to generate the phylogenetic tree and phyloseq / ggplot2 were used to create the NMDS plots. Faecal slurry samples were subsampled to 500 (the number of reads in the sample with the lowest number of reads).

4.4.1.3 Data Analysis

Data were statistically analysed using STAMP version 2.1.3 (statistical analysis of taxonomic and functional profiles, Donovan Parks and Robert Beiko, 2015). Data were analysed at all taxa levels available up to genus. When comparing two groups of samples (such as timepoint 0 hours *vs.* 24 hours) a White's non-parametric t-test, two-sided, with a CI method of bootstrap 0.95 and with a multiple test correction of Benjamini-Hochberg was used. When doing a multiple comparison (between soy, chicken, quorn and control) a Kruskal-Wallis H-test, post-hoc test of Tukey-Kramer 0.95 and effect size Eta-squared, with a multiple test correction of Benjamini-Hochberg was used. A q-value (corrected p-value) was considered statistically significantly different when ≤0.05, in all analyses.

Data were also analysed using the online tool Microbiome analyst (https://www.microbiomeanalyst.ca/) to analyse for ecology diversity indexes.

Sequences from statistically significant genera were further explored using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to extrapolate taxonomic information to the species level. Metabolic profiles from BLAST hits were explored in the literature to draw potential correlations to this study's findings.

4.5 Metabolomics Analysis

Metabolomics analysis is an analytic method that detects molecules involved in metabolic pathways (metabolites). Generally, this is achieved via a multi-step process involving sample preparation, extraction of compounds (targeted or untargeted), derivatization, separation, detection, and data treatment (using specific software and compound databases) (Cevallos-Cevallos *et al*., 2009).

4.5.1 Metabolomic Analysis of *In vitro* Fermentation Batch Culture Samples using Liquid Chromatography with Tandem Mass Spectrometry

In this research project I have used a Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) upon derivatisation to 3-nitrophenylhydrazone to analyse 10 SCFAs which were: acetate, butyrate, propionate, lactate, valerate, isovalerate, isobutyrate, 2-OH-butyrate, 2-methylbutyrate, and hexanoate, following a modification of an existing methodology by Han *et al*., 2015. Such modification will be published by Valdivia-Garcia (manuscript under preparation).

The general basic principle underpinning the LC-MS/MS technique is that a stationary phase (LC column) will be the phase through which a sample will be moving through (by a mobile phase) at high pressure. Depending on the nature of the compounds found in the sample, both phases will affect the elution rates for each compound. The effluent is then directed to a mass spectrometer which has an ionisation source where the effluent is ionised creating charged particles. These charged particles are exposed to a magnetic field that will generate ions and deflect them (separate them) by their mass-to-charge ratio (m/z). This is a unique property for each compound, therefore allowing its qualification. Compounds are also analysed in a quantitative way since MS also detects the relative abundance of m/z.

This analysis was performed in the Dept. of Cancer and Surgery, Sir Alexander Fleming Building, South Kensington Campus, Imperial College London.

4.5.2.1 Reagents

Formic acid of analytical grade, acetonitrile, and isopropanol were purchased from Fisher Scientific (NH,USA). All SCFAs to be tested, and N-(3-dimethylaminopropyl)-N0 -ethylcarbodiimide (EDC) were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). The ethanol LC-MS was purchased from Honeywell Riedel-de Haën (Seelze, Germany). [13C,D3]-acetic acid, [D5]-propionic acid, and [D7]-butyric acid were from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Stock solutions of the SCFAs, including the labelled compounds, were prepared in ultrapure LC/MS grade water and were stored at −80ºC.

4.5.2.2 Samples Extraction Preparation and derivatisation

Faecal slurry samples were thawed at room temperature and vortexed for few seconds prior to take a 100 µL aliquot of 100 into a microcentrifuge tube. Next, 200 µL of cold isopropanol (pure) was added and mixture was vortexed for few seconds and centrifuge at 13000 RPM for 15 min at 4-8 ºC. 250 µL of the supernatant were transferred into a new microcentrifuge tube tube and store at -80 ºC until derivatisation. In a 96 well plate (Eppendorf, UK) (2 mL), 10 µL of extracted supernatant, and 90 µL of HPLC grade water were added, vortexed and covered with a silicone mat (60180-M150, Thermo Scientific, UK) and stored at 4-8ºC until derivatisation. Overall, a 30x dilution was used. For the derivatisation, 50 µL of internal standard-Solution A (Appendix 2) was added, and immediately covered with silicone mat. Next, 50 µL of internal standard-Solution B (Appendix 2), and immediately covered with silicone mat. The plate was then transfered to a MixMate (Eppendorf, UK) for 30 seconds at

1400 RPM and kept at 4-8°C until derivatisation. Next, 40µL of 200 mM 3-NPH solution was added to each well and immediately covered with silicone mat. 40µL of 120mM EDC solution was added to each well and plate was covered with the silicone mat, and mixed in the MixMate for 30 secs at 1400 RPM. Then, it was centrifuged at 4000 RCF for 5min at 4°C. Next, the plate was placed on a block heater for 30 minutes at 40°C. The linear dynamic range of the ten SCFAs and the internal standard solutions are described in Table 15.

Table 15. Linear Dynamic Range for ten SCFAs and stable labelled internal standard solutions used in calibration curves

Analyte	Dynamic range µM	Labelled IS µM
Lactic acid	0.1-100	60 (Sol. A)
Acetic acid	2.5-100	60 (Sol. A)
Propionic acid	0.1-100	60 (Sol. B)
2-Hydroxybutyric acid	0.1-100	60
Isobutyric acid	0.1-100	6 (Sol. B)
Butyric acid	0.1-100	6 (Sol. B)
2-methylbutyric acid	0.1-10	6**
Isovaleric acid	0.1-10	6 (Sol. B)
Valeric acid	0.1-10	6 (Sol. B)
Hexanoic acid	0.1-10	6 (Sol. B)

(*Propionic acid 1-13C; ** Isovaleric acid D9). Reference: Garcia *et al*., 2021 [Manuscript in Preparation].

3.5.2.3 LC/MS Spectroscopy Treatment

20mL of 0.1% formic acid solution were charged into a multichannel solution basin and 400µL were pipetted into the 96-well plate. The plate was then placed on MixMate for 30 seconds at 1400 RPM. Then, it was centrifuged at 4000 RCF for 10 min at 4°C. Using a multichannel pipette, 200 µL of supernatant were transferred to a new 300 µL round bottom 96-well plate. Plates were sealed with aluminium foil at 60°C for 10 seconds prior to the injection. Quality control samples at a dilution of 10x and 5x were prepared independently by pooling 10 µL of each sample.

4.5.2.4 Faecal SCFAs Compound Identification

Ten faecal SCFAs were separated following a reverse phase separation with multiple monitoring reaction experiments. SCFA detection was performed on a Waters Xevo TQ-S (Waters, Milford, USA) on negative electrospray ionisation polarity. Samples were analysed using MassLynx version 4.2 (Waters Laboratory Informatics, MA, USA). The peak areas of the chromatograms of the endogenous and stable isotope labelled internal standard compounds were adjusted manually. The lower limit of detection for the SCFAs was 0.1 µmol/L (except for acetate which was 5 µmol/L). MassLynx integrated the areas of the peaks and these were used in the following calculations to calculate the concentration of the SCFAs. SCFAs concentration were calculated by interpolating from the standard curve the ratio (correlation divided by the internal standard area). The upper limit of detection that surpassed the maximal ratio in the calibration curves are 100 µmol/L for acetate, lactate, propionate, butyrate, isobutyrate and 2-hydroxybutyrate. The upper limit of detection for the rest was 10 µmol/L.

4.5.2.5 Statistical Analysis

The normality of the data were checked using a Q-Q plot using GraphPad Prism version 9 (Graphpad Software, San Diego, CA, USA). For non-normally distributed data, data were transformed. Data were plotted as the change from baseline and at 24 hours using GraphPad Prism version 9. Changes between the four groups were analysed using a repeated measures one-way ANOVA with a Geisser-Greenhouse correction (since equal variability of differences was not assumed). Holm-Sidak test was used for multiple comparison test. A two-tailed P-value ≤0.05 was considered statistically significant. Analyses with all the data and without outliers was performed. Outliers were considered to be those values that were outside two SD. The data are expressed as mean ± SEM, unless otherwise stated. The statistical analysis was done using GraphPad Prism version 9.

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CHAPTER 5: The effects of mycoprotein and guar gum on glycaemic control and appetite in south Asian and white European adults with Type 2 Diabetes

5.1 Introduction

5.1.1 Ethnic differences in glycaemic control

Some studies have shown different metabolic responses between ethnicities upon the same dietary challenge. For example, a short-term study feeding a 5-day high-fat high-calorie diet (usual diet supplemented with 375 mL/day of cream), mimicking a western diet, showed that healthy south Asians exhibited a significant increased fasting blood glucose, insulin levels and insulin resistance compared to matched-white Europeans (Bakker *et al*., 2014). To explain these ethnic differences mechanistically, the authors performed muscle biopsies to explore whether there were differences in the insulin/mammalian target of rapamycin signalling, metabolic gene expression, and mitochondrial respiratory chain content of the skeletal muscle and found no ethnic differences in these parameters.

The differences between ethnicities in the postprandial responses to the same food, are also observed in acute (single-meal) interventions. Most of the evidence comes from studies performed in the late 2000s, looking at whether international glycaemic index (GI) values of foods (obtained from studies mostly conducted on white European cohorts) are applicable to south Asian countries. All studies consistently show that the postprandial blood glucose response (iAUC, mmol/L x min) was significantly higher in those south Asian compared to healthy matched-white Europeans. For example, the increase in blood glucose iAUC in the south Asians was +28% (p<0.01) for glucose drink, +38% (p<0.01) for cereal breakfast (Venn *et al*., 2010), and +62% (p=0.03) for malted wheat cereal (high-fibre food) (Henry *et al*., 2008), compared to matched-white Europeans. However, regarding people with T2D, no evidence on postprandial metabolic responses to different foods was found in south Asian *compared* to white Europeans. These data highlight that south Asians have a distinct metabolic adaptation to the same meal and diet, perhaps making them more prone to elevated postprandial blood glucose and insulin, which can increase their risk of T2D, cardiovascular disease and T2D-related complications. This may be explained by the thrifty phenotype hypothesis in which poor foetal and post-natal nutrition leads to impaired pancreatic beta-cell function which in an obesogenic environment it would result in a disadvantage in the way to handle energy (Hales *et al*., 1992). Poor foetal nutrition is reflected with low birth ways which correlated with T2D development later in life in a cohort of babies in the UK (Hales *et al*., 1991). It is likely that the thrifty phenotype hypothesis is true in south Asians since these babies, compared to white European counterparts have a

low birth weight (Anand *et al*., 2016). This is relevant since south Asians typically follow a diet high in carbohydrate content (45% *vs* 25% compared to white Europeans) (Burden *et al*., 1994, Greenhalgh *et al*., 1997, Misra *et al*., 2004) and in which a beta-cell impairment would lead to an inadequate handling of this carbohydrate, and therefore increased postprandial blood glucose response. Furthermore, south Asians when migrated, they are exposed to westernised unhealthy foods with detrimental effects to their metabolism, there is a need to develop ethnic-specific staple foods which can reduce postprandial blood glucose levels in the south Asian community, similarly, to white Europeans, which are acceptable for the consumer.

5.1.2 Body composition, feeding habits and eating frequency between south Asians and white Europeans

South Asians have been shown to have a "thin-fat phenotype" (anthropometrically thin but metabolically obese), in which for a normal BMI (according to western criteria in between 18.5-25 kg/m^2), they have a greater percentage of body fat and low percentage of muscle mass, and this is evident even from infancy (Yajnik *et al*., 2003). The south Asian subgroup, they present a higher waist-to-hip ratio (indicating central obesity), subscapular skinfold thickness as well as body, subcutaneous and intra-abdominal visceral fat (including liver fat) (Hills *et al*., 2018). Visceral fat and truncal fat (central fat) correlates with insulin action in south Asians (Banerji *et al*., 1999, Sandeep *et al*., 2010, Chandalia *et al*., 2007).

Food intake plays an important role in postprandial metabolism. South Asians who migrate to high-income countries experience a change in dietary habits which depend on a variety of factors related to country of origin, urban/rural residence, socio-economic and cultural factors and situation in host country. Nevertheless, a review has identified that the main dietary changes are an increase in energy and fat intake, reduction in carbohydrates, a partial replacement from whole grains and pulses to refined carbohydrates resulting in reduced dietary fibre intake, increase in meat and dairy foods (Holmboe-Ottensen *et al*., 2012). These changes are more prominent even in young populations in these ethnicity (Donin *et al*., 2010). These dietary changes could be a result of “acculturation” in the host country by the migrant south Asians which consists of adopting elements of their recipient countries’ diet resulting in a reduction in fibre and increased intake of energy, fat, meat and highly processed foods (Raj *et al*., 1999). Dietary macronutrients are linked with blood lipids increasing their cardiovascular risk (Donin *et al*., 2020) and also are linked to increased

postprandial blood glucose, particularly if they have basal insulin resistant states, leading to compensatory hyperinsulineamia and risk for type 2 diabetes. Elevated serum insulin and 2h postprandial glucose response were associated positively with carbohydrate intake and inversely with alcohol intake, but not with saturated fatty acid intake (Sevak *et al*., 1994).

Eating frequency is also a factor that influences glyceamic control since a more frequent eating pattern would increase postprandial blood glucose and insulin responses and truncate the previous postprandial period which otherwise would be likely returning to baseline levels. Nevertheless, a study compared postprandial insulin, GIP and glucose responses following a high (6 meals a day) vs. low (3 meals a day) frequency eating pattern, observing no differences in insulin, GIP and glucose to insulin ratio (Kanaley et al., 2014). Regular (6 occasions a day) vs. irregular (chaotic from 3-9 meals a day) eating pattern effect on postprandial responses was also studied (Farshchi et al., 2005). This study showed that while regular eating was associated with lower energy intake, postprandial thermogenesis and fasting total LDL cholesterol, there were no differences in fasting glucose and insulin values, but peak insulin were lower than in the chaotic meal. It remains to be studied whether south Asians who have migrated have a more chaotic eating frequency than white Europeans which may make them more prone to hyperinsulinaemia.

5.1.3 The effect of fibre-rich foods on glycaemic control in south Asians

South Asians are responsive to fibre-rich foods (e.g. legume-enriched foods providing 9-11 g of fibre and basmati rice) in the sense that they present a lower blood glucose response compared to more high-GI foods (e.g. glucose, idli rice, jasmine rice) (Tan *et al*., 2015 and 2018). Supplemented chapattis with high-fibre powders (vegetable and pulses) given acutely significantly reduced the postprandial glucose and insulin response by 44-46% and 47-59%, respectively in healthy Pakistanis (Akhtar *et al*., 2019). More specifically, enriching legume flour-based flatbreads with 2 or 4 g of guar gum resulted in similar significant reductions over 30% in PPG in healthy south east-Asians (Boers *et al*., 2017). A posteriori study by the same authors analysed glucose in venous blood samples of healthy subjects following the intake of flatbreads with 3 and 4% of guar gum. This time, the significant reductions were still shown for PPG and PPI, although of less magnitude than the previous study (by 15% and 28%, respectively) (Boers *et al*., 2017). Interestingly, no effects on self-reported appetite

feelings were detected following the consumption of fibre-rich flat breads (Boers *et al*., 2017 and Zafaar *et al*., 2013).

Furthermore, the scarce data on mycoprotein, a high-fibre and protein ingredient used in Quorn foods, show to improve insulin sensitivity and to reduce the first phase blood glucose secretion, compared to matched animal-derived protein equivalents in healthy subjects of white European ethnicity in its majority. No data on the effects of mycoprotein on healthy or with T2D within the south Asian specific community has been found.

In summary, south Asians have an increased postprandial blood glucose response than matched-white Europeans. Healthy south Asians have improved blood glucose response with fibre-rich foods. Flat bread or chapati is a staple food within the south Asian community and its enrichment with fibre is acceptable to the consumer and induces a physiologically relevant decrease in blood glucose (Kanaya *et al*., 2010, Zafaar *et al*., 2013)**.** This suggests that it could be an effective strategy for diabetes management, along with other fibre-rich foods such as mycoprotein. Nevertheless, studies investigating this on subjects with T2D are currently lacking.

5.2 Purpose of the Study

5.2.1 Hypothesis

I hypothesised that the ingestion of acute mycoprotein has a beneficial effect on postprandial blood glucose, insulin, appetite and energy intake in south Asian and white European adults with T2D. In addition, this effect may be greater when mycoprotein is combined with guar gum. Furthermore, it is possible that there are differences in blood glucose management between the two ethnicities.

5.2.2 Aims

The overall aim of this exploratory study was to investigate the effect of mycoprotein and guar gum on postprandial blood glucose, insulin, appetite and energy intake in south Asian and white Europeans adults with T2D.

5.2.3 Outcomes Measures

The primary outcome measure is:

1. Plasma glucose concentrations

The co-secondary outcome measures are:

1. Serum insulin concentrations
2. Subjective appetite responses
3. Energy intake at *ad libitum* and post-visit
4. GLP-1 and PYY concentrations

5.3 Methodology Study Design

This study was a randomised, double-blind, crossover, controlled trial involving 6 separate study visits and was approved by London – Bromley Research Ethics Committee (REC reference number 19/LO/0476).

5.3.1 Volunteers Recruitment

Several volunteer recruitment strategies were employed which are detailed in Appendix 3. Of these, the most successful strategies were:

1. Social media – Facebook advertisement, Imperial College Yammer, Nextdoor.co.uk and Twitter feeds.
2. Charities: Diabetes UK and Diabetes.co.uk - an advertisement was placed on the website of these charities.
3. Newspaper advertising – adverts were put twice (November 2019 and January 2020) in the *Metro* and *Evening Standard.*
4. Newspaper article – informing about the study details was placed in the Imperial College newspaper *Felix* (August 2019).
5. Community groups: Age UK – members and staff were approached by researcher personally to inform about the study.

Interested potential participants contacted the researcher via phone/email and were asked pre-screening questions to pre-assess their eligibility. Pre-eligible potential participants were sent via post/email the Participant Information Sheet (PIS) (Appendix 4). After reading the PIS, participants who further expressed their interested in volunteering were invited to attend the Imperial Clinical Research Facility at Hammersmith Hospital, Du Cane Road, London, W12 0NN for a screening visit to discuss the study, obtain written and informed consent (Appendix 5) and fully assess their eligibility for the study.

The eligibility was assessed by collecting information about the volunteer's lifestyle and health history, completing an adult eating behaviour questionnaire (Appendix 6) and performing some examinations. These examinations included anthropometric measurements, blood pressure, an electrocardiogram and a blood sample (HbA1c, full blood count, lipids, liver function test, and urea and electrolytes). Pregnancy tests were performed to women of reproductive age. Eligible women of reproductive age were only invited to the facility on the first phase of their menstrual cycle to control for glycaemic control and appetite (Valdes *et al*., 1991, Jarrett *et al*., 1968, Buffenstein *et al*., 1995). In addition, as an adaptation test, screened volunteers were asked to consume a test meal.

5.3.3.1 Inclusion criteria

Volunteers were suitable to participate in the study if they fitted the following inclusion criteria: diagnosed with T2D, of south Asian (Afghanistan, Bangladesh, Bhutan, Maldives, Nepal, India, Pakistan and Sri Lanka) or white European ethnicity, and aged 18 to 70 years old.

5.3.3.2 Exclusion criteria

Volunteers were not suitable to participate in the study if they ascribed to any the following exclusion criteria: had mixed ancestors, was taking any of the following medications: insulin, injectable hypoglycaemics or orlistat, had a gastrointestinal, heart, pancreas disease, cancer, infection requiring antibiotics, any condition involving the imbalance of hormones, was a shift worker, had a weight change of ≥5% of total kilograms in the preceding 3 months, were currently smoking, had history of alcohol or drug abuse. A complete list of the exclusion criteria can be found in Appendix 7.

5.3.2 Randomisation and Blinding

Volunteers who upon the screening visit were classified as eligible, were randomised to an order of intervention. Randomisation was performed using an online validated tool named Sealed Envelope provided by Sealed Envelope Company (www.sealedenvelope.com).

Both patients and researcher were blinded to the test food types. Test foods were manufactured with labels A, B, C and chapati bread types with 1 and 2. Test foods were made in a way they tasted and looked identical. Unblinding of the test foods happened after the data analysis.

5.3.3 Sample size

A total of 24 patients with T2D and non-insulin treated (12 white European and 12 south Asian) adult male or female volunteers were recruited. The sample size is obtained based on the recommendations by Julious, 2005 for pilot studies (n=12 per group) since no study on the effects of mycoprotein on glycaemic control in people with T2D has ever been published, so there is no study to base the sample size on.

5.3.4 Test meals

In this study, the test meals consisted of 3 protein sources (in form of mince) which were soy (positive control), chicken (negative control) and processed mycoprotein (Quorn). The meals were accompanied of chapatti bread (carrier for guar gum) enriched with or without guar gum. In total, the test meals tested are:

1. Soy mince with guar gum-enriched chapati,
2. Chicken mince with guar gum-enriched chapati,
3. Processed mycoprotein (Quorn) mince with guar gum-enriched chapati,
4. Soy mince with plain chapati,
5. Chicken mince with plain chapati,
6. Processed mycoprotein (Quorn) mince with plain chapati

The test meals were manufactured as ready meals by NPD Direct Ltd (Lincolnshire, UK). Before serving, meals were prepared as per manufacturer instructions which involved reheating in the microwave for 3 minutes for the mince and toasting for 2 min for the chapati. The meals were energy and macronutrient matched as closely as possible. Table 16 describes the nutrient profile of the test meals and Appendix 8 shows the meals appearance. Chapati with guar gum provided 5 g of providing 5 g of fibre.

Table 16. Nutritional composition of the mince test meals

Per serving (wet weight)	**Soy with chapati (400 g)**	**Chicken with chapati (400 g)**	**Quorn with chapati (350 g)**
Energy (kcal)	473	461	461
Carbohydrates (g)	57.40	57.85	55.30
Fat (g)	6.90	9.55	9.00
Protein (g)	35.79	30.65	30.30
Fibre (g)	19.30	10.75	19.00

G, grams; kcal, kilocalories.

5.3.4 Study visit overview

5.3.4.1 Study Visit Preparation

Prior to the study visits, volunteers were asked to refrain from strenuous physical activity 72 hours before the study visit and to avoid alcohol and caffeine consumption 24 hours before the study visit. Furthermore, to control for the second-meal effect (Thorburn *et al.*, 1993), volunteers were asked to standardise their evening meal by choosing a ready-meal of their choice prior to the study visit. In addition, all those volunteers that were taking oral hypoglycaemic medication were asked to skip their diabetic medication in the morning of the study visit, but to continue it as usual after the study visit. Volunteers were allowed to take any medication not related to diabetes as usual.

5.3.4.2 Study Visit Protocol

On the study visit, volunteers were asked to attend the NIHR/ Imperial Clinical Research Facility at 08:00am, following an overnight fast. Next, researcher checked with volunteers the compliance of the study visit preparation with a series of questions (e.g. are you fasted?) and an international physical activity questionnaire (IPAQ) (Geneva, 1998). If the answers were satisfactory, blood pressure was taken. Following, volunteers were encouraged to empty their bladder before their body composition measurements were performed using bioelectrical impedance analysis (BIA) (Tanita® BC-418 analyzer; Tanita Corporation, Japan). Body composition measurements were performed at the same time to avoid intra-day variation. Measurements were not always possible to be performed on the same day of the week to control for intra-day variation, but factors that would affect the measurements from day-to-day were controlled for (e.g. heavy sweating from vigorous exercise, alcohol intake, etc.). The principle behind the BIA method lies on the natural resistance (impedance) of corporal fluids (such as water, fat and fat free mass) to a low electrical current that is transmitted through the electrodes present in BIA scales. The impedance measures can then be used to infer the amount of water, fat and fat free mass in the body (body composition). These are calculated using formulas which are owned by Tanita and not openly available.

Next, an intravenous cannula is placed in the antecubital fossa of volunteer for blood sampling. At time point –15 and –5 minutes relative to the test meal intake, 2 fasting blood

samples are drawn. Next, a test meal was served and allowed 15 minutes to consume it. After its consumption, volunteers were asked to rate the palatability of the meal on a scale from 1 to 10, with a score 1 representing “not good at all”, and a 10 “extremely palatable”. Following, 7 postprandial blood samples were drawn at timepoints 15-, 30-, 45-, 60-, 90-, 120- and 180-minutes relative to meal intake. 8 mL of blood were taken at each timepoint, totalling 72 mL (8x9). Figure 11 depicts the study visit timeline of events. With every blood sample, volunteer was asked to complete a 100 mm visual analogue scale (VAS) to assess subjective appetite feelings. After the last blood sample, cannula was removed and participant was asked to consume an *ad libitum* meal consisting of homogenous pasta with tomato sauce and oil to assess *ad libitum* energy intake. Volunteer was instructed to consume until they felt comfortably full and to avoid distractions (e.g. looking at phone) while eating.

After this, volunteer was asked to keep a record of their dietary intake for the next 48 hours by using a food diary to assess energy intake post-visit. In addition, volunteer was requested to provide a stool sample passed on the next day of the study visit in a stool kit provided to assess for gut microbiota and SCFA production.

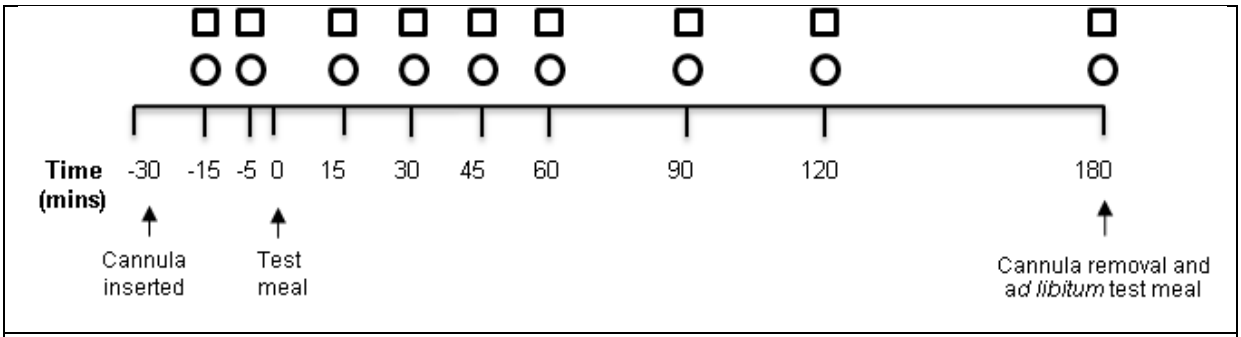

Figure 11: Timeline of a study visit. Open circles represent the timepoint at which blood samples were taken, whereas open squares represent the timepoint at which VAS ratings were measured.

5.3.4.3 Samples Collection, Processing, Storage and Analysis

Please refer to Methods section 4.1.1 for blood samples collection, processing, storage and analysis.

5.3.5 Statistics

Normality of the data were checked using visual inspection of Q-Q plots (Appendix 9). For non-parametric data, data were transformed prior to carrying out parametric statistical tests.

Data were treated as following:

- For blood glucose, insulin and VAS data were analysed as incremental area under the curve (iAUC). iAUC is an estimation measure that correlates with continuous glucose monitoring (Ugi *et al*., 2016) and is calculated using the trapezoidal method using Graphpad Prism v.9.0. Incremental stands for the fact that values used to calculate AUC are the datapoints from which the baseline measurement (average of timepoints -15 and -5 and represented as 0) has been subtracted, presenting the change from baseline for each datapoint. iAUC is useful to understand the sensitivity of a system and it allows the normalisation of values when there is high inter-variability in the metabolic parameters.
- For gut hormone data, since the majority of the two fasting baseline values for a same individual had and intra-variability (coefficient of variability) ≥20% suggesting that the baseline measure has a high error, the change from baseline for each timepoint will not be calculated to avoid carrying the error to the further calculations and avoid obtaining an iAUC high in errors. Total AUC will be used instead using the raw values. Total AUC informs of total hormonal output in the system. iAUC and total AUC calculated accounted for positive peaks above the baseline from which negative peaks below baseline had been subtracted (net AUC).

Data were analysed as following:

- Ethnic differences in baseline food diary data, baseline participant characteristics and metabolic rate were analysed using a T-test, with a two-tailed p-value considering samples to be heteroscedastic. This statistical analysis was performed on Excel (Microsoft Office). Metabolic rate was calculated using the Schofield equation (Schofield, 1985 adapted by YMCA fit) which considers age, body weight and gender, as well as accounting for physical activity level.
- For iAUC, tAUC, mean and timeline data for glucose, insulin, gut hormones, VAS, *ad libitum* and post-visit energy and nutrient intake a linear mixed model was used. Protein, bread, timepoint and ethnicity factors were considered as fixed factors and participant as a random factor. If there was a main effect of the aforementioned factors or interaction, post-hoc multiple comparisons were made using Bonferroni's

corrections. P-value was considered to be statistically significant if ≤ 0.05. The linear mixed model was performed using Stata v.16.0 (Stata Corp LLC, Texas, USA).

- Homeostatic model assessment – insulin resistance (HOMA-IR) was correlated with repeated measures to the glucose $iAUC_{0\text{-}180}$ following each test meal to assess the effect of the insulin-resistant status of the participants on their glucose response to the different test meals (Matthews *et al*., 1985).

Data were presented as following: AUC data are presented as box and whiskers as median and 5-95% percentiles range as well as the individual datapoints. Quantitative raw data in tables is presented as mean and 95% confidence interval, unless otherwise stated. Time series data and other data will be presented as mean and SEM in bar graphs. Graphs were performed using Graphpad Prism v9.0.

5.4 Results

5.4.1 Participant's characteristics

Figure 12 summarises the CONSORT Flow Diagram of the study participant's recruitment, randomisation, allocation and analysis.

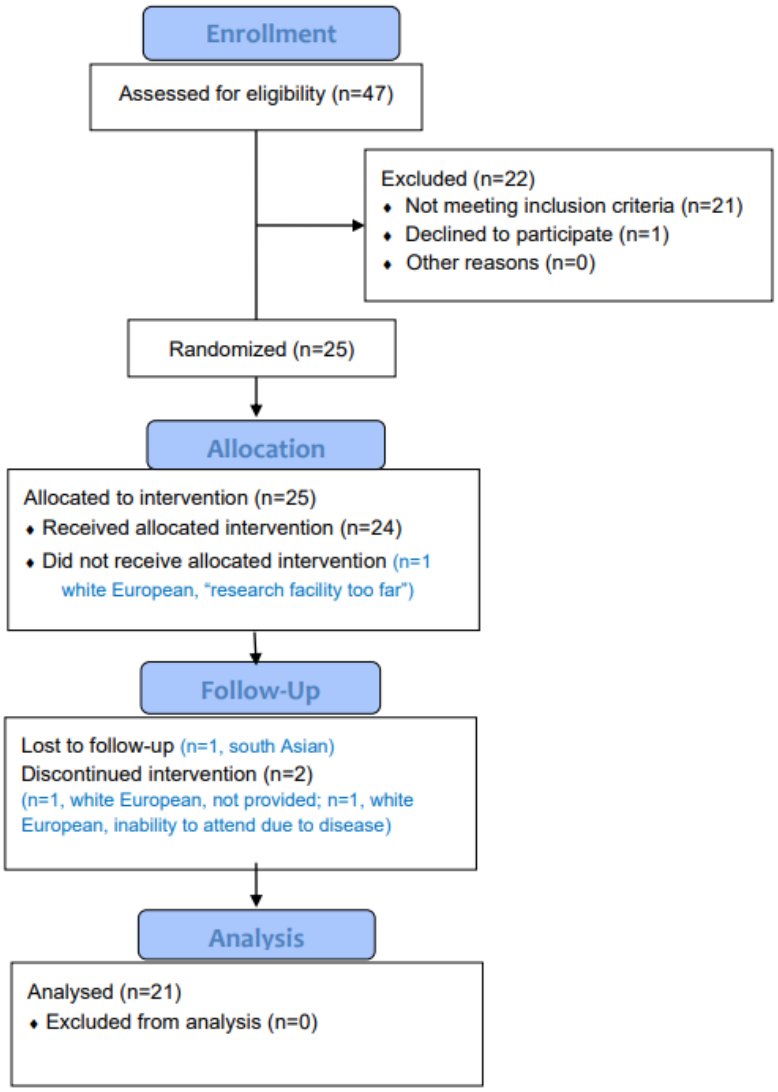

Figure 12. CONSORT of the flow of subjects through the study. CONSORT, Consolidated Standards of Reporting Trials.

Participants who had completed at least the 83% of the study visits (5 visits out of 6) were included in the analysis. Of those who completed the study, 2 south Asians were vegetarian, so only 4 visits (soy and mycoprotein with and without guar gum) were performed on these participants.

The demographic baseline characteristics of participants who completed at least the 83% of the study visits is captured in Table 17. Further baseline blood analytes such as urea and electrolytes are described in the Appendix 10. The south Asian group had a statistical

significantly decreased body weight, body mass index (BMI), body fat (kg), body muscle mass (kg), TAGs, and FBG, compared to the white European at baseline. Care should be taken at interpreting raw BMI values between ethnicities since there is ethnic-specific classification of it. For south Asian ≥23 kg/m^2 and ≥25 kg/m^2 is classed as overweight and obese, respectively, by the Indian Consensus Group (Misra *et al*., 2015), whereas for white European, ≥25 kg/m^2 and ≥30 kg/m^2 is classed as overweight and obese, respectively (WHO, 2021). In the south Asian group, 3 out of 10 participants were reported to be second generation (south Asians born in the UK) from migrant south Asians, whereas 7 out of 10 were the first generation (moved to the UK when adults) of migrant south Asians in the UK.

Table 17. Demographic characteristics of participants

	Total (N=21)		**White European (n=11)**		**South Asian (n=10)**		**P-value**
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	
Age	61.05	1.65	62.27	2.30	59.70	2.42	0.45
Sex (male/female)	-		11 (10/1)		10 (9/1)		-
Body weight (kg)	78.26	3.58	88.55	4.54	66.93	2.72	<0.01
BMI (kg/m^2)	26.00	0.76	27.70	0.99	24.12	0.87	0.01
Ethnic-specific BMI categories*							
Healthy (%)	-	-	18.18		30		-
Overweight (%)	-	-	54.55		50		-
Obese (%)	-	-	27.27		20		-
Body fat (kg)	19.36	1.50	22.85	2.06	14.45	0.84	<0.01
Body fat (%)	24.08	0.99	25.56	1.49	22.03	0.86	0.06
Body muscle mass (kg)	58.14	2.39	62.45	2.96	52.17	2.81	0.02
Body muscle mass (%)	70.66	1.72	70.77	1.43	70.41	3.15	0.91
Visceral fat score	11.28	0.53	12	0.78	10.22	0.71	0.21
HDL (mmol/L)	1.22	0.06	1.29	0.08	1.13	0.07	0.19
LDL (mmol/L)	2.23	0.16	2.26	0.25	2.18	0.22	0.81
TAGs (mmol/L)	1.04	0.08	1.37	0.12	0.85	0.11	0.01
ALTs (IU/L)	27.75	2.81	30	4.65	25	3.43	0.40
Fasting blood glucose (mmol/L)	7.39	0.31	8.12	0.07	6.48	0.08	<0.01
HbA1c (mmol/mol)	52.76	1.55	51.82	1.64	53.80	2.77	0.53
Years since T2D diagnosis	6.99	1.13	6.32	1.30	7.73	1.95	0.54
T2D management							
Only lifestyle (%)	9.52		18.18		0		-
Metformin (%)	85.71		72.72		100		-
Sulfonylurea (%)	28.57		27.27		30		-

DPP4i (%)	19.04	27.27	10	-
SGLT-2i (%)	9.52	18.18	0	-

*BMI of ≥23 kg/m^2 and ≥25 kg/m^2 as overweight and obese, respectively, by the Indian Consensus Group (Misra *et al*., 2015). Visceral fat score: fat in the internal abdominal cavity, surrounding vital organs in the trunk. Rating from 1-12 means healthy levels whereas levels 13-59 indicate excess level of visceral fat. Visceral adipose tissue (VAT) calculated with Tanita correlates with VAT from MRI (R=0.84, for males and 0.88 for females, P<0.001, for both) (Tanita, Columbia University College of Physicians and Surgeons, 2004). SEM, standard error of the mean; BMI, body mass index; HbA1c, glycated A1c haemoglobin; T2D, type 2 diabetes; DPP4i, dypeptidyl 4 inhibitor; SGLT-2i, sodium glucose transporter 2 inhibitor. T-test were performed between south Asians and white Europeans. P-value ≤0.05 is considered significant.

5.4.2 Baseline dietary intake

Table 18 shows the baseline dietary intake of the participants on average in a day. For all subjects regardless of ethnicity, their energy intake a day was of 1798.94 ± 71.70kcal, the carbohydrate intake was of 46.26 ± 1.82% a day, of fat intake was of 38.24 ± 1.62%, of protein 17.03 ± 0.66% and 20.41 ± 1.15g of fibre a day. There were no statistical differences in the intake at baseline between the two ethnic groups.

Table 18. Baseline dietary intake of participants per day

	All (N=21)		**White European (n=11)**		**South Asian (n=10)**		
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**P-value**
Energy (kcal)	1798.94	71.70	1746.44	103.14	1687.11	200.26	0.44
CHO (g)	208.08	11.83	194.71	18.13	203.80	24.45	0.23
Total Sugars (g)	65.72	5.76	63.17	7.25	62.68	10.92	0.65
CHO (%)	46.26	1.82	44.60	70.30	48.32	48.85	-
Fat (g)	77.00	5.01	75.25	6.28	71.85	10.75	0.72
Fat (%)	38.24	1.62	38.78	54.80	38.33	48.32	-
SFA (g)	27.44	2.08	30.53	3.27	21.62	2.78	0.11
CN6-PUFA (g)	7.49	1.94	7.59	3.25	6.96	2.02	0.96
CN3-PUFA (g)	1.07	0.26	1.09	0.46	0.99	0.18	0.94
MUFA (g)	25.08	1.77	25.28	2.43	22.60	3.42	0.91
PUFA (g)	12.89	1.32	11.21	1.43	13.60	2.52	0.18
Trans Fats (g)	0.97	0.11	1.11	0.15	0.74	0.16	0.19
Protein (g)	75.05	2.63	75.30	3.93	67.68	7.87	0.92
Protein (%)	17.03	0.66	17.25	15.26	16.05	15.72	-
Fibre (g)	20.41	1.15	18.94	1.51	20.14	2.60	0.17
Starch (g)	138.32	8.22	128.08	12.40	137.00	16.60	0.19
Alcohol (g)	3.49	1.95	4.83	3.38	1.99	1.60	0.47

T-test were performed between south Asians and white Europeans. P-value ≤0.05 is considered significant. CHO, carbohydrate; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SEM, standard error of the mean.

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The amount of chapati consumed during participants' daily life was also assessed by ethnicity and presented in Table 19. South Asians were greater in number of chapati consumers (60%) compared to white Europeans (18.18%) in their daily lives. The number of chapatis a day consumed were 2.14 for the south Asians compared to 1.52 for the white Europeans, providing 62.04±6.43g and 56.73±18.15g of carbohydrate a day, respectively.

Table 19. Consumption of chapatis per ethnicity at home.

	White Europeans		**South Asians**	
Consumers (number)	2		6	
Consumers (%)	18.18		60	
	Mean	**SEM**	**Mean**	**SEM**
Chapatis a day	1.52	0.18	2.14	0.99
Chapati (g)	91.25	11.28	128.44	13.32
Protein (g)	8.52	2.04	10.40	1.08
Fat (g)	6.09	4.16	16.44	1.71
CHO (g)	56.73	18.15	62.04	6.43
Energy (kcal)	301.35	39.07	421.28	43.70
Starch (g)	55.45	18.30	59.72	6.19

CHO, carbohydrate; g, gram; kcal, kilocalorie; SEM, standard error of the mean.

5.4.3 Test meal palatability

The palatability score of the test meals was as described in Figure 13 and Table 20. In a scale from 0 to 10, soy was rated with (mean±SEM) 6.69±0.45, soy and guar gum with 7.13±0.57, chicken with 7.81±0.32 and 6.96±0.35 with guar gum, mycoprotein with 5.68±0.99 and 6.42±0.39 with guar gum. They were all categorised as good, except mycoprotein which was acceptable. By ethnicity, white Europeans found the food more appealing than south Asians with the 66% of the meals being good, 16% with very good and 16% acceptable compared to 50% of the meals being good and 50% acceptable. No statistical significances were found for protein, bread, ethnicity, or any interaction.

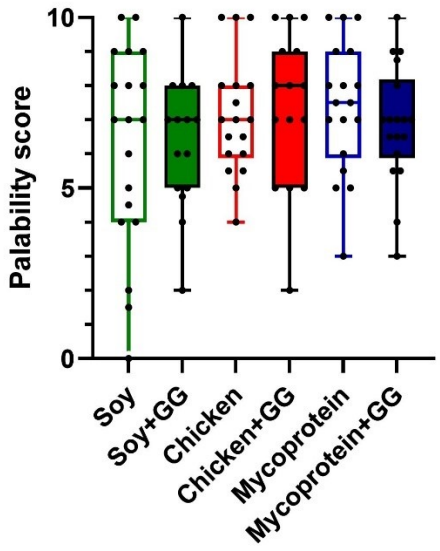


Figure 13. Palatability score following the consumption of soy, chicken and mycoprotein with or without guar gum for all subjects (N=21). Data represents mean±SEM and individual datapoints. Palatability score ranged from 1 (not good at all) to 10 (extremely palatable). The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the boxes represent the type of chapati, these being plain chapatis (empty boxes) and guar gum-enriched chapatis (filled boxes). GG, guar gum; n, sample size; SEM, standardise error of the mean.

Table 20. Palatability of test foods.									
	All (N=21)			White Europeans (n=11)			South Asians (n=10)		
	Mean	**SEM**	**Category**	**Mean**	**SEM**	**Category**	**Mean**	**SEM**	**Category**
Soy	6.69	0.45	Good	5.75	1.60	Acceptable	5.57	1.00	Acceptable
Soy+GG	7.13	0.57	Good	6.89	0.53	Good	5.82	0.54	Acceptable
Chicken	7.81	0.32	Good	7.05	0.55	Good	6.14	0.77	Good
Chicken+GG	6.96	0.35	Good	8.13	0.52	Very good	6.00	0.98	Acceptable
Mycoprotein	5.68	0.99	Acceptable	8.00	0.42	Good	7.50	0.51	Good
Mycoprotein+GG	6.42	0.39	Good	7.25	0.48	Good	6.54	0.50	Good
GG, guar gum; SEM, standard error of mean. 0-2, very poor; 2.1-4, poor; 4.1-6, acceptable; 6.1-8, good; 8.1-10, very good.									

5.4.4 Blood glucose

5.4.4.1 Blood glucose $iAUC_{0-180}$

The mean blood glucose $iAUC_{0-180}$ for each intervention for all participants is represented in Figure 14 and Appendix 11. Soy led to a blood glucose $iAUC_{0-180}$ of 399±38.91, soy+GG to 251.9±58.51, chicken to 567.4±59.22, chicken+GG to 313.0±33.22, mycoprotein to 435.6±49.75 and mycoprotein+GG to 257.2±41.71 mmol/L x min. A statistical significance for protein (p<0.01), bread (p<0.01) and ethnicity (p<0.01) was found. The effect of protein type was significantly decreased for mycoprotein relative to the chicken protein (mean [95% CI]) (-129.66 [-218.64, -40.68] mmol/L x min, p=0.001), and for soy relative to chicken (-159.87 [-248.85, -70.89], p<0.01). The effect of soy protein compared to mycoprotein was similar and was not statistically different (-30.21 [-111.33, +50.90] mmol/L x min, p=1.0). The effect of bread type on blood glucose $iAUC_{0-180}$ was significantly decreased for the guar gum-enriched type relative to the plain type (-182.71 [-238.9 1, -126.51] mmol/L x min, p<0.01). Ethnicity had a significant effect on blood glucose $iAUC_{0-180}$ where the south Asian group had a significant increase in blood glucose $iAUC_{0-180}$ relative to the white European group (+203.98 [84.51, 323.45] mmol/L x min, p<0.01).

No statistical significant difference was found for interaction between any of these factors (protein, bread and ethnicity).

All subjects (N=21)
Main effect of protein: p<0.01Main effect of bread: p<0.01
Main effect of ethnicity: p<0.01

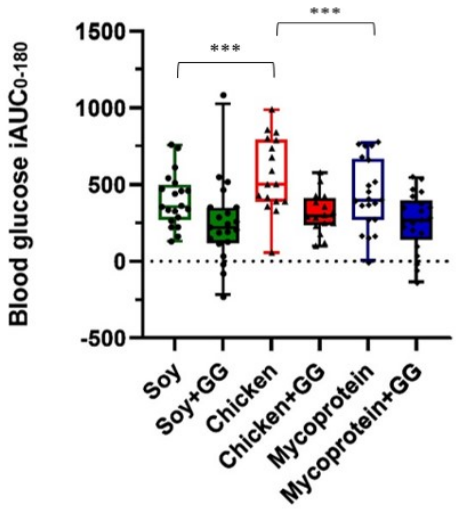


Figure 14. Blood glucose $iAUC_{0-180}$ following the consumption of soy, chicken and mycoprotein with or without guar gum for all subjects (N=21). Data represents median±5-95%

percentile and individual datapoints. For all subjects, the analysis was performed for 20,21,17,16,21 and 21 subjects for soy, soy+GG, chicken, chicken+GG, mycoprotein and mycoprotein + GG.The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the boxes represent the type of chapati, these being plain chapatis (empty boxes) and guar gum-enriched chapatis (filled boxes). ***p-value≤0.001. iAUC, incremental area under the curve; GG, guar gum; n, sample size.

The mean blood glucose $iAUC_{0-180}$ for each intervention by ethnicity is represented in Appendix 12. No statistical significant difference was found for interaction between any of these factors (protein, bread and ethnicity).

The individual percentages of change in blood glucose $iAUC_{0-180}$ induced by the addition of guar gum to each protein type is illustrated in Figure 15. The statistical analysis showed that there was an ethnicity main effect (p=0.04), in which south Asians had a significant increase in the percentage change of blood glucose $iAUC_{0-180}$ by +30.88% induced by the addition of guar gum, compared to white Europeans (p=0.03). There was no interaction between protein and ethnicity.

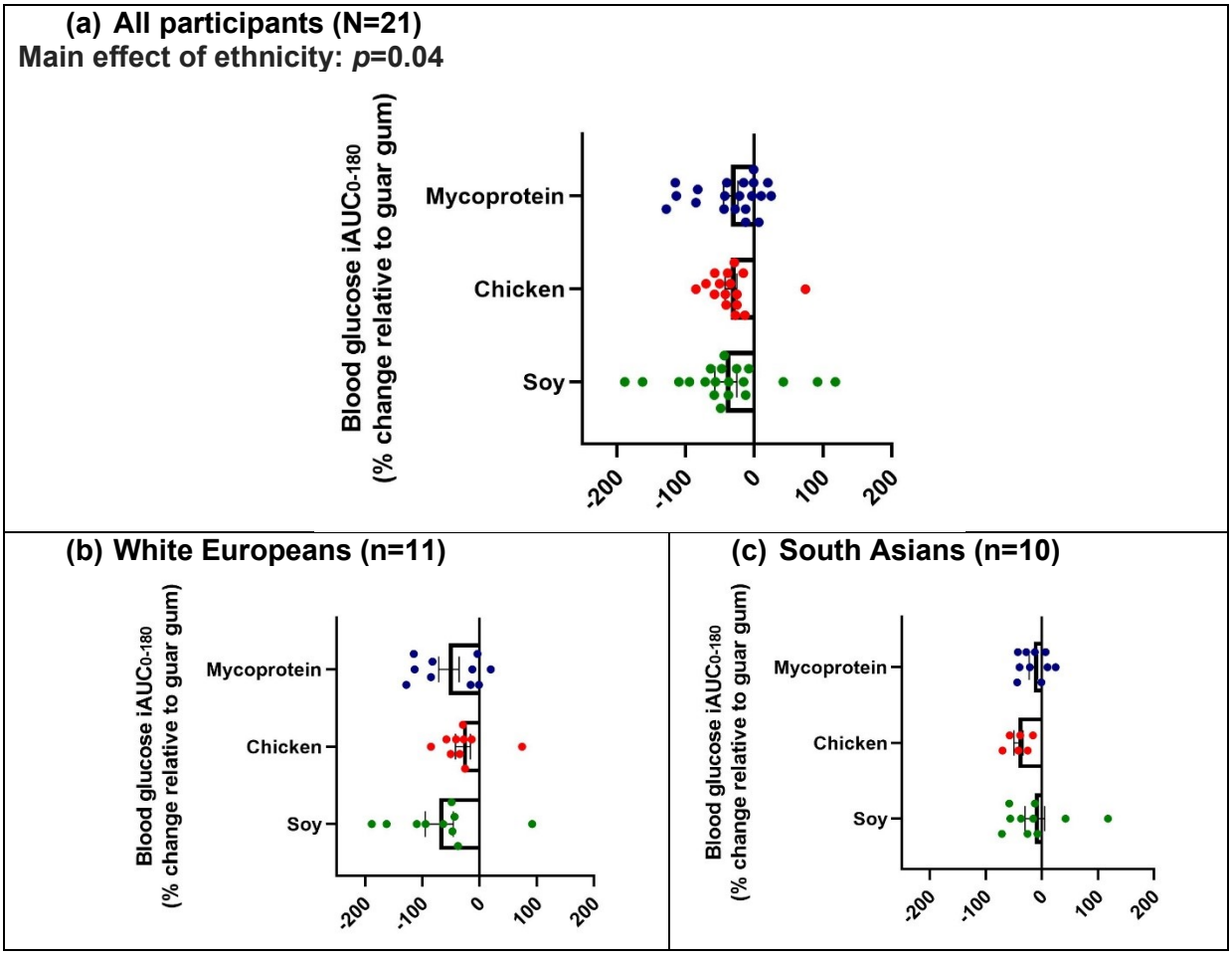


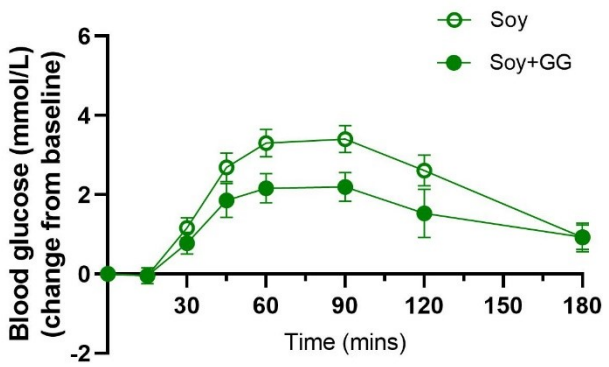
Figure 15. Change of blood glucose $iAUC_{0-180}$ relative to the guar gum-enriched chapati per each type of protein tested. (a) All participants (N=21), (b) white Europeans (n=11), (c) south Asians (n=10). Data are represented as mean±SEM and individual datapoints. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). SEM, standard error of the mean; n, sample size.

5.4.4.2 Blood glucose over time

The postprandial blood glucose changes from baseline up to 180 min are represented in Figure 16. The mixed model revealed an effect of protein (p<0.01), bread (p<0.01), ethnicity (p<0.01), interaction between protein and bread (p<0.01), protein, bread and ethnicity (p=0.04), timepoint (p<0.01), protein and timepoint (p=0.02), bread and timepoint (p<0.01), and of ethnicity and timepoint (p<0.01). For the main effect of protein, soy and mycoprotein had a significant reducing effect of -0.76 and -0.75 mmol/L compared to chicken (p<0.01), whereas for soy and mycoprotein were no statistically different. For the main effect of bread, guar gum-enriched chapati had a significant lowering effect of -0.90 mmol/L compared to plain chapati (p<0.01). For the ethnicity effect, south Asians had a significantly increase of +0.90 mmol/L compared to white Europeans (p<0.01). There was no interaction between protein, bread and timepoint.

All participants (N=21)
Main effect of protein: *p*<0.01
Main effect of bread: *p*<0.01
Main effect of ethnicity: *p*<0.01
Main effect of timepoint: *p*<0.01
Main interaction effect of protein*bread: *p*<0.01
Main interaction effect of protein*bread*ethnicity: *p*=0.04
Main interaction effect of protein*timepoint: *p*=0.02
Main interaction effect of bread*timepoint: *p*<0.01
Main interaction effect of ethnicity*timepoint: *p*<0.01

(a) Soy



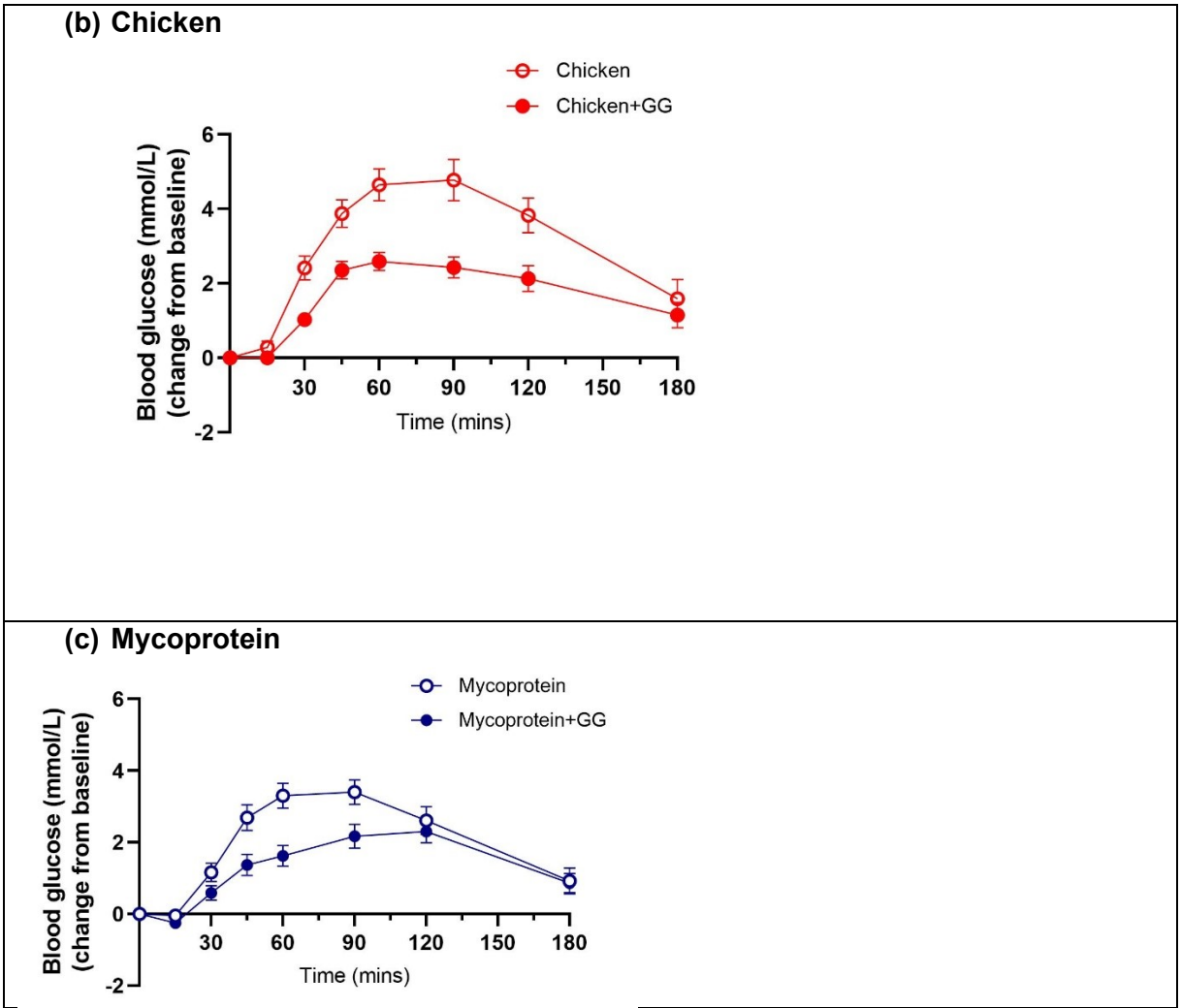


Figure 16. Blood glucose over 180 min following the consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). Change from baseline of postprandial blood glucose over time up to 180 min following the ingestion of (a) soy, (b) chicken, and (c) mycoprotein with and without guar gum in all participants (N=21). Test food was given at timepoint 0 min. Data represents mean±SEM. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the circles represent the type of chapati, these being plain chapatis (empty circles) and guar gum-enriched chapatis (filled circles). GG, guar gum; SEM, standard error of the mean; n, sample size.

The postprandial blood glucose changes from baseline up to 180 min for white Europeans and south Asians are represented separately in Figure 17. Overall, the addition of guar gum led to lower postprandial responses of blood glucose over time compared to the proteins alone in both ethnic groups. Notably, white Europeans are closer to returning to baseline blood glucose levels at minute 180 min compared to south Asians. As mentioned before, there was no interaction between protein, bread, ethnicity and timepoint.

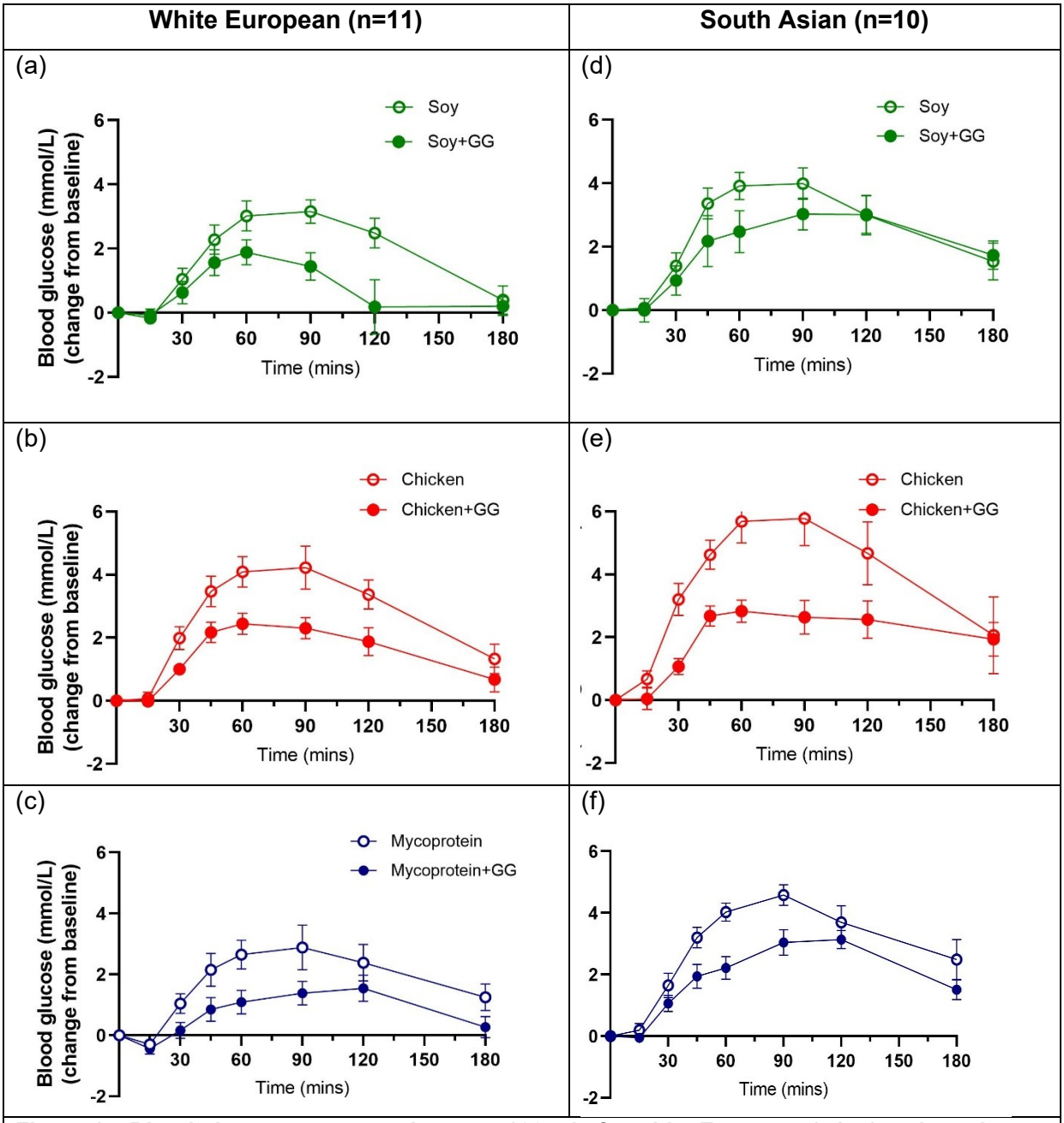
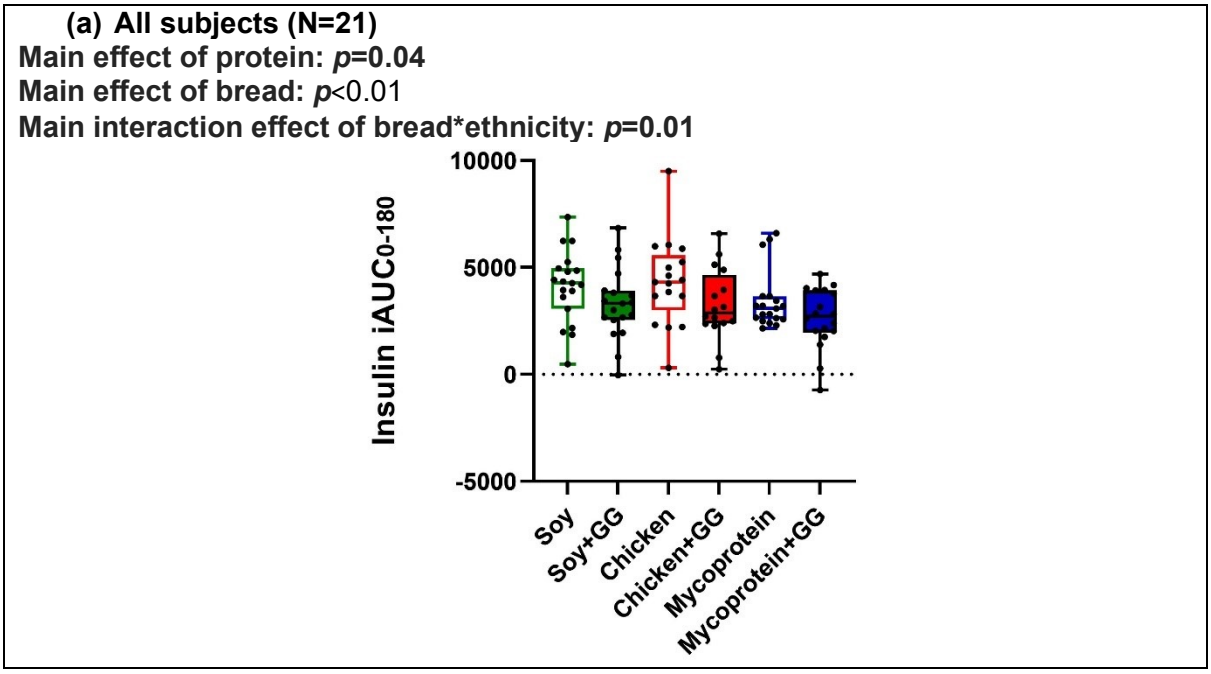


Figure 17. Blood glucose concentration over 180 min for white European (a,b,c) and south Asian (d,e,f) following the consumption of soy, chicken and mycoprotein with or without guar gum. Change from baseline of blood glucose over time up to 180 min following the ingestion of (a) soy, (b) chicken, and (c) mycoprotein with and without guar gum in white Europeans and (d) soy, (e) chicken, and (f) mycoprotein in south Asians. Test food was given at timepoint 0 min. Data represents mean±SEM. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the circles represent the type of chapati, these being plain chapatis (empty circles) and guar gum-enriched chapatis (filled circles). GG, guar gum; SEM, standard error of the mean; n, sample size.

5.4.5 Postprandial insulin

5.4.5.1 Postprandial insulin iAUC

The insulin $iAUC_{0\text{-}180}$ for all participants is represented in Figure 18. Soy led to insulin iAUC0-180 min of 4103±385.7, soy+GG to 3297±379.2, chicken to 4324±495.7, chicken+GG to 3247±420.6, mycoprotein to 3435±312.5 and mycoprotein+GG to 2628±332.7 mmol/L x min. A statistical significance for protein (p<0.01), bread (p<0.01) and interaction between bread and ethnicity (p=0.01) was found. For the effect of protein, post-hoc analysis for multiple comparison using Bonferroni showed no statistical differences between proteins. For the effect of bread, guar gum-enriched chapati led to a decrease in insulin $iAUC_{0\text{-}180}$ by -897.67 [95% CI -1377.23, -418.11] mmol/L x min (p<0.01). For the bread and ethnicity interaction effect, post-hoc analysis for multiple comparison showed a statistical decrease by -1888.44 [95% CI -3103.27, -673.60] mmol/L x min with guar-gum enriched chapati in the south Asian group (p<0.01).



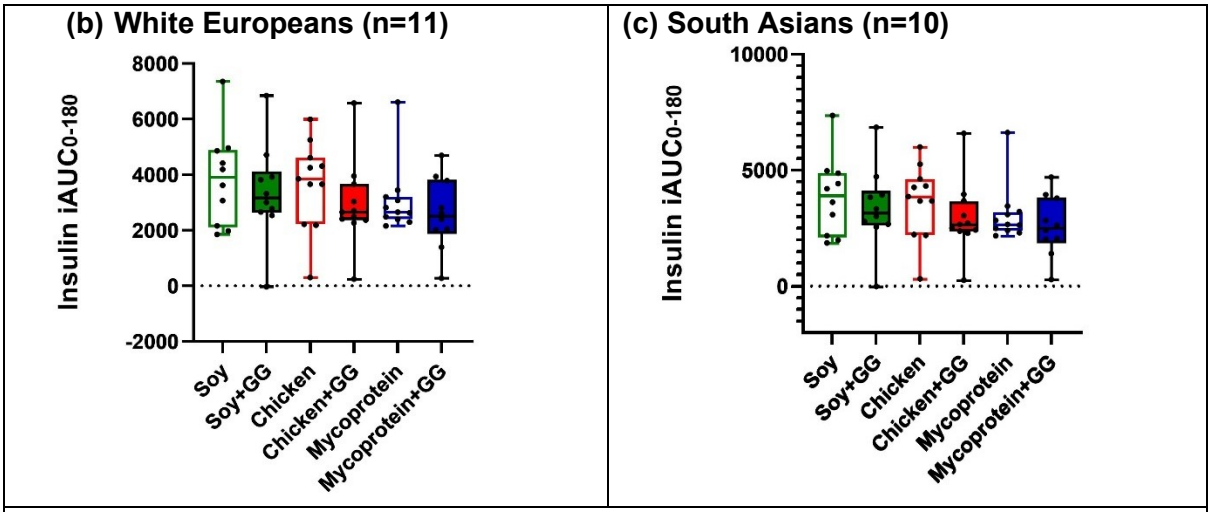


Figure 18. Insulin $iAUC_{0-180}$ following the consumption of soy, chicken and mycoprotein with or without guar gum. (a) All subjects (N=21), (b) White Europeans (n=11), (c) South Asians (n=10). Data represents median±5-95% percentile and individual datapoints. For all subjects, the analysis was performed for 19,19,17,16,19 and 19 subjects for soy, soy+GG, chicken, chicken+GG, mycoprotein and mycoprotein + GG. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the boxes represent the type of chapati, these being plain chapatis (empty boxes) and guar gum-enriched chapatis (filled boxes). iAUC, incremental area under the curve; GG, guar gum; SEM, standard error of the mean; n, sample size.

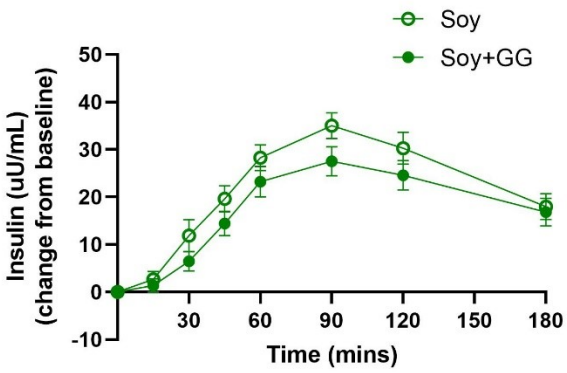
Homeostatic model assessment for insulin resistance prior to each study visit was also calculated and reported in Appendix Figure 13. The results showed that there was an ethnicity significant main effect ($p<0.01$), in which south Asians had a difference of -2.43 units compared to white Europeans. The average values for each ethnicity were: 5.32 (white European) and 2.89 (south Asian). The relationship between the glucose $iAUC_{0-180}$ and their insulin resistant state prior the visit was analysed using a repeated measure correlation and is shown in Appendix Figure 14. The results showed that there was no significant correlation.

5.4.4.2 Postprandial insulin over time

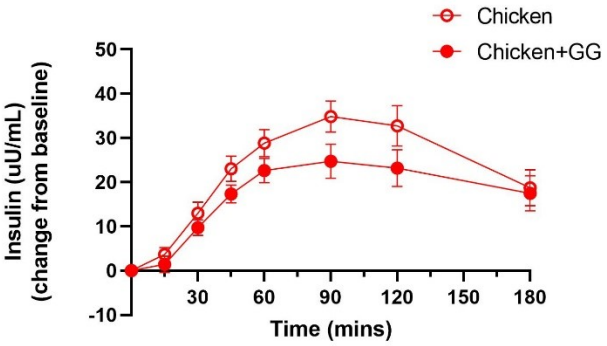
The insulin changes from baseline up to 180 min for soy, chicken and mycoprotein with and without guar gum are represented in Figure 19. Notably, overall individuals do not return to baseline insulin levels at minute 180 min, suggesting poor insulin clearance capacity. The mixed model revealed an effect of protein (p<0.01), bread (p<0.01), and an interaction between ethnicity and bread (p<0.01).

All participants (N=21)
Main effect of protein: *p*<0.01
Main effect of bread: *p*<0.01
Main interaction effect of ethnicity*bread: *p*<0.01

(a) Soy



(b) Chicken



(c) Mycoprotein

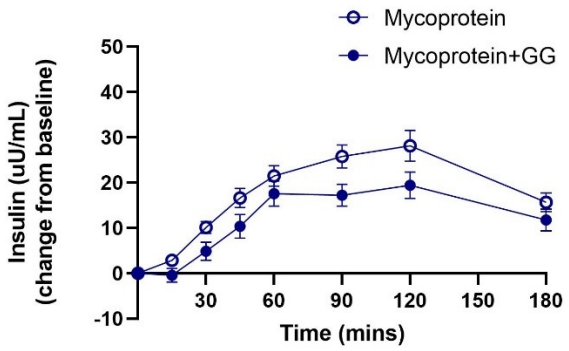


Figure 19. Insulin change from baseline over 180 min following the consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). Change from baseline of insulin over time up to 180 min following the ingestion of (a) soy, (b) chicken, and (c) mycoprotein with and without guar gum in all participants (N=21).Test food was given at timepoint 0 min. Data represents mean±SEM. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the circles represent the type of chapati, these being plain chapatis (empty circles) and guar gum-enriched chapatis (filled circles). SEM, standard error of the mean; GG, guar gum; n, sample size.

5.4.4.3 First-phase insulin secretion

The first-phase insulin secretion (insulin $iAUC_{0-30}$) was also analysed to determine whether the differences in blood glucose between ethnicities and proteins were due to differences in first insulin secretion output. Soy led to insulin iAUC0-30 min of 130.1±48.18, soy+GG to 70.46±33.13, chicken to 152.6±35.47, chicken+GG to 94.37±37.69, mycoprotein to 119±20.20 and mycoprotein+GG to 33.63±39.13 mmol/L x min. Figure 20 and Appendix 13 summarise the findings for the first-phase insulin secretion following the test meals intake. The mixed model revealed an effect of bread (p<0.01) only. For the bread effect, guar gum-enriched chapati induced a significant decrease of -66.23 mmol/L x min compared to plain chapati.

All participants (N=21)
Main effect of bread: *p*<0.01

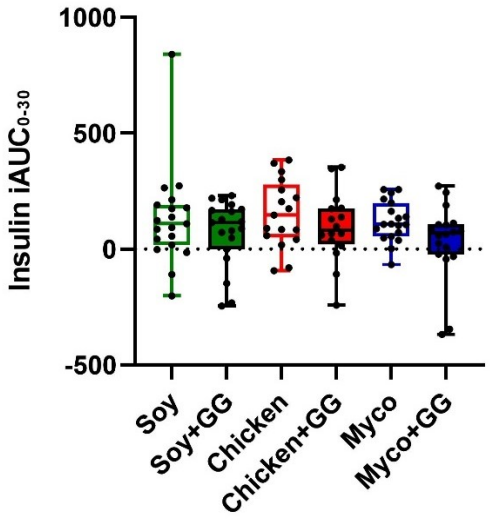


Figure 20. Insulin $iAUC_{0-30}$ following the consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). Data represents median±5-95% percentile and individual datapoints. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the boxes represent the type of chapati, these being plain chapatis (empty boxes) and guar gum-enriched chapatis (filled boxes). iAUC, incremental area under the curve; GG, guar gum; SEM, standard error of the mean; n, sample size.

5.4.4.4 Insulin sensitivity index (ISI 0,120)

Insulin sensitivity index (ISI0,120) is an insulin sensitivity surrogate measure using oral glucose tolerance test postprandial values which correlates with the euglycaemic hyperinsulinaemic clamp (r=0.63, p<0.01) developed by Gutt and team (Gutt *et al.*, 2000). ISI is calculated in this study as the cohort of study are heterogenous in the medication they use (e.g. 3 participants take sulfonylureas which indicate that participant have a greater degree of impairment in insulin secretion compared to the other participants). For this reason, glucose and insulin changes are looked at together via ISI 0,120 (Figure 12). It is not expected to observe differences in insulin sensitivity as this is an acute study so it is highly unlikely to observe physiological improvements in insulin sensitivity which are often linked to chronic lifestyle interventions. The results showed a significant main effect of bread (p<0.01) in which the guar gum-enriched chapati had a greater increase in ISI 0,120 of +0.03 compared to plain chapati (p=0.01).

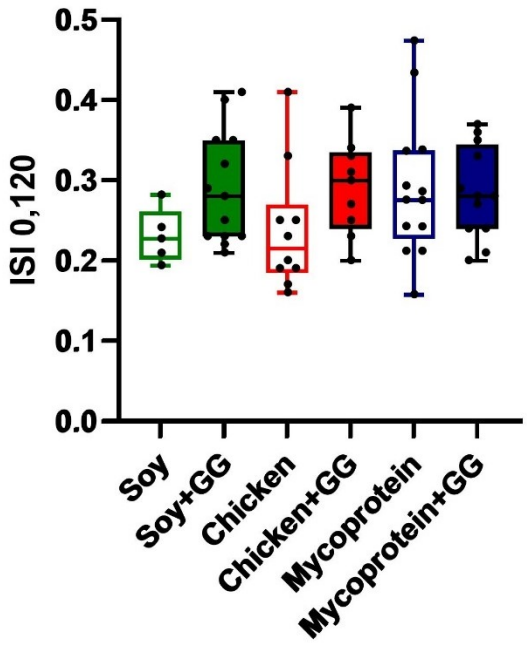


Figure 21. ISI 0,120 following consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). Data represents median±5-95% percentile and individual datapoints. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the boxes represent the type of chapati, these being plain chapatis (empty boxes) and guar gum-enriched chapatis (filled boxes). GG, guar gum; ISI 0,120: insulin sensitivity index 0,120; n, sample size. Missing values: 16 for soy, 8 for soy+GG, 11 for chicken, 12 for chicken + GG, 8 for mycoprotein, 9 for mycoprotein+GG.

5.4.6 GLP-1

The GLP-1 total $AUC_{0\text{-}180}$ for all participants is represented in Figure 22 and described in Appendix 14. Soy led to GLP-1 total iAUC0-180 min of 7607±627.5, soy+GG to 8055±794.0, chicken to 6736±611.9, chicken+GG to 6807±815.8, mycoprotein to 6914±674.1 and mycoprotein+GG to 7912±840.5 mmol/L x min. No statistical significances were found for protein, bread, ethnicity or any interaction.

All participants (N=21)

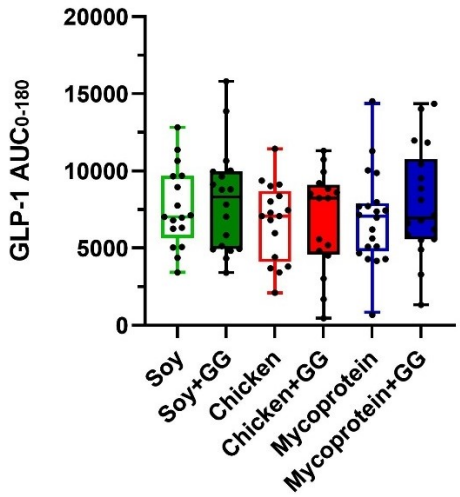
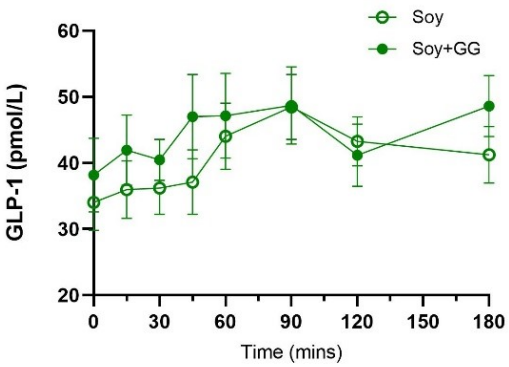


Figure 22. GLP-1 total AUC_{0-180} following consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). Data represents median±5-95% percentile and individual datapoints. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the boxes represent the type of chapati, these being plain chapatis (empty boxes) and guar gum-enriched chapatis (filled boxes). AUC, area under the curve; GLP-1, glucagon-like peptide-1; n, sample size.

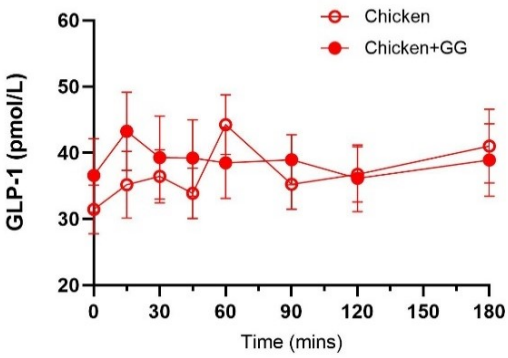
The postprandial GLP-1 changes over time up to 180 min are represented in Figure 23. The mixed model revealed an effect of timepoint (p=0.0008).

All participants (N=21)
Main effect of timepoint: p=0.0008

(a) Soy



(b) Chicken



(c) Mycoprotein

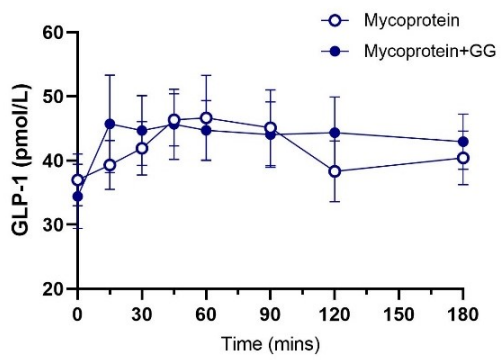


Figure 23. GLP-1 concentration following consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). GLP-1 levels over 180 min following the ingestion of (a) soy, (b) chicken and (c) mycoprotein with or without guar gum, respectively. Test food was given at timepoint 0 min. Data represents mean±SEM The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the circles represent the type of chapati, these being plain chapatis (empty circles) and guar gum-enriched chapatis (filled circles). AUC, area under the curve; GLP-1, glucagon-like peptide-1; SEM, standard error of the mean; n, sample size.

5.4.7 PYY

The PYY total $AUC_{0\text{-}180}$ for all participants and by ethnicity is represented in Figure 23 and described in Appendix 15. Soy led to PYY total iAUC0-180 min of 5537±1057, soy+GG to 5876±874.6, chicken to 5391±852.8, chicken+GG to 4536±998.6, mycoprotein to 5805±1518 and mycoprotein+GG to 5434±1074 mmol/L x min. There was a statistical interaction effect of protein and ethnicity (p=0.02). Post-hoc analysis showed no further statistical differences for any protein and ethnicity combination.

(a) All participants (N=21)
Main interaction effect of protein*ethnicity: p=0.02

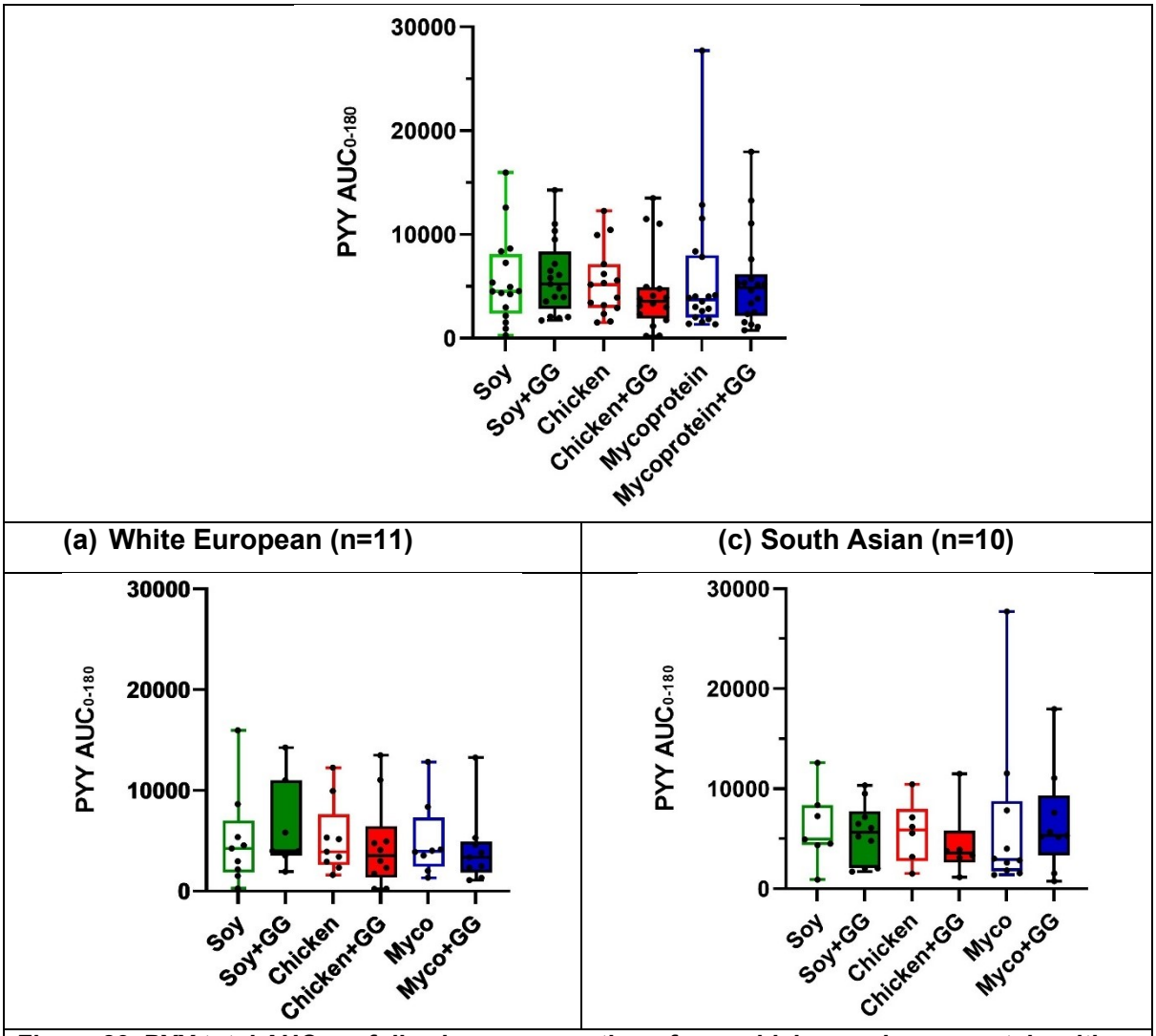


Figure 23. PYY total $AUC_{0\text{-}180}$ following consumption of soy, chicken and mycoprotein with or without guar gum. PYY total $AUC_{0\text{-}180min}$ following ingestion of soy, chicken and mycoprotein with or without guar gum, for (a) all participants (N=21), (b) white Europeans (n=11), (c) south Asians (n=10). Data represents median±5-95% percentile and individual datapoints. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the boxes represent the type of chapati, these being plain chapatis (empty boxes) and guar gum-enriched chapatis (filled boxes). AUC, area under the curve; PYY, peptide tyrosine tyrosine; n, sample size.

The postprandial PYY changes over time up to 180 min is represented in Figure 24. The mixed model revealed an effect of protein (p=0.03). For the protein effect, mycoprotein showed a significant increased by +4.7 [+0.12, +9.44] compared to chicken (p=0.04). For the protein and ethnicity interaction, in white Europeans chicken significantly decreased by -7.17 pmol/L compared to soy (p<0.01) and mycoprotein also significantly decreased by -8.12 pmol/L compared to soy (p<0.01).

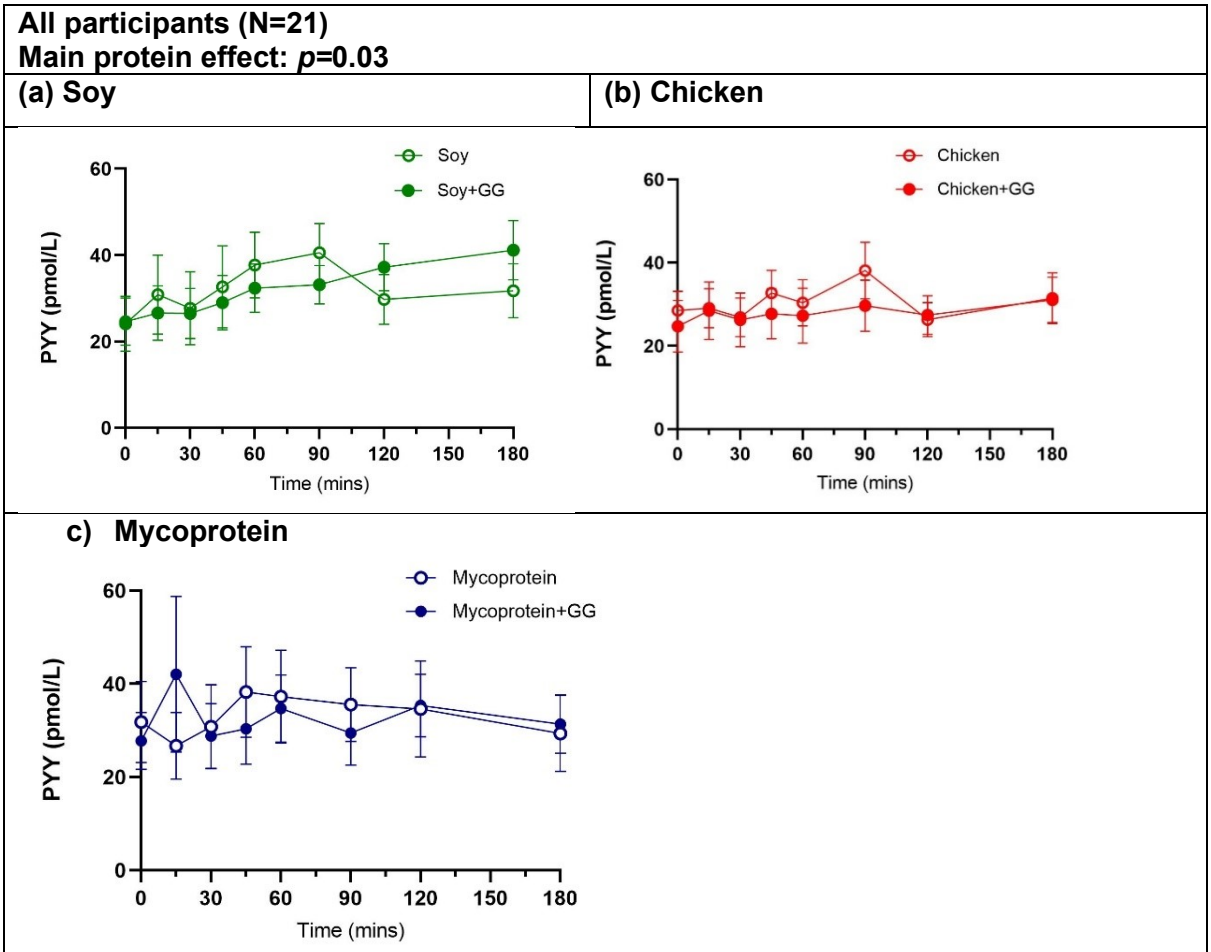


Figure 24. PYY concentration following consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). PYY levels over 180 min following the ingestion of (a) soy, (b) chicken and (c) mycoprotein with or without guar gum, respectively. Test food was given at timepoint 0 min. Data represents mean±SEM. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the circles represent the type of chapati, these being plain chapatis (empty circles) and guar gum-enriched chapatis (filled circles). AUC, area under the curve; PYY, peptide tyrosine tyrosine; SEM, standard error of the mean; n, participants.

5.4.8 Visual analogue scales

5.4.8.1 Composite appetite score (CAS)

Composite appetite score (CAS) measures [appetite for a meal+ desire to eat + hungry + (100-fullness)]/4 $AUC_{0\text{-}180min}$ and score over time are represented in Figure 25. Soy led to a CAS $AUC_{0\text{-}180min}$ of 6351±585, soy+GG to 6383±668.8, chicken to 6191±591.4, chicken+GG to 6731±755.4, mycoprotein to 6983±743.8 and mycoprotein+GG to 6316±683.4 mmol/L x min. No statistical significance was found for protein, bread, ethnicity, or interaction.

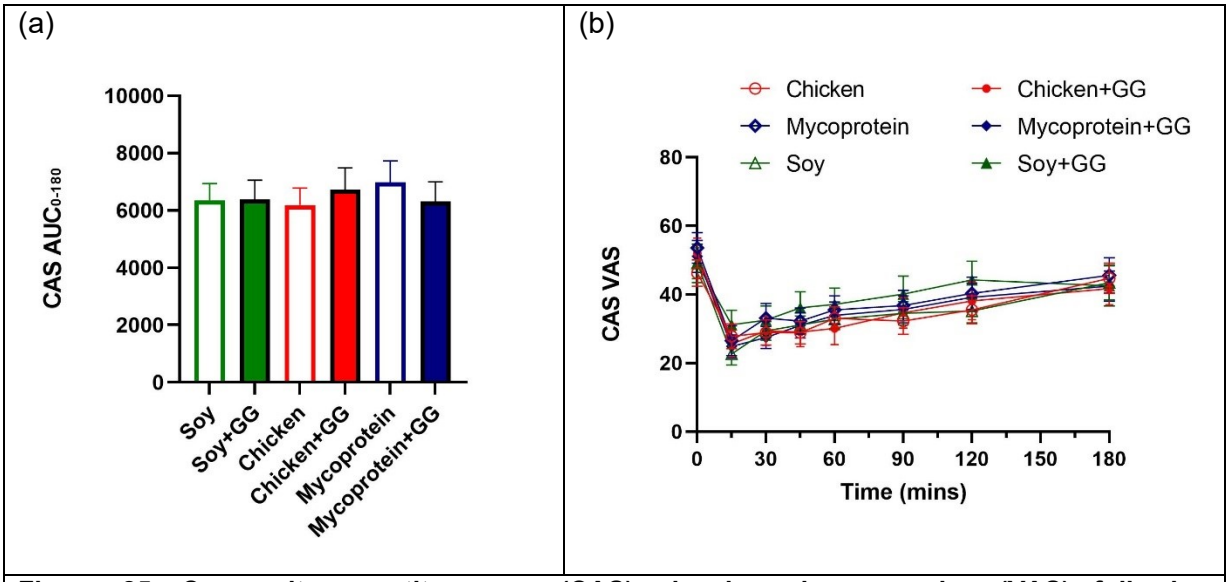


Figure 25. Composite appetite score (CAS) visual analogue scales (VAS) following consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). (a) Area under the curve (AUC) of the change from baseline 0-180 min of the composite appetite score (CAS) following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents median±5-95% percentile and individual datapoints. (b) Change from baseline composite appetite score (CAS) of visual analogue scale (VAS) over 180 min following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents mean±SEM. Test food was given at timepoint 0 min. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). In the timeline graph, the geometry represents the type of proteins, these being soy (triangle), chicken (circle), and mycoprotein (square). The filling of the geometric datapoints represent the type of chapati, these being plain chapatis (empty) and guar gum-enriched chapatis (filled). AUC, area under the curve; CAS, composite appetite score; VAS, visual analogue scale; n, sample size.

5.4.8.2 Sickness score

	A	B	C	D	E	F	G
	Soy	Soy+GG	Chicken	Chicken+GG	Mycoprotein	Mycoprotein+GG	
1	Number of values	20	18	18	17	20	20	
2							
3	Minimum	0.000	0.000	0.000	0.000	0.000	0.000	
4	25% Percentile	0.000	0.000	0.000	0.000	0.000	0.000	
5	Median	0.000	0.000	0.2500	0.000	0.5000	0.000	
6	75% Percentile	2.875	4.750	4.125	4.750	3.375	2.500	
7	Maximum	25.50	32.00	41.00	22.50	21.00	34.00	
8	Range	25.50	32.00	41.00	22.50	21.00	34.00	
9								
10	Mean	3.325	4.139	4.778	4.118	2.675	4.225	
11	Std. Deviation	7.164	8.426	10.78	7.369	5.386	9.227	
12	Std. Error of Mear	1.602	1.986	2.541	1.787	1.204	2.063	
13								
14								
15								
16								
17								
18								

Sickness $AUC_{0\text{-}180min}$ and score over time represented in Figure 26. Soy led to sickness iAUC0-180 min of 3.32±1.60, soy+GG to 4.13±1.60, chicken to 4.77±2.54, chicken+GG to 4.11±1.78, mycoprotein to 2.67±1.20 and mycoprotein+GG to 4.22±2.06 mmol/L x min. No statistical significance was found for protein, bread, ethnicity, or interaction.

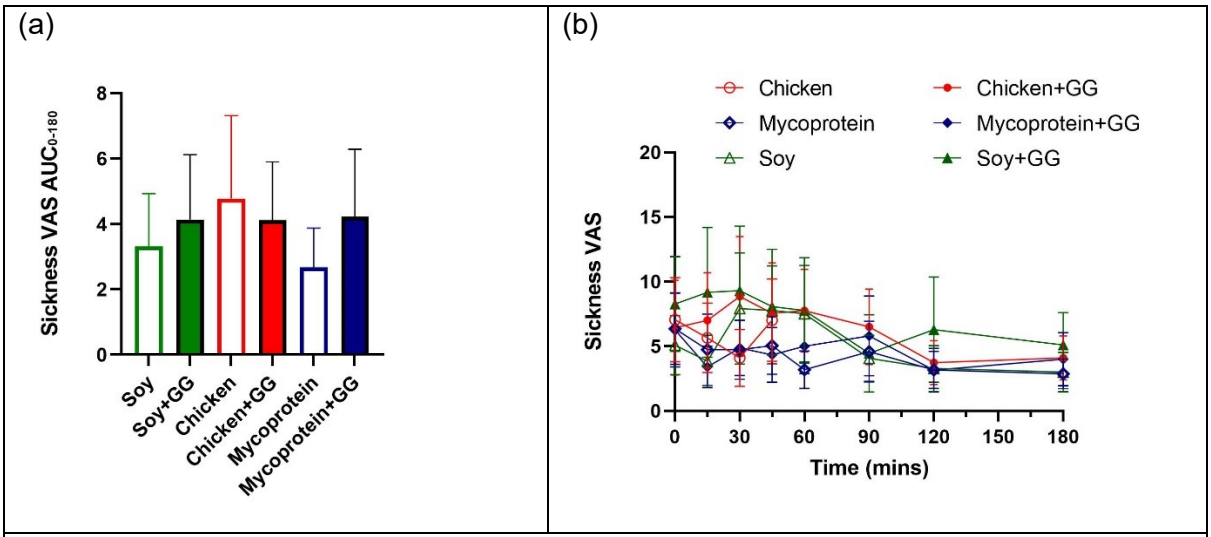


Figure 26. Sickness VAS following consumption of soy, chicken and mycoprotein with or

without guar gum for all participants (N=21). (a) Area under the curve (AUC) of the change from baseline 0-180 min of the sickness VAS following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents median±5-95% percentile and individual datapoints. (b) Change from baseline sickness VAS over 180 min following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents mean±SEM. Test food was given at timepoint 0 min. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). In the timeline graph, the geometry represents the type of proteins, these being soy (triangle), chicken (circle), and mycoprotein (square). The filling of the geometric datapoints represent the type of chapati, these being plain chapatis (empty) and guar gum-enriched chapatis (filled). AUC, area under the curve; VAS, visual analogue scale; n, sample size.

5.4.8.3 Hungry score

Hungry $AUC_{0\text{-}180min}$ and score over time represented in Figure Appendix 16. No statistical significance was found for protein, bread, ethnicity or interaction.

5.4.8.4 Desire to eat score

Desire to eat $AUC_{0\text{-}180min}$ and score over time represented in Figure Appendix 17. No statistical significance was found for protein, bread, ethnicity or interaction.

5.4.8.5 Appetite for a meal score

Appetite for a meal $AUC_{0\text{-}180min}$ and score over time are represented in Figure Appendix 18. No statistical significance was found for protein, bread, ethnicity, or interaction.

5.4.8.6 Appetite for a sweet score

Descriptive statistics	A Soy	B Soy+GG	C Chicken	D Chicken+GG	E Mycoprotein	F Mycoprotein+GG	G
1 Number of values	20	19	18	17	19	20	
2							
3 Minimum	5.625	0.000	0.000	0.000	0.000	7.500	
4 Maximum	16058	15176	14736	15555	16155	15454	
5 Range	16052	15176	14736	15555	16155	15447	
6							
7 Mean	2573	2875	3449	3080	3541	3776	
8 Std. Deviation	3946	4470	4546	4672	4912	4657	
9 Std. Error of Mean	882.4	1026	1071	1133	1127	1041	
10							
11 Coefficient of variatior	153.4%	155.5%	131.8%	151.7%	138.7%	123.3%	
12							
13							

Appetite for a sweet $AUC_{0\text{-}180min}$ and score over time are represented in Figure 27. Soy led to appetite for a sweet AUC0-180 min of 2573±882.4, soy+GG to 2875±1026, chicken to

3449±1071, chicken+GG to 3080±1133, mycoprotein to 3541±1127 and mycoprotein+GG to 3776±1041 mmol/L x min. The only statistical significance was found for protein effect (p<0.01), where soy induced a significant decrease by -768.93 and -896.80 mm x min, compared to chicken and mycoprotein, respectively.

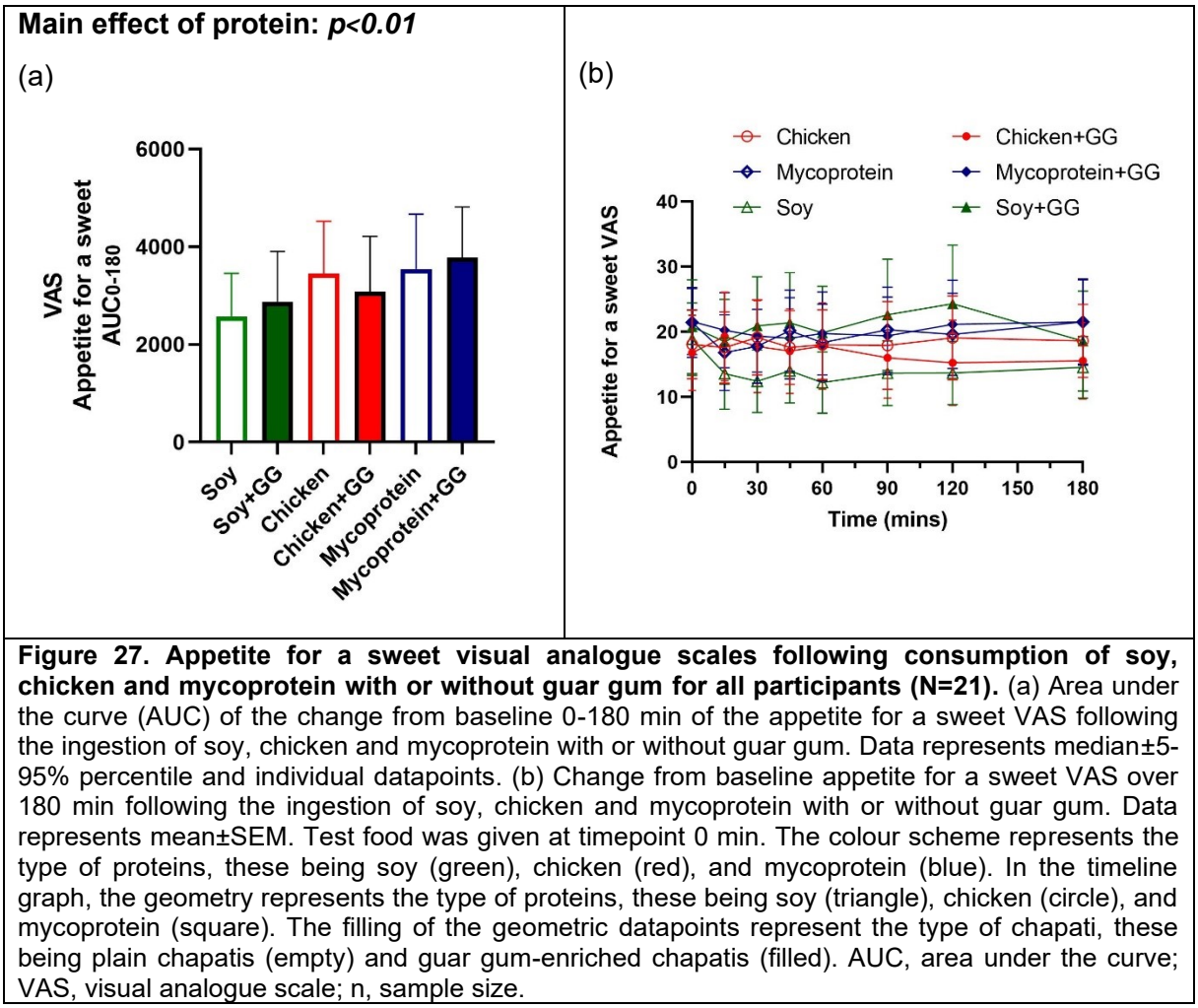


Figure 27. Appetite for a sweet visual analogue scales following consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). (a) Area under the curve (AUC) of the change from baseline 0-180 min of the appetite for a sweet VAS following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents median±5-95% percentile and individual datapoints. (b) Change from baseline appetite for a sweet VAS over 180 min following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents mean±SEM. Test food was given at timepoint 0 min. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). In the timeline graph, the geometry represents the type of proteins, these being soy (triangle), chicken (circle), and mycoprotein (square). The filling of the geometric datapoints represent the type of chapati, these being plain chapatis (empty) and guar gum-enriched chapatis (filled). AUC, area under the curve; VAS, visual analogue scale; n, sample size.

5.4.8.7 Fullness score

Fullness $AUC_{0\text{-}180min}$ and score over time are represented in Figure Appendix 19. No statistical significance was found for protein, bread, ethnicity, or interaction.

5.4.9 Energy intake

The effect of the test meals on *ad libitum* energy intake is illustrated in Figure 28.a and Appendix 19. Soy led to an *ad libitum* energy intake (kcal) of 721.9±109.0, soy+GG to 748.0±72.37, chicken to 699.1±68.93, chicken+GG to 730.1±95.60, mycoprotein to

640.0±65.57 and mycoprotein+GG to 689.1±83.10. No statistical difference for protein, bread, ethnicity, or interaction was found. The effect of the test meals on post-visit energy intake is illustrated in Figure 28.b and on Appendix 20. Soy led to a post-visit energy intake (kcal) of 2038±165.1, soy+GG to 1893±193.8, chicken to 1834±150.0, chicken+GG to 2024±156.2, mycoprotein to 1738±156.6 and mycoprotein+GG to 1927±174.3. No statistical difference for protein, bread, ethnicity, or interaction was found.

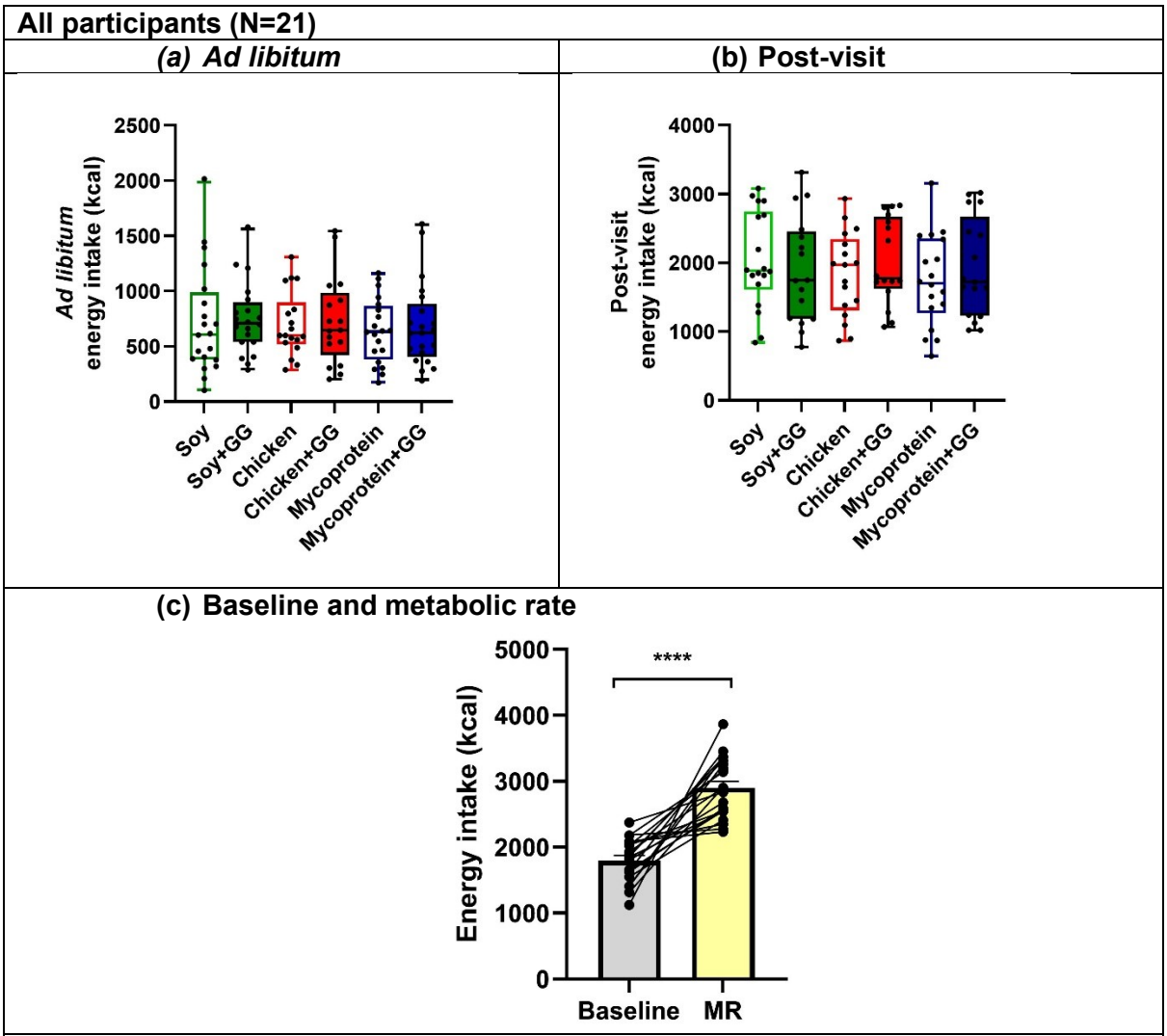


Figure 28. *Ad libitum* and post-visit energy intake following consumption of soy, chicken and mycoprotein with or without guar gum for all participants and baseline and metabolic rate (N=21). (a) *Ad libitum* energy intake and (b) post-visit energy intake per day following test meals, (c) energy intake reported at baseline prior first study visit and the energy intake required based on metabolic rate and physical activity level calculated using the Schofield equation. Data represents median±5-95% percentile and individual datapoints for (a) and (b). Data represents mean±SEM and individual datapoints for (c). Kcal, kilocalorie; MR, metabolic rate; SEM, standard error of the mean; n, sample size. ****p≤0.0001.

The average effect and statistical effect of the test meals on macronutrient and nutrient intake post-visit is described in Table 21. There was a statistically significant interaction between bread and ethnicity for energy from carbohydrate (%) (p=0.01) and for energy from protein (%) (p<0.01). Post-hoc multiple comparison analysis showed no significantly differences between any combination of bread and ethnicity for both energy from protein and carbohydrate.

Table 21. Post-visit dietary intake of participants per day.

		Energy (kcal)	CHO (%)	Fat (%)	Protein (%)	Fibre (g)
Soy	**Mean**	2038.15	31.02	25.55	10.44	20.44
	SEM	165.07	1.43	1.36	0.41	2.04
Soy+GG	**Mean**	1892.67	30.52	26.23	10.82	15.49
	SEM	193.76	1.39	1.43	1.05	2.01
Chicken	**Mean**	1833.80	31.96	24.38	10.28	21.44
	SEM	150.01	1.46	1.05	0.46	2.17
Chicken+GG	**Mean**	2024.46	30.77	25.49	10.77	21.59
	SEM	156.15	1.60	1.36	0.61	2.40
Mycoprotein	**Mean**	1737.89	29.25	26.05	11.82	17.75
	SEM	156.60	2.15	1.65	0.66	2.41
Mycoprotein+GG	**Mean**	1926.75	32.67	24.32	11.28	21.50
	SEM	174.35	1.63	1.50	0.71	1.87

CHO, carbohydrate; g, grams; GG, guar gum; kcal, kilocalories; SEM, standard error of the mean; NSSD, non-statistically significant different.

5.5 Discussion

In this study the effects of mycoprotein on blood glucose and appetite were investigated for the first time in people with T2D of south Asian and white European ethnicities.

5.5.1 Summary of findings

- There were no differences of tests foods on palatability, and overall, test foods were widely accepted amongst participants and were rated as “good”.
- For postprandial blood glucose:
    - Mycoprotein and soy led to a similar decreased response in postprandial blood glucose compared to chicken.
    - Guar gum-enriched chapati decreased postprandial blood glucose compared to plain chapati.
    - Ethnicity had an effect on blood glucose, in which the south Asian group had a higher postprandial blood glucose compared to white Europeans.
    - There was an effect of ethnicity on the percentage of blood glucose reduction with guar gum, in which guar gum induced in south Asians a significant increase compared to white Europeans.
    - No effect of interaction between protein, bread and ethnicity on postprandial blood glucose.
- For insulin response:
    - There was an effect of protein on postprandial insulin response but post-hoc analysis showed no differences.
    - Guar gum-enriched chapati decreased postprandial insulin response compared to plain chapati.
    - There was an interaction between bread and ethnicity, where guar gum-enriched chapati decreased postprandial insulin response in the south Asian.
    - There was a decreasing effect of guar gum-enriched chapati on the first-phase insulin response.
- For appetite feelings:
    - There was no effect of protein, bread, ethnicity, or interaction for the visual analogue scale feelings of hunger, fullness, desire to eat, appetite for a meal and sickness.

    - There was an effect of protein on appetite for a sweet, in which soy induced a significant decrease compared to chicken and mycoprotein.
    - There was no effect on GLP-1 secretion.
    - There was an interaction effect of protein and ethnicity on PYY secretion but multiple comparisons showed no effect.
- For food intake:
    - There was no effect on *ad libitum* energy intake.
    - There was no effect on post-visit energy intake.
    - There was an interaction effect of bread and ethnicity on percentage of energy from carbohydrate and protein.

5.5.2 Detailed discussion

In this study, 21 participants with T2D were recruited and ask to consume 3 protein types with chapati with or without guar gum acutely.

5.5.2.1 The effect of mycoprotein and guar gum on palatability

The results on palatability showed no effect of protein, bread or ethnicity, nor interaction on palatability, and overall, test foods were widely accepted amongst participants and were rated as “good”. The greatest score was for chicken and the lower score was for mycoprotein.

Considering that south Asians are widely chapati consumers (60% of participants consumed an average of 2 units a day), and it has been reported to be a staple food in their culture, it is of value to use guar gum-enriched chapatis as a method to increase this populations’ fibre intake by 5 g. Furthermore, the inclusion of the test meals into their daily diets constitutes an addition of 19 g of fibre into their daily diets, representing 63% of the DRI (30 g/day, according to WHO) provided in one single meal. About <10% of the UK adults, irrespective of ethnicity, consume 30 g a day (SACN, 2021). The latest evidence on UK-free living population dietary intake suggests that a small increase in fibre intake over the last years have occurred partly due to increases in whole foods such as brown rice and pasta (Greisser *et al*., 2021). This is suggestive that people tend to increase fibre from western-looking foods. The test foods provided in the present study are western-looking foods that could be widely accepted in the population and could make an impact at increasing people’s dietary intake unnoticeably, in a palatable and acceptable way.

5.5.2.2 The effect of mycoprotein and guar gum on postprandial blood glucose

Consistent with other findings, there was an effect of protein in which soy and mycoprotein induced a similar decreased response in postprandial blood glucose compared to chicken. There are only two studies which have investigated the acute postprandial responses in blood glucose following mycoprotein intake in healthy subjects. One has shown how mycoprotein administered in liquid reduced blood glucose iAUC at 60 min but was non-significantly different at 180 min compared to a milkshake (Turnbull *et al*., 1995), which goes in line with the present findings. Another study that tested similar doses of 88 and 132 g of mycoprotein, did not find significant changes in blood glucose response compared to chicken. This may be because the population used was overweight and obese, whereas in the present study the ratio of healthy, overweight, and obese ratios was more evenly distributed. The soy protein used in the present study had isoflavone content. Isoflavones such as genistein have decreased fasting glucose and insulin following chronic interventions in humans (Ricci *et al*., 2010). Also, phyto-oestrogens present in soy proteins may inhibit glucose uptake in the intestine and regulate insulin secretion in pancreatic β-cell via cAMP pathway (Cederroth *et al*., 2008).

There was an effect of bread type, in which guar gum-enriched chapati induced a decreased postprandial glucose response compared to plain chapati. This is relevant since guar gum in a solid matrix has been reported to not be as effective at affecting physiology as within a liquid matrix due to a loss of viscous properties. For individuals with T2D, doses ranging from 7.6-16 g of guar gum incorporated into bread co-ingested with carbohydrate loads reduced glucose iAUC by 26-51% compared to controls (Jenkins *et al*., 1976, Jenkins *et al*., 1980, Gatenby *et al*., 1996), although most of these studies are confounded by the existing high-fibre content in these breads.

Of note, individual data from the condition soy and guar gum show a wide error bar with outliers going in both directions. It is possible that for those individuals who had the lowest iAUC values did not consume all the chapati or meal provided compared to the other individuals. In the study visit, the researcher made sure the meals were eaten in full but in some counted occasions this was not possible. Furthermore, during the chapati standard preparation in the microwave, on some occasions the chapati presented burnt patches when others did not, which may have led to decreased ingestion of available CHO. Nevertheless,

T2D have certain variability in their blood glucose, coupled with natural inter-variability (e.g., day to day) (Molnar *et al*., 1972) of physiological response and other factors that may have played a role (e.g., stress) could be behind this observation (Goetsch *et al*., 1990). As for the individual that presents the highest iAUC value (ID 23), specifically a value two SDs greater, also presented outlier values two SDs greater for soy and mycoprotein alone and one SD greater for mycoprotein and guar gum (no chicken data available), being the worst responder from all participants for all tested conditions. An explanation could be that his ability to manage glycaemia postprandially was greatly impaired, possibly due to the fact he had been the longest diagnosed with T2D (15 years) and had the highest baseline HbA1c (64 mmol/mmol) compared to the other individuals. Coupled to this, his south Asian ethnicity may have also contributed to a wore insulin resistance and his overall worst metabolic response to the same dietary challenge.

Overall, the results agree with the first hypothesis in which mycoprotein in combination with guar gum-enriched chapati would result in an additive effect at reducing blood glucose levels as both protein and guar gum-enriched chapati had a significant main effect at reducing blood glucose. However, there was no synergistic effect between mycoprotein and guar gum as there was no interaction effect between protein and guar gum at reducing blood glucose level compared to mycoprotein with plain chapati. The guar gum-enriched chapati

Consistently with the second hypothesis, it was found that south Asians had an increased blood glucose postprandial response compared to white Europeans. Furthermore, a statistically significant effect was detected for ethnicity on the percentage change in blood glucose with the addition of guar gum, in which the response of south Asian's was lower compared to white Europeans. This is interesting since participants from the two ethnic groups had similar demographic characteristics (age and gender ratio), similar diabetic control (HbA1c) and years since T2D diagnosis. However, in terms of metabolic characteristics, white Europeans had a significantly worst metabolic profile since they had increased body fat, triacylglycerides in blood, fasting glucose, raw BMI,and body weight which would suggest increased insulin resistance (less glucose tolerance), which was confirmed with significantly increase HOMA-IR values at baseline compared to south Asians. Despite a significantly increased raw BMI of 27.70 kg/m^2 in the white Europeans (classed as overweight), south Asians reported to be in average 24.12 kg/m^2 which according to the ethnic-specific cut-off corresponds to being overweight too and actually being closer to the

cut-off for obese compared to the white European (0.88 *vs.* 2.30 kg/m^2 units of difference for the obese cut-off, respectively) (Misra *et al*., 2015), (WHO, 2021).Nevertheless, white Europeans had a significantly higher muscle mass (kg) compared to south Asians. In healthy individuals, the skeletal muscle is the largest tissue in the body and the main organ site for postprandial glucose disposal. The contribution of skeletal muscle on glucose disposal on the post-absorptive state is 20%, whereas on the post-prandial state is 27% (Bayol *et al*., 2014). Evidence has consistently shown that south Asians have a decreased skeletal muscle mass or lean body mass compared to white Europeans (Banjeri *et al*., 1999, Lear *et al*., 2009, Rush *et al*., 2009, Anand *et al*., 2011, Hall *et al*., 2010). Muscle can uptake glucose independently of insulin action upon contraction/exercise (Merry et al., 2009). In this study, participants were in a resting position during the study visits, hence contraction/exercise induced glucose insulin-independent uptake may have not explained the significantly lower postprandial blood glucose responses in the WE. For this reason, other factors such as increased blood flow compared to SA may have played a role but were unexplored (Alvim *et al*., 2015, Ferrannini *et al*., 2018). A difference in muscle mass in the present study's participants may have decreased the capacity of glucose disposal in the post-prandial state compared to the white European cohort, which may have explained the ethnic differences in blood glucose response.

5.5.2.3 The effect of mycoprotein and guar gum on postprandial insulin

There is a significant reducing effect of protein and guar gum-enriched chapati independently on postprandial insulin $iAUC_{0\text{-}180}$ which agrees with the significant decreasing effect of protein on postprandial blood glucose. Insulin is secreted in response to an elevation of glucose in the blood hence a significant decrease of blood glucose would not trigger an elevation in insulin. Post-hoc multiple comparison analysis showed no statistical significance for any protein type, suggesting they act similarly despite having slightly different digestibility scores (0.91, 0.95 and 0.99 for soy, chicken and mycoprotein), but perhaps similar insulinotropic amino acid content. These proteins had no effect on the first-phase insulin secretion, whereas guar gum-enriched chapati induced a decrease in insulin output at 30 minutes. First-phase insulin secretion is related to the immediately releasable pool of pre-synthesised and stored insulin in the β-cells. This is suggestive that in the first 30 minutes of digestion guar gum's viscous properties may be at their maximum efficacy and as digestion goes on, the bolus is exposed to different mixing and grounding forces (e.g. mouth,

50-200 /s); (Sherman *et al*., 1976), so it is possible that guar gum viscosity properties have declined during digestion post-30 minutes. This may have led to an increase in the digestion of protein into amino acid and carbohydrate into glucose, and increased nutrient sensing, leading to the appearance of glucose and insulin in the blood. For instance, an *in vitro* experiment showed that when guar gum was exposed to different shear force rates (2 unaligned forces pushing a part of a body in opposite directions) an exponential decline in guar gum's viscosity from 80 mPa/s to 10 mPa/s with 100 to over 1000 shear rate (/s) was observed (Blackburn *et al*., 1981).

Increased insulin levels in south Asian *vs.* white European were expected since south Asians tend to have increased insulin resistance. Some authors attribute this to the fact that this subgroup has less dynamic intramyocellular lipid droplets which leads to the accumulation of visceral fat and insulin resistance (Gemmink *et al*., 2017). However, in the present south Asian cohort, indicators of insulin resistance such as visceral fat score, saturated fatty acid intake and liver function metabolites (e.g. alanine transferase) were not significantly different compared to the white Europeans, potentially explaining the lack of effect of ethnicity. Instead, an interaction effect between south Asian ethnicity and guar gum-enriched chapati at lowering insulin output was found compared to white Europeans. Since guar gum exerts a mechanical effect in the digestive tract, particularly in the stomach, it is difficult to think there are ethnic differences in how guar gum is digested between ethnicities. There is no evidence investigating different digestion patterns between these two cohorts. Considering that GIP is secreted in response to nutrient absorption (Ebert *et al*., 1980), which can be a surrogate measure of digestion, it would have been interesting to measure GIP response overtime to investigate any differences between ethnicities that may suggest different nutrient absorption patterns between the ethnicities, therefore explaining the beneficial effect of guar gum in the south Asians over the white Europeans.

5.5.2.4 The effect of mycoprotein and guar gum on gut hormone release

Only one study has been published in healthy humans assessing the effect of mycoprotein (at doses 66,88,132 g) on GLP-1 and PYY output relative to matched-chicken in which no differences in tAUC were found (Bottin *et al*., 2016). These observations are in line with the findings in the present study. The reason for this may be because all test foods' protein content trigger GLP-1 release similarly, with a rise as soon as 15-min post-intake in all conditions which is in line with the literature (Van der Klaauw *et al*., 2013, Rizi *et al*., 2018).

Moreover, in Bottin's study, they found that there was an effect of timepoint for GLP-1, but not for PYY release which is in accordance with the observations in the present study. In Bottin's and the present study, GLP-1 levels are observed to remain high at 180 min and to not return to baseline, which is consistent with the secretion patterns in the literature (Van der Klaauw *et al*., 2013, Rizi *et al*., 2018). A study using ^{13}C-labelled isotope flatbread with 4% guar gum (similar to the 5% content of guar gum in the present study) showed an elevated peak of abundance in isoleucine product of the flatbread protein content breakdown at 130 min (Schlicker *et al*., 2019). Considering that isoleucine has been found to trigger GLP-1 response, this suggests that the isoleucine content product of flatbread breakdown could have contributed to the long-lasting increased effect of GLP-1.

Here we reported total AUC instead of incremental, therefore informing of total hormonal output in the system. Because there was great inter-participant variability in the gut hormonal response (e.g. some participants having curves ranging within 10 units and some above), this may have reduced the chances of detecting statistical significances due to the large variation. Furthermore, it should be acknowledged that this study was a pilot study, and it was not powered accounting for this variance to detect gut hormone changes was made. A way to address this that it was considered, would be to have done gut hormonal change from baseline. However, in the present data, the two baselines' measurements (-15 and -5 datapoints) within participant and test food had an intra-CV higher than 20%, therefore the decision was made to not do change from baseline to avoid carrying the error to further timepoints and calculate an iAUC high in error.

5.5.2.5 The effect of mycoprotein and guar gum on appetite

There was no effect of mycoprotein alone or in combination with guar gum on subjective appetite feelings measured by VAS, which is in line with the lack of an effect in the gut hormone measured (PYY and GLP-1). However, other gut hormones that play a role in appetite such as CCK, PP and IAPP should have been explored and may elicit an effect that has not been captured as they were not measured. We tested meals containing a dose of 37 g of protein, which represents a high dose of protein. Protein in varying doses has been shown to decrease VAS feelings (Fischer *et al*., 2004, Harper *et al*., 2007, Johnson and Vickers, 1993, Latner and Schwartz, 1999, Lluch *et al*., 2007, Poppitt *et al*., 1998, Porrini *et al*., 1995, Rolls *et al*., 1988, Stubbs *et al*., 1996, Vandewater and Vickers, 1996), whereas other authors could not replicate this (Blom *et al*., 2006, Porrini *et al*., 1997, Raben *et al*.,

2003). The literature suggests that 30 g of protein is enough to detect differences in satiety (Leidy *et al*., 2015), however, we did not detect differences between proteins, perhaps because all have the same amount of protein and similar digestibility score (PDCAAS: soy: 0.91; mycoprotein: 0.99; chicken: 0.95) (Van Vliet *et al*., 2015, Boye *et al*., 2012, Finnigan *et al*., 2017). Acute interventions using guar gum have also been shown to decrease appetite feelings measured via VAS (French and Read, 1994, Hoad *et al*., 2004, Lavin and Read, 1995), with the exception of some authors who did not detect statistical decreases in appetite (Adam and Westerterp-Plantenga, 2005, Burley *et al*., 1987). VAS is a tool that it is widely used to assess subjective appetite feelings, however, it has its drawbacks such as the fact that every participant may rate differently a feeling that could be detected as physiologically the same, creating variance, that may have explained the lack of differences.

Nevertheless, there was an effect of soy at reducing appetite for sweet compared to both chicken and mycoprotein. This is in line with Williamson and team's finding in which they used a macronutrient self-selection paradigm methodology in which 18 foods of varying macronutrient composition were presented to the participants 4.5 hours after they had mycoprotein and tofu (Williamson *et al*., 2006). They found that tofu intake led to significantly less high-calorie/high/fat choice of food compared to chicken and mycoprotein.

5.5.2.6 The effect of mycoprotein and guar gum on *ad libitum* energy intake

There was no effect of protein, bread, ethnicity and interaction on *ad libitum* intake. A similar amount of mycoprotein intake (132 g) resulted in reduced *ad libitum* intake by -8% (-48 kcal) 180-min post-intake compared to matched-chicken in healthy individuals (Bottin *et al*., 2016). Another study using a dose of 44 g of mycoprotein induced a decrease of *ad libitum* energy intake by -12%, 20-min post-intake, compared to chicken, and similarly to tofu (Williamson *et al*., 2006). Although this effect was observed 20-min post intake which is not comparable to the 180 min post intake frame used in the present study. The same authors added a latter *ad libitum* intake given at 270 min post mycoprotein intake (similar to the time frame used in the present study) which showed no statistically significant differences compared to chicken or tofu (Williamson *et al*., 2006), perhaps due to the smaller dose (44 g) compared to the dose used in the present study (100 g). Only one study was found to investigate the acute effects of guar gum on energy intake (Chow *et al*., 2007) showing that guar gum-supplemented bars significantly reduced energy intake by -20% compared to matched-control bars, although

these bars had other types of fibre in it. Other evidence using 2 g of partially hydrolysed guar gum in combination with protein (8 g) (as a yogurt) showed that it reduced energy intake at subsequent *ad libitum* meal (Lluch *et al*., 2010).

The lack of significant changes could be because all test proteins with chapati had similar satiating power. One study using a 50 g liquid preload of different protein sources (i.e. soy, whey and gluten) showed to reduce *ad libitum* intake by -10% in a similar way at 3 hours post intake compared to glucose (Bowen *et al*., 2006), and this was mediated partly by an increase in GLP-1 release.

On the other hand, the lack of significant changes could also be due to the lack of differences in the GLP-1 and PYY response which are both anorexigenic hormones and their secretion following meal intake influences appetite and energy intake. Nevertheless, the levels of other gut hormones that play a role in appetite such as CCK, PP and IAPP following the test meal intake should have been explored since they may elicit an effect that has not been captured as they were not measured. Furthermore, the lack of differences may be due to methodological issues in the *ad libitum* meal served. It is possible that the *ad libitum* meals were not palatable enough for some participants to be consumed until comfortably full leading to an underconsumption not in line with the physiological feeling of hunger in the participants. This is in line with the energy intakes of 3 participants (2 white Europeans and 1 south Asian) who ate <350 kcal each study visits when their energy intake per meal according to their basal metabolic rate and physical activity was 2.50-3.20 times greater of what they consumed at *ad libitum* for each study visit. Ideally, participants should have been asked at screening visit to taste between different *ad libitum* food trials. Those foods rated with an intermediate score (not too low-to avoid lack of palatability, nor too high-to avoid hedonic intake and overconsumption) should have been chosen and be served to the same participant. However, the study performed by Bottin *et al*., used the same type of pasta meal and yet statistical differences were found, although a higher slightly dose and type of population were used (132 g of mycoprotein and healthy overweight and obese *vs.* 100 g of mycoprotein and T2D of different ethnicities).

It could also have been that some participants were being aware of how much they were eating by having a visual reference (by eating from the wall edge of the bowl rather than the centre) and restricted themselves from eating each time similarly, even though pasta was

served in excess. The researcher gave no indication on eating from the centre of the bowl nor continuous refilling of pasta was employed. These may be some limitations of this study that should be addressed in further studies investigating *ad libitum* energy intake.

5.5.2.7 The effect of mycoprotein and guar gum on post-visit energy intake

For the post-visit energy intake, there was no effect of protein, bread, ethnicity, and interaction. Soy protein intake in the form of liquid has been shown to significantly decrease energy intake compared to carbohydrate and low soy protein intake suggesting a dose-dependent manner (Nepocatych *et al*., 2019). Another study showed that soy protein intake accompanied of carbohydrate induced a reduced energy intake similarly to other protein types such as pea, wheat, egg, albumin, casein (Lang *et al*., 1998). It has been shown that soy protein is rich in cysteine which is then metabolised in taurine in the body. Taurine elevations over time have been observed following soy protein intake in humans. Taurine has also been correlated to decreased feeding in rodent animals and it is present in fish food which is known to be satiating. It would be interesting to understand the protein digestion dynamics into aminoacids of each test food to understand better the lack of differences between proteins, as well as the thermic effect of each test food since thermic effect of food has been related to satiety (Crovetti *et al*., 1998). Studies using similar doses of mycoprotein to the one tested in this study (100 g) show contradicting results. One study using the same load of carbohydrate as this study (77-80 g carbohydrate) in healthy lean individuals testing 130 g of mycoprotein leading to a significant decrease in post-visit energy intake by -16% (Turnbull et al., 1995). However, another study using 132 g of mycoprotein (+25-30 g carbohydrate) did not show such effect on post-energy intake compared to energy and macronutrient-matched chicken (in healthy overweight and obese individuals) (Bottin *et al*., 2016).

A random-effects meta-analysis (n=3) showed that guar gum-enriched foods did not have an effect on energy intake (Salleh *et al*., 2019). However, when studies were examined individually it was observed how guar gum within a liquid matrix had a significant reducing effect compared to a solid matrix (e.g.cookie). Liquid matrices may preserve the viscous properties of guar gum, whereas this may not be true for solid matrices such as the one used in the present study (chapati), hence explaining the lack of a statistical effect by bread type.

The lack of a statistical effect of protein and guar gum may stem from having relied on self-reported food diaries to measure energy intake which may have led to under- and misreporting. The present data exemplifies this assumption since there was a significant reduction in their reported baseline energy intake per day compared to their calculated energy requirements per day (calculated based on metabolic rate and physical activity). If their baseline energy intake was accurately recorded, participants would be on energy deficit and body weight loss would have been detected along the study duration when body weight was taken. However, no significant change in body weight was found over the course of the study for any participant, suggesting there was a strong tendency to under report in food diaries. This is a widely acknowledged limitation within the self-reported food diaries approach for energy intake measurement (Schoeller *et al*., 1990). A study using doubly labelled water to measure total energy expenditure in free-living subjects revealed how participant's self-reported intakes were lower than their actual energy intake. This tendency is greater with obese individuals (Heitmann *et al*., 1995), for which the present study has 27 and 20% within the white European and the south Asian cohort, respectively. This may explain the lack of differences between the test foods. Therefore, future studies assessing energy intake should consider using better methodologies such as wearable, and unnoticeable technology which is able to track what we eat or sophisticated and accurate metabolic profiling that reflects diet intake. However, these approaches are still underway at the time of writing this thesis.

5.6 Limitations

Despite the novel findings reported in this study, there were some limitations. This study was not powered to detect statistical differences on glycaemic, appetite and energy intake outcomes in people with T2D of south Asian or white European descent. However, data from this pilot trial is useful to inform future acute studies using similar interventions and people with T2D of south Asian and European ethnicities.

In this study, while some baseline demographic characteristics were similar and non-statistically different (i.e. age, gender ratio, HbA1c and years since T2D diagnosis, in terms of metabolic characteristics, white Europeans had a significantly worst metabolic profile since they had increased body fat, triacylglycerides in blood, fasting glucose, raw BMI and body weight which would suggest increased insulin resistance (less glucose tolerance). Furthermore, there were 3 people in each ethnic group who were on sulfonylurea which

promotes insulin secretion from the pancreatic beta-cells, suggesting worse glycaemic control and different insulin responses following the meal intakes, compared to the other participants mainly on metformin which would not have insulin secretion problems to the same extent as people on sulfonylureas. These may have contributed to increase the variability explaining the lack of main effect for some variables and outcomes.

The energy intake post-visit data were collected using unweighted self-reported food diaries by participants who were not trained to do so. This may have led to reporting bias giving rise to inaccurate estimates of energy intake, which was in agreement with an observed discordance between participant's reported baseline energy intake and their estimated energy requirements per day (calculated based on metabolic rate and physical activity). Energy intake miss reporting via self-reported food diaries is a widely recognised limitation within the nutrition research field (Schoeller *et al*., 1990). Therefore, future studies assessing energy intake should consider using better approaches for measuring dietary intake as accurately as possible. These can be the following used separately or in combination: weighted food diaries having trained participants beforehand, metabolic profiling from urine (to-date only accurate when determining dietary patterns (Posma *et al*., 2020)), wearable and unnoticeable devices (e.g. glasses with a camera) that can track what we eat (currently under development). However, these approaches are still have their own limitations.

The main limitation of the human study is that it is an acute intervention. While acute interventions are useful to determine the postprandial response to a mixed meal test within a very tightly controlled environment, they provide little information on whether the acute effects observed are also present with chronic intakes. For example, continuous reductions of postprandial blood glucose over time are more physiologically relevant than at one-single time blood glucose reduction for people with T2D. Considering the beneficial nutritional profile of mycoprotein, the sparse trial data on glycaemia and appetite and the results of the epidemiological study, it is suggestive that chronic intakes of mycoprotein may have a role in regulating blood glucose, appetite, and energy intake. Therefore, high-quality longer-term trials investigating the effect of chronic mycoprotein intake on both PPG and Hb1Ac changes, as well as insulin sensitivity using the gold-standard hyperinsulinaemic-euglycemic clamp, in people with T2D are needed to explore this.

5.7 Conclusions

This is the first study to investigate the effect of mycoprotein on glycaemia in a cohort with a compromised blood glucose metabolism such as people with T2D. The novel findings of the present study are that soy and mycoprotein induce a similar decreasing effect on postprandial blood glucose compared to chicken. Enriching chapati with guar gum induces a decreased blood glucose response. South Asians present an increased postprandial glucose response compared to white Europeans, despite a better demographic and metabolic profile and mechanisms underpinning these are unknown. Guar gum-enriched chapati has an effect at reducing postprandial insulin response, particularly in the south Asian, and does not have an effect on appetite, and energy intake probably due to the solid matrix it is contained in leading to a loss of viscous properties. There is no interaction effect between protein, bread and ethnicity on blood glucose, insulin, appetite, or energy intake. The study has some limitations such as the acute nature of the intervention, the use of self-reported food diary data that should be addressed in future studies. This study adds on to the previous literature on the favourable effect of mycoprotein and guar gum on glycaemic control. This study is of value as it is the first one conducted in people with T2D. This study is important because it informs about the potential of a culturally-adapted ready meal, that is well accepted within the south Asians, that may make them improve postprandial glycaemia. There is the potential that should this meal be included in their diet's, may help them manage their diabetes. However, high-quality longer-term trials investigating the effect of chronic mycoprotein intake on both PPG and Hb1Ac changes, as well as insulin sensitivity (using the gold-standard hyperinsulinaemic-euglycemic clamp) in people with T2D are needed to explore this.

5.8 Bibliography of Chapter 5

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CHAPTER 6: The effects of mycoprotein on gut microbiota, SCFA production and metabolomics: *In vitro* study

6.1 Introduction

6.1.1 Gut microbiota

The human gut is inhabited by trillions of microbial cells constituting a large proportion of our genes (3.3 million), outnumbering the host own genes by 100 times (Qin *et al*., 2012). Traditional roles of gut microbiota include the production of vitamins and secondary bile acids, modulation of host immunity, gut endocrine function and neurological signalling, modification of drug action, elimination of toxins and mediation of host and substrate metabolism. Furthermore, recent evidence suggests that gut microbiota is related to a myriad of disease conditions (Vijay *et al*., 2021). A “healthy” microbiome at a taxonomic level has not been established yet, however, it is known that high microbial richness (number of different species), diversity (relative abundance of each specie) and stable microbiome functional cores are characteristics of a healthy phenotype (Turnbaugh *et al*., 2009). A high microbial diversity has been linked to improved insulin sensitivity and metabolic health in human studies (Turnbaugh *et al*., 2006), whereas microbial dysbiosis (disruption of gut microbiota homeostasis) is linked to the development to a range of metabolic conditions including obesity (Shen *et al*., 2013).

The gut microbiome has a unique composition for every individual. The composition is determined by genetics, early-life determinants, the environment, and lifestyle choices (e.g. medication, exercise, diet, etc.), that largely correlate to ethnicity (Deschasaux *et al*., 2018, Leeming *et al*., 2019, Monda *et al*., 2017). In particular, diet explains ~20% of gut microbiome composition (Leeming *et al*., 2019). Food-borne bacteria colonises the gut (David *et al*., 2014) as well as undigested food reaches the proximal and distal gut and serves as energy substrate for bacterial growth, therefore modulating the bacterial communities. Interestingly, changes in diet for as short as 2 days can alter bacterial communities in humans (David *et al*., 2014). This was shown in a study using young, healthy humans (BMI 19-32 kg/m^2) in which it was shown that a switch of 15% to 30% of daily kcal of protein intake for 4 days, significantly changed the gut microbiota β-diversity (relative abundance (%) of each species) at day 1, with an increased abundance of bile-tolerant bacteria and decrease of bacteria that digest fibre.

6.1.2 Diet and gut microbiota

6.1.2.1 Fermentation of dietary protein

Although protein digestion in the mammalian digestive tract is very efficient, 10% of a normal protein intake (this being 0.8 g of protein/kg body weight/day, according to the dietary recommended allowance (DRA)), will not be completely digested in the stomach. Similarly, >10% of a high protein intake (defined as >1.2g of protein/kg body weight/day (>150% of DRA)) will not be completely digested in the stomach. In both cases, residual peptides and proteins will be mainly fermented in the distal gut by proteolytic gut microbiota. This yields a wide range of metabolites (e.g., ammonia, phenolic compounds, indoles, hydrogen sulphide, branched-chain fatty acids (BCFA)), which have been shown to be detrimental to host metabolism (Portune *et al*., 2016). For example, elevated BCAAs such as leucine, isoleucine and valine are strong biomarkers for insulin resistance and increased risk of T2D. Insulin resistant individuals have an increased abundance of *Prevotella copri* and *Bacteroides vulgatus* which have increased capacity to synthetise BCFA (Wang *et al*., 2011). Interestingly, some of these proteolytic products serve as a substrate to other gut bacteria (cross-feeding), therefore disturbances in the bacterial communities can result in the absorbance of these detrimental metabolites by the host (Canfora *et al*., 2019).

6.1.2.2 Fermentation of dietary fibre

Dietary fibre cannot be digested by the host enzymes and moves down to the lower gut where it can be fermented by saccharolytic gut microbiota. Unlike the human host, gut bacteria possess a wide range of enzymatic tools (e.g. proteolysis, β-glucuronidases, β-lyases) that are able to digest fibre-rich substrates to host-absorbable secondary metabolites (Den Besten et al., 2013). These are mainly the SCFAs acetate, propionate, butyrate, which are produced in a ratio of 3:1:1 (Canfora et al., 2015). Butyrate is mainly used as an energy-substrate for colonocytes, regulates colonocyte proliferation and differentiation, and represents an important anti-inflammatory molecule (Liu et al., 2018). While acetate and propionate can either be partly oxidised by colonocytes, they can also act as ligands of the free fatty acid receptor (FFAR) 2 and FFAR3 expressed in the colon or be absorbed by the host (Morrison et al., 2016), and metabolised by the liver (e.g., lipogenesis or gluconeogenesis), and act systemically since they can be sensed by peripheral tissues via FFAR2 and FFAR3 (van der Hee et al., 2021). These receptors are expressed in several tissue types including the liver, muscle, adipose tissue, and pancreas (van der Hee et al.,

2021). In the colon, SCFAs stimulate the release of GLP-1 and PYY hormones, which play a role in glucose homeostasis and appetite regulation (van der Hee et al., 2021). *In vitro* work has suggested the role of SCFAs such as sensitising muscle cells to insulin (Frampton *et al*., 2020) and inhibiting pancreatic β-cell apoptosis (Pingitore *et al*., 2016). Furthermore, an association between plasmatic levels of acetate and glycaemic markers have been found in people with T2D, but not for obese and lean healthy subjects (Todesco *et al*., 1993). This evidence is suggestive that SCFAs may have a role in blood glucose homeostasis in humans.

6.1.3 Gut microbiota derived-SCFAs on blood glucose in humans

6.1.3.1 Acetate

Only studies investigating the acute effects – but not chronic - of acetate in humans were found in the literature (Van der Beek *et al*., 2016, Scheppach *et al*., 1998, Mettler *et al*., 2009, Laurent *et al*., 1995, Freeland *et al*., 2010). Overall, studies show that acute acetate leads to a non-significant pooled effect on postprandial blood glucose iAUC and insulin iAUC in humans. In general, studies investigating the acute effects of acetate had a small sample size and employed heterogenous routes of administration. Interestingly, the consistent data on the acute effect of acetate stimulating fatty acid oxidation pathways, suggests that acetate administered chronically may have an indirect effect on maintaining insulin sensitivity which could be protective against insulin resistance. However, further studies are needed to elucidate this.

6.1.3.2 Butyrate

Chronic butyrate supplementation of 4g/day x 4 weeks showed to improve peripheral and hepatic insulin sensitivity as assessed with hyperinsulinaemic-euglycaemic clamps in healthy but not in participants with metabolic syndrome (Bouter *et al*., 2018). The lack of effect in metabollicaly diseased populations, is in agreement with another chronic study in subjects with T2D (Roshanvaran *et al*., 2017) in which 600 mg/day sodium butyrate intake for 45 days did not have any effect on fasting, 2 hours postprandial blood glucose, HbA1c, fasting insulin and the quantitative insulin sensitivity check index (QUICKI) compared to the placebo (starch). This is suggestive that metabolically compromised individuals may metabolise SCFAs differently to healthy counterparts. Interestingly, a later study by the same authors observed that chronic butyrate administration led to an increase in the acetate-producing bacteria *Akkermansia muciniphila* (Roshanvaran *et al*., 2018), and for which an inverse

relationship with glycaemic control has been reported elsewhere (Dao *et al*., 2016). However, caution should be taken with this study as they did not adjust for baseline *A. mucinphila* levels and did not control the diet for each of the two parallel groups, which may confound the findings on gut microbiota.

6.1.3.3 Propionate

Overall, studies investigating acute administration of propionate showed no significant pooled effect on postprandial blood glucose and insulin iAUC in humans compared to placebo (Laurent *et al*., 1995, Todesco *et al*., 1991, Darwiche *et al*.,2001, Darzi *et al*., 2012, Byrne *et al*., 2016, Chambers *et al*., 2018, Tirosh *et al*., 2019). However, the sample size calculations for the majority of these studies were not powered to detect significant differences in blood glucose or insulin following propionate intake. Some studies using high doses such as 222 mmol/L observed decreased glycemic and insulinemic responses within 60 minutes. It has been suggested that propionate can decrease gastric emptying (Darwiche *et al*., 2001, Liljeberg *et al*., 1996) and inhibit human salivary α-amylase potentially contributing to a decrease in glucose absorption *in vivo*. Likewise, studies investigating chronic administration of propionate in overall have shown a non-significant pooled effect on postprandial blood glucose and insulin iAUC, as well as on fasting blood glucose and insulin in humans (Chambers *et al*., 2015, Venter *et al*., 1990, Todesco *et al*.,1991, Byrne *et al*., 2019, Chambers *et al*.,2019).

6.1.5 Gut microbiota fermentation of mycoprotein

One-week intervention replacing meat for mycoprotein-based foods showed a 40% increase in blood acetate levels relative to baseline, which was 5 times greater than the plasma acetate increase observed in the control group (one-week of meat intake) suggesting *in vivo* fermentation of mycoprotein (Coelho *et al*., 2020). *An in vitro* study investigating mycoprotein fermentation by the human gut microbiota (Harris *et al*., 2019) observed that whole mycoprotein did not increase SCFA compared to control. However, isolated mycoprotein fibre increased SCFAs production by almost 6 times compared to control, similarly to other fermentable fibres such as laminarin, rhamnose, and oligofructose. Nevertheless, the substrates used in this study were not pre-digested, whereby using a pre-digested substrate is a more realistic approach of conducting the study as mycoprotein is digested *in vivo* when it reaches the gut for microbial fermentation. Furthermore, in the aforementioned study,a

small sample size (n=3) was used which may lead to inaccurate and non-representative results.

6.2 Purpose of the Study

Although the scarce and weak evidence may indicate a possible potential for mycoprotein to be fermented by human gut microbiota, stronger evidence needs to be built to support this by increasing the sample size and using pre-digested mycoprotein which would represent a more realistic approach of what the human gut microbiota is exposed to *in vivo*.

6.2.1 Hypothesis

I hypothesise that pre-digested mycoprotein can be fermented by the human gut microbiota and there is SCFAs production as a by-product of such fermentation.

6.2.2 Aims

The overall aim of this exploratory study is to investigate the mechanism by which mycoprotein may exert beneficial effects on blood glucose homeostasis and appetite regulation.

6.2.3 Outcomes Measures

The primary outcome measures are:

1. SCFAs production

The co-secondary outcome measures are:

1. Bacterial composition (α- and β-diversity)
2. Batch culture pH levels
3. Metabolomic profile of the digestion of substrates

6.3 Methodology and Study Design

A proof-of-concept study was performed to assess the feasibility of the whether mycoprotein is fermentable in humans. Next, an *in vitro* batch culture fermentation study using healthy faecal donations was carried out. This last study was approved by Imperial College Research Ethics Committee (ICREC reference number: 19IC5605).

6.3.1 Proof of concept study: Fermentability of Quorn

6.3.1.1 Participants

Participants were eligible if they were deemed to be healthy, non-vegan/vegetarian, non-heavy alcohol consumers (<14 units per week), not being currently treated with antibiotics or drugs known to influence intestinal permeability such as paracetamol, ibuprofen, and pre- or probiotics. Participants were asked to standardise their evening meals and to avoid fibre (avoid vegetables, whole grain foods, fruit, legumes) on their evening meal prior to the study day and during the study day.

6.3.1.2 Study visit

A proof-of-concept study was performed between September and October 2018 to test Quorn mince fermentation in healthy humans (N=3, 2 male and 1 female of BMI 18.5-25 kg/m^2) using a breath hydrogen analyser for a period of 8 hours in a controlled environment and low-fibre diet. Participants were asked to conduct two study visits separated by 7 days in which they had to first consume 12.5 g of inulin (Beneo-Orafti HP, Kreglinger Europe, Antwerpen, Belgium) dissolved in 250 ml of water (providing 10 g of fibre) which served as a positive control for fermentation and 182 g of Quorn mince (Sainsbury's) (providing 10 g fibre). Quorn mince was cooked as per manufacturer instructions. A standard low-fibre lunch was provided during the day. Breath hydrogen measurements were taken with a Gastrolyzer (Bedfont, UK) every 60 min for 480 min.

6.3.2 Pilot Study: *In vitro* Batch Culture Fermentation

6.3.2.1 Preparation of substrates

The substrates used in this experiment were protein sources of soy (VegiDeli Quarter Pounders, VBites, Northants, UK), chicken (chicken breast, Sainsbury's, UK) and processed mycoprotein (Quorn) (Quorn mince, Marlow Foods Ltd, Stokesley, UK) which were commercially available in the supermarket. The nutritional profile of the substrates are described in Table 22.

Table 22. Nutritional profile of substrates per 100 g.

	Soy	**Chicken**	**Quorn**
Energy (kcal)	183	127	105
Carbohydrates (g) (sugars, g)	6.2 (1.2)	<0.5 (<0.5)	4.5 (0.6)
Fat (g) (saturates, g)	8.7 (0.7)	3.0 (0.6)	0.6 (2.0)

Protein (g)	19.3	24.8	14.5
Fibre (g)	4.4	<0.5	5.5
G, grams; kcal, kilocalories.			

Prior to the standardised cooking preparation of the substrates, the leanest part of the breast chicken was prepared by removing fat strips following mince processing using a household chopper (500W 2-in-1 Hand Blender Set, Breville, Australia).

Substrates were cooked individually using a standard cooking procedure consisting of pan-frying at medium heat for 5 min while stirring every minute. This was performed to achieve a realistic setting of a product for human consumption.

6.3.2.2 Pre-digestion of substrates

Cooked substrates were digested according to an adapted *in vitro* protocol (Mills *et al*., 2008), a protocol that was previously used for digesting proteins. The protocol consists of 3 consecutive phases, described below:

Phase 1 (Oral phase): 30 g of cooked substrate was placed into a plastic bag (Standard Bag BA6141/5, Seward Limited, UK) and gronded using a mortar and pestle until the size of the substrate minced parts were visually small (approximately 4 mm) and uniform. Next, 100 mL of double distilled water was added to the mixture and placed into a stomacher (Stomacher®400 Circulator, Seward Limited, UK) for 5 min at 250 rpm. 10 mg of alfa-amylase (A 4551, Sigma, UK) in 3.125 mL of $CaCl_2$ (0.001 M) solution was added to the mixture at pH 7.0 and incubated at 37°C for 30 min in a shaker (Excella E25, New Brunswick Scientific, Eppendorf) at 129 rpm.

Phase 2 (Gastric phase): 1.35 g of pepsin (P 7000, Sigma, UK) in 12.5 mL of HCl (0.1 M) solution was added to the mixture at pH 2.0 and incubated at 37°C for 2 hours in a shaker (Excella E25, New Brunswick Scientific, Eppendorf) at 129 rpm.

Phase 3 (Intestinal phase): Following, 280 mg of pancreatin (P 8096, Sigma, UK) and 1.75 g of bile (B 8631, Sigma) in 62.5 mL of $NaHCO_3$ (0.5 M) solution was added to the mixture at pH 7.0 and incubated at 37°C for 3 hours in a shaker (Excella E25, New Brunswick Scientific, Eppendorf) at 129 rpm.

Next, in order to obtain substrate dry matter, the mixture was transferred to a 1 kDa molecular weight cut-off regenerated cellulose dialysis tubing (Spectra/Por 6, Spectrum Europe, Netherlands) and dialysed for 15 hours against NaCl (0.01 M) at 5°C to remove low molecular mass digestion products. After, the dialysis fluid was renewed, and dialysis was allowed for an additional 2 hours. The remaining substrate was stored at –80°C for 2 days prior to being freeze dried (Frozen in Time Ltd., North Yorkshire, UK) for approximately 5 days.

6.3.2.3 Sample size

The sample size was calculated on the primary outcome, an increase in SCFAs production, using the study by Harris *et al*. 2019. Using an *in vitro* batch culture fermentation of 10 mg/mL of mycoprotein fibre supplemented with 32% faecal slurry, they reported a significant 81% increase in SCFA with mycoprotein fibre isolate (mean ± SEM) (61.15 ± 15.73) compared to control (blank) (11.13 ± 1.61). With a level of statistical significance set at α= 0.05 and a power of 95%, the sample size needed for this study was 4. To account for a 30% dropout, a minimum of 6 participants were recruited to take part in this study.

6.3.2.4 Volunteers Recruitment

Volunteers were recruited through a study advert put up in public spaces including the Imperial College London campus premises.

Interested individuals contacted the researcher to express their interest via email. Researcher sent a PIS (Appendix 21) back to the interested individuals. If after reading the PIS, the potential participant was interested, the researcher scheduled a screening visit at the 10th floor of Queen Elizabeth the Queen Mother (QEQM) Wing building, South Wharf Road, London W2 JNY. The purpose of the screening visit was to discuss the study, obtain written and informed consent (Appendix 22) and fully assess their eligibility for the study. The eligibility was assessed by asking health questions in relation to gastrointestinal diseases, intake of probiotics, having diabetes, having taken laxative or antibiotics in the past 3 months to assess compliance with inclusion and exclusion criteria.

6.3.2.5 Inclusion Criteria

Volunteers were eligible for the study if they fitted all the following inclusion criteria: deemed to be healthy (not diagnosed with T2D), and were aged 18-75 years old.

6.3.2.6 Exclusion Criteria

Volunteers were not eligible for the study if they fit any of the following exclusion criteria: taking antibiotic course in the preceding 3 months, have any gastrointestinal condition, taking any medication that affects gut microbiota fermentation, taking probiotics, prebiotics or laxatives in the past month, have a mixed ethnicity.

6.3.3 Study visit overview

6.3.3.1 Preparation for the study visit

Eligible volunteers were invited to the 10th floor of QEQM Wing building, South Wharf Road, London W2 JNY for a study visit for a stool donation. Prior to this visit, volunteers were asked to fill a food diary for the 3 days preceding the study visit.

6.3.3.1 Study visit – Collection of faecal inoculum

On the study visit day, volunteer was asked to donate and collect their fresh faeces using a sterile, lidded faeces container (Fecotainer, RAL 9003, Excretas Medical BV, Netherlands) which was immediately sealed and placed in the anaerobic chamber (AW 400TG, Electrotek, UK) (atmosphere of 90% N_2, 5% CO_2 and 5% H_2, set at 70% humidity and 37°C) within 5-10 min of collection. Faeces was provided in a bathroom in proximity to the laboratory where the anaerobic chamber (AW 400TG, Electrotek, UK) used to process the faeces was located.

6.3.3.2 Preparation of faecal inoculum

To generate 25% (w/v) faecal slurry, 16 g of faeces were placed in a strainer plastic bag (Strainer Bag BA6141/STR, Seward Limited, UK) with 64 mL of degassed sterile phosphate-buffered saline (PBS) solution (J75889-K2, Thermo Scientific, UK) and homogenised for 1 min at 230 rpm using a stomacher (Stomacher®400 Circulator, Seward Limited, UK). Next, faecal slurry was used as the inoculum for this study.

6.3.3.3 Preparation of bacterial growth media

The sterile bacteria growth media composition is described in Appendix 23. This medium was used based on previous studies using a chemostat to mimic the human gut (Olano-Martin et al., 2000).

Briefly, solution A and E were autoclaved at 121°C for 2 hours to achieve sterility. In the biosafety cabinet, 1 mL of hemin and 1 mL of minadcone were added into solution B. Next,

unprecipitated L-cysteine was added into solution B. Following, solutions B, C and D were combined and transferred into solution A using a vacuum filter (431118, Scientific Laboratory Supplies, UK) to achieve sterility. Last, 1mL of Tween 80 (P1754, Sigma Aldrich) was added to the mixture. Sterile growth media was stored at 4°C and renewed every 14 days.

6.3.4 *In vitro* batch culture fermentation (static)

6.3.4.1 Principle

A static *in vitro* batch culture is a methodology which is used to evaluate gut microbiota fermentation, composition, and diversity in a single vessel in which a faecal slurry is inoculated into the vessel and incubated with a substrate of interest under anaerobic conditions. It is a methodology that allows investigation of gut microbial changes in a controlled environment, and it is simple, cheap, and reproducible (Pérez-Burillo *et al*., 2019). Unlike dynamic models, static *in vitro* batch culture fermentation it is not pH-controlled and does not have multiple vessels connected and motored by pumps which would be more representative of gut physiology.

6.3.4.2 Experiment protocol

The *in vitro* batch culture fermentation was performed by following an adapted protocol by McDonald (2013) in anaerobic conditions and using an anaerobic chamber (AW 400TG, Electrotek, UK) at conditions 80% N_2, 10% CO_2 and 10% H_2. The procedure consisted of inoculating sterile falcon tubes (Corning, USA) containing 13.5 mL of sterile growth medium (Appendix 23) with 1.5 mL of faecal slurry (25%). Next, 150 mg of each substrate (pre-digested soy, chicken or Quorn) was transferred to the falcon tubes containing 15 mL of inoculated growth media, to achieve a final concentration of 10 g/L. For the negative controls (absence of faecal slurry), the same procedure was repeated replacing the faecal slurry for sterile phosphate bovine serum (PBS) (J75889.AE, Alfa Aesar, UK). In addition, a positive (faecal slurry + growth media) and negative control (PBS + growth media) was generated.

In total, there were 8 experimental conditions per faecal inoculum, as described below.

1. Positive control: faecal slurry + growth media
2. Negative control: PBS + growth media
3. Soy +: faecal slurry + growth media + soy pre-digested substrate
4. Chicken +: faecal slurry + growth media + chicken pre-digested substrate
5. Quorn +: faecal slurry + growth media + Quorn pre-digested substrate

6. Soy -: PBS + growth media + soy pre-digested substrate
7. Chicken -: PBS + growth media + chicken pre-digested substrate
8. Quorn -: PBS + growth media + Quorn pre-digested substrate

6.3.4.3 Samples Collection and Storage for Analysis

1 mL of batch culture sample was collected at 0 and 24 hours to measure pH levels as an indicator of fermentation, using a calibrated pHmeter (HI 2210, Hanna, Spain).

200 µL of batch culture sample were collected at 0 and 24 hours to seed fastidious anaerobic agar plates (NCM0014A, Neogen, USA) (supplemented with 5% defibrinated horse blood (EOLADHB500N500500, Fisher Scientific, UK) to visually check for bacterial growth as a quality control for the protocol.

In addition, 4 x 1 mL of batch culture sample were collected at 0 and 24 hours and aliquoted into sterile microcentrifuge tubes (Eppendorf safe-lock tubes, Eppendorf) and stored at –80°C for subsequent DNA extraction (2 x 1 mL) (section 4.4.1.1) and metabolomic profiling analysis (2 x 1 mL) (section 4.5).

6.4 Statistics

Normality of the data were checked visually using QQ plots. Breath hydrogen data were transformed into incremental AUC from 180-480 min, which is when a notorious rise in breath hydrogen was observed in both test foods. iAUC was calculated using the trapezoidal method using Graphpad Prism v.9.0. A two-way ANOVA was used to compare inulin iAUC from Quorn iAUC, using a Sidak's multiple comparison test. A p-value ≤0.05 was considered statistically significant.

pH and SCFA data were analysed using a linear mixed model. Protein factor was considered as fixed factors and participant as a random factor. If there was a main effect of the aforementioned factors or interaction, post-hoc multiple comparisons were made using Bonferroni's corrections. P-value was considered to be statistically significant if ≤ 0.05. The linear mixed model was performed using Stata v.16.0 (Stata Corp LLC, Texas, USA).

For the 16S rRNA gene sequencing and SCFAs data, the data treatment and statistical analysis employed has been described in Section 4.5 and 4.6 (Chapter 4: Experimental Methods). Q-values (false discovery rate adjusted P-values) ≤0.05 were considered

statistically significant. For the α-diversity index (Shannon), an ANOVA was performed. For the β-diversity index (Bray-Curtis), an PERMANOVA was performed.

6.5 Results

6.5.1 Proof of concept

6.5.1.1 Breath Hydrogen

The results on breath hydrogen are described in Figure 29. An increase in breath hydrogen was observed for the inulin condition starting at timepoint 240 min for 1 individual, and at 360 min for the other 2 individuals. The statistical test revealed a statistically significant decrease in breath hydrogen $iAUC_{180\text{-}480\ min}$ following Quorn intake compared to inulin. The mean difference between Quorn and inulin is -6420 ppm [95% CI -9603.48, -3236.52]. The mean breath hydrogen $iAUC_{180\text{-}480\ min}$ concentration for inulin is 10025 ppm [4446,15604] and for Quorn is 2948 [1019,4877].

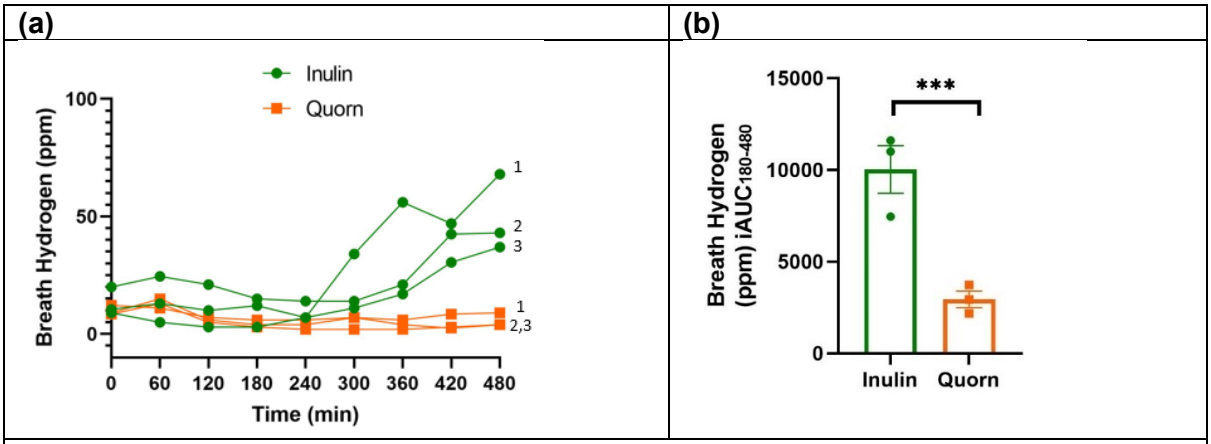

Figure 29. Breath hydrogen concentrations following inulin and Quorn intake (N=3). (a) Breath hydrogen concentration (ppm) over the course of 480 min. Test food was given at timepoint 0 min. Each line represents one individual labelled 1,2,3. Green line represents inulin, and orange line represents Quorn. (b) Breath hydrogen (ppm) $iAUC_{180\text{-}480\ min}$. Data are represented as mean±SEM and individual datapoints. Green represents inulin, and orange represents Quorn. ***P-value<0.001. n, sample size.

6.5.2 *In Vitro* Batch Culture Fermentation Study

6.5.2.1 Participant's baseline characteristics

Table 23 summarises the demographic characteristics of the eligible participants recruited for the fresh stool donations for the *in vitro* batch culture fermentation study.

Table 23. Baseline characteristics of participants (N=8)		
Sex (M/F)	(3/5)	
Ethnicity		
White Europeans	5 (1/4)	
South Asians	3 (2/1)	
	Mean	**SEM**
BMI	23.22	4.67
Age	27.75	3.61
BMI, body mass index; M, male; F, female; N, sample size; SEM, standard error of the mean.		

6.5.2.2 Participant's baseline dietary intake

Table 24 describes the baseline dietary intake per day of the 8 participants.

Table 24. Baseline dietary intake per day		
	Mean	SEM
Energy (kcal)	1785.24	322.52
CHO (g)	193.25	32.51
Total sugars (g)	87.53	17.78
Fat (g)	86.34	16.84
Saturated fats (g)	28.97	6.94
Protein (g)	67.79	17.50
Starch (g)	93.28	18.35
Fibre (g)	22.76	3.94
CHO, carbohydrate; g, gram		

6.5.2.3 pH

Figure 30 and Table 25 capture the results on pH change over 24 hours of incubation with soy, chicken, mycoprotein and control (only growth media with faecal slurry) under anaerobic conditions. The mixed model showed no statistically significant main effect of type of protein (p=0.896), meaning that there were no differences between soy, chicken, Quorn and the control. However, there was a statistically significant main effect of the individual (p=0.011).

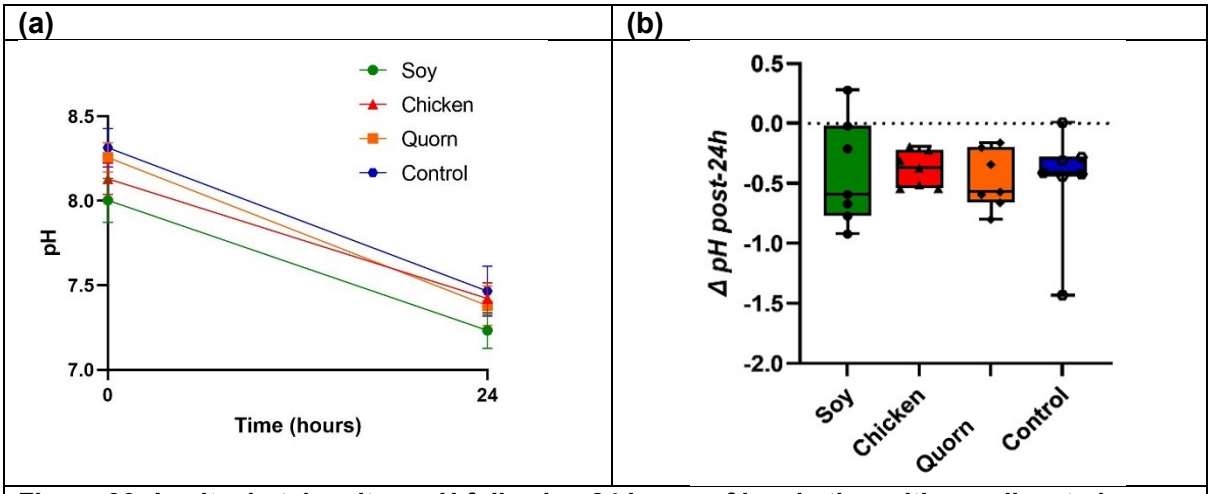

Figure 30. *In vitro* batch culture pH following 24 hours of incubation with pre-digested soy, chicken and Quorn (N=8). (a) pH levels over the course of 24 hours for pre-digested soy (green), chicken (red), Quorn (orange) and control (growth media, with faecal inoculum, without added substrate) (blue). Data represents mean±SEM. (b) pH change post-24 hours relative to baseline (timepoint 0) for pre-digested soy (green), chicken (red), Quorn (orange) and control (growth media without substrate) (blue). pH values are corrected for the pH change in the negative controls for soy, chicken, Quorn and control. Data represents the median and the 5-95% percentile and the

Table 25. pH net change following 24 hours of incubation.					
		Soy	**Chicken**	**Quorn**	**Control**
	Mean	-0.41	-0.38	-0.47	-0.47
CI	**Lower 95%**	-0.82	-0.52	-0.70	-0.89
	Upper 95%	-0.01	-0.24	-0.25	-0.05
CI, confidence interval.					

individual datapoints. Δ, increment; n, sample size.

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6.5.2.4 SCFAs

Table 26 summarises the averages of acetate, butyrate, propionate, and total SCFAs levels at 0 hours, 24 hours and the change from baseline following the incubation with soy, chicken, Quorn substrates and control (growth media and faecal slurry). There was a statistically significant effect of protein for propionate change of baseline (p<0.01). The Bonferroni post-hoc showed that chicken led to a greater propionate production *vs.* soy (+19.59 mmol/L, p=0.03) and *vs.* control (+23.19 mmol/L, p<0.01). There was no statistically significant effect of protein on the change from baseline for acetate and butyrate. The values for the remaining SCFAs (2-OH-butyrate, isovalerate, valerate, 2-methyl-butyrate, hexanoate) were below range of detection.

Table 26. Short chain fatty acid concentrations (mM) in the *in vitro* batch culture at 0 hours, 24 hours following incubation and change from baseline (N=8).

		0 hours		24 hours		Change from baseline	
		Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Acetate (mM)	**Soy**	0.00	0.00	70.08	12.60	70.08	12.87
	Chicken	0.61	0.44	75.97	16.81	75.36	16.78
	Quorn	6.88	6.03	75.66	15.87	68.78	17.03
	Control	0.00	0.00	44.13	12.73	44.13	12.73
Propionate (mM)	**Soy**	1.65	0.49	32.43	9.15	30.78	9.06
	Chicken	1.47	0.29	51.85	11.62	50.38	11.47
	Quorn	2.16	1.02	42.45	12.63	40.29	12.18
	Control	1.15	0.37	28.33	4.74	27.19	4.87
Butyrate (mM)	**Soy**	1.35	0.28	4.70	3.68	3.35	3.49
	Chicken	1.50	0.29	11.89	10.22	10.39	10.02
	Quorn	0.97	0.20	8.68	7.25	7.71	7.14
	Control	0.96	0.31	10.17	6.23	9.21	5.97
Total SCFAs (mM)	**Soy**	3.00	0.72	107.21	20.53	104.21	20.06
	Chicken	3.58	0.88	139.70	36.31	136.13	35.98
	Quorn	10.01	7.02	126.79	34.13	116.78	33.74
	Control	2.11	0.62	82.63	22.06	80.53	21.81

SCFAs, short chain fatty acids; SEM, standard error of the mean; mM, millimolar; n, sample size.

Figure 31 and Table 27 shows the percentage change in acetate, propionate, butyrate as well as the total SCFAs (mM) at 24 hours post-incubation. There was no statistically significant effect of protein in the percentage of acetate, propionate or butyrate. For total SCFAs, chicken protein significantly increased by +57.07 [+106.15,+7.98] mmol/L, compared to the control (p=0.01).

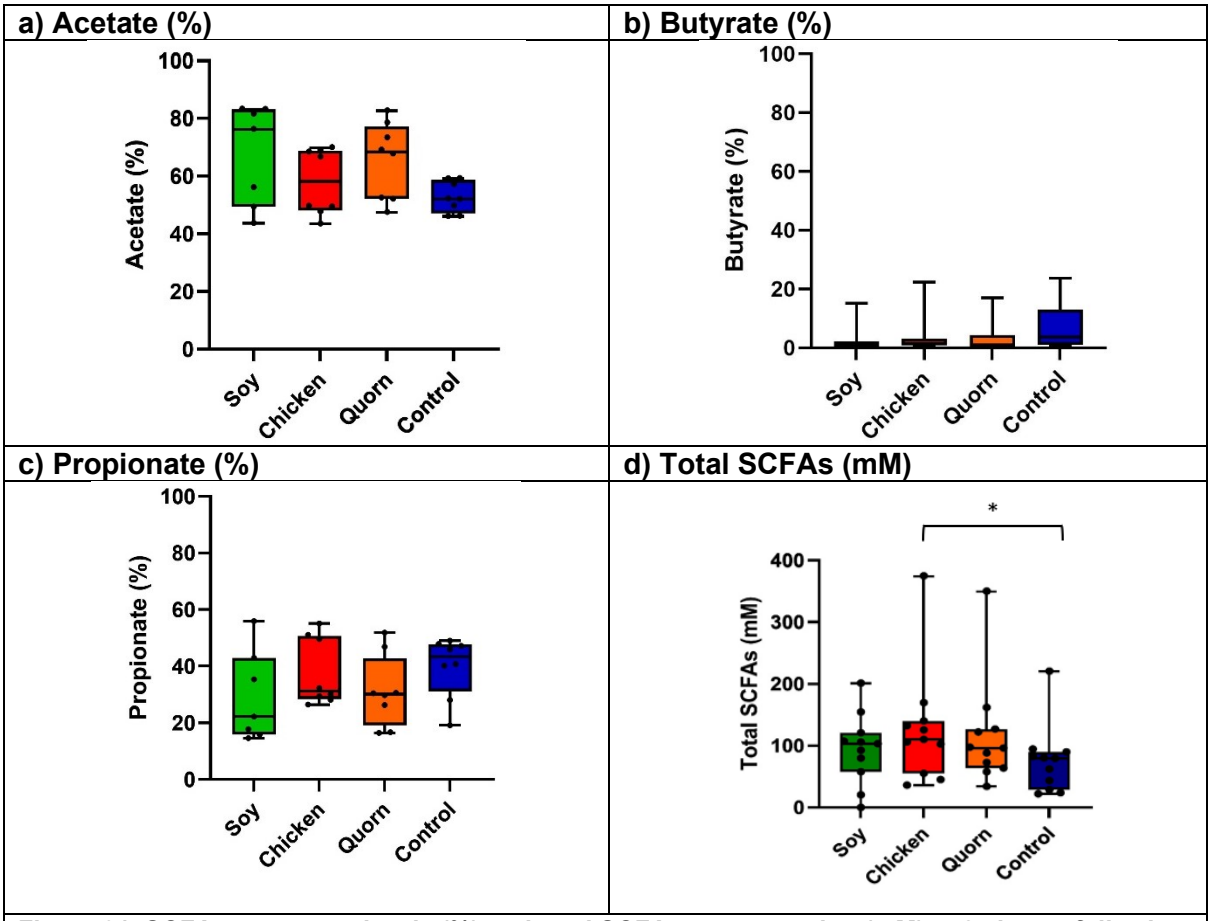

Figure 31. SCFA percentage levels (%) and total SCFAs concentration (mM) at 24 hours following the in vitro batch fermentation culture incubation with pre-digested soy, chicken and Quorn (N=8). Percentage levels (%) of a) acetate; b) butyrate; c) propionate, and d) total SCFA concentration (mM) being the sum of acetate, propionate and butyrate for pre-digested soy (green), chicken (red), Quorn (orange) and control (growth media, with faecal inoculum with no added substrate) (blue). Data represents the median and the 5-95 percentile and individual datapoints. Percentages are calculated over the total SCFAs (sum of acetate, propionate and butyrate). *P-value≤0.05. Mm, milimolar; n, sample size; SCFAs, short chain fatty acids.

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Table 27. Total SCFAs concentration (mM) and percentage of acetate, propionate and butyrate at 24 hours post-incubation.								
	Total SCFA (mM)		**Acetate (%)**		**Propionate (%)**		**Butyrate (%)**	
Substrate	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Control	80.53	21.81	52.76	1.66	39.78	3.81	7.45	2.83
Soy	104.21	20.06	59.20	10.20	25.62	5.87	2.68	1.79
Chicken	136.13	35.98	58.07	3.47	37.79	3.44	4.14	2.61
Quorn	116.78	33.74	65.46	4.27	31.13	3.90	3.42	2.01
Values are represented as mean±SEM. SCFAs, short chain fatty acids; mM, millimolar; SEM, standard error of the mean.								

The CV of the assay is reported in Appendix 24.

6.5.2.5 Bacterial composition

The results in Figure 32 show the change in faecal slurry microbiota at the phylum level for all participants following *in vitro* batch culture fermentation from 0 hours to 24 hours post-incubation of the pre-digested substrates soy, chicken, Quorn and control (growth media, with faecal inoculum, and no added substrate). At the phylum level, participants' stool microbiota shifted from SV_rare dominance at baseline to Proteobacteria predominance at 24-h post incubation for all four conditions. The SV_rare taxa represent the clustering together of a number of bacteria that had a low number of sequencing reads.

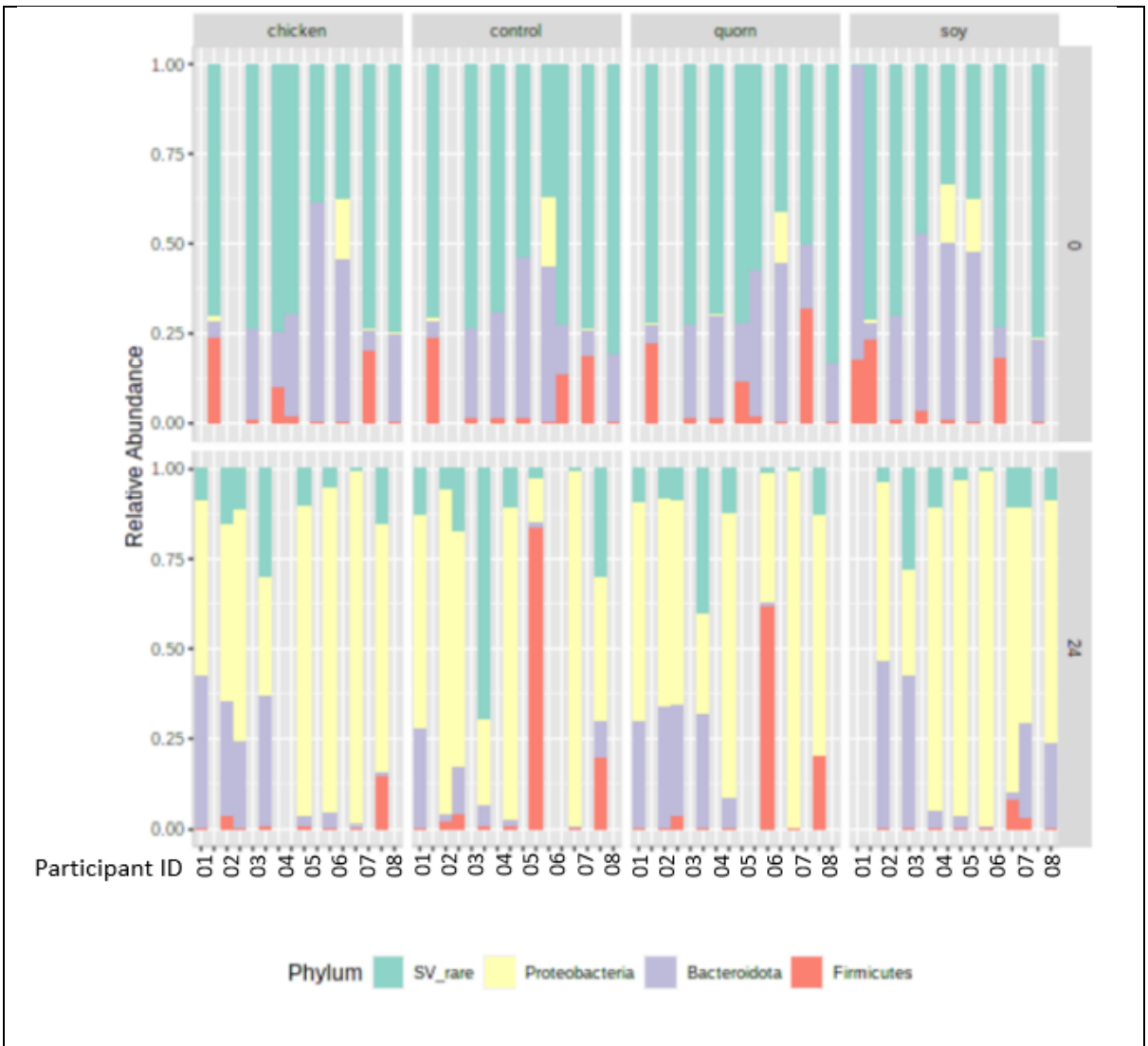


Figure 32. Faecal metataxonomic changes at the phlym level in relation to the *in vitro* batch culture fermentation of the pre-digested substrates chicken, control, Quorn and soy, (N=8). 16S rRNA gene sequencing of DNA extracted from faecal slurries, presented as relative abundance plots. Top horizontal bar graphs shows the timepoint of 0 hours, whereas bottom

horizontal bar graph shows the timepoint of 24 hours. All samples are positive samples (with faecal inoculum). Control is composed of growth media, with faecal inoculum, and no added substrate. SV_, structural variants; n, sample size.

At the family level, there were statistically significant changes in the relative abundance of Enterobacteriaceae, Lahnospiraceae, Clostridiaceae, Fusobacteriaceae, and Ruminococcaeceae from 0 hours to 24 hours in all conditions (see Figure 33). In particular, at 24h post-incubation there was an increase in Enterobacteriaceae by 40% and more modest increases for Clostridiaceae and Fusobacteriase (by 1%), whereas there was a decrease of Lachnospiraceae and Ruminococcaeceae (both by 5%). Since the biggest change over the course of 24h was for an increase in Enterobacteriaceae, changes at a deeper taxa level such as genera were explored. It was found that the abundance of the genus Escherichia-Shigella increased by +42.9% post-24 hours on average in all conditions (p=0.01). There were no statistically significant differences in the relative proportion for all other taxa at a genera level between the different protein types of post-24 hour incubation.

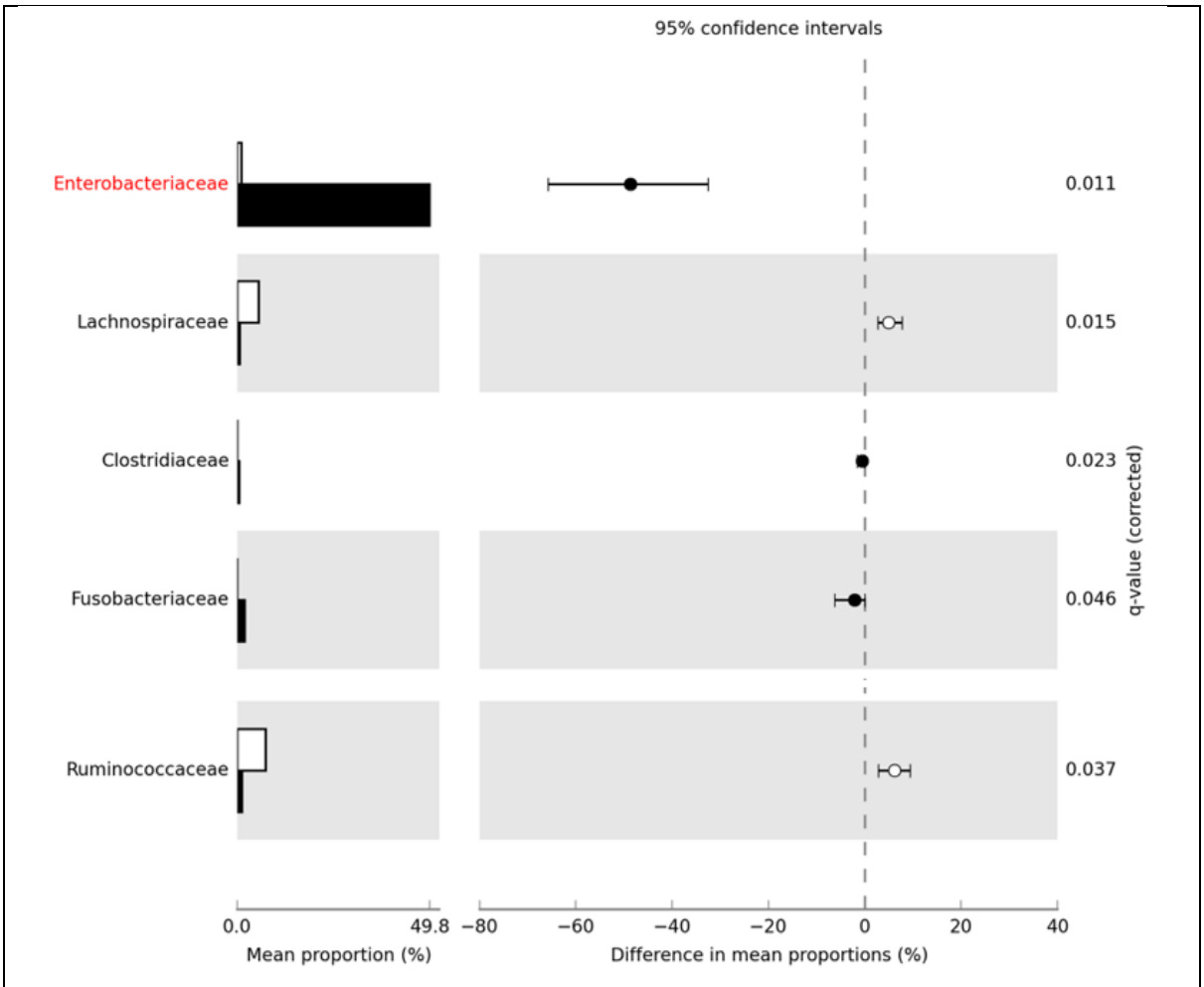
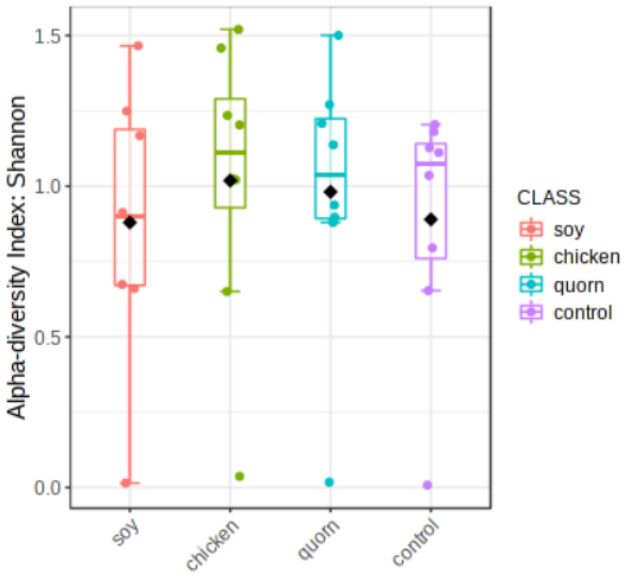


Figure 33. Mean proportion (%) (left panel) and mean proportion difference at the family level (%) (right panel) from 0 hours (white) to 24 hours (black) post-incubation in all

substrates (N=8). Only families with statistically significant differences are shown. Data shown as mean (left panel) and as mean [95% confidence interval]. Q-value (corrected) ≤0.05 is statistically significantly different. n, sample size.

The results in Figure 34 show the α-diversity and β-diversity of genus at 24 hours post-incubation for each condition (soy, chicken, Quorn and control). There were no statistically significant differences between the groups for any taxa.

(a) α-Diveristy



(b) β-diversity

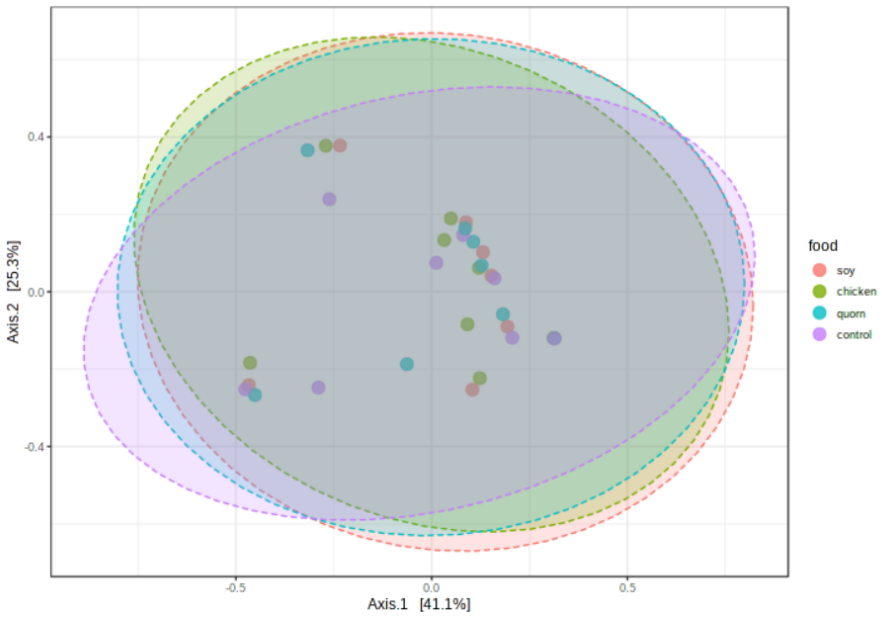


Figure 34. α- and β-diversity indexes of genus at 24 hours post-incubation for each

condition (soy (red), chicken (green), Quorn (blue) and control (purple)) (N=8). (a)α-divesity of soy (red), chicken (green), Quorn (blue) and control (growth media with faecal inoculum but not added substrate) (purple) following 24h of incubation. Data represents the median and the 5-95 percentile and individual datapoints. (b)β-diversity of soy (red), chicken (green), Quorn (blue) and control (growth media with faecal inoculum but not added substrate) (purple) following 24h of incubation. Data shown as mean±95% confidence interval and individual datapoints. N, sample size.

The results in Figure 35 show the statistically significant change in the relative abundance in detected at the taxa level of Family at 24 hours post-incubation. A statistically significant increase (+0.2%) in the proportion of Clostridiaceae was found in Quorn relative to soy (p=0.046). Furthermore, at the genus level, there was a positive trend for *Clostridium senso stricto 1* in being increased in the Quorn group compared to the soy group. The assigned sequence variant was assessed using the NCBI's database BLAST, and it indicated a high similarity to *C. beijerinckii*.

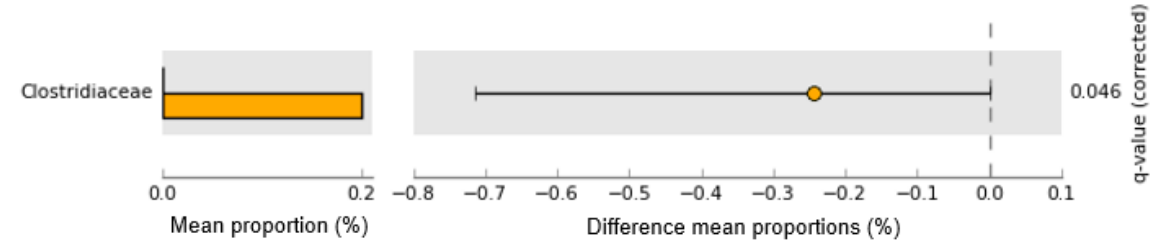


Figure 35. Mean proportion (%) (left panel) and mean proportion change (%) (right panel) of of the relative abundance of Clostridiaceae at 24 hours post-incubation in Quorn (orange) *vs.* soy (green – not visible) (N=8). Data shown as mean (left panel) and as mean [95% confidence interval]. Q-value (corrected) ≤0.05 is statistically significantly different. n, sample size.

6.6 Discussion

In this study it was investigated for the first time whether pre-digested mycoprotein was fermented by gut microbiota and there were SCFAs were and bacterial changes. This study was adequately powered based on previous data on SCFAs changes.

6.6.1 Summary of findings

- There was a significant decrease in breath hydrogen production in the Quorn condition compared to the positive control (inulin).
- There was no effect of any protein on pH change from baseline.
- There were no significant differences in pH for any protein at 24 hours compared to baseline, and for the difference of pH over time compared between each type of protein.

- There was an effect of chicken on SCFAs change from baseline and of chicken on propionate percentage at 24 hours-post incubation. The control had a higher butyrate percentage at 24 hours post-incubation compared to the other substrates.
- The α- and β-diversity did not significantly differ between protein treatments. Clostridium significantly differed in abundance when comparing Quorn and soy protein treatment, but there were no other significant differences detected between the remaining protein conditions.

6.6.2 Detailed discussion

6.6.2.1 The effect of mycoprotein fermentation on breath hydrogen

Hydrogen is a by-product of most gut microbiota carbohydrate fermentation (Levitt *et al*., 1968). Measuring breath hydrogen is an indirect and non-invasive technique that has been employed in many studies as a marker of gut transit and gut microbiota fermentation (Metz *et al*., 1976). In the present proof-of-concept study, the 3 participants showed a significant decrease in hydrogen production compared to the positive control of inulin following 480 min (8 hours) and following a low-fibre diet through the study day and evening prior. This finding is in line with an internal report found in the grey literature in which *in vitro* batch culture of mycoprotein did not lead to the production of hydrogen (Cummings *et al*., 1990). It has been reported that some strains of Bacteroides and anaerobic streptococci are not able to produce hydrogen and some bacterial species may produce insufficient hydrogen to be detected in the breath (Metz *et al*., 1976). Therefore, care should be taken in drawing conclusions on whether Quorn is fermentable *in vivo*. Altogether, this suggests that mycoprotein-derived food such as Quorn may not be fermented by the gut microbiota in humans *in vivo.* However, further experiments *in vivo* should be conducted to validate this as not all bacterial fermentation leads to hydrogen production.

6.6.2.2 The effect of mycoprotein fermentation on pH

In this study, pre-digestion of the substrates prior to the fermentation was employed which represents a novelty in the methodology compared to the methodology of previous studies on the topic (Harris et al., 2019). This was done to mimic physiological conditions in which digested Quorn would be first digested by the gastrointestinal tract and exposed to digestive enzymes and bile acids before it is exposed to the gut bacteria. The results showed that there was a significant drop in pH compared to baseline for all substrates independently. However, the average drop observed following 24 hours of incubation was 0.68 units which

is relatively low when compared to the 1.5 units drop in pH in other studies using pre-digested substrates (e.g. beta-glucan from oats) (Mischie 2020, unpublished). There was no effect of substrate added (soy, chicken, Quorn or control-growth media with no substrate-) on the pH change from baseline. A decrease in pH can be an indicator of media's acidity, and increased acidification over time would be suggestive of gut microbiota fermentation into products that acidify the media such as SCFAs (Campbell et al., 1997). In this study, we used an eutrophic growth media which included peptone water, yeast extract, several salts, bile salts, hemin and menadione. Peptone water represents an extra source of nitrogen for the bacteria on top of the test protein substrate added. Bacterial by-products of fermentation depend on the carbohydrate/protein ratio (Portune *et al*., 2016). Higher ratios would lead to the use of CHO as an energy substrate for the bacteria, whereas protein would be used for bacterial growth building blocks (Portune *et al*., 2016). In this case, the media is low in carbohydrates, meaning that the protein present is used as energy (catabolism) and detrimental products will be generated such as indoles, hydrogen sulfides, ammonia, etc. For this reason, it is possible that products from amino acid catabolism were generated and may have buffered, the pH decreased as a result of SCFAs production. Nevertheless, metabolites other than SCFAs were not assessed in this study, and they warrant consideration in future research.

6.6.2.3 The effect of mycoprotein fermentation on SCFAs production

In line with the findings on pH, there was no effect of any substrate on SCFAs (total, acetate, butyrate, and propionate) change from baseline, except for chicken which led to increased propionate. Notably increased levels of acetate are found at baseline levels of the Quorn condition. This increase may be related to the presence of calcium acetate as reported on the ingredients' labelling of Quorn products, as this component is used in its processing and shaping of raw mycoprotein into Quorn meat-alike products. Nevertheless, statistical comparisons used in this section are made from the changes from baseline (so any potential increases of SCFAs found at baseline derived from their ingredient recipe and not gut microbiota fermentation itself, are corrected for).

Total SCFAs at 24h showed one outlier which had the highest SCFAs values for chicken, Quorn and control. This was identified to be individual number 5 who had the greatest intake of fibre a day (mean±SD) (32.68±6.45g/day) which could explain the elevated SCFAs levels.

Mycoprotein fermentation has been previously studied using *in vitro* batch culture for 24 hours using healthy human faecal samples (Harris *et al*., 2019). In this study, they showed that mycoprotein fibre increased total SCFAs compared to control by 6 times (61.2 mM vs. 11.13 mM, respectively) and similarly to the rest of the tested fibres (positive controls). The mycoprotein fibre that Harris and team used was a fibre-rich substrate that had 75% fibre available for fermentation. The authors obtained it via an in-house purification method, and administered it in powder, which is not representative of the Quorn-products consumed *in vivo*, which contain more than fibre (e.g. protein). This is highlighted with the finding that the fermentation efficiency of the 75% fibre-rich mycoprotein fibre was 5 times less than whole mycoprotein, in which 4 mM of SCFAs were produced per gram of whole mycoprotein, suggesting that more components than just fibre are important for gut microbiota fermentation.

In the present study, Quorn substrate as sold in the supermarket (with 6% of fibre) was used and underwent an *in vitro* digestion protocol that simulates human digestion physiology and freeze-drying (pre-digestion). The pre-digested Quorn substrate we used led to a non-significant increase of total SCFAs compared to control by 1.45 times (116.78 mM vs. 80.53 mM, respectively). This was in line with the findings of Harris when a similar Quorn product (retail chicken style pieces) (whole mycoprotein) containing 6% of fibre was assessed which led to a non-significant increase of total SCFAs compared to control by 2.2 times (to 24.87 mM), although of a magnitude 10 times lower.

The findings of this study disagree with our initial hypothesis that pre-digested Quorn would lead to increased SCFAs production. The results of this study build on previous literature suggesting that it is possible that higher fibre concentrations (around 75%) do lead to increases in SCFAs production using a faecal inoculum of 32%. However, more realistic Quorn substrate (pre-digested and with lower concentrations of fibre such as 6%) do not lead to SCFAs production using a faecal inoculum ranging 25-32%. It is unlikely that during the *in vitro* digestion the fibre fraction of the substrates was removed during the process since the enzymes employed were α-amylase, pepsin, pancreatin which are not able to break β-glycosidic bonds in β glucan or within the chitin glucan complex. Furthermore, a dialysis tubing of pore size 1 kDa was used and the chitin-glucan unbroken are high molecular complexes of about 100-200 kDa (EFSA Journal 2011), so it would have not been lost from the matter during the dialysis osmotic step used for *in vitro* fermentation.

The lack of SCFA production in this study may be attributed to other factors that were not measured. There are many factors that influence specific-SCFAs production from gut microbiota *in vitro*. Amongst them are solubility, where soluble fibres generally are more fermentable than those which are not. In the case of Quorn, the fibre content is composed of 1/3 chitin and 2/3 fungal β-glucan. Fungal β-glucans are typically polymers covalently bound to chitin via hydrogen bonds, forming a network within the β-glucan matrix which results in a strong cell wall that is insoluble (Rosenberger *et al*.,1976). Also, it is important to consider the starting pH in which the *in vitro* batch culture fermentation takes place as it may influence specific-bacterial growth and SCFAs ratio production (Belenger *et al*., 2007, Walker *et al*., 2005, Duncan *et al*., 2009). For example, butyrate-producing bacteria can be detected at pH 5.5, but not at pH 6.7 (Duncan *et al*., 2009). Colonic pH depends on dietary intake and gut area but it oscillates around 5.5 (distal gut) to 7.5 (proximal gut) (Farmer *et al*., 2014). Experts recommend adjusting the pH to 7.0 with HCl 0.1 M for *in vitro* batch culture fermentation (Pérez-Burillo *et al*., 2019), however, in the present study, the starting growth media pH was around 8-8.5, despite having used a defined bacteria growth media recipe, which may have not been optimal for bacterial growth and fermentation. In addition, the same authors recommend using a 32% faecal inoculum to assure optimal bacterial density whereas in the present study a 25% was used as it was previously used before by McDonald (2013) for the same type of experiment.

In Harris and team's study, both types of mycoprotein (fibre-rich fraction and whole mycoprotein) tested showed a similar proportion of acetate, propionate and butyrate conforming approximately the 55,22,22% of total SCFAs, respectively (Harris *et al*., 2019. This is in line with the percentages found in this study of 39,27,33%. In the present study, there were no changes in SCFAs percentage at 24 hours-post incubation. One difference was observed for acetate percentage that was increased in soy, chicken and Quorn protein types compared to control (no protein substrate added), although the absolute values in mM of acetate and total SCFAs (acetate + propionate + butyrate) were not different in any protein compared to the control.

6.6.2.4 The effect of mycoprotein fermentation on bacterial composition

In line with the findings on pH and SCFAs, there were no changes in gut microbiota at 24 hours across the different protein sources and negative control. This was against the initial hypothesis. A period of 48 hours was enough to observe a significant change in β-diversity

following a switch to a high-protein diet (15 to 30% of daily kcal from protein), given that 24 hours were needed for food to reach the colon where gut bacteria is most abundant (measured with tracing dye) (David *et al*., 2014). However, this was not observed for the high-fibre diet (10 g to 25 g fibre/day) group, which is in line with these findings. In this study, we used a high-fibre and protein substrate Quorn (20 kcal/100 kcal of protein) and we exposed it to bacteria for 24 hour (a 48 hour period incubation time was not possible due to the fact that the batch culture was not pH-controlled and 24 hours to allow substrate reach the bacteria was not needed as it was a direct exposure). The ability for bacteria to grow to niche environments created by the addition of a substrate for which bacteria is already adapted for, such as a protein increase from 15 to 30% of daily kcal from protein, may be a fast process that may explain the contrasting results from this study and the literature. However, in the present experiment, Quorn substrate was used, which represents an unusual source of dietary fibre (fungal β-glucan complexed with chitin) for which baseline bacteria may have not been adapted to. For this reason, it can be that the baseline bacteria from the subjects may be naïve to its fermentation and usage due to a lack of essential enzymes capable of breaking down β-glucan and chitin complexes. Such adaptation may require more time than 24 hours, hence the lack of significant change in bacterial diversity (α – referring to the number of species present; and β – referring to the relative abundance between communities (Walters *et al*., 2020). In this context, only one participant was self-reported as Quorn consumer, however, the regularity of Quorn-products consumption was once a month, which may have not been enough to have Quorn-specific species present in that sample and detect a capacity of Quorn fermentation and increase in SCFAs production.

Nevertheless, a significant change was detected for the Quorn protein type compared to soy in which the family Clostridiaceae was increased. The family of Clostridiaceae are strict anaerobic, saccharolytic species able to use chitin and to be proteolytic (Maukonen, 2012). They are butyro- and acetogenic but it depends on the substrate they use (Maukonen, 2012). There was an almost significance with *Clostridium senso stricto 1*, for which its sequence was blasted and resulted to be closely matched to the specie *C. beijerinckii*. Nevertheless, this could be a chance finding as the p-value was close to being non-significant and a deeper analysis of the individual data shows that only one person had increased relative proportion of this family.

6.7 Limitations

In this study a static *in vitro* batch culture was used to conduct a powered study based on the preliminary results of a pilot trial using 3 participants. While this methodology is useful for initial trials such as the present one, it has some drawbacks such as the short-period time of incubation it allows (up to 48h) and the high-risk of infection due to multiple sampling. In this study, we limited the incubation time to 24 hours to prevent detrimental metabolites to accumulate and cause changes to pH that could hamper normal bacterial growth. Furthermore, strict aseptic measures were used throughout the experiment to control for external contamination. One of them was seeding fastidious agar plates with each experimental condition, to visually check that there was no bacterial growth in the negative controls (test protein + growth media). This was in line with the 16S and DNA concentration data on the negative controls.

The gold-standard *in vitro* methodology to assess gut microbiota fermentation is the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). This is a continuous system which closely replicates physiological intestinal conditions. The two main characteristics of this system is that there is movement of the digesta through pumped vessels as it would happen with the faecal matter along the different areas of the gut *in vivo*, and that pH of the system is tightly controlled (as different gut areas have different pH (Farmer *et al*., 2014), allowing longer incubation periods (Molly *et al*., 1993). The downside is that this equipment is expensive and complex, meaning that it is not widely available and requires a high level of expertise. However, the ideal model should contemplate a gut epithelium lining, dissolved oxygen (concentrations depending on gut area), complex gut microbiota, anaerobic/microaerophilic conditions, a mucus layer and physiological conditions of pH, and fluid retention times. Furthermore, it would be interesting to first understand the intestinal gastric time of each test food for each individual and culture bacteria in such model for as the length period of each participants' specific intestinal transit time. Nevertheless, it is important to keep in mind that the best approach is to be able to assess gut fermentation directly from human via non-invasive methods. At the time of writing this thesis, only one approach is currently under trial, which is real-time gut microbiota fermentation technology employed by Professor Ellen's Blaak team in the University of Maastricht, in collaboration with TSE systems and Sensus companies.

The present static *in vitro* batch culture fermentation study used a medium that was low in fibre, fat and carbohydrate, therefore gut bacteria were switched to a different growth medium from that in the human gut. The study could have been improved by using a growth medium that better reflected a typical human diet as gut microbiota metabolism and therefore taxa changes are influenced by the carbohydrate to protein ratio (Portune *et al*., 2016). In a free-living population protein is usually consumed in combination with carbohydrate, perhaps promoting a bacterial usage of nitrogen towards building blocks rather than energy, and this is a scenario we did not reproduce in the *in vitro* experiment in which we fed the test protein alone.

In this study, we did not assess other products of fermentation which are typical from protein fermentation such as indoles, sulphide hydrogen, ammonia which would have led to differences in percentage of acetate, butyrate and propionate over the total metabolites produced by the gut bacteria (including products from protein fermentation). These are important metabolites since they have an impact on the metabolism. Relativising the SCFAs concentrations to the total gut microbiota-derived metabolites can highlight what the relevance of SCFAs in the host metabolism is and their likelihood to be sensed by the FFAR in the gut. Furthermore, studying other metabolites inform of cross-feeding processes (bacterial growth promoted by the by-products of the fermentation of a main substrate).

6.8 Conclusions

In conclusion, the novel finding from this study is that Quorn intake did not increase breath hydrogen production *in vivo*, and that pre-digested Quorn did not change pH,SCFAs concentration nor microbiota *in vitro*, suggesting that Quorn may not be fermented by the human gut microbiota. This study adds value to the existing scarce literature via a statistically powered (main outcome change in SCFAs) study that used a realistic substrate (pre-digested). Furthermore, it contributes to expanding the understanding of such unsual type of fibre in our diets (complex beta-glucan and chitin). Since this fibre is mostly insoluble, and insoluble fibres generally have a role at modulating bacterial communities, an adequately powered study to detect differences at a bacterial level is needed, which can be based from the results of this study. This study has some limitations such as a short incubation time (24h) due to the use of an *in vitro* static batch culture fermentation, which does not resemble features of the *in vivo* gut physiology (pH controlled and movement of digesta along different gut vessels), and that a positive fermentable control was not used.

Therefore, future studies should be repeated using better fermentation approaches such as SHIME using *in vivo* content sampling from the lower gut and a positive fermentable control.

6.9 Bibliography of Chapter 6

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Chapter 7: General Discussion

7.1 Summary of Findings

- This is the first-time a crossectional study investigating the association of being a Quorn consumer with health parameters is performed using a database of free-living population such as the NDNS. Being a Quorn consumer was significantly associated with HDI, DASH score, fibre, total and food energy intake, and a negative associated with energy density, sugars, alcohol intake, and FBG and HbA1c.

- This is the first-time the available evidence on mycoprotein and glycemia and appetite/energy intake is systematically reviewed. The results suggested that there was a consistent significant effect of acute mycoprotein intake at decreasing appetite and energy intake, and a less clear effect on glycaemic control, with potential to improve insulin sensitivity.

- This is the first-time that the acute effects of mycoprotein along with guar gum on glycemia, appetite and energy intake are tested in subjects with T2D. Acute intake of mycoprotein decreased postprandial blood glucose compared to chicken and guar gum-enriched chapati decreased postprandial blood glucose compared to plain chapati in people with T2D. Ethnicity had an effect on blood glucose, in which the south Asian group had a higher postprandial blood glucose compared to white Europeans. There was no effect of mycoprotein or guar gum on appetite or energy intake. The mechanisms behind the effects on blood glucose may not be driven in changes in gut hormones.

- This is the first-time the potential of Quorn at producing breath hydrogen (marker of fermentation) is tested in free-living humans for 8 hours. Quorn did not show to increase breath hydrogen in healthy humans compared to inulin.

- This is the first-time that pre-digested Quorn is tested in an *in vitro* batch culture fermentation to understand its gut microbiota fermentability potential. Pre-digested Quorn did not lead to a change in pH, bacterial communities and SCFAs production followed by 24h of *in vitro* batch culture incubation, suggesting that Quorn foods cannot be fermented by the human gut microbiota.

7.2 Introduction

T2D is a metabolic disease affecting millions of people worldwide, with a high prevalence in south Asian countries. The blood glucose response following the same dietary challenge is different between matched south Asians and white Europeans. Dietary fibre such as guar gum and protein play a role at regulating blood glucose and appetite in healthy and people with T2D. One of the main mechanisms underpinning this is via gut hormone release stimulated by gut microbiota fermentation derived products such as SCFAs. Mycoprotein is a meat-replacement sold under the name of Quorn. Mycoprotein is naturally high in both fibre and protein content, and it has been consumed by the UK population since 1985. Nevertheless, it has been in the supermarkets for nearly 30 years without knowing the benefits that Quorn may have on health. Understanding the impact of consuming mycoprotein-based products in the UK populations' health is important, and this has constituted the Study 1. Evidence from studies in humans suggest that mycoprotein has a beneficial effect on blood glucose and appetite, but this evidence needed to be systematically reviewed and meta-analysed, which has constituted the Study 2. From the results of the systematic review, studies using metabolically compromised individuals such as T2D were lacking. Therefore, in Study 3 the effect of mycoprotein and guar gum in combination with guar gum was studied using a RCT in people with T2D of south Asian and white European ancestry, as the food ingredient guar gum has consistently been shown to decrease blood glucose levels in people with T2D. Furthermore, understanding the potential mechanisms by which mycoprotein has an effect on blood glucose and appetite were explored in Study 4. Considering that high-fibre and protein foods stimulate gut microbiota fermentation to SCFAs and that these have an impact on blood glucose and appetite regulation, an *in vitro* batch culture fermentation study was conducted.

7.3 Discussion and Findings

In Study 1, for the first-time a crossectional study investigating the association of being a Quorn consumer with health parameters (i.e., nutritional intake, healthy diet scores, demographic variables, NCD's risk markers) was performed using a database of free-living population such as the NDNS. It was confirmed for the first time that being a mycoprotein consumer was associated with greater diet quality scores, higher fibre in the free-living population, which was in favour of the initial hypothesis. Furthermore, mycoprotein consumers were shown to be negatively associated with energy density, sugars, alcohol intake as well as FBG, HbA1c, the latter being NCD's risk markers. This was also in

agreement with the initial hypothesis. These findings were in line with a study in which a 1-week intervention with Quorn led to a decrease in FBG (Coelho *et al*., 2020). The results of the associations suggesting a relationship between being a mycoprotein consumer and better diet scores and health outcomes could be possibly related to a healthy consumer bias. This was confirmed when the demographic profile of mycoprotein consumers was explored, in which there were more likely to be women, white, younger, living in England, belonging to a higher socio-economic status, have greater dietary quality scores and less likely to be current smokers relative to non-consumers. This raised an important imperative of finding ways to increase consumption of mycoprotein-based foods within males, older individuals, Black, Asian or minority ethnicities, which are demographic groups at great risk of chronic diseases such as T2D. The addition of mycoprotein foods into their diets could represent a health benefit at reducing their metabolic risk to chronic diseases such as T2D. Furthermore, being a mycoprotein consumer was associated with a greater total and food energy intake which was against the initial hypothesis. This finding is in line with the literature in which acute mycoprotein interventions led to a decreased energy intake (Williamson *et al*., 2006, Bottin *et al*., 2016). Despite Study 1 in general showing the associations of including mycoprotein in one's diet on positive health outcomes, there was still a need to understand whether these effects were replicated in a controlled setting and whether mycoprotein alone had an impact on glycaemic control and appetite. Therefore, Studies 2 and 3 were conducted.

In Study 2, for the first-time a systematic review on mycoprotein and glycaemia, appetite and energy intake in humans was conducted. Systematic reviews are at the top of the pyramid of evidence, which pools empirical and critically appraised data that fits determined eligibility criteria to answer a specific question (Cochrane Handbook). They also help identifying gaps and flaws of available studies to date and identifying future areas of research. The results of Study 2 did not show a clear effect of acute mycoprotein on blood glucose levels, but it showed a decrease in insulin levels, which did not agree with the initial hypothesis that mycoprotein led to a reduced postprandial blood glucose, due to a lack of evidence. Acute mycoprotein intake also showed to decrease energy intake at an *ad libitum* meal and post-24 hours in healthy lean, overweight, and obese humans which was in favour of the initial hypothesis. The evidence in regards the mechanisms underpinning these effects are scarce and inconclusive. While there may be a suggestion that the insoluble and hyphal structure of

mycoprotein may delay gastric emptying, having an impact on both glycaemic control and appetite, the paracetamol method did not show differences between mycoprotein and chicken (even though such technique was not the gold-standard to measure the gastric emptying of solid food). This was coupled to the lack of GLP-1 response (hormone that modulates gastric emptying), suggesting no effect on gastric emptying. However, other evidence such as the digestion pattern of the protein content following mycoprotein intake was suggestive of an effect on gastric emptying which should be explored in future studies with adequate methodology. All in all, evidence came from a very limited number of heterogeneous studies. Further well-controlled and low-bias-risked studies are needed to elucidate the short- and long-term effects of mycoprotein intake on glycaemic control and energy intake, in healthy and metabolically compromised people such as people with T2D, as well as the mechanisms underpinning these effects.

In Study 3, for the first-time, the acute effects of mycoprotein along with guar gum are tested on glycemia, appetite and energy intake are tested in subjects with T2D via a RCT. The subjects of study were people with T2D of south Asian descent compared to those with T2D of white European descent. This presents another novelty, since to-date subjects of study in Quorn investigations were mostly white European, and poorly represented ethnicities (which have worst metabolic handling of food) were not studied. A third novelty was the design of a palatable, ethnic-adapted ready-meal (i.e. spices and chapati bread) which in one go was providing the 66% of the DRI of fibre. The rationale behind this came from the results of Study 1 and 2, where it was identified that there was a need and lack to test the effect of mycoprotein on vulnerable ethnicities and metabolically compromised individuals such as people with T2D. The intervention of this study was accompanied with guar gum to study whether there was an additive effect of adding two sources of fibre-rich foods on the outcomes. These attributes made the study be the first one of this kind to be performed. The results showed that mycoprotein induced a similar decreasing effect on postprandial blood glucose to soy and a decreased response compared to chicken which was in line with the hypothesis. This could be due to the fibre content of both soy and mycoprotein compared to chicken. However, there was no interaction effect between mycoprotein and guar gum as it was hypothesised. On the other hand, the next finding showed that south Asians presented an increased postprandial glucose response compared to white Europeans, despite a better demographic and metabolic profile than white Europeans. I hypothesised that differences in

postprandial blood glucose would occur between the two ethnicities. Mechanisms underpinning these are unknown, but this could be due to the south Asian group having lower muscle mass, therefore less capacity to metabolise glucose or a higher degree of insulin resistance, although markers such as ALT and visceral fat were not unfavourable for the south Asian group. These ethnic differences in the way these fibre-rich protein is metabolised are important as not only they build up onto the scarce available data on the topic, but it highlights that not all dietary management are equally as efficient at decreasing blood glucose in an ethnic community and that ethnicity is a factor to be considered for T2D dietary management. Nevertheless, enriching chapati with guar gum induces a decreased blood glucose response in all participants. Guar gum-enriched chapati had an effect at reducing postprandial insulin response, particularly in the south Asian, and does not have an effect on appetite, and energy intake probably due to the solid matrix it was contained in leading to a loss of the viscous property. In study 3, lnly gut hormones GLP-1 and PYY were investigated as mechanisms driving the effects. It was observed that there was no effect of protein, bread or ethnicity type on their levels. This was a similar finding to that of Bottin's (Bottin et al., 2016) were a similar amount of acute mycoprotein led to no changes in these gut hormones compared to chicken in healthy overweight and obese individuals. Therefore, the mechanisms underpinning the effects of mycoprotein were unclear.

In Study 4, for the first-time pre-digested Quorn was tested in an *in vitro* batch culture fermentation to understand its gut microbiota fermentability potential. This was performed to to understand the mechanisms behind the potential chronic effects of mycoprotein on glycaemia and appetite observed in Study 1. Study 4 presented several novelties when compared against existing evidence on the effects of mycoprotein on SCFAs production (Harris *et al*., 2019):

- First, it was an adequately powered study.

- Secondly, an *in vitro* digestion protocol was used to pre-digest the substrates. This was done to have a better approximation of a real-life setting in which pre-digested food travels down the colon to be fermented, rather than whole food.

- Thirdly, we assessed changes in gut microbial communities and in pH to have a more holistic picture of the potential gut fermentation of Quorn.

The results showed that Quorn intake does not lead to increase in hydrogen production in humans suggesting that Quorn may not be fermented in human gut. In addition, *in vitro* assessments have not showed that pre-digested mycoprotein leads to a decreased pH and increase in SCFAs concentration and clinically significant change in bacterial community compared to the negative control. This was against the hypothesis that mycoprotein gut fermentation would lead to increased SCFAs production. The growth media used was low in carbohydrate, meaning that the protein present may have been used as energy (catabolism) and detrimental products will be generated such as indoles, hydrogen sulfides, ammonia, etc. For this reason, it is possible that products from amino acid catabolism were generated and may have buffered the growth media. This may have affected optimal bacterial growth and SCFAs production. Furthermore, there are many factors that influence specific-SCFA production from gut microbiota *in vitro* such as solubility and starting pH. The solubility of mycoprotein is not defined, and the literature suggests that fungal β-glucan forms a strong insoluble network with chitin (Turnbull *et al*., 1998). In addition, starting pH may have been too low to allow optimal growth of butyrate-producing bacteria which can be detected at pH 5.5, but not at pH 6.7, which explains the lack of butyrate differences for instance. This may have been the case for the other types of SCFAs as well.

7.4 Limitations

7.4.1 Epidemiological study

The epidemiological study is a cross-sectional study, meaning that data are studied at a specific timepoint. This limits the ability to infer cause-and-effect relationships. For this reason, longitudinal studies, which include measurements of the same individual over time, are needed to capture the real effect of chronic exposure to mycoprotein-based foods in each individual. Furthermore, NDNS is a survey-database in which participants self-reported their dietary intake for 4 days. Despite, the food was weighed, participants were not trained to do such task and self-reported energy intake is regarded by the scientific community as a high-risk biased methodology to report (Lichtman *et al*.,1992, Schoeller *et al*., 1990 and 1995). Instead, objective food intake measures should be employed but at the time of writing this thesis, no objective method has been yet developed and validated against the gold standard.

In this study, it was not possible to adjust for physical activity levels because the NDNS database lacked a standard measurement for it across the survey years. Physical activity

has been linked to glycaemic control, metabolic parameters and energy intake in a wide range of studies (Frampton *et al*., 2021). Using other databases such as the AIRWAVE or NHANES database with a standard measure for physical activity was not possible because they did not capture specifically Quorn branded products. Importantly, the NDNS database was not complete as some blood markers were missing in almost 50% of the participants for both consumer and non-consumer group. This is an inevitable and intrinsic consequence of the nature of mass-databases, and it may have been due to either a refusal by the participants to have the nurse visit or due to issues during the laboratory analysis. To assess whether the missing data may have led to biased results, a post-hoc analysis was performed and showed some statistically significant differences in nutrient intakes between participants with and without missing data. Therefore, it is possible that should the full dataset for all parameters had been available, the real associations of being a mycoprotein-based food consumer on blood parameters and blood pressure may differ from the results obtained in this study. A way to address the missing data problem was using multiple imputation. However, this was carefully considered and disregarded as imputing data for such high number of missing participants from the available participants' data may have led to a decreased group variance, increasing the chances of detecting statistical differences between groups, this leading to biased results.

Furthermore, the demographic profile observed for mycoprotein consumers was more likely to be women, white, younger, living in England, belong to a higher socio-economic status, have greater dietary quality scores and less likely to be current smokers relative to non-consumers. This may have led to a demographic unrepresentativeness of other population groups such as men, black, and of lower socio-economic status which may have represented a limitation. Therefore, care should be taken when extrapolating these results in the real-world population.

7.4.2 Systematic review

In the systematic review the evidence found from the systematic search was limited in number of studies (n=3, for glycaemic outcomes and n=4, for energy intake outcomes). Furthermore, data were heterogenous as these had diverse ways of reporting the glycaemic outcomes (e.g. mean glucose at specific time frames and iAUC) and energy intakes (post-20 min and post-180 min) which could not be pooled together for a meta-analysis as they are not physiologically comparable. Meta-analysing the results is important to improve precision

of estimates of the overall effect and to settle controversies arising from conflicting studies. This highlights the need for further research on the acute effects of mycoprotein on glycaemia and energy intake in a diverse range of populations (healthy, metabolically compromised and with T2D) using physiologically relevant and similar ways to report the outcome so meta-analyses are possible. Chronic studies are important to understand the health impact of a long-term exposure to mycoprotein as they provide a realistic context within a free-living setting. Such studies should be high-quality, meaning that they should abide by PROSPERO protocols, follow CONSORT reporting guidelines to avoid risk-of-bias, and have an adequate control of outcome co-founders. Furthermore, high-quality studies also mean employing gold-standard methodologies for glucose management assessment such as euglycaemic-hyperinsulinaemic clamps and energy intake such as automatic re-fillable *ad libitum* meals in a controlled environment (Wanskin *et al*., 2005) and free-living energy intake reporting using objective measures, which are still under-development at the time of writing this thesis. Only with high-quality studies it is possible to generate high-quality and trustable data from meta-analysis.

Equally as important, uncovering the mechanisms relating to mycoprotein-induced decreases in subjective appetite, acute and short-term energy intake and any potential effect glycaemic control is key to inform re-development of mycoprotein-containing foods or the use of different mycoprotein variants with characteristics that can optimise health outcomes in the population. So far, data are scarce and inconsistent regarding the mechanisms underpinning mycoprotein's effects, and studies investigating gut hormone release, gastric emptying, gut microbiota fermentation following mycoprotein intake are greatly required.

7.4.3 Human study

This human study was not powered to detect statistical differences on glycaemic, appetite and energy intake outcomes in people with T2D of south Asian or white European descent. However, data from this pilot trial is useful to inform future studies using the same intervention and type of population.

Energy intake post-visit was assessed using unweighted self-reported food diaries and this may have led to reporting bias constituting an important limitation of the study. The data highlights this as a significant reduction in their reported baseline energy intake per day was found when compared to their calculated energy requirements per day (calculated based on metabolic rate and physical activity). This is a widely acknowledged limitation within the self-

reported food diaries approach for energy intake measurement (Schoeller *et al*., 1990). Therefore, future studies assessing energy intake should consider using better methodologies such as wearable, and unnoticeable technology which is able to track what we eat or sophisticated and accurate metabolic profiling that reflects diet intake. However, these approaches are still underway at the time of writing this thesis.

Nevertheless, the main limitation of the human study is that it is an acute intervention. While acute interventions are useful to determine the postprandial response to a mixed meal test within a very tightly controlled environment, they provide little information on whether the acute effects observed are also present with chronic intakes. For example, continuous reductions of postprandial blood glucose over time are more physiologically relevant than at one-single time blood glucose reduction for people with T2D. Considering the beneficial nutritional profile of mycoprotein, the sparse trial data on glycaemia and appetite and the results on the epidemiological study, it is suggestive that chronic intakes of mycoprotein may have a role at regulating blood glucose, appetite, and energy intake. Therefore, high-quality longer-term trials investigating the effect of chronic mycoprotein intake on both PPG and Hb1Ac changes in people with T2D are needed to explore this.

7.4.4 *In vitro* study

In this study a static *in vitro* batch culture was used to conduct a powered study based on the preliminary results of a pilot trial using 3 participants. While this methodology is useful for initial trials such as the present one, it has some drawbacks such as the short-period time of incubation it allows (up to 48h) and the high-risk of infection due to multiple sampling. In this study, we limited the incubation time to 24 hours to prevent detrimental metabolites to accumulate and cause changes to pH that could hamper normal bacterial growth. Furthermore, strict aseptic measures were used throughout the experiment to control for external contamination. One of them was seeding fastidious agar plates with each experimental condition, to visually check that there was no bacterial growth in the negative controls (test protein + growth media). This was in line with the 16S and DNA concentration data on the negative controls.

The gold-standard *in vitro* methodology to assess gut microbiota fermentation is the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). This is a continuous system which closely replicates physiological intestinal conditions. The two main characteristics of this system is that there is movement of the digesta through pumped

vessels as it would happen with the faecal matter along the different areas of the gut *in vivo*, and that pH of the system is tightly controlled (as different gut areas have different pH (Farmer *et al*., 2014), allowing longer incubation periods (Molly *et al*., 1993). The downside is that this equipment is expensive and complex, meaning that it is not widely available and requires a high level of expertise. However, the ideal model should contemplate a gut epithelium lining, dissolved oxygen (concentrations depending on gut area), complex gut microbiota, anaerobic/microaerophilic conditions, a mucus layer and physiological conditions of pH, and fluid retention times. Furthermore, it would be interesting to first understand the intestinal gastric time of each test food for each individual and culture bacteria in such model for as the length period of each participants' specific intestinal transit time. Nevertheless, it is important to keep in mind that the best approach is to be able to assess gut fermentation directly from human via non-invasive methods. At the time of writing this thesis, only one approach is currently under trial, which is real-time gut microbiota fermentation technology employed by Professor Ellen's Blaak team in the University of Maastricht, in collaboration with TSE systems and Sensus companies.

The present static *in vitro* batch culture fermentation study used a medium that was low in fibre, fat and carbohydrate, therefore gut bacteria were switched to a different growth medium from that in the human gut. The study could have been improved by using a growth medium that better reflected a typical human diet as gut microbiota metabolism and therefore taxa changes are influenced by the carbohydrate to protein ratio (Portune *et al*., 2016). In a free-living population protein is usually consumed in combination with carbohydrate, perhaps promoting a bacterial usage of nitrogen towards building blocks rather than energy, and this is a scenario we did not reproduce in the *in vitro* experiment in which we fed the test protein alone.

In this study, we did not assess other products of fermentation which are typical from protein fermentation such as indoles, sulphide hydrogen, ammonia which would have led to differences in percentage of acetate, butyrate and propionate over the total metabolites produced by the gut bacteria (including products from protein fermentation). These are important metabolites since they have an impact on the metabolism. Relativising the SCFAs concentrations to the total gut microbiota-derived metabolites can highlight what the relevance of SCFAs in the host metabolism is and their likelihood to be sensed by the FFAR

in the gut. Furthermore, studying other metabolites inform of cross-feeding processes (bacterial growth promoted by the by-products of the fermentation of a main substrate).

7.5 Future work recommendations

7.5.1 Epidemiological study

Despite the results of the crossectional study suggesting that there are positive associations with Quorn consumers and health, this was taken from data of individuals in a specific time in their lives and assuming they were regular consumers based on a very non-strict cut-off (average > 1kcal of mycoprotein a day). Therefore, there is still a gap of knowledge in understanding any potential cause-effect relationship between being a Quorn consumer and health parameters (e.g. risk of developing a metabolic chronic disease) in the long term. This should be studied by conducting epidemiological longitudinal studies using follow-up data to better understand the long-term impact of mycoprotein-based foods in the NCDs' risk profile of people. To-date and to my knowledge, these databases still need to be generated and are unavailable at the time of writing this thesis. Furthermore, conducting an epidemiological cross-sectional study using other databases in other countries that are not the UK are encouraged to contrast the present findings, specifically using databases that have physical activity data so health outcomes can be adjusted for this cofounder.

7.5.2 Systematic review

At the time of finalising the systematic review (in 2020) it was pinpointed the lack of chronic mycoprotein interventions and their long-term impact on health. However, later on, in 2021, a chronic study was published which investigated the effect of replacing meat products for Quorn for a week on health parameters including glycaemic outcomes in healthy adults (Coelho *et al*., 2021). Despite this, there is still a need to conduct adequately powered, well-controlled, low-biased chronic (longer than a week-long) interventional studies in both healthy and metabolically impaired (e.g. prediabetic, T2D) populations. Furthermore, the systematic review highlighted that the potential mechanisms underpinning the effects of mycoprotein on glycaemic control and appetite come from sparse and biased studies and therefore still not fully understood and future research should uncover them. These are some recommendations for future investigations on the acute mechanisms:

- **Investigating the effects of mycoprotein on gastric emptying:** One *in vitro* study (Marks et al., 2004b) show that filament length of mycoprotein could affect gastric

emptying. Another study (Bottin *et al*., 2016) measured gastric emptying using the paracetamol method and found no significant differences. Nevertheless, the paracetamol method is not thought to be the adequate method to measure gastric emptying in solid food as it soluble and follows a liquid phase. Paracetamol method is regarded to be better for liquid foods, whereas for solids the gold-standard methodology is the ^{13}C-octanoid acid breath test. Therefore, the recommendation is to study the effect of solid mycoprotein on gastric emptying using the ^{13}C-octanoid acid breath test.

- **Investigating the effect of mycoprotein food structure on digestive enzyme activities (e.g. α-amylase, pepsin) *in vivo*:** It has been already shown in i*n vitro* studies that the hyphae has a role at inhibiting digestive enzymes, potentially mediating decreased absorption of nutrients in the gut, including glucose (Colosimo et al., 2020a and b). However, *in vitro* studies have many limitations and are not realistic of the *in vivo* physiology. For this reason, this needs to be validated using *in vivo* human digesta. The use of nasoenteral feeding tubes, which have been already used in other studies, could be a feasible approach to undertake these investigations as they allow the *in vivo* sampling of digestive samples throughout long-period of digestion (up to 8 hours) (Byrne et al., 2019).
- **Investigating the effect of mycoprotein on the secretion of gut hormones such as GIP, CCK, islet amyloid polypeptide and pancreatic polypeptide.** Evidence from in healthy subjects as well as the present evidence show no difference in GLP-1 and PYY secretion compared to chicken meals. Nevertheless, other important gut hormones such as the ones mentioned, in particular GIP which has been seen to drive the effects of insoluble fibre on glycaemic control (Weickert et al., 2005) are needed to be explored following the intake of mycoprotein.

Furthermore, investigations on whether the gut microbiota can ferment mycoprotein are greatly encouraged. These investigations could provide insights into the effects of mycoprotein on blood glucose and appetite in the longer-term. The recommendations on these investigations are better detailed in section 7.5.4.

Furthermore, a systematic review of the effects of mycoprotein on health outcomes should be revised in a few years-time when more studies become available which may allow an assessment of the main effects via a meta-analysis.

7.5.3 Human study

Currently, only one recent study looking at the mid-term effects of Quorn intake which lasted one week (Coelho et al., 2021) and was performed in healthy individuals. Therefore, there is still a gap in knowledge in regard to the longer-term effects (i.e. 3 months) of mycoprotein intake in health in both healthy and diseased populations (e.g. prediabetic, T2D). There is still a need for high-quality, adequately powered, longer-term trials investigating the effect of chronic mycoprotein intake on both postprandial blood glucose, insulin sensitivity (using gold-standard hyperinsulinaemic euglycaemic clamps) and Hb1Ac changes in people with T2D of both south Asian and white European ethnicities. Future human studies should plan carefuly for the measurement of variables that inform about the mechanisms behind such effects (e.g. gastric emptying, wide range of gut hormone release involved in appetite and glucose regulation such as CCK, IAPP, PP, GIP) as the mechanisms of action are still unknown. The differences between ethnicities on the effects of guar gum on blood glucose should also be explored, for example, by analysing differences in glucose-dependent insulinotropic polypeptide (GIP) levels, which is a hormone that is released in response to nutrient transport in the gut epithelium.

7.5.4 *In vitro* study

There is still a need to advance the mechanistic understanding of mycoprotein's effect on blood glucose and appetite regulation. For this reason, *in vivo* human sample collected from the stomach, duodenum and ileum using a feeding tube could be used in future *in vitro* fermentation studies. Future *in vitro* fermentation studies should use pH-controlled fermentation systems that allow longer incubation periods, these being more realistic to an *in vivo* physiology, based on the prior comprehension of the intestinal transit time of mycoprotein. The gold-standard *in vitro* methodology to assess gut microbiota fermentation is the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). This is a continuous system which closely replicates physiological intestinal conditions. The two main characteristics of this system is that there is movement of the digesta through pumped vessels as it would happen with the faecal matter along the different areas of the gut *in vivo*, and that pH of the system is tightly controlled (as different gut areas have different pH (Farmer *et al*., 2014), allowing longer incubation periods (Molly *et al*., 1993).

It would be interesting to delve into the question of whether human gut microbiota can ferment the deemed insoluble chitin-β-glucan complex in people who are regular Quorn

consumers whose gut microbiota may have been exposed to this particular substrate for a longer time and may be adapted to it. SCFAs as well as metabolites other than SCFAs (e.g. protein fermentation derived metabolites) should be assessed. Another unanswered question is whether regular Quorn consumption leads to beneficial shifts in the gut microbiota.

7.6 Conclusion

To conclude, this is the first time it has been shown that mycoprotein-based food consumers have a better nutritional profile, health diet index and metabolic markers than non-mycoprotein consumers. The mechanisms by which this may happen are largely unknown and gut microbiota fermentation into SCFAs from mycoprotein is unclear. Furthermore, the data presented in this thesis is the first to establish a role of mycoprotein at decreasing blood glucose levels in people with T2D of white European and south Asian ethnicity. Guar gum-supplemented chapati has been shown to be a good approach to decrease postprandial blood glucose. Importantly, the two different ethnicities do not have the same metabolic response to the same test food and this should be taken into account when doing T2D dietary management.

The 4 inter-disciplinary studies reported in this thesis have some limitations such as the lack of a complete dataset for blood markers, the sparce evidence available to sustain a meta-analysis, the acute interventional nature which limits the extrapolation of the findings to a population that consumes mycoprotein chronically, the methods employed to assess appetite and energy intake as well as the lack of using a dynamic *in vitro* batch culture fermentation or to track real time fermentation in humans.

Of importance, the present findings must be validated and contrasted with further research studies. Future studies must take into account the limitations hereby reported and avoid them to prevent a loss in time, funding and human resources and advance in our understanding on mycoprotein faster. When possible, the gold-standard approaches such as longitudinal study, meta-analysis, chronic interventional study and SHIME model, should be used to investigate the effects of mycoprotein on glycaemic control, appetite, and energy intake. Future studies should be high-quality, low-risk bias studies, controlling as tightly as possible for potential cofounders.

7.7 Bibliography of Chapter 7

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Appendices

Appendix 0. Sensitivity analyses for BMI, blood pressure and blood metabolites.

Table S1. Differences between missing and non-missing data for blood markers within each consumer group.												
	Non-consumers (N=5316)						Mycoprotein consumers (N=191)					
	missing data (n=2600)		non-missing data (n=2716)		% difference vs. non-missing	P-value	missing data (n=108)		non-missing data (n=83)		% difference vs. non-missing	P-value
(percentage of total energy, kcal)	mean	SE	mean	SE			mean	SE	mean	SE		
Age	49.28	0.38	50.69	0.35	-2.86	<0.01	44.00	1.65	46.00	1.72	-4.55	0.29
Food energy (kcal)	1721.16	11.42	1814.41	12.23	-5.42	<0.001	1767.00	57.70	1678.83	58.01	4.99	0.28
Total energy (kcal)	1636.38	10.41	1726.07	11.32	-5.48	<0.001	1833.18	60.50	1743.15	58.98	4.91	0.29
Energy density (kcal/g)	0.67	0.00	0.65	0.00	2.31	0.01	0.63	0.02	0.60	0.02	5.07	0.30
Protein	16.80	0.08	16.57	0.08	1.38	0.04	15.01	0.39	15.18	0.41	-1.08	0.78
Fat	33.16	0.14	33.54	0.14	-1.13	0.05	32.56	0.69	32.75	0.75	-0.59	0.85
Saturated fatty acids	12.40	0.07	12.51	0.07	-0.88	0.29	12.06	0.38	11.80	0.39	2.17	0.63
CMUFA	12.00	0.06	12.15	0.06	-1.23	0.08	11.39	0.31	11.56	0.35	-1.45	0.73
CN6PUFA	4.66	0.03	4.76	0.03	-2.06	0.03	5.01	0.17	5.29	0.21	-5.61	0.30
CN3PUFA	0.96	0.01	0.98	0.01	-1.77	0.18	1.00	0.04	1.00	0.05	0.23	0.97
Trans fatty acids	0.58	0.01	0.57	0.01	2.77	0.05	0.54	0.03	0.54	0.03	0.43	0.96
Carbohydrate	45.77	0.16	45.52	0.16	0.55	0.26	48.98	0.76	48.40	0.82	1.18	0.61
Total Sugars	19.14	0.14	19.51	0.14	-1.92	0.06	20.92	0.64	20.15	0.72	3.68	0.43
Starch	26.60	0.13	25.98	0.14	2.33	<0.001	28.02	0.69	28.23	0.72	-0.72	0.84
Non milk extrinsic sugars	11.60	0.14	11.58	0.13	0.20	0.90	12.63	0.69	11.58	0.67	8.30	0.28
Free Sugars	11.28	0.13	11.26	0.13	0.19	0.90	12.54	0.67	11.41	0.65	9.02	0.23
Alcohol (g)	12.11	0.49	12.62	0.47	-4.20	0.45	9.45	1.44	9.19	1.62	2.80	0.90
Fibre (g)	17.07	0.13	18.56	0.14	-8.75	<0.001	24.07	0.77	22.31	0.81	7.29	0.12
HDI	5.84	0.02	5.93	0.02	-1.63	<0.001	6.59	0.13	6.54	0.14	0.67	0.82
DASH score	1.35	0.02	1.42	0.02	-5.14	0.01	2.00	0.12	1.89	0.11	5.71	0.49

P≤0.05 statistically different. A t test, considering unequal variance was performed. CMUFA, monounsaturated fatty acids; CN6PUFA, CN6 poly unsaturated fatty acids; CN3PUFA, CN3 poly unsaturated fatty acids; HDI, healthy diet index; DASH, Dietary approaches to stop hypertension score; SE, standard error of the mean.

Table S2. Differences between missing and non-missing data for body mass index within each consumer group.												
	Non-consumers (N=5316)						Mycoprotein consumers (N=191)					
	missing data (n=247)		non-missing data		% difference vs. non-missing	P-value	missing data (n=15)		non-missing data (n=176)		% difference vs. non-missing	P-value
(percentage of total energy, kcal)	mean	SE	mean	SE			mean	SE	mean	SE		
Age	58.00	0.93	49.00	0.24	15.52	<0.001	46.69	13.00	44.66	15.39	4.35	0.60
Food energy (kcal)	1543.63	24.12	1688.80	7.53	-9.40	<0.001	1558.75	141.86	1743.75	42.81	-11.87	0.25
Total energy (kcal)	1601.31	25.94	1775.21	8.18	-10.86	<0.001	1617.46	141.26	1809.71	44.50	-11.89	0.23
Energy density (kcal/g)	0.66	0.01	0.66	0.00	0.16	0.92	0.53	0.06	0.62	0.02	-17.21	0.16
Protein	17.19	0.21	16.70	0.06	2.86	0.02	15.36	1.06	15.06	0.29	1.99	0.79
Fat	33.32	0.32	33.46	0.09	-0.42	0.68	30.24	1.58	32.85	0.54	-8.65	0.15
Saturated fatty acids	12.92	0.18	12.47	0.05	3.55	0.02	10.71	0.91	12.06	0.29	-12.58	0.19
CMUFA	11.83	0.14	12.14	0.04	-2.63	0.03	10.34	0.68	11.56	0.25	-11.87	0.12
CN6PUFA	4.41	0.07	4.74	0.02	-7.59	<0.001	5.36	0.50	5.11	0.14	4.55	0.66
CN3PUFA	0.96	0.02	0.98	0.01	-2.14	0.34	0.94	0.10	1.00	0.03	-6.82	0.56
Trans fatty acids	0.60	0.01	0.56	0.00	5.48	0.02	0.41	0.05	0.55	0.02	-33.34	0.03
Carbohydrate	46.52	0.38	45.52	0.11	2.16	0.01	50.72	2.98	48.55	0.54	4.27	0.50
Total Sugars	19.07	0.35	19.22	0.09	-0.78	0.68	23.94	2.18	20.29	0.48	15.21	0.14
Starch	27.43	0.32	26.28	0.09	4.22	<0.001	26.79	2.45	28.23	0.50	-5.37	0.59
Non milk extrinsic sugars	10.82	0.32	11.49	0.09	-6.18	0.05	15.61	2.40	11.88	0.48	23.88	0.17
Free Sugars	10.80	0.31	11.18	0.09	-3.56	0.23	15.42	2.35	11.76	0.47	23.74	0.17
Alcohol (g)	8.24	0.94	12.34	0.32	-49.78	<0.001	8.39	5.34	9.42	1.09	-12.34	0.86
Fibre (g)	16.35	0.31	18.01	0.10	-10.10	<0.001	21.90	1.80	23.43	0.59	-7.01	0.45
HDI	5.81	0.05	5.90	0.02	-1.49	0.08	6.54	0.26	6.57	0.10	-0.49	0.91
DASH score	1.38	0.04	1.37	0.01	0.46	0.89	2.15	0.28	1.93	0.09	10.26	0.49

P≤0.05 statistically different. A t test, considering unequal variance was performed. CMUFA, monounsaturated fatty acids; CN6PUFA, CN6 poly unsaturated fatty acids; CN3PUFA, CN3 poly unsaturated fatty acids; HDI, healthy diet index; DASH, Dietary approaches to stop hypertension score; SE, standard error of the mean.

Table S3. Differences between missing and non-missing data for blood pressure within each consumer group.												
	Non-consumers (N=5316)						Mycoprotein consumers (N=191)					
	missing data (n=1870)		non-missing data		% difference vs. non-missing data	P-value	missing data (n=72)		non-missing data (n=119)		% difference vs. non-missing data	P-value
(percentage of total energy, kcal)	mean	SE	mean	SE			mean	SE	mean	SE		
Age	48.59	0.39	51.16	0.30	-5.29	<0.001	42.39	15.48	46.28	14.81	-9.18	0.08
Food energy (kcal)	1654.92	11.73	1691.26	9.12	-2.20	0.01	1707.93	71.23	1742.25	49.80	-2.01	0.70
Total energy (kcal)	1744.19	12.94	1771.71	9.80	-1.58	0.09	1760.08	72.70	1816.04	52.16	-3.18	0.54
Energy density (kcal/g)	0.67	0.00	0.65	0.00	3.29	<0.001	0.62	0.03	0.61	0.02	1.82	0.72
Protein	16.63	0.09	16.81	0.07	-1.06	0.11	15.39	0.46	14.89	0.36	3.27	0.39
Fat	33.29	0.14	33.56	0.12	-0.81	0.14	32.26	0.88	32.89	0.62	-1.95	0.56
Saturated fatty acids	12.48	0.08	12.52	0.06	-0.34	0.67	11.78	0.48	12.06	0.33	-2.36	0.63
CMUFA	12.07	0.06	12.15	0.05	-0.62	0.35	11.24	0.38	11.61	0.29	-3.35	0.44
CN6PUFA	4.66	0.03	4.75	0.03	-2.12	0.02	5.16	0.22	5.12	0.17	0.80	0.88
CN3PUFA	0.95	0.01	0.99	0.01	-4.21	<0.001	1.00	0.05	1.00	0.04	0.41	0.95
Trans fatty acids	0.57	0.01	0.56	0.00	0.79	0.55	0.55	0.04	0.53	0.02	3.30	0.68
Carbohydrate	45.73	0.17	45.52	0.14	0.45	0.34	49.36	0.87	48.33	0.71	2.09	0.36
Total Sugars	19.00	0.15	19.34	0.12	-1.82	0.06	21.17	0.79	20.22	0.60	4.50	0.34
Starch	26.71	0.14	26.15	0.11	2.08	<0.001	28.16	0.81	28.08	0.64	0.26	0.94
Non milk extrinsic sugars	11.78	0.15	11.20	0.11	4.90	<0.001	12.91	0.80	11.71	0.61	9.26	0.24
Free Sugars	11.45	0.14	10.95	0.10	4.32	<0.001	12.79	0.78	11.58	0.59	9.43	0.22
Alcohol (g)	12.75	0.56	11.49	0.35	9.88	0.06	7.45	1.48	10.54	1.50	-41.51	0.15
Fibre (g)	17.19	0.15	18.32	0.12	-6.57	<0.001	23.17	0.82	23.39	0.76	-0.96	0.84
HDI	5.84	0.02	5.93	0.02	-1.51	<0.001	6.44	0.15	6.65	0.13	-3.13	0.30
DASH score	1.37	0.02	1.38	0.02	-0.17	0.93	2.06	0.14	1.88	0.10	8.95	0.30

P≤0.05 statistically different. A t test, considering unequal variance was performed. CMUFA, monounsaturated fatty acids; CN6PUFA, CN6 poly unsaturated fatty acids; CN3PUFA, CN3 poly unsaturated fatty acids; HDI, healthy diet index; DASH, Dietary approaches to stop hypertension score; SE, standard error of the mean.

Appendix 1. Search Strategy used in databases

Appendix 1. Search Strategy used in databases	
Google Scholar	allintitle: "mycoprotein" OR "quorn" AND "glycemia" OR "glucose" OR "food intake" OR "energy intake"
EMBASE	(mycoprotein or quorn).mp. and (glycemia or glucose or food intake or energy intake).m_titl. [m*p*=title, abstract, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword, floating subheading word, candidate term word]
Web of Knowledge	(TI=(mycoprotein OR quorn) AND TI=(glycemia OR glucose OR food intake OR energy intake)) AND LANGUAGE: (English) AND DOCUMENT TYPES: (Article)

Appendix 2. Internal standard-Solution A and B.

Internal standard-Solution A

Material	Supplier	Product	Density	Concentration of IStd-Stock	Concentration of IStd-Sol A
			(g/mL)	M	µM
Acetic acid 1,2-13C2	Sigma	282022-250MG	**0.986**	**1**	**60**
Lactic acid 3-13C	Sigma	799548-1G	**n.a.**	**1**	**60**

Internal standard-Solution B

Material	Supplier	Product Number	Density (g/mL)	Concentration of IStd-Stock	Conce IS
				mM	
Propionic acid 1-13C	Sigma	2824448-1G	**0.993**	**1000**	
Butyric acid 1,2-13C2	Sigma	491993-100MG	**0.986**	**1**	
Isobutyric acid-D6	Molecular Dimensions	TRC-1789182	**1.024**	**1**	
Valeric acid 5-13C	Sigma	605662-100MG	**0.948**	**1**	
Isovaleric acid-D9	Sigma	808997-100MG	**1.022**	**1**	
Hexanoic acid-D3	Sigma	489727-100MG	**0.951**	**1**	

Appendix 3. Volunteer recruitment strategies employed in the human study.

Appendix 3. Recruitment strategies	
Strategy	**Specifications**
Newspaper advertising	2 x 2-day adverts were put in the *Evening Standard* and *Metro*
Social media	Advertisement on Facebook, Imperial College Yammer, Nextdoor.co.uk and Twitter feeds.
Newspaper article	informing about the study details was placed in the Imperial College newspaper *Felix* (August 2019).
Diabetes UK and Diabetes.co.uk	An online advertisement was placed in the website of these charities.
Age UK	Members and staff were approached by researcher personally to inform about the study.
Posters and leaflets community places	Placed in public spaces such as university campus, libraries, GP practices, supermarket boards, hindu temples, churches and ethnic
South Asian local recruiter	Was engaged to inform their community about the study
DESMOND*	People attending the DESMOND courses were approached by researcher to inform them about the study. In addition, when researcher not available, leaflets were handed out by educators at the end

	of the sessions.
General practicionairs, surgeries and Clinical Dietitians	8 GP within the North West London area actively informed suitable potential volunteers during endocrinology and bariatric clinics
Diabetes UK meetings	One evening informative meetings organised by Diabetes UK was attended, where members were informed about the study.
Databases	From Imperial Clinical Research Facility
Specialist Consultants	3 consultants within the North West London area actively informed suitable potential volunteers during endocrinology and bariatric clinics
Website	via Imperial College London Translational Nutrition (www.) and by the Imperial College research facility (www.), website gumtree.
Other studies scavenging	unsuitable or suitable and finished participants from other on-going trials such as Aspire-DNA, Endo-Barrier, Kisspeptin were sent an email with information about the study.
*DESMOND, Diabetes Education and Self-Management for On-going and Newly Diagnosed.	

Appendix 4. Participant Information Sheet used for in the human study.

Section for Nutrition Research, Department of Metabolism, Digestion and Reproduction
6th Floor Commonwealth Building
Imperial College London
Hammersmith Hospital Campus, Du Cane Road
London W12 0NN

INFORMATION SHEET FOR RESEARCH PARTICIPANTS

A Double-Blind Randomised Controlled Trial Investigating the Acute Effects of Mycoprotein on Glycaemic Control and Appetite in South Asian and Caucasian Adults with Type 2 Diabetes Mellitus

You will be given a copy of this Information Sheet and a signed copy of your consent form to keep, should you decide to participate in the study.

You are being invited to take part in a research study. This study is being carried out as part of a doctoral degree. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year.

You are free to withdraw at any time without explanation.

Thank you for reading this

What is the PURPOSE of this study?

Type 2 Diabetes Mellitus (T2DM) is a disease in which blood sugar is not well controlled. This is because the body fails to produce or sense insulin, which is the hormone that controls blood sugar. Research studies have reported that South Asians are nearly four times more prone to develop T2DM and have a poorer blood sugar control than people with European origins.

Diet is the cornerstone treatment for T2DM. People with T2DM are recommended to modify their diets towards a healthy and balanced diet including low glycaemic index and fibre-rich foods. Foods high in fibre reduce the incidence of T2DM and helps controlling blood sugar in people with T2DM.

Mycoprotein is a meat-free food high in both fibre and protein as well as low in fat, which is sold in many supermarkets across the UK under the brand name of "Quorn" in different forms (e.g. nuggets, mince, etc.). We have previously demonstrated that mycoprotein improves blood sugar control in healthy overweight and obese humans. However, we do not know what are the effects of mycoprotein in people with T2DM and if there is a different effect between South Asians and people of European origin.

In addition, guar gum is a type of fibre that is known to be very good at controlling blood sugar in people with T2DM.

Therefore, in this study we will investigate the effects of mycoprotein with and without guar gum on blood sugar control and appetite in South Asian and people with European origins with T2DM. The aim of this study is to see whether mycoprotein given only once (acutely) improves blood sugar control in these group of people and whether this effect is greater with guar gum.

Why have I been invited?

We have asked you to take part because you are an adult from South Asian and European origin with T2DM. We think your participation will help us to find out how meat-free food can help managing blood sugar levels in people T2DM of South Asian and European origin.

You are SUITABLE to participate if you meet the following criteria:

- Diagnosed with Type 2 Diabetes Mellitus
- South Asian (Afghanistan, Bangladesh, Bhutan, Maldives, Nepal, India, Pakistan and Sri Lanka) or European ancestry
- Aged 18 to 70 years

You are NOT SUITABLE to participate if you meet any of the following criteria:

- Non diagnosed with Type 2 Diabetes Mellitus
- Mixed ancestors
- Diabetic taking any of the following medications:
    - Insulin
    - Diabetic medication (Except from metformin and oral hypoglycaemic which are allowed)
    - Orlistat
- Have a gastrointestinal, heart, pancreas disease
- Cancer

- Infection requiring antibiotics
- History of alcohol or drug abuse
- Any condition involving the imbalance of hormones
- Are currently participating in another research study or have taken part within 3 month of study entry
- Currently smoking
- Allergy to breathing mould, penicillin, egg, soy
- Asthmatic
- Hypothyroidism
- Weight change of ≥ 5% of total kilograms in the preceding 3 months
- Shift workers
- Vegetarian
- Medical implants that require batteries such as heart pace makers.

Do I have to take part?

It is completely up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form.

If you decide to participate you are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

Visit 1: Health Screening

If you agree to take part in this study, you will first be invited to attend a health screening with one of the researchers involved in this study. This will involve a blood test (20 ml which are equivalent to four teaspoons of blood approximately), height and weight measurements as well as blood pressure will be taken. You will also have done an electrocardiogram (ECG). This is a non-invasive test to look at the health of your heart. Women will be asked to do a urine test for pregnancy. As long as these medical checks are satisfactory and you are still happy to participate, you will then be enrolled onto the study.

In addition, you will be asked to consume a test food within 15 minutes as a little trial to mimic the conditions of the study visits so that you can get familiar with the procedure.

The health screening should last approximately 1 hour and will take place in the Imperial Clinical Research Facility (NIHR Imperial CRF) at Hammersmith Hospital.

It should be noted that the health screenings performed as part of this study cannot be viewed as a comprehensive health check.

Visits 2-7: Study Visits

If you do decide to participate, you will be randomised to one of these two groups:

- **Group 1:** test foods provided as soup (in order to test raw mycoprotein)

- **Group 2:** test foods provided as mince (in order to test processed mycoprotein called Quorn)

The only difference between groups is the format in which protein source is given (raw mycoprotein as soup and processed mycoprotein called Quorn as mince).
Once assigned in your group, you will be asked to come for six separate study day visits (visits 2-7) in which you will receive test food in a random order. There will be at least 3 to 7 days between visits 2-7 (Figure 1).

The test foods you are going to be randomised to are:

A. Soy soup B. Chicken soup C. Mycoprotein soup D. Soy with guar gum soup E. Chicken with guar gum soup F. Mycoprotein with guar gum soup	OR	A. Soy mince B. Chicken mince C. Quorn mince D. Soy mince with guar gum E. Chicken mince with guar gum F. Quorn mince with guar gum

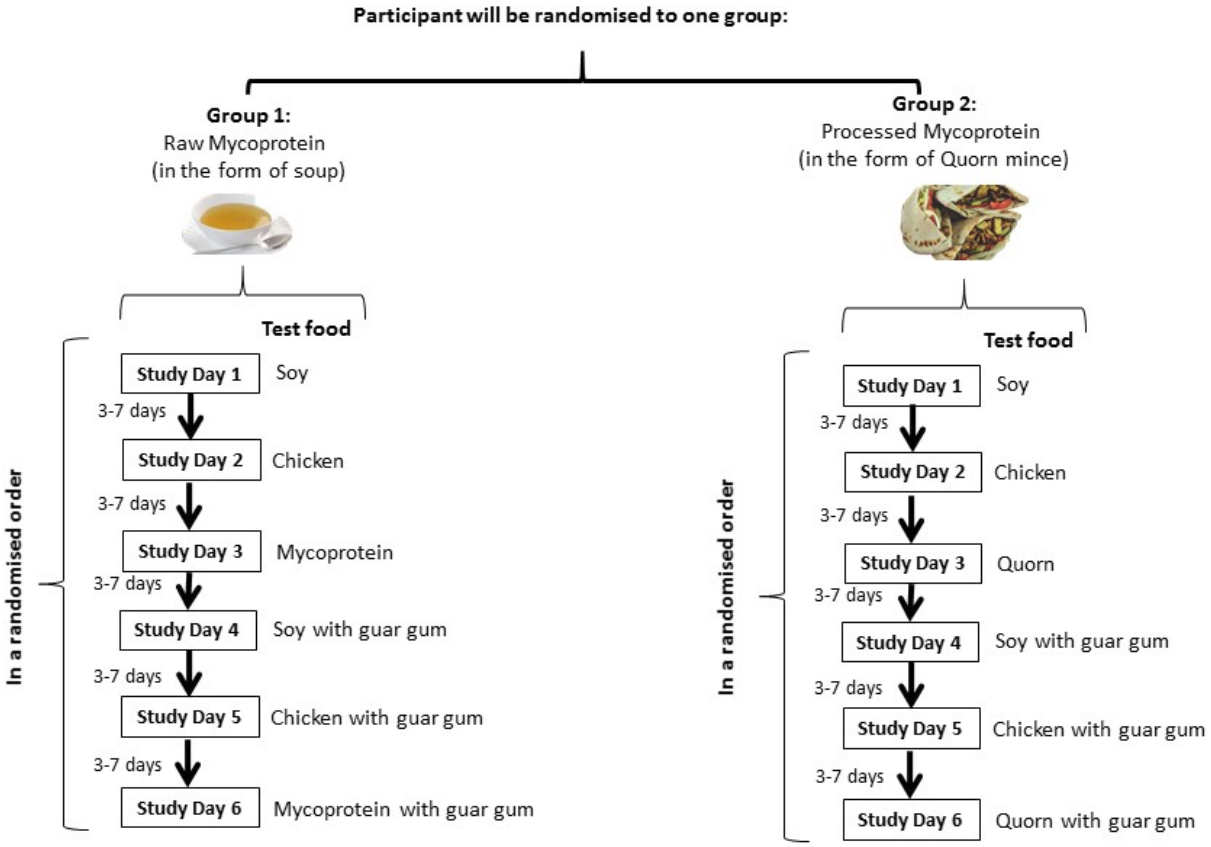


Figure 1. Study time-line scheme.

Test foods:

In each group, the protein source of the test food will be: soy, chicken and mycoprotein. All test foods will be accompanied by chapatti bread. In the meals with guar gum, the chapati bread will have guar gum incorporated into the dough. The test foods served have been carefully manufactured by Marlow Foods Ltd to make all of them with the same energy and nutrient content. In addition, all test foods (soups and mince foods) will be of similar appearance and taste across the six meals. This is done to make you unaware of which food is given to you, which could influence your blood sugar levels. In addition, the food will be served to you with an anonymous labelling of A,B,C,D,E,F. This is done because it is a double-blind study meaning that neither the researcher or you will know what the test food is.

About mycoprotein

Mycoprotein is a food made of a fungus named *Fusarium venenatum*. Mycoprotein is sold under the name brand of "Quorn", which is a meat-substitute food brand commercially available in many supermarkets across the UK. Quorn has a wide range of meat-free products (e.g. mince, nuggets, burgers, etc.) that have a texture and flavour that may remind you of their equivalent meat products.

Preparation before each study visit:

Before the first study visit we will ask you to complete a 4-day food diary in which you will have to write down all the food and drink that you have consumed during those past 4 days. Additionally, 72 hours before the study visit you will be asked to refrain from strenuous exercise. On the day before each study visit, you will also be asked to refrain from caffeine, alcohol. In addition, the evening before each study visit, you will be asked to consume a ready-meal consisting of rice and chicken and bring the packaging/sleeve with you on the next day. Should you wish, we can provide you with a ready-meal to consume on the evenings before each study visit.

After the evening meal, you will be asked to fast overnight for 12 hours approximately from 8 pm of the evening before the study visit to 9 am of the next day (you are only allowed to drink plain water). If you are on hypoglycaemic medication, you will be asked to have the last dose at 8 pm of the day before each study visit. However, you should continue taking any other medication prescribed. You will also be asked to bring a stool sample with you before the first study day. We will explain to you in detail how to do this and you will be given appropriate containers.

Study day:

On the study day, you will be asked to arrive at the NIHR Imperial Clinical Research Facility at Hammersmith Hospital in a fasted state (from 8 pm on the evening before the study day) at approximately 8:00 am. You will be asked to empty your bladder (and in case of woman, have a pregnancy test) and have your body weight and composition measured in a bioelectrical impedance scale (not invasive). After this, at approximately 8:30 am a small plastic tube (cannula) will be inserted into your arm by a nurse. This will be in place for the duration of the study day and will be used to take blood samples without causing you any further discomfort.

At approximately 8:45 am and 8:55 am, two fasting blood samples will be taken within 10 minutes apart. Following this, a test food will be served and you will be expected to finish within approximately 15 minutes. Next, 7 blood samples will be taken at the time-points: 15, 30, 45, 60, 90, 120 and 180 minutes relative to the start of the test food intake. A total of 100 ml of blood will be taken (about 17 teaspoons of blood) during the entire study day. These will be taken through the plastic cannula.

Additionally, you will be asked to complete questionnaire after each blood sample to assess subjective feelings of hunger, fullness and nausea.

The final blood sample will be collected at approximately 12:00 am. Once this has been collected the cannula will be removed and you will be served a buffet meal made of a bowl of pasta with tomato sauce. After this, you will be given a 72-hour food diary to complete during the next 3 days and expected to be brought back on your next visit or by post after your last visit. Additionally, whenever it is possible for you, you will be asked to collect your next stool sample after each study visit. Stool sample kit and instructions on how to do so will be given to you. Finally, you will be able to go home. Figure 2 depicts the study day time-line of events.

During the course of the study day you will be able to read or work on your laptop.

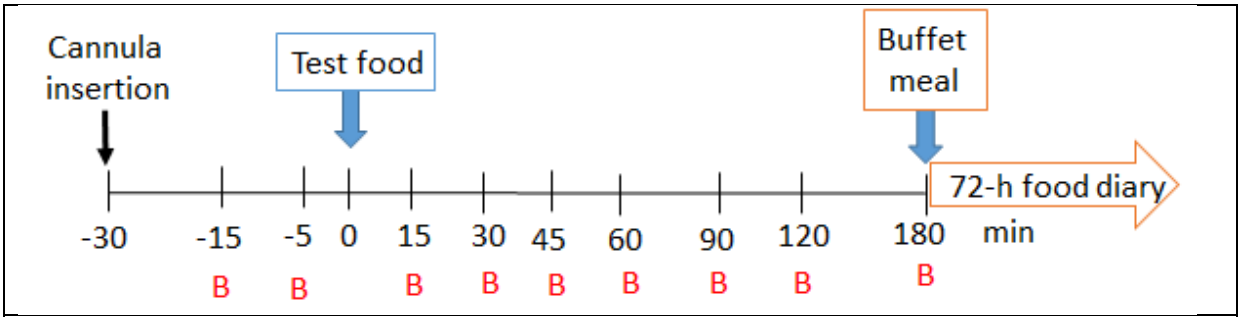


Figure 2. Study day time-line. B: blood sample taken.

What are we analyzing?

We are measuring blood sugar and insulin in blood to assess your blood sugar control. Additionally, to assess your appetite, we will measure your gut hormone levels in blood and ask you to complete questionnaires rating your feelings of hunger. Furthermore, we will serve you a buffet meal and ask you to complete a 72-hour food diary to measure energy intake.

What are the possible disadvantages and risks of taking part?

Consumption of Quorn foods have not been generally found to be linked to any serious side effects. In rare occasions an allergic cross-reaction can occur if you are allergic to breathing mold, penicillin or you have asthma. Soy and traces of egg can be found in the test food. Some people may be sensitive to Quorn food and experience allergic reactions such as hives, vomiting, diarrhoea and

anaphylaxis. In such case, participant will be attended by expertise clinical staff in a fully equipped research facility, in the highly unlikely event of allergic reaction. However, to avoid this from happening we will exclude you if you have a known allergy to any of the ingredients of which test food is composed of. During the screening visit we will show you a list of the test food ingredients for you to determine whether you are allergic to any.

Some people also experienced a bloating sensation after consuming mycoprotein. This sensation should go away in 1 to 5 hours.

Insertion of the cannula into your arm on each of the study visits may cause minor discomfort and possible bruising. In rare occasions, when the needle of the cannula is inserted is possible that a localised infection develops. These procedures will only be carried out by experienced nurses and medical staff under sterile conditions to minimise all these risks.

It should be noted that the health screenings performed as part of this study cannot be viewed as a comprehensive health check. However, vary rarely, unexpected observations may be detected which may need further investigation. In this event, you will be informed and a report will be sent to your GP, who will arrange further tests and coordinate your further care.

If any of the screening questionnaires or blood tests reveal any medical problems (e.g. kidney or liver problems), your GP will be informed so that they can coordinate your further care, arrange any further tests, and refer you on to Hospital Doctors if necessary.

Taking part in the study will provide no direct benefit for you. The information that we get from this study will help us to better understand blood sugar control and appetite regulation in people with T2DM with European and South Asian origin, and may help us in the future to better advice about diet to people who suffer from T2DM.

Will my taking part in this study be kept confidential?

Personal identifiable data (name, address, telephone, email, etc.) will be stored in Imperial College London computer at the NIHR/Wellcome Trust Clinical Research Facility at Hammersmith Hospital. Other identifiable data (consent form, enrolment log, etc.) will be kept in a secured and locked filling cabinet at the NIHR/Wellcome Trust Clinical Research Facility at Hammersmith Hospital, Imperial College Healthcare NHS Trust. Non-identifiable data will be stored in secured computers at Imperial College London. All data stored electronically will be password protected. Only the study team will have access to the study data.

Participants will be assigned a unique ID which they will be referred to so as to keep their identifiable information and samples, anonymised. Samples will be stored for future research.

Recruitment of patients will be registered at NIHR/Welcome Trust Clinical Research Facility at Hammersmith hospital, Imperial College Healthcare NHS Trust.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the study. If this happens, your research doctor will tell you about it and discuss with you whether you want to continue in the study. If you decide to continue in the study, you will be asked to sign an updated consent form. Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study.

What happens when the research study stops?

Once the study has finished, the results of the study can be made available to you and/or your GP should you wish. If you have any problems immediately following the study, then you should contact one of the research doctors on the numbers provided on page 13.

What will happen to the results of the research study?

The results are likely to be published in the year following the study. Your confidentiality will be ensured at all times and you will not be identified in any publication. At the end of the study, the results of the study can be made available to you and/or your GP should you wish.
Anonymised results of this study will be presented at relevant conferences and symposiums (e.g. Diabetes UK conferences) as a means of early communication. Full reports and papers will then be prepared for publication in high impact medical, nutrition, and diabetes related journals (e.g. British Medical Journal, British Journal of Clinical Nutrition, Diabetes, and American Journal of Clinical Nutrition). The reports and papers that are published about the research will not identify you or any other person who participated in this research.

If a participant, who has given informed consent, loses capacity to consent during the study, the participant and all identifiable data or tissue collected would be withdrawn from the study. Data or tissue which is not identifiable to the research team may be retained.

Who is organising and funding the research?

This study is undertaken as part of a PhD. This study is being organised by the Section for Nutrition Research, of the Department of Metabolism, Digestion and Reproduction, Imperial College London. The funding is confirmed, and it is funded by Imperial College London.

Who has reviewed the study?

This study has been reviewed by the Health Research Authority. This study was given a favourable ethical opinion for conduct in the NHS by the London-Bromley Research Ethics Committee.

Will I be reimbursed for my travel expenses/time?

You will be reimbursed £30 per visit up to a maximum of £180 on completion of the study. In addition, you will be able to claim for the cost of the evening standard meals (maximum of £5/day) as well as for travel expenses to/from the research facility from/to participant's home (maximum of

£25/day). To make such claims, you will have to present a receipt. You will not have to pay for the test foods provided during the study visits.

What if something goes wrong?

Imperial College London holds insurance policies which apply to this study. If you experience harm or injury as a result of taking part in this study, you will be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Principal Investigator *(*Professor Gary Frost, Email: g.frost@imperial.ac.uk; Telf.: +44 (0) 7872 850308). The normal National Health Service mechanisms are also available to you such as contacting the local Patient Advice Liaison Services (PALS; pals@imperial.nhs.uk, 020 3313 0088). A member of the team will be able to give you their contact information upon request. If you are still not satisfied with the response, you may contact the Imperial College, Joint Research Compliance Office.

When do I have to decide by if I want to take part in this study?

You can take up to 2 weeks to decide if you wish to take part in this study.

Contact for further information

If you have any further questions, please contact the doctorate student or research doctors in charge, during working hours:

PhD student: Anna Cherta	Email: protein@imperial.ac.uk Telephone: +44 07522887666 Address: 6th Floor, Commonwealth Building, Hammersmith Hospital, Du Cane Road, London, W12 0NN
Research Doctor: Anne Dornhorst	Telephone: +44 07885586030 Email: a.dornhorst@imperial.ac.uk

The researchers and doctors involved in the study, Professor Gary Frost, will be also available by telephone

During working hours through Professor Gary Frost's secretary 020 8383 3242 **At all other times** through Hammersmith Hospital switchboard 020 8383 1000

We would like to thank you for reading the Participant Information Sheet and for considering taking part in this study.

Please feel free to talk to your family and friends about the study.

If you have any further questions, please feel free talk to the study doctor before considering entry into this study.

If you experience any problems during this study, you may withdraw at any stage.

Thank you for taking the time to read this.

TRANSPARENCY NOTICE

Imperial College London is the sponsor for this study based in the United Kingdom. We will be using information from you and your medical records created for this study in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Imperial College London will keep identifiable information about you.

- 10 years after the study has finished in relation to data subject consent forms.
- 10 years after the study has completed in relation to primary research data.

Further information on Imperial College London's retention periods may be found at https://www.imperial.ac.uk/media/imperial-college/administration-and-support-services/records-and-archives/public/RetentionSchedule.pdf.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.
You can find out more about how we use your information:

Professor Gary Frost
Head of the Section for Nutrition Research
Department of Metabolism, Digestion and Reproduction
Imperial College Hammersmith Campus
Du Cane Road
London
W12 0NN
Tel +44 (0) 20 7594 0959
Mobile +44 (0) 7872 850308
Email g.frost@imperial.ac.uk

Legal Basis

As a university we use personally-identifiable information to conduct research to improve health, care and services. As a publicly-funded organisation, we have to ensure that it is in the public interest when we use personally-identifiable information from people who have agreed to take part in research. This means that when you agree to take part in a research study, we will use your data in the ways needed to conduct and analyse the research study.

Health and care research should serve the public interest, which means that we have to demonstrate that our research serves the interests of society as a whole. We do this by following the UK Policy Framework for Health and Social Care Research.

International Transfers

There may be a requirement to transfer information to countries outside the European Economic Area (for example, to a research partner). Where this information contains your personal data, Imperial College London will ensure that it is transferred in accordance with data protection legislation. If the data are transferred to a country which is not subject to a European Commission (**EC**) adequacy decision in respect of its data protection standards, Imperial College London will enter into a data sharing agreement with the recipient organisation that incorporates EC approved standard contractual clauses that safeguard how your personal data are processed.

Contact us

If you wish to raise a complaint on how we have handled your personal data or if you want to find out more about how we use your information, please contact Imperial College London's Data Protection Officer via email at dpo@imperial.ac.uk, via telephone on 020 7594 3502 and via post at Imperial College London, Data Protection Officer, Faculty Building Level 4, London SW7 2AZ.

If you are not satisfied with our response or believe we are processing your personal data in a way that is not lawful you can complain to the Information Commissioner's Office (ICO). The ICO does recommend that you seek to resolve matters with the data controller (us) first before involving the regulator.

Imperial College Healthcare NHS Trust will collect information from you for this research study in accordance with our instructions.

Imperial College Healthcare NHS Trust will use your name, NHS number and contact details to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from Imperial College London and regulatory organisations may look at your medical and research records to check the accuracy of the research study. Imperial College Healthcare NHS Trust will pass these details to Imperial College London along with the information collected from you. The only people in Imperial College London who will have access to information that identifies you will be people who need to contact you for the research study or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, NHS number or contact details.

Imperial College Healthcare NHS Trust will keep identifiable information about you from this study for 10 years after the study has finished.

Data to be used for future research

When you agree to take part in a research study, the information about your health and care may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this country or abroad. Your information will only be used by organisations and researchers to conduct research in accordance with the UK Policy Framework for Health and Social Care Research**.**

This information will not identify you and will not be combined with other information in a way that could identify you. The information will only be used for the purpose of health and care research and cannot be used to contact you or to affect your care. It will not be used to make decisions about future services available to you, such as insurance.

Appendix 5. Informed consent form used in the human study.

IRAS ID: 260347

REC Reference: 19/LO/0476

Department of Investigative Medicine

Hammersmith Hospital Campus
Imperial College London
6th Floor, Commonwealth Building
Du Cane Road,
W120NN
Tel 020 838 33242
Fax 020 838 33142

CONSENT FORM

A Double-Blind Randomised Controlled Trial Investigating the Acute Effects of Mycoprotein on Glycaemic Control and Appetite in South Asian and Caucasian Adults with Type 2 Diabetes Mellitus

		Initials
1.	**I confirm that I have read and understood the information sheet *"A Double-Blinded Randomised Controlled Trial* Investigating the Acute Effe of Mycoprotein on Glycemic Control and Appetite in South Asian and Cauca Adults with Type 2 Diabetes Mellitus – *Version* ____ – *Date*_____" *for the above study*. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.**	
2.	**I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.**	
3.	**I understand that sections of any of my research notes may be looked at by responsible individuals from Imperial College, Imperial College NHS Healthcare Trust, and regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.**	
4.	**I agree to have my GP informed about my participation in this study.**	
5.	**I agree to have my GP forwarded my results in the event of these being abnormal.**	
6.	**I understand that the information collected (samples and data) about me will be used to support other research in the future and may be shared anonymously with other researchers to have my collected tissue samples stored and used for this study.**	

7. **I wish to have a summary of the main findings sent at the end of the study and therefore agree to have my contact details stored for study result dissemination purposes only.**		
8. **I agree to be contacted again by the investigators to be invited to participate in future research.**	Yes	No
9. **I agree to take part in this study.**		

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Appendix 6. Adult Eating Behaviour Questionnaire

Adult Eating Behaviour Questionnaire

Please read each statement and tick the box most appropriate to you

	Strongly disagree	Disagree	Neither agree or disagree	Agree	Strongly agree
I love food	☐	☐	☐	☐	☐
I often decide that I don't like a food, before tasting it	☐	☐	☐	☐	☐
I enjoy eating	☐	☐	☐	☐	☐
I look forward to mealtimes	☐	☐	☐	☐	☐
I eat more when I'm annoyed	☐	☐	☐	☐	☐
I often notice my stomach rumbling	☐	☐	☐	☐	☐
I refuse new foods at first	☐	☐	☐	☐	☐
I eat more when I'm worried	☐	☐	☐	☐	☐
If I miss a meal I get irritable	☐	☐	☐	☐	☐
I eat more when I'm upset	☐	☐	☐	☐	☐
I often leave food on my plate at the end of a meal	☐	☐	☐	☐	☐
I enjoy tasting new foods	☐	☐	☐	☐	☐
I often feel hungry when I am with someone who is eating	☐	☐	☐	☐	☐
I often finish my meals quickly	☐	☐	☐	☐	☐
I eat less when I'm worried	☐	☐	☐	☐	☐
I eat more when I´m anxious	☐	☐	☐	☐	☐
Given the choice, I would eat most of the time	☐	☐	☐	☐	☐

	Strongly disagree	Disagree	Neither agree or disagree	Agree	Strongly agree
I eat less when I'm angry	☐	☐	☐	☐	☐
I am interested in tasting new food I haven't tasted before	☐	☐	☐	☐	☐
I eat less when I'm upset	☐	☐	☐	☐	☐
I eat more when I'm angry	☐	☐	☐	☐	☐
I am always thinking about food	☐	☐	☐	☐	☐
I often get full before my meal is finished	☐	☐	☐	☐	☐
I enjoy a wide variety of foods	☐	☐	☐	☐	☐
I am often last at finishing a meal	☐	☐	☐	☐	☐
I eat more and more slowly during the course of a meal	☐	☐	☐	☐	☐
I eat less when I'm annoyed	☐	☐	☐	☐	☐
I often feel so hungry that I have to eat something right away	☐	☐	☐	☐	☐
I eat slowly	☐	☐	☐	☐	☐
I cannot eat a meal if I have had a snack just before	☐	☐	☐	☐	☐
I get full up easily	☐	☐	☐	☐	☐
I often feel hungry	☐	☐	☐	☐	☐
When I see or smell food that I like, it makes me want to eat	☐	☐	☐	☐	☐
If my meals are delayed I get light-headed	☐	☐	☐	☐	☐
I eat less when I'm anxious	☐	☐	☐	☐	☐

Appendix 7. List of the exclusion criteria used in the eligibility process in the human study.

•Not diagnosed with Type 2 Diabetes Mellitus

•Mixed ancestors

•Taking any of the following medications:

•Insulin

• Diabetic medication (except from Metformin or oral hypoglycaemic which are allowed).

• Orlistat

• Any gastrointestinal disease that interferes with bowel function and nutritional intake (e.g. Diabetes related constipation or diarrhoea secondary to neuropathy or chronic inflammatory bowel disease, gastrectomy, etc.)

•Significant heart (New York Heart Association class IV), hepatic (transaminase levels greater than 3 times normal) or renal disease (requiring dialysis)

•Cancer

•Pancreatitis

•Major infections (requiring antibiotics) within 3 weeks before study entry

•Concomitant therapy with acarbose, meglitinides, insulin, systemic glucocorticoids or within 2 weeks prior to study entry

•History of alcohol and/or drug abuse

•Investigator's uncertainty about the willingness or ability of patient to comply with the protocol requirements

•Participation in other nutrition trials within 3 months of study entry or drug trials within 5 months of study end

•Current smokers

•Allergic to test food or traces contained in the test food, breathing mould, penicillin, egg, soy

•Asthmatics.

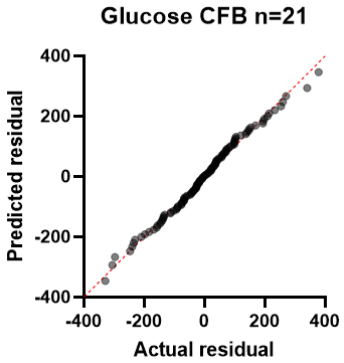
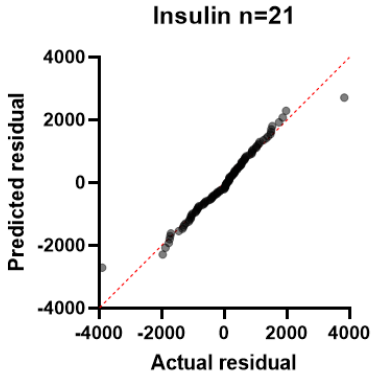
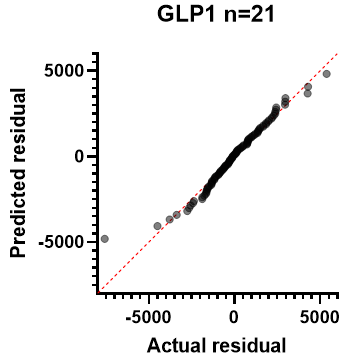
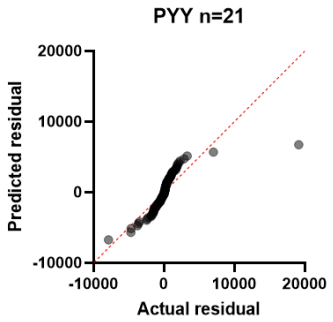
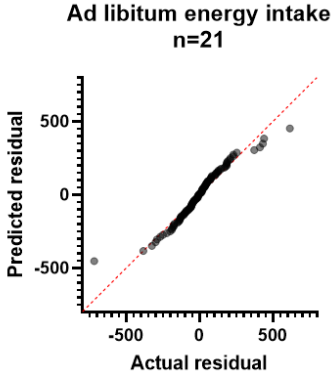
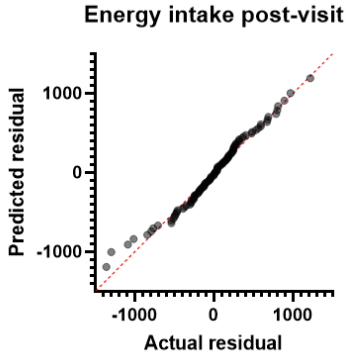
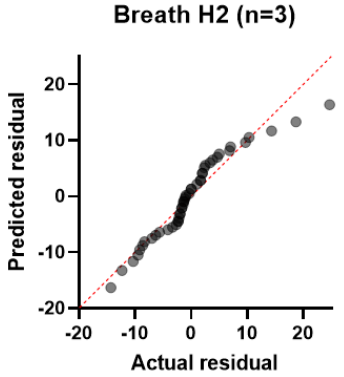
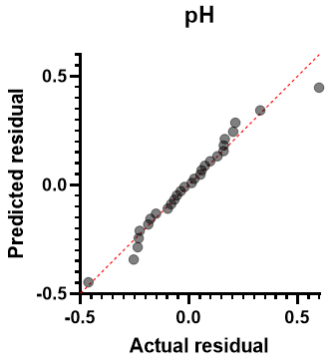
•Any condition involving the imbalance of hormones
•Hypothyroidism.
•Weight change of ≥ 5% in the preceding 3 months
•Shift workers
•Vegetarian
• Medical implants that require batteries such as heart pacemakers

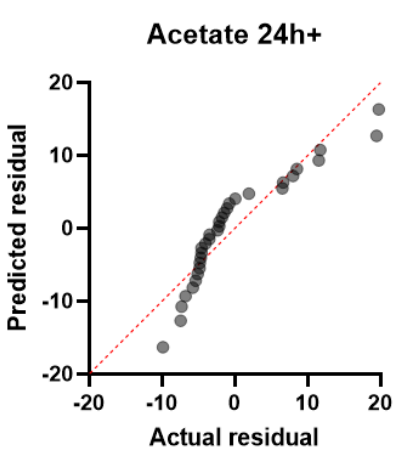
Appendix 8. Meal appearance of the test meals given in the human study.

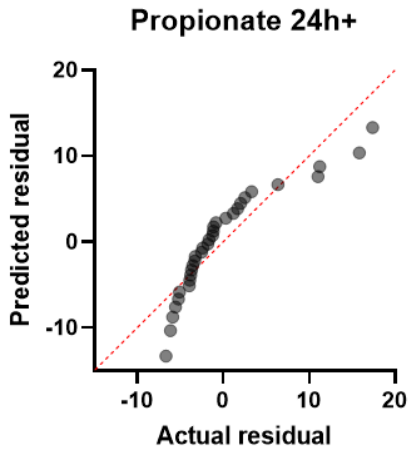
Meal A1	Meal B1	Meal C1
		
Meal A2	**Meal B2**	**Meal C2**
		

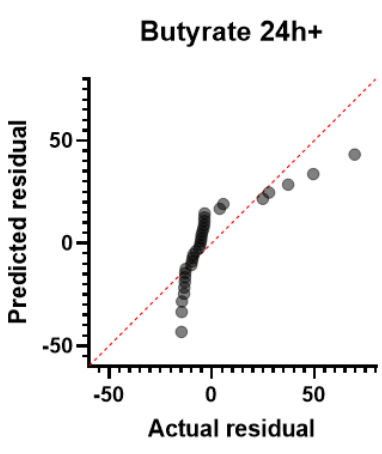
Appendix 9. Q-Q plots of glucose, insulin, GLP-1, PYY, energy intake, breath hydrogen, pH, SCFAs.

Appendix 9. Q-Q plots of glucose, insulin, GLP-1, PYY, energy intake, breath hydrogen, pH, SCFAs.

Glucose	Insulin	GLP-1	PYY
			
Energy intake *ad libitum*	Energy intake post-visit	Breath hydrogen	pH
			
Acetate	Propionate	Butyrate	


Acetate 24h+
Predicted residual
Actual residual
20
10
0
-10
-20
-20
-10
0
10
20


Propionate 24h+
Predicted residual
Actual residual
20
10
0
-10
-10
0
10
20


Butyrate 24h+
Predicted residual
Actual residual
50
0
-50
-50
0
50

Appendix 10. Baseline blood analytes obtained from the screening visit in the human study.

Appendix 10. Baseline screening blood characteristics							
	white European			South Asian			P-value
	Mean	SD		Mean	SD		
sodium	140.64	1.35	0.41	140.44	2.01	0.64	0.80
potassium	4.33	0.33	0.10	4.37	0.19	0.06	0.76
creatinine	77.73	10.53	3.18	81.67	19.23	6.09	0.56
chloride	104.73	2.59	0.78	104.75	2.25	0.71	0.98
urea	4.90	0.69	0.21	5.74	1.15	0.36	0.06
eGFR	86.64	6.36	1.92	84.67	12.89	4.08	0.66
Alanine aminotrasferase	30.00	14.70	4.44	25.00	11.37	3.60	0.40
Alkaline phosphatase	81.36	13.04	3.94	66.56	20.35	6.44	0.06
Albumin	41.00	3.07	0.93	42.56	2.19	0.69	0.20
Total bilirubin	15.36	7.07	2.14	16.67	10.27	3.25	0.75
Cholesterol	4.18	0.83	0.25	3.70	0.84	0.27	0.21
Triglycerides	1.38	0.40	0.12	0.85	0.38	0.12	0.01
HDL	1.29	0.28	0.09	1.13	0.23	0.07	0.19
LDL	2.26	0.80	0.24	2.18	0.73	0.23	0.81

Total cholesterol:HDL ratio	3.38	1.00	0.30	3.35	0.82	0.26	0.94
non HDL cholesterol	2.89	0.86	0.26	2.57	0.80	0.25	0.40
HbA1c	52.36	5.54	1.67	52.89	8.77	2.77	0.88
Fasting blood glucose	8.12	0.23	0.07	6.48	0.26	0.08	0.00
Visceral fat score	12.00	2.58	0.78	10.22	2.24	0.71	0.21
Egfr, Estimated glomerular filtration rate; HDL, high density lipoprotein; LDL, low density lipoprotein; HbA1c, glycated haemoglobin A1c							

Appendix 11. The mean blood glucose $iAUC_{0\text{-}180}$ for all participants and by ethnicity.

Appendix 11. Blood glucose iAUC0-180 min for all participants, white Europeans and south Asians.									
	All (N=21)			White Europeans (n=11)			South Asians (n=10)		
	Mean	95% CI		Mean	95% CI		Mean	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper
Soy	399	317.5	480.4	334.5	237.7	431.2	463.4	326.9	599.9
Soy+GG	251.9	129.8	373.9	133.5	-5.559	272.6	382.1	184.6	579.5
Chicken	567.4	441.9	693	494.6	349.1	640.1	700.9	435.7	966.1
Chicken+GG	313	242.2	383.8	276.8	176	377.6	373.3	267.1	479.5
Mycoprotein	435.6	331.9	539.4	335.5	171.8	499.3	545.8	435.5	656.1
Mycoprotein+GG	257.2	170.2	344.2	152	33.05	271	373	277.1	468.8
GG, guar gum; CI, confidence interval.									

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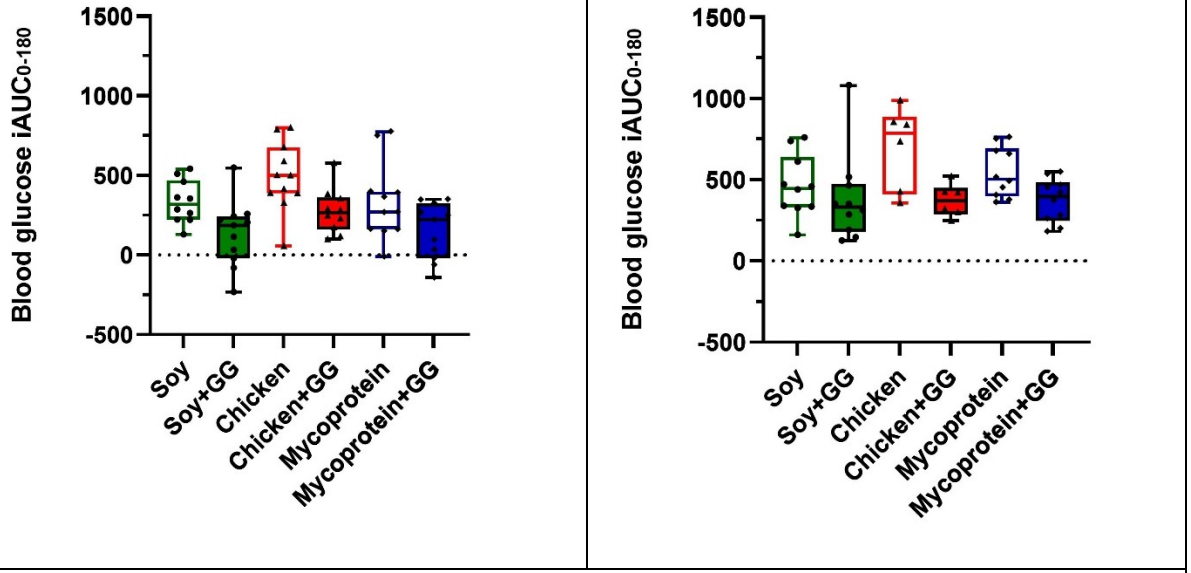
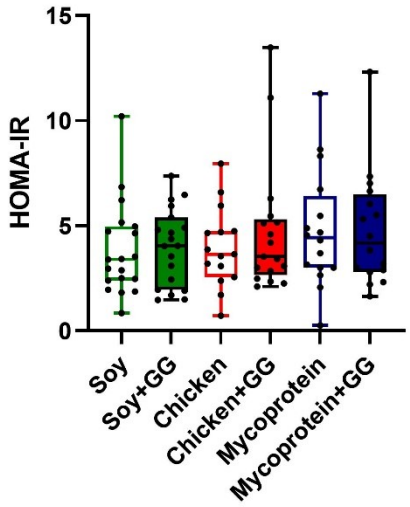
Table Appendix 11. Percentage change in blood glucose iAUC0-180 min due to guar gum addition.									
	All (N=21)			White Europeans (n=11)			South Asians (n=10)		
	Mean	95% CI		Mean	95% CI		Mean	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper
Soy	-41.38	-75.12	-7.646	-70.33	-125.6	-15.07	-12.44	-52.53	27.65
Chicken	-33.74	-52.29	-15.18	-28.98	-58.75	0.781	-41.67	-62.68	-20.65
Myco-protein	-34.14	-55.94	-12.35	-53.52	-93.69	-13.35	-14.77	-32.14	2.595
GG, guar gum; CI, confidence interval.									

Appendix 12. Blood glucose iAUC0-180min following the consumption of soy, chicken and mycoprotein with or without guar gum by ethnicity.



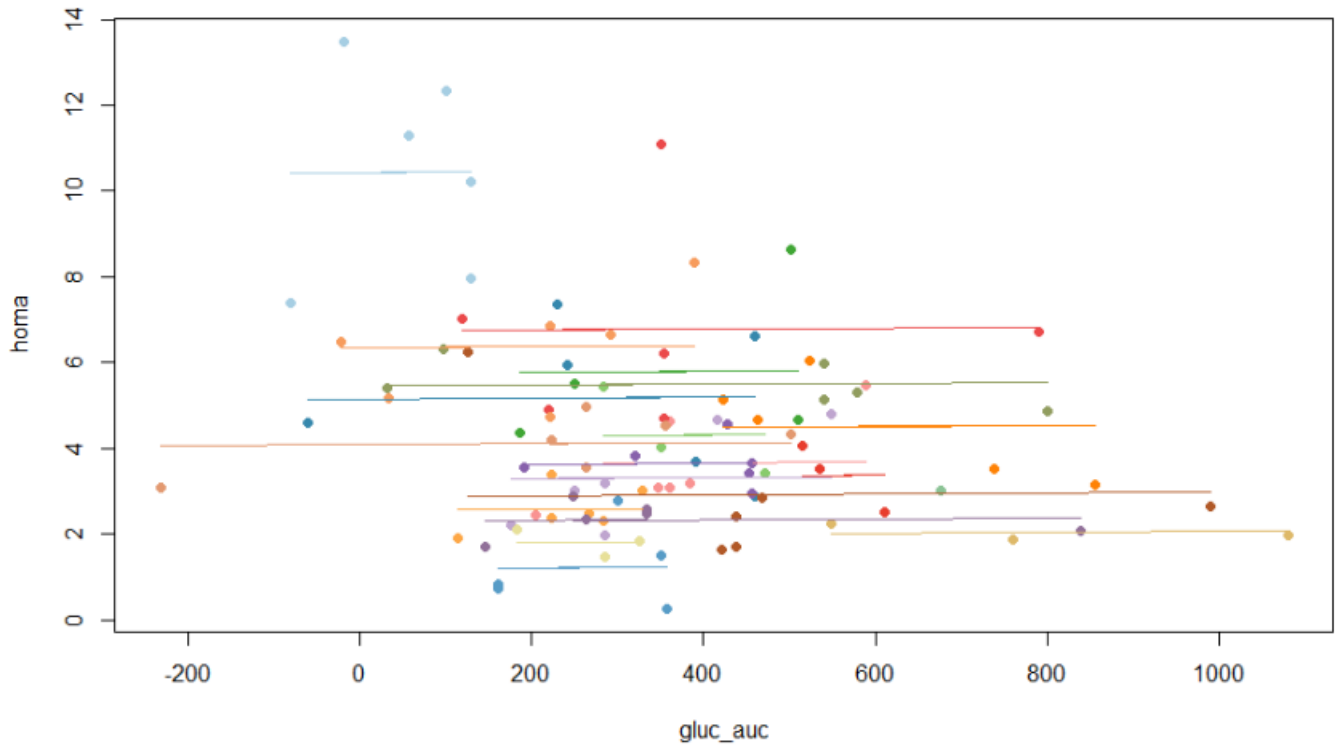
Appendix 12. Blood glucose iAUC0-180min following the consumption of soy, chicken and mycoprotein with or without guar gum by ethnicity. (a) White Europeans (n=11), (b) south Asians (n=10). Data represents median±5-95% percentile and individual datapoints. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the boxes represent the type of chapati, these being plain chapatis (empty boxes) and guar gum-enriched chapatis (filled boxes). iAUC, incremental area under the curve; GG, guar gum; n, sample size.

Appendix 13. The HOMA-IR before each study visit.



Appendix 13. HOMA-IR before each study visit. f for all subjects (N=21). Data represents median±5-95% percentile and individual datapoints. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). The filling of the boxes represent the type of chapati, these being plain chapatis (empty boxes) and guar gum-enriched chapatis (filled boxes). GG, guar gum; HOMA-IR, homeostatic model assessment-insulin resistance; n, sample size.

Appendix 14. The correlation between Glucose iAUC0-180 with HOMA-IR at baseline of each study visit.



Appendix 14. Repeated measures correlation between glucose iAUC0-180 and HOMA-IR at baseline of each study visit.

Appendix 15. The first-phase insulin secretion (insulin $iAUC_{0\text{-}30min}$) for all participants.

Table Appendix 15. Insulin iAUC0-30min.			
	All (N=21)		
	Mean	95% CI	
		Lower	Upper
Soy	130.1	28.9	231.3
Soy+GG	70.46	0.856	140.1
Chicken	152.6	77.37	227.8
Chicken+GG	94.37	14.04	174.7
Mycoprotein	119.4	76.91	161.8
Mycoprotein+GG	33.63	-48.92	116.2
GG, guar gum; CI, confidence interval.			

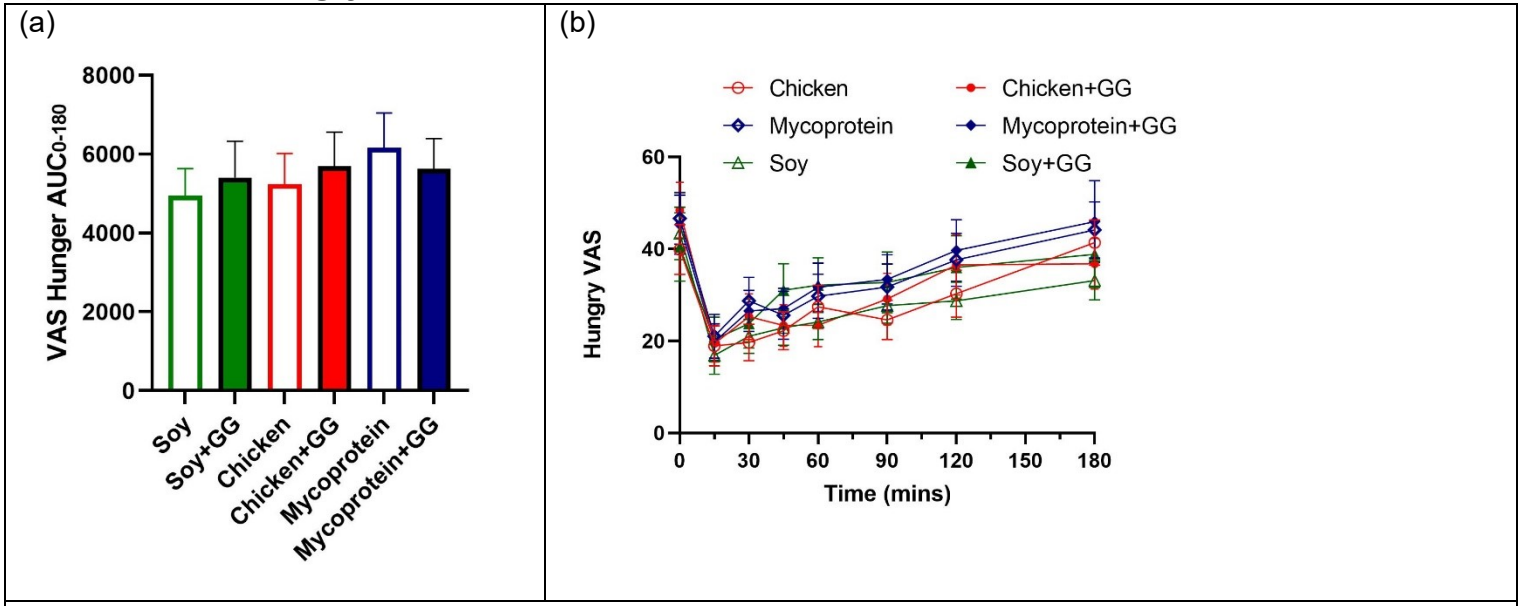
Appendix 16. The GLP-1 $AUC_{0\text{-}180\ min}$ for all participants and by ethnicity.

Appendix 16. GLP1 AUC0-180 min.									
	All (N=21)			White Europeans (n=11)			South Asians (n=10)		
	Mean	95% CI		Mean	95% CI		Mean	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper
Soy	7607	6277	8937	7227	5576	8878	8150	5334	10966
Soy+GG	8055	6380	9730	7925	5485	10366	8185	5309	11060
Chicken	6736	5439	8033	6464	5108	7821	7234	3636	10832
Chicken+GG	6807	5069	8546	7369	5434	9305	5870	1534	10207
Mycoprotein	6914	5503	8325	6302	4347	8257	7662	5248	10075
Mycoprotein+GG	7912	6138	9685	8313	5533	11094	7510	4679	10342
GG, guar gum; CI, confidence interval.									

Appendix 17. The PYY $AUC_{0\text{-}180\ min}$ for all participants and by ethnicity.

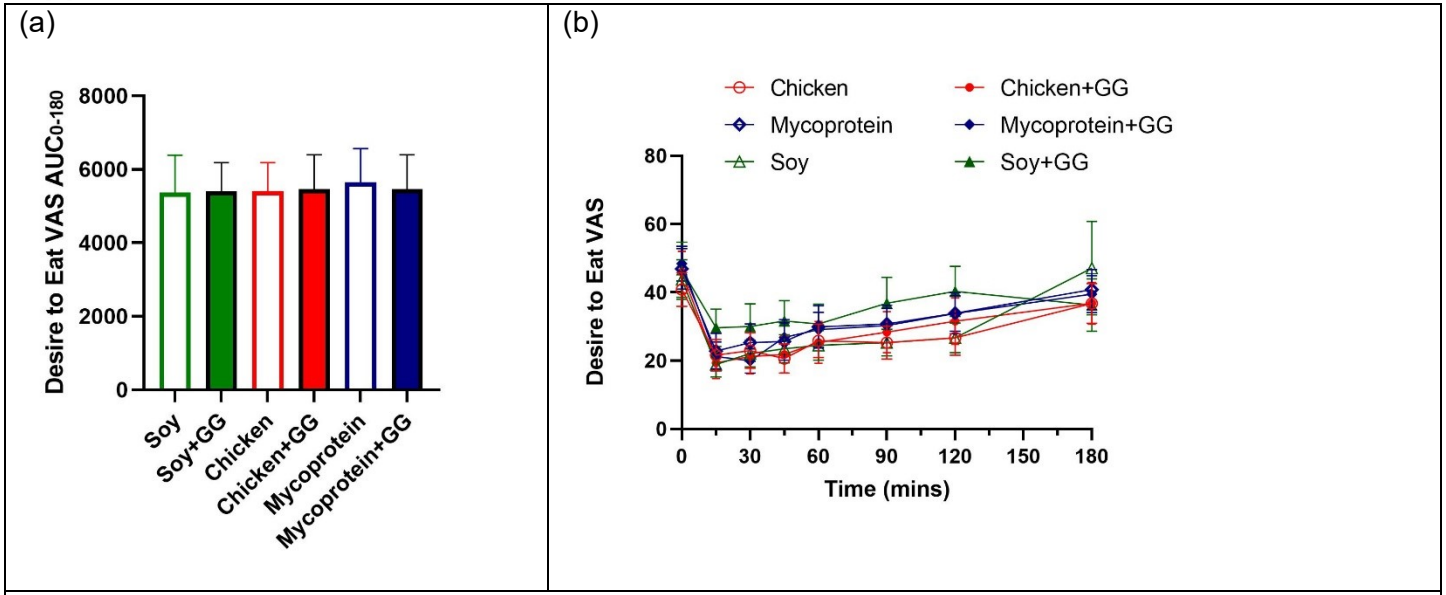
Appendix 17. PYY AUC0-180 min.									
	All (N=21)			White Europeans (n=11)			South Asians (n=10)		
	Mean	95% CI		Mean	95% CI		Mean	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper
Soy	5537	3285	7789	5071	1412	8729	6136	2713	9560
Soy+GG	5876	4021	7730	6362	2175	10549	5535	3372	7698
Chicken	5391	3562	7220	5206	2444	7968	5668	2394	8942
Chicken+GG	4536	2407	6664	4578	1422	7733	4466	706.8	8226
Mycoprotein	5805	2602	9007	5020	1859	8180	6433	611.2	12254
Mycoprotein+GG	5434	3168	7701	4169	1338	7000	6699	2707	10692
GG, guar gum; CI, confidence interval.									

Appendix 18. Hungry $AUC_{0\text{-}180\ min}$.



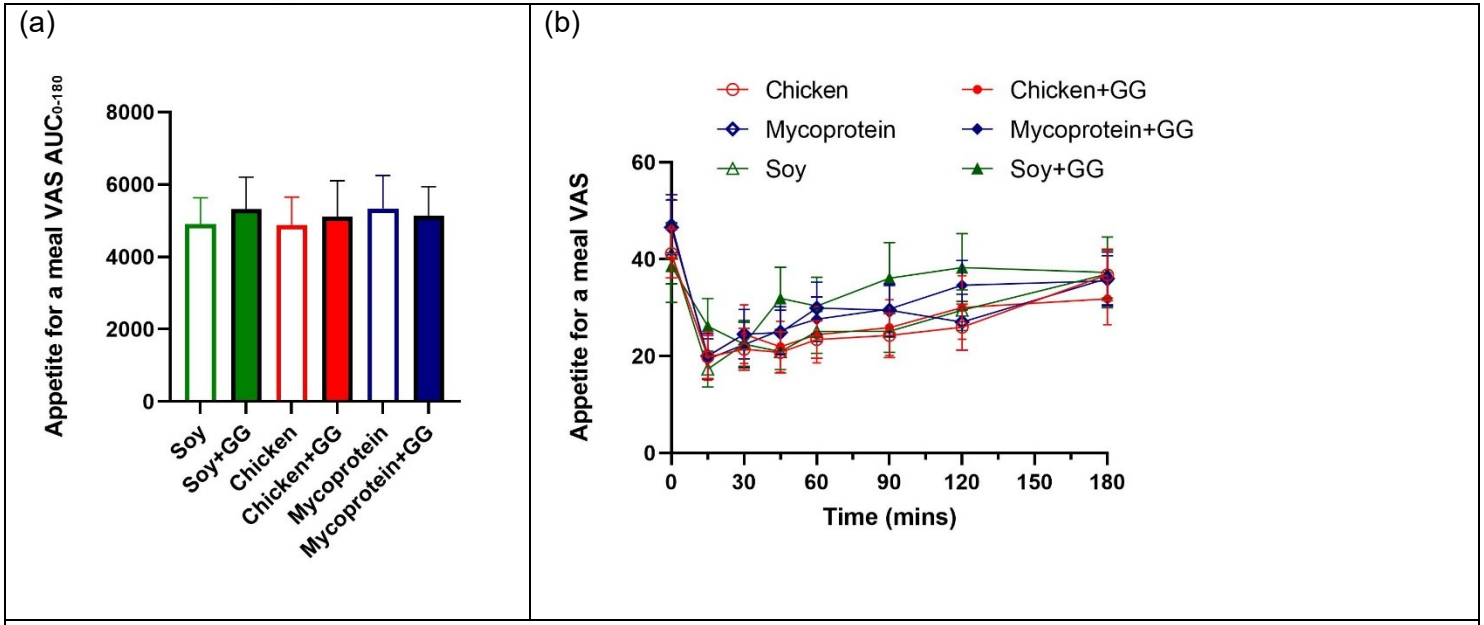
Appendix 18. Hungry visual analogue scales following consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). (a) Area under the curve (AUC) of the change from baseline 0-180 min of the hunger VAS following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents median±5-95% percentile and individual datapoints. (b) Change from baseline appetite for a hunger VAS over 180 min following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents mean±SEM. Test food was given at timepoint 0 min. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). In the timeline graph, the geometry represents the type of proteins, these being soy (triangle), chicken (circle), and mycoprotein (square). The filling of the geometric datapoints represent the type of chapati, these being plain chapatis (empty) and guar gum-enriched chapatis (filled). AUC, area under the curve; VAS, visual analogue scale; n, sample size.

Appendix 19. Desire to eat $AUC_{0\text{-}180\ min}$.



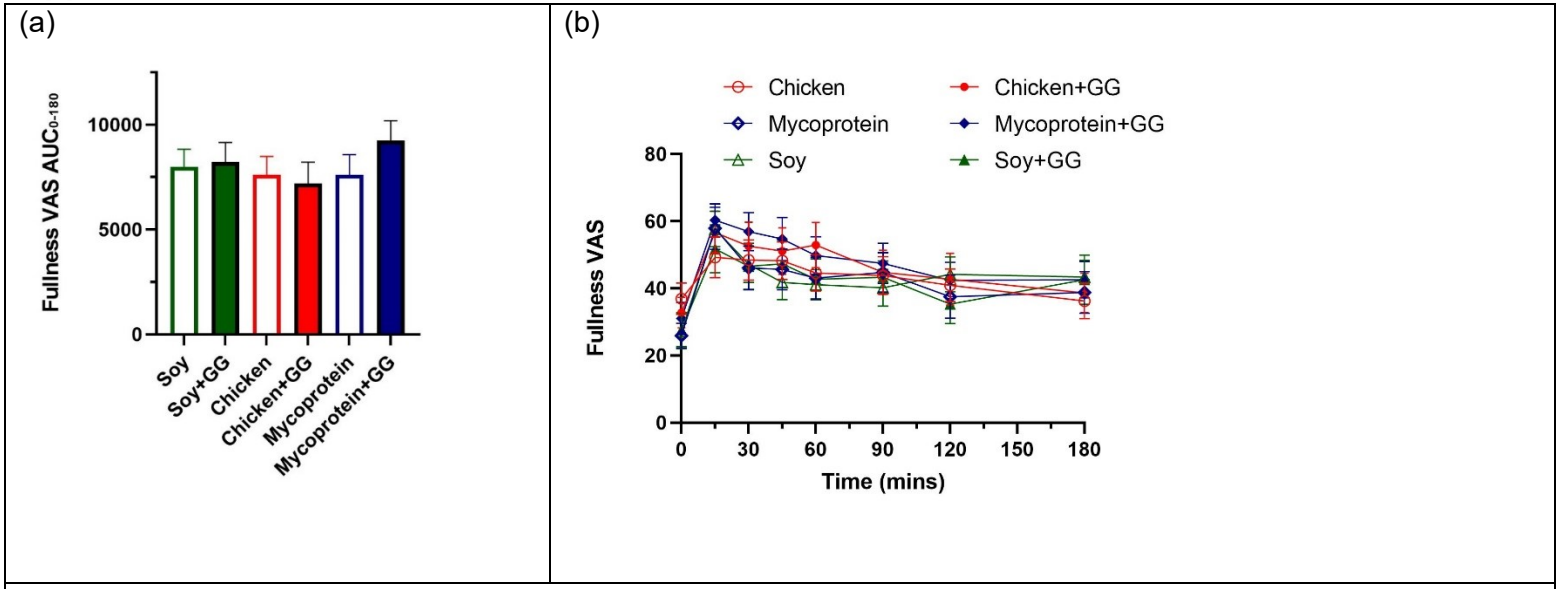
Appendix 19. Desire to eat visual analogue scales following consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). (a) Area under the curve (AUC) of the change from baseline 0-180 min of the desire to eat VAS following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents median±5-95% percentile and individual datapoints. (b) Change from baseline desire to eat VAS over 180 min following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents mean±SEM. Test food was given at timepoint 0 min. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). In the timeline graph, the geometry represents the type of proteins, these being soy (triangle), chicken (circle), and mycoprotein (square). The filling of the geometric datapoints represent the type of chapati, these being plain chapatis (empty) and guar gum-enriched chapatis (filled). AUC, area under the curve; VAS, visual analogue scale; n, sample size.

Appendix 20. Appetite for a meal $AUC_{0\text{-}180\ min.}$



Appendix 20. Appetite for a meal visual analogue scales following consumption of soy, chicken and mycoprotein with or without guar gum for all participants (N=21). (a) Area under the curve (AUC) of the change from baseline 0-180 min of the appetite for a meal VAS following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents median±5-95% percentile and individual datapoints. (b) Change from baseline appetite for a meal VAS over 180 min following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents mean±SEM. Test food was given at timepoint 0 min. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). In the timeline graph, the geometry represents the type of proteins, these being soy (triangle), chicken (circle), and mycoprotein (square). The filling of the geometric datapoints represent the type of chapati, these being plain chapatis (empty) and guar gum-enriched chapatis (filled). AUC, area under the curve; VAS, visual analogue scale; n, sample size.

Appendix 21. Fullness $AUC_{0\text{-}180\ min.}$



Appendix 21. Fullness score visual analogue scales following consumption of soy, chicken and mycoprotein with or without guar gum. (a) Area under the curve (AUC) of the change from baseline 0-180 min of the fullness VAS following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents median±5-95% percentile and individual datapoints. (b) Change from baseline fullness VAS over 180 min following the ingestion of soy, chicken and mycoprotein with or without guar gum. Data represents mean±SEM. Test food was given at timepoint 0 min. The colour scheme represents the type of proteins, these being soy (green), chicken (red), and mycoprotein (blue). In the timeline graph, the geometry represents the type of proteins, these being soy (triangle), chicken (circle), and mycoprotein (square). The filling of the geometric datapoints represent the type of chapati, these being plain chapatis (empty) and guar gum-enriched chapatis (filled). AUC, area under the curve; VAS, visual analogue scale; n, sample size.

Appendix 22. The effect of the test meals on *ad libitum* energy intake.

Appendix 20. Energy intake for all participants (N=21)									
	***Ad libitum* energy intake**								
	Mean	**95% CI**					**Mean**	**95% CI**	
		Lower	Upper					Lower	Upper
Soy	721.9	493.8	950				-	-	-
Soy+GG	748	596.5	899.5				-	-	-
Chicken	699.1	553.7	844.5				-	-	-
Chicken+GG	730.1	527.4	932.8				-	-	-
Mycoprotein	640	502.7	777.2				-	-	-
Mycoprotein+GG	689.1	515.8	862.5				-	-	-
Baseline	-	-	-	-	-	-	1799	1645	1953
Metabolic rate	-	-	-	-	-	-	2900	2694	3106
GG, guar gum; CI, confidence interval.									

Appendix 23. The effect of the test meals on energy intake post-visit.

Appendix 23. Energy intake for all participants (N=21)									
				Post-visit energy intake					
				Mean	95% CI		Mean	95% CI	
					Lower	Upper		Lower	Upper
Soy				2038	1690	2386	-	-	-
Soy+GG				1893	1480	2306	-	-	-
Chicken				1834	1516	2152	-	-	-
Chicken+GG				2024	1692	2357	-	-	-
Mycoprotein				1738	1407	2068	-	-	-
Mycoprotein+GG				1927	1557	2296	-	-	-
Baseline				-	-	-	1799	1645	1953
Metabolic rate				-	-	-	2900	2694	3106
GG, guar gum; CI, confidence interval.									

Appendix 24. Participant information sheet for the *in vitro* batch culture fermentation study.

Participant Information Sheet

Myco2 PIS v3 18/01/202 **19IC5605**

Study title

Investigating the effects in vitro of pre-digested mycoprotein on gut microbiota changes, fermentation profile and metabolomics in South Asian and Caucasian adults.Investigating the effects in vitro of pre-digested mycoprotein on gut microbiota changes, fermentation profile and metabolomics in South Asian and Caucasian adults

You are being invited to take part in a research study that aims to improve understanding of how mycoprotein (a food component present in Quorn foods which is rich in both fibre and protein) affects the bacteria (bugs) in your gut and the chemicals they produce. Before you decide to take part, it is important for you to understand why the research is being done and what it will involve.

Please take some time to read the following information carefully and discuss it with others if you wish.

Please ask us if there is anything that is unclear to you or if you would like to receive more information. Take time to decide whether you want to take part in the study.

Thank you for reading this.

Why is this study important?

Type 2 Diabetes (T2D) is a disease in which blood sugar levels are not well controlled. Millions of people are affected worldwide by T2D and almost 25% of the cases are South Asians. Diet is the cornerstone treatment for T2D. However, the blood sugar response of people of South Asians ancestry is different compared to European counterparts when exposed to the same dietary challenge. Dietary fibre is known to improve blood sugar levels. Mycoprotein is a food regarded as a vegetarian meat replacement, sold under the brand name of Quorn in many supermarkets across the UK. Mycoprotein has a high content in fibre and has been shown to improve blood sugar levels in healthy people. However, we do not understand how this happens. It is known that fibre content of foods is not digested and it travels down to the lower part of your gut almost intact. There it is processed by the bugs resident in your gut called gut microbiota. As a result, gut microbiota produces what we call short-chain fatty acids (SCFA) that are molecules that have been proven beneficial for regulating blood sugar levels. In this study, we would like to investigate how mycoprotein affects gut microbiota in healthy (not diagnosed with type 2 diabetes) people and people with type 2 diabetes, and which molecules are generated. We would also like to see if there are differences in gut microbiota amongst people of South Asian and European ancestry too.

To understand which gut microbiota are present in the gut in response to mycoprotein, we will incubate your stool (where gut microbiota lives) with dietary proteins and then analyse the genetic code (DNA) of the microbiota. Although DNA analysis will be performed on the stools (faeces), the DNA that will be analysed will be bacterial and not human. Hence, your DNA will not be analysed. We will also analyse the chemicals that the gut microbiota in your stool generates.

The results of this study could help us understand how mycoprotein improves blood sugar levels and the differences between people of South Asian and European ancestry.

This study is part of a PhD project at Imperial College London.

Why have I been chosen?

You are being invited to participate in this study because you fulfil **ALL** of the study's inclusion criteria:

- Your ancestry is either South Asian (Bangladesh, India, Sri Lanka, Pakistan) or Caucasian (European)
- You are either healthy (do not suffer from type 2 diabetes) or have been diagnosed with type 2 diabetes
- You do not have any gastrointestinal condition
- Your age is 18-75 years
- You have not been on a course of antibiotics in the past 3 months
- You are not on a course of probiotics
- You have not taken and are taking prebiotic supplements in the past month
- You have not taken laxatives in the past month
- You have not a mixed ethnicity

Do I have to take part?

No, it is up to you to decide whether you wish to take part in this study. If you do decide to take part you will be given this Participant Information Sheet to keep and be asked to sign an informed consent form which you will also be able to keep. If you decide to take part you are still free to withdraw at any time and without giving a reason. Your participant status will not be affected if you decide to withdraw from the study.

What do I have to do?

Once you read carefully this Participant Information Sheet and ask any questions about the study that you might have, you will be given 2 weeks to decide whether you wish to participate in the study. If you decide to participate, you will sign the Consent Form in which you will agree with what participation in this study involves during a screening visit at the 10th floor of Queen Elizabeth the Queen Mother (QEQM) Wing building. After this, the researcher will check with you your eligible criteria. Then, a study visit will be arranged on the same site. Before the study visit, you will have to complete a 3-day food diary. The template with some instructions on how to do this will be given to you for you to fill in. On the study visit, you will be given a stool collection kit that you will use to collect your faeces. You will be given an instruction sheet on how to collect your faeces. You will have to carefully read these instructions to preserve adequately the stool. The study visit is estimated to last 20 minutes although it is dependent on the time it will take your body to donate your stool sample.

You will donate your faeces in the stool collection kit in a toilet on the 10th floor of Queen Elizabeth the Queen Mother (QEQM) Wing building. The stool collection instructions will explain clearly how you need to do this. Specifically, once your faeces are in the collection kit, you will need to lock the container with a lid by screwing it in. This is done to seal the container immediately to avoid any air exposure and contamination. After this, you will have to wash effectively your hands according to the instruction. Next, you will have to give the sealed container with the stool inside to the researcher as soon as possible at an agreed meeting point in the same area.

For demographic data purposes, you will be asked your age, body mass index and ethnicity.

Before the study visit, you will have to complete a 3-day food diary and food frequency questionnaire.

Please remember that you can ask as many questions as you wish, if in doubt, and that you can withdraw from the study at any time point.

What are the possible disadvantages and risks of taking part?

Stool self-collection is associated with a minimum risk of participant's contamination with stool samples. However, this risk has been minimised by providing you with a collection and hygiene manual after stool sample collection.

What are the possible benefits of taking part?

While there are no clinical benefits for taking part, you will be compensated with £15 for your travel and time costs. Also, you will contribute to advancing knowledge of how mycoprotein affects gut bugs and what chemicals they secrete that may regulate blood sugar levels in different ethnicities, through the stool samples you will provide.

What if something goes wrong?

Imperial College London holds insurance policies that apply to this study. If you experience harm or injury as a result of participating in this study, you will be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If you are harmed due to someone's negligence, then you may have grounds for legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during this study then you should immediately inform the Investigator (Prof Gary Frost, g.frost@imperial.ac.uk). The normal National Health Service mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial College, Joint Research Compliance Office.

Will my taking part in this study be kept confidential?

If you decide to participate in this study, all the information collected in this study will be kept strictly confidential. Information needed for this study's purpose will be collected in a highly secure electronic database. This information is linked-anonymised ensuring that you cannot be personally identified.

This means that we will allocate a code to your information and stored samples, and we will keep your personal details separate from your stored data and samples but linked by that code. We will make sure that only researchers have access to that code. You will be allocated a unique identifier number that will be used to track all clinical data and samples taken during the study.

Authorised individuals from the research team, study sponsor (Imperial College London) or regulatory bodies, may inspect relevant medical records. This is to ensure that the study is being carried out correctly and safely.

If you wish to raise a complaint on how we have handled your personal data or if you want to find out more about how we use your information, please contact Imperial College London's Data Protection Officer via email at dpo@imperial.ac.uk, via telephone on 020 7594 3502 and via post at Imperial College London, Data Protection Officer, Faculty Building Level 4, London SW7 2AZ.

If you are not satisfied with our response or believe we are processing your personal data in a way that is not lawful you can complain to the Information Commissioner's Office (ICO). The ICO does recommend that you seek to resolve matters with the data controller (us) first before involving the regulator.

What will happen to the results of this research study?

The results of this study will be analysed by the research team and will be included in a PhD thesis whose aim is to investigate the effects of mycoprotein on gut microbiota changes, fermentation profile and metabolomics in both South Asian and Caucasian individuals who are both healthy (not diagnosed with type 2 diabetes) or diagnosed with type 2 diabetes. Results will be presented at relevant gut microbiota and nutrition conferences and journals for the wider community to reference. No identifiable information of the participant will be included in the PhD thesis, conferences or journal articles; therefore, you will not be identified. Your identity will

be maintained strictly confidential. Your stool samples will be disposed of securely at the end of this study and will not be used for any future experiments.

If you wish to find out the outcomes of this study, you are welcome to contact the organisers of the study.

Who is organising and funding the research?

This study is organised by the Section for Nutrition Research, Department of Medicine at Imperial College London. This study is funded by Quorn and Imperial College London. Quorn is not involved in any part of the research process.

Who has reviewed the study?

This study has been reviewed by the Head of Department, Professor Mark Thrusz. This study has also been given ethical approval by ICREC, approval number: 19/LO/0476.

Contact for Further Information

If you have any further queries regarding the study, please do not hesitate to contact us:

Anna Cherta, 7th Floor Commonwealth Building, Hammersmith Campus, Imperial College London, Du Cane Road, W12 0NN, e-mail: anna.cherta-murillo17@imperial.ac.uk

Prof Gary Frost, 6th Floor Commonwealth Building, Imperial College Hammersmith Campus DuCane Road, London W12 ONN, e-mail: g.frost@imperial.ac.uk

Thank you for taking part in this study!

Transparency notice

Will my taking part in this study be kept confidential?

Imperial College London is the sponsor for this study based in the United Kingdom. We will be using information from you in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Imperial College London will keep identifiable information about you.

- *10 years after the study has finished in relation to data subject consent forms.*
- *10 years after the study has completed in relation to primary research data.*

For College studies included the following link: Further information on Imperial College London's retention periods may be found at https://www.imperial.ac.uk/media/imperial-college/administration-and-support-services/records-and-archives/public/RetentionSchedule.pdf.

Your rights to access change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information by contacting Professor Gary Frost, Email g.frost@imperial.ac.uk, Tel 020 7594 0959.

The Section for Nutrition Research will keep your name and contact details confidential and will not pass this information to Imperial College. The Section for Nutrition Research will use this information as needed, to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Certain individuals from Imperial College and regulatory

organisations may look at your medical and research records to check the accuracy of the research study. Imperial College will only receive information without any identifying information. The people who analyse the information will not be able to identify you and will not be able to find out your name or contact details.

The Section for Nutrition Research will keep identifiable information about you from this study for 10 years after the study has finished.

LEGAL BASIS

As a university we use personally-identifiable information to conduct research to improve health, care and services. As a publicly-funded organisation, we have to ensure that it is in the public interest when we use personally-identifiable information from people who have agreed to take part in research. This means that when you agree to take part in a research study, we will use your data in the ways needed to conduct and analyse the research study.

Health and care research should serve the public interest, which means that we have to demonstrate that our research serves the interests of society as a whole. We do this by following the UK Policy Framework for Health and Social Care Research.

INTERNATIONAL TRANSFERS

There may be a requirement to transfer information to countries outside the European Economic Area (for example, to a research partner). Where this information contains your personal data, Imperial College London will ensure that it is transferred in accordance with data protection legislation. If the data are transferred to a country which is not subject to a European Commission (EC) adequacy decision in respect of its data protection standards, Imperial College London will enter into a data sharing agreement with the recipient organisation that incorporates EC approved standard contractual clauses that safeguard how your personal data are processed.

CONTACT US

If you wish to raise a complaint on how we have handled your personal data or if you want to find out more about how we use your information, please contact Imperial College London's Data Protection Officer via email at dpo@imperial.ac.uk, via telephone on 020 7594 3502 and via post at Imperial College London, Data Protection Officer, Faculty Building Level 4, London SW7 2AZ.

If you are not satisfied with our response or believe we are processing your personal data in a way that is not lawful you can complain to the Information Commissioner's Office (ICO). The ICO does recommend that you seek to resolve matters with the data controller (us) first before involving the regulator.

Appendix 25. Informed consent form for the *in vitro* batch culture fermentation study.

Informed Consent Form for Participants v6

Myco2 17/02/2020 19IC5605

Full Title of Study: Investigating the effects in vitro of pre-digested mycoprotein on gut microbiota changes, fermentation profile and metabolomics in South Asian and Caucasian adults.

	Consenting Information	Initials
1.	I confirm that I have read and understood the Participant Information Sheet version 3 dated 18/01/2020 for the above study and have had the opportunity to ask questions which have been answered fully.	
2.	I understand that my participation is voluntary, and I am free to withdraw at any time, without giving any reason and without my legal rights being affected.	
3.	I give permission for Imperial College London to access my records that are relevant to this research.	
4.	I give/do not give (delete as applicable) consent for samples collected during this study.	
5.	I give/do not give (delete as applicable) consent for information collected about me to be used to support other research in the future, including those outside of the EEA.	
6.	I give/do not give (delete as applicable) consent to being contacted to potentially taking part in other research studies.	
7.	I consent to take part in the above study.	

Participant Name Date Signature

Name of researcher taking consent Date Signature

Upon completion, one (1) copy will be kept by the participant and one (1) copy will be kept by the principal investigator.

Appendix 26. Bacteria growth media composition for 500 mL total volume.

Solution A (425 mL ddH_2O):

0.05g sodium chloride

0.005g calcium chloride (anhydrous)

1g peptone water

1g yeast extract

1g sodium bicarbonate

Solution B (25 mL ddH_2O):

0.02g potassium phosphate, monobasic

0.02g potassium phosphate, dibasic (P3786)

0.005g magnesium sulfate heptahydrate:

0.5mL hemin stock solution*

0.5mL menadione stock solution*

Solution C (12.5 mL ddH_2O):

0.25g bile salts (for microbiology)

Solution D (12.5 mL ddH_2O):

0.25g L-cysteine hydrochloride, monohydrate

*Hemin stock solution (100 mL of a 5 mg/mL solution)

0.5 g hemin (Sigma, Cat. No. 51280-1G)

5 mL 2M sodium hydroxide*

95 mL ddH_2O

Sterilized by autoclaving at 121ºC for 20 minutes

*Menadione stock solution (100 mL of a 1 mg/mL solution)

0.1 g menadione (Sigma, Cat. No. M9429-25G)

100 mL 100% ethanol (Sigma, Cat. No. 32221-2.5L)

Filter sterilized using a 150 mL 0.22 µm bottle top filter (Appleton Woods, Cat. No. BC602)

*2M Sodium hydroxide (100 mL)

8 g sodium hydroxide pellets (Fisher, Cat. No. 10743591)

100 mL ddH_2O

Filter sterilized using a 150 mL 0.22 µm bottle top filter (Appleton Woods, Cat. No. BC602)

Appendix 27. Coefficient of variation of the SCFAs analysis.

SCFAs analysis CV%										
	Acetate	Lactate	Propionate	2-OH-butyrate	Isobutyrate	Butyrate	2-Me-butyrate	Isovalerate	Valerate	Hexanoate
Quality control 1	23.47	11.90	15.78	n.d	0.08	3.60	0.03	0.07	n.d	n.d
SD	0.41	0.12	0.22	n.d	0.00	0.07	0.00	0.00	n.d	n.d
CV%	1.76	1.04	1.42	n.d	2.88	1.97	5.40	3.11	n.d	n.d
Quality control 2	29.93	12.12	16.37	n.d	0.11	4.08	0.04	0.08	n.d	n.d
SD	0.47	0.12	0.23	n.d	0.00	0.04	0.00	0.00	n.d	n.d
CV%	1.58	1.00	1.38	n.d	0.96	1.04	3.39	2.23	n.d	n.d
n.d, non detectable levels for those concentrations below 0.1 uM (lowest limit of detection); CV, coefficient of variability; SD, standard deviation. For acetate, lactate, butyrate the levels are mM. For the remaining compounds the levels are uM.										

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Author: Anna Cherta-Murillo , Gary S. Frost

Publication: British Journal of Nutrition

Publisher: Cambridge University Press

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Author:
Anna Cherta-Murillo , Aaron M. Lett , James Frampton , Edward S. Chambers , Tim J. A. Finnigan , Gary S. Frost

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