Gut Microbiota in Obesity of Different Aetiology; Cause or Effect?

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Author's declaration

I declare that the original work presented in this thesis is the work of the author Dr. Muhammad Jaffar Khan. I have been responsible for the organisation, recruitment, sample collection, laboratory work, statistical analysis and data processing of the whole research, unless otherwise stated.

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Abbreviations

%CV	Percentage coefficient of variation
%EAI	Proportion of estimated actual energy intake
%EAR	Proportion of estimated average requirement
%RNI	Proportion of recommended nutritional intake of protein
Acc	Acetyl Co-A carboxylase
AGRP	Agouti related peptide
ALSPAC	Avon longitudinal study for parents and children
AMP	Adenosine monophosphate
AMPK-P	Phosphorylated AMP kinase
ANGPTL4	Angiopoitein-like protein 4
ANOVA`	Analysis of variance
ARA/EPA	Arachidonic acid/ecosapentaenoic acid
BCFA	Branched chain fatty acids
BMI SDS	Body mass index standard deviation scores
BMR	Basal metabolic rate
C2	Acetate
C3	Propionate
C4	Butyrate
C5	Valeric acid
C6	Caproic acid
C7	Hexanoic acid
C8	Octanoic acid
CD	Cluster of differentiation
ChREBP	Carbohydrate response element binding protein
Cox-2	Cyclooxygenase 2
Cpt-1	Carnitine palmitoyl transferase-1
D/L ratio	D and L lactate ratio
DEXA	Dual energy X-ray absorptiometry
DF	Dietary fibre
DNBC	Danish National Birth Cohort
eCB	Endocannabinoid
ELISA	Enzyme-linked immunosorbant assay
EU	European Union
F/B ratio	Firmicutes to Bacteroidetes ratio
FAO	Food and Agricultural Organization
FDA	Federal drug agency
FFAR2 &3	Free-fatty acid receptor 2 and 3
FFM	Fat free mass
FFQ	Food frequency questionnaire
Fiaf	Fasting induced adipocyte factor
FISH	Fluorescent in situ hybridization
FM	Fat mass in kg
FXR	Fernesoid x receptor
GC-FID	Gas chromatography with flame ionisation detector
GC-MS	Gas chromatography-mass spectrometry
GH	Growth hormone
GHS type 1a	Growth hormone secretagogue receptor type 1a
GLP-1	Glucagon like peptide-1
GOS	Galacto-oligosaccharide
GPR43 &41	G protein coupled receptor 43 and 41
HC	Healthy lean control
HDL	High density lipoprotein

HL	Hypothalamic lean
HMG-CoA	3-hydroxy, 3-mehtylglutaryl Co-enzyme A
HMP	Human microbiome project
HNMR	High-resolution proton nuclear magnetic resonance imaging
HO	Hypothalamic obese
HOMA IR	Homeostatic model assessment for insulin resistance
HPLC	High performance liquid chromatography
HR	Hazard ratio
hsCRP	High sensitivity C reactive protein
Ht	Height
iAP	Intestinal alkaline phosphatase
iC4	Iso-butyric acid
iC5	Iso-valeric acid
IKK	Inhibitor of NFkB Kinase
INFABIO	Acronym for "Effect of diet and lifestyle on risk of gastrointestinal infection and allergy in early life; consumer knowledge, attitudes and needs"
IQR	Interquartile range
ISAAC	International Study of Asthma and Allergies in Childhood
KOALA	Dutch acronym for "Child, parents and health: lifestyle and genetic constitution"
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MCR4	Melanocortin 4 receptor
MetaHIT	Metagenome of the human intestinal tract
MS	Mass spectrometry
NAFLD	Non-alcoholic fatty liver disease
NDNS	National Diet and Nutrition Survey
NFkB	Nuclear factor kappa B
NICE	National institute of clinical excellence
NLRPs	Nucleotide binding domain leucine-rich-repeat-containing proteins
NMDS	Non-metric multidimensional scale
NO	Nitric oxide
NOD	Nucleotide oligomerisation domain
OGTT	Oral glucose tolerance test
OR	Odds ratio
OTUs	Operational taxonomic units
PAL	Physical activity level
PPARγ	Peroxisome proliferator-activated receptor gamma
PWS	Prader Willi Syndrome
PYY	Peptide YY (appetite hormone)
qPCR	Quantitative polymerase chain reaction
R	Spearman rank correlation
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal RNA
RS	Resistant starch
SACN	UK Scientific Advisory Committee on Nutrition
SCFA	Short chain fatty acids
SDS	Standard deviation scores
SIGN	Sottish intercollegiate network
SIMD	Scottish index of multiple deprivation
SO	Simple obese
SPME-GCMS	Solid phase microextraction-gas chromatography mass spectrometry

SRB	Sulphate reducing bacteria
SREBP1	Sterol response element binding protein 1
TAE	Tris-acetate EDTA
TAG	Tri-acyl glycerol
TG	Triglycerides
TGGE	Temperature gradient gel electrophoresis
TH2	Type II cells
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TrKB	Tyrosine kinase-B
VLDL	Very-low-density lipoprotein
WHO	World health organisation
WoREC	West of Scotland research Ethics committee
Wt.	Weight
ZO-1,ZO-2	Zonula Occludin-1&2

Abstract

Thus far, none of the several factors attributed to the recent global epidemic of obesity fully explain the aetiology of obesity. Recently, changes in the gut microbiota composition have been causatively related to the aetiology of obesity in adults as well as children via several proposed mechanisms such as production of short chain fatty acids. However, it is not yet clear whether differences in the gut microbiota composition between lean and obese people are a cause of obesity or if it is an effect of different dietary patterns between lean and obese individuals.

The aim of this observational study was to explore the possibility of "reverse causality" by comparing the gut microbial composition, metabolic activity, and fermentation capacity in children with obesity of different aetiology.

For this purpose, children/young adults with "simple" obesity (due to an unknown cause) and hypothalamic obesity (due to a known cause, such as Prader-Willi syndrome or craniopharyngioma), hypothalamic lean children/young adults (with Prader-Willi syndrome) from endocrine and dietetic clinics and healthy lean children/young adults from the community were recruited (chapter 2). Two faecal samples at interval of 2-3 months with anthropometric, body composition, and 24 h dietary data were collected from each participant. For each faecal sample, the gut microbial metabolic activity was measured by faecal short chain fatty acids (SCFA), hydrogen sulphide, D and L lactate, and ammonia. The fermentative capacity or energy harvesting capability of the gut microbiota from each subject group was assessed with 24 h *in-vitro* batch culture fermentations using 5 different dietary substrates (apple pectin, raw potato starch, wheat bran, raftilose and maize starch). The V4 region of the 16S rRNA gene was sequenced on the illumina® MiSeq platform to explore differences in bacterial community taxonomy between the groups.

Anthropometric and body composition (chapter 3) in the simple and hypothalamic obese patients differed from each other in the nature of their obesity; hypothalamic obese patients being shorter and with lower fat mass compared to the simple obese. Under-reporting on behalf of the obese participants and the nature of dietary assessment method employed in this study limited the real association of dietary intake with body composition.

No significant differences in faecal SCFA, hydrogen sulphide, ammonia, and D & L lactate concentrations were observed between participants with obesity of different aetiology (chapter 4). Obese ("simple" & hypothalamic together) participants had significantly higher concentration of propionate than lean (healthy lean& lean hypothalamic together) participants both at the time of recruitment and after 2-3 months. Moreover, SCFA concentrations were positively correlated with BMI z-score. Our results

suggested that metabolic activity of the gut microbiota is not different between obesity of different aetiology which contradicts the causative role of gut microbiota in the aetiology of obesity. Moreover, higher SCFA concentration in obese (simple & hypothalamic obese) than lean (healthy & hypothalamic lean) phenotype in our study indicated that previously observed differences in the concentration of SCFA are likely to be the result of differences in dietary intake.

SCFA in faecal samples are a net result of production versus absorption in the gut and therefore may not accurately reflect the energy harvesting capability of the gut microbiota. To address this question, we conducted *in-vitro* batch culture fermentation studies to assess fermentation capacity of the gut microbiota with 5 different dietary fibres (chapter 5). Our results suggested that fermentation capacity of the gut microbiota did not differ between obesity of different aetiology implicating that factors other than gut microbiota energy harvesting capability may be causally related to the aetiology of obesity. Obese groups (simple and hypoth. obese together) tended to produce higher SCFA than lean groups (healthy and hypoth. lean together). However, small sample size and large inter-individual variations particularly in the two obese groups may have obscured any significant differences between lean and obese phenotype. Significantly higher rate of propionate production in obese vs. lean groups was in confirmation with our findings from faecal samples. Furthermore, processing time of the samples negatively affected the production of SCFAs independent of phenotype and pathology.

Whether no differences in the gut microbial metabolic activity and fermentation capacity between obesity of different aetiology were extending to the gut microbiota composition was further assessed by high-throughput next-generation sequencing (chapter 6). Although the major bacterial phyla i.e. Firmicutes and Bacteroidetes were similar between simple and hypothalamic obesity, hypothalamic obese group had a higher relative abundance of Proteobacteria compared to simple obese. Overall, the two obese groups had lower rarefied OTU richness and diversity than the two lean groups. Furthermore, there was a highly significant correlation of obesity with community structure and a slight impact of pathology explaining 5% of total variance. A higher frequency of *Dorea* and *Collinsella* and a lower frequency of *Veillonella* and *Alistipes* were observed in obese groups than lean groups. Moreover, a significant relationship between the OTU community composition and weight gain was observed. These results indicated that the presence of similar bacterial metabolic activity in hypothalamic vs. simple obese is not fully translated into their structural diversity at all taxonomic levels. This suggests that composition of the gut microbiota may not be related to the similarity in “functional” diversity and similarities at phylum level may not indicate absence of differences at lower taxonomic levels. Similar

metabolic function may suggest identical or comparable dietary patterns in obese people regardless of the aetiology of obesity.

Overall, these results do not support the role of the gut microbiota in the aetiology of obesity but provide strong evidence to suggest that the findings reported in this and previous studies are the result of obesity and likely to be due to different dietary patterns and intake between lean and obese children (chapter 7). Further studies are needed to investigate whether gut microbiota composition and their metabolic products in our cohort are related to the expression of functional genes in metabolic pathways.

Chapter 1: Gut Microbiota and Obesity

1.1 Purpose and outlines

The chapter discusses the burden of obesity, its risk factors, and consequences as well as a discussion of the gut microbiota composition and metabolic activity in the colon and how their diversity changes over the course of life. Further critique of the evidence on the composition and functionality of gut microbiota in obesity aetiology from different animal and human studies and the proposed mechanisms relating gut microbiota and obesity is given. Lastly, the rationale for the current study, study group, and study objectives are briefly discussed.

1.2 Obesity

Historically, an apple shaped body in humans was a sign of wealth, good health and availability of resources. However, the past few centuries have seen transitions of human behaviour, culture, economy, and health in many perspectives. Gross changes in lifestyle over recent decades have resulted in an increasing incidence of obesity in the developed world followed by increases in developing nations. Obesity has nearly doubled in the past 30 years and is growing in pandemic proportions worldwide. It is now considered "the disease of the millennium" by the International Obesity Task Force. Obesity is the leading cause of many preventable causes of death such as type 2 diabetes, coronary heart disease, hypertension, osteoarthritis, and cancer (1). Overall, it is the fifth leading cause of death and is associated with many short and long term morbidities.

Many criteria have been used to define obesity since 1920 in both American and British health surveys. In general terms, obesity is defined as the accumulation of excessive body fat to an extent that causes harmful effects. For epidemiologic convenience, obesity is expressed as body mass index (BMI) rather than total body fat. For adults over 18 years of age, BMI is expressed as weight in kilograms divided by height in meters squared. A BMI $\geq$30 kg/m^2 in adults is defined as obesity. In children, the body mass index is expressed as standard deviation scores (SDS) because median BMI (in kg/m^2) varies substantially with age and the gender based specific growth of the child. However, currently there is no single internationally recognized cut off to delineate overweight and obesity in children. The World Health Organization (WHO) defines obesity as BMI SD scores of >3 SDS from birth to age 5 years and >2 SDS for 5-19 years

above WHO growth standards median (2). The Centre for Disease Control and Prevention (CDC) in the United States defines a child as obese at ≥95th centile between ages 2-19 years and ≥97.7th centile for obese children less than 2 years of age which is a modified version of the WHO reference criteria (3). The International Obesity task Force (IOTF) recommends the use of BMI cut points which converge to the adult BMI cut offs of 30 kg/m^2 for obese and 25 kg/m^2 for overweight. BMI ≥2 SDS is therefore defined as obesity for children age 2-18 years (4).

1.3 Epidemiology of obesity

1.3.1 Obesity in adults

In 2008, the WHO reported 1.4 billion people, age 20 years or older, as overweight and obese globally. Of these, 500 million (200 million men and nearly 300 million women) were obese (5). The Health Survey of England 2011 recorded an increase in obesity from 13% to 24% in adult male and from 16% to 26% in adult female population between 1993 and 2011. The proportion of those with normal weight has decreased from 41% to 34% in men and from 50% to 39% in women between 1993 and 2011(6). The Scottish Health Survey 2012 reported an increase in obesity from 17.2% in 1995 to 26.1% in 2012 in adults aged 16-64 years. Overall 64.3% of the Scottish adult population (16 years and over and men more likely than women) were overweight or obese (7).

1.3.2 Obesity in children

Obesity in childhood has been linked with obesity in adulthood and hence obesity related complications (8). The number of children at risk of obesity is also increasing. In 2011, over 40 million children aged less than 5 years were reported as overweight worldwide (9). The Health Survey of England 2011 recorded obesity in 17% boys and 16% girls and overweight and obesity in 31% boys and 28% girls (6). In Scotland, although some reports suggested a decrease in obesity in school children from 14.2% in 1997 to 10.2% in 2004 in Aberdeen (10), overall there has been an upward trend in overweight and obesity prevalence. In the Scottish Health Survey 2012, 16.6% children were at risk of obesity (at or above 95^{th} centile). There was an increase in obesity from 14.5% in 1998 to 19.7% in boys' age 2-15 years, while 13.7% girls aged 2-15 were obese in 2012. In addition to these figures, a further 13.8% children were at risk of overweight (between 85^{th} and 95^{th}centile).

The risk of obesity was higher in older children aged 12-15 years than younger ones (26.1% of boys, 18.3% of girls) (7). Another report published in May 2012 by the Scottish Government reported 22% children at age 6 to be overweight (including 9% obese) (11).

1.3.3 The plateau effect

The prevalence of obesity pandemic in children and adolescents has now been observed to level off, stabilize, or even decrease in some developed countries after extensive campaigns to limit obesity including Australia, Europe, USA and Russia (1). A strong decrease in the prevalence of obesity was seen in children and adolescents in Japan (1). Data from the USA have shown a stabilization plateau phase between 2003-04 and 2009-10 after an initial rise from 9.1% in 1988 to 16.9% in 2003-2004 in adolescents' age 12-17 years (12). However, it is worth noting that the trend of increasing obesity in adults is variable within many of these countries (12).

1.3.4 Risk factors contributing to childhood obesity

Obesity is a multifactorial disorder caused by many known (genetic and acquired) and unknown factors. Known causes of obesity could include genetic hormone deficiency such as genetic deficiency of the leptin gene (whose expression is responsible for the synthesis of hormone leptin involved in the regulation of glucose and lipid metabolism) or it can be associated with the malfunctioning of the hypothalamic satiety centre. Malfunctioning of satiety centre could either be due to a chromosomal abnormality such as Prader-Willi Syndrome (PWS) or due to erosion by a tumour such as craniopharyngioma. Obesity due to a genetic or acquired malfunction of the hypothalamic satiety centre is referred to as "hypothalamic obesity" in this thesis. However, obesity related to a known cause contributes only a small proportion to the global obesity epidemic, most of which is not attributable to a definitive risk factor. For the purpose of this thesis, such forms of obesity are referred to as "simple" or "classical obesity".

1.3.4.1 Risk factors contributing to simple obesity

Although genetic factors contribute to the prevalence of obesity in a small proportion of children (~5-8%), such as genetic defects in leptin and its receptors, pro-opiomelanocortin (POMC), prohormoneconvertase 1, melanocortin receptor-4 (MCR4), and neurotrophin TrKB (13, 14), the recent disproportionate boom in the prevalence of obesity is not solely explained by these polygenic factors. Childhood obesity by and large is attributed to many putative risk factors, some of which are consistently associated with obesity. However, all

are only potential and not well established (15). Several studies have highlighted risk factors contributing to childhood obesity, some of which are summarised in Table 1.1. Out of more than 20 potential risk factors, parental obesity, early adiposity-rebound at <5.5 years age, rapid growth, weight SDS at age 8 and 18 months, more than 8 h television watching per week at three years age, weight gain in first year, and sleep deprivation in first 3 years of age have been associated with childhood obesity in multiple regression models (15). Others have suggested childhood obesity is a consequence of events in three phases of life; prenatal phase, phase of adiposity rebound, and adolescence phase (16), all of which include similar risk factors as suggested by Reilly *et al.* (2005) except for maternal under or over nutrition during pregnancy. It is interesting to note that both over and under-nutrition in early life can lead to obesity via catch-up growth which follows a period of deficient nutrient requirement. The relationship of weight with adiposity may therefore be J shaped rather than linear (17).

Association of adult obesity risk factors with mortality and morbidity related complications is well studied (18). Freedman *et al.* (2001) showed in the Bogalusa heart study (n=2617, age 2-17 years) that up to 80% of obese children followed for 17 years become obese adults; however, the association of childhood obesity with adult coronary heart disease risk factors such as plasma lipids, insulin and blood pressure was very weak. Furthermore the levels of these risk factors for coronary heart disease did not change with childhood weight status or age of obesity onset (19). The link between childhood obesity and the predictors of adverse cardiovascular health in adult obese individuals is therefore missing. Park *et al.* (2012) recently conducted a systematic review of 39 studies to explore this link. Although several adult diseases or adult disease-related risk factors such as type 2 diabetes, coronary heart disease, stroke, kidney and colorectal cancers, and all-cause mortality were associated with childhood BMI, the association did not exist after adjustment for adult BMI (20). This suggested other unknown factors that could potentially contribute to the link between childhood obesity and adult disease risk factors independent of adult BMI status. Additionally there are limited long-term studies to give any conclusive evidence on association of disease related risk factors in adulthood with obesity in childhood while accounting for the adult BMI (21).

1.3.5 Hypothalamic obesity (mechanism of obesity in Prader-Willi syndrome and craniopharyngioma)

1.3.5.1 Obesity in Prader-Willi Syndrome

Prader-Willi Syndrome (PWS) is a genetic neurological disorder due to loss of function in the long arm (q11-q13) of paternally derived chromosome 15 occurring in 1 in 16000 live births. This disease is characterised prenatally by decreased fetal movements, polyhydromnios and post-natally by hypotonia ("floppy child"), feeding problems, and failure to thrive in early infancy. This is followed by general growth delay, low IQ (intelligence quotient), severe forms of obesity due to hyperphagia, sleep abnormalities, behavioural problems and hypogonadism. There are some phenotypic features peculiar to most but not all PWS patients such as short stature, small hands and feet, narrow nasal bridge, almond shaped palpebral fissure, thin upper lip, narrow bifrontal diameter, scoliosis, eye abnormalities, thick saliva, hypopigmentation, and cat like cry (22).

Prader Willi syndrome is the most common cause of syndromal obesity and a major cause of metabolic complications in this group. Obesity in PWS is hallmarked by the insatiable hunger which inculcates behavioural changes in children to get excess food. Ghrelin is a gut hormone released from the stomach and found in higher concentration in plasma in the fasting state stimulating food intake (hence called orexogenic). Ghrelin is found in plasma in an acylated (active) and de-acylated forms. The levels of ghrelin are reduced after food intake in healthy people and hence may determine meal size and help in short term control of food intake (23). The first evidence regarding persistently increased orexogenic ghrelin levels in PWS vs. normal children came from the study of DelParigi and colleagues (24). Ghrelin levels remained high even after meals which lead to a delayed sense of fullness and persistent drive to eat. Many other studies have suggested higher plasma ghrelin levels in PWS obese patients compared with simple non-PWS obese, healthy lean, leptin deficient, and melatonin receptor 4 deficient patients (25, 26) (Figure 1.1).

Table 1.1: Summary of studies investigating factors associated with onset of obesity in children

Reference	Study type	Country/region	studies included (n)	(Range of) No. (n)	Factors predicting childhood obesity
(27)	Longitudinal study	USA (Hawaii)	n/a	n= 9,439 age 5-7 years	Strong association with maternal gestational diabetes (OR (95% CI): 1.82 (1.15-2.88), p<0.0001) (adjusted for maternal age, ethnicity, weight gain, parity)
(28)	Longitudinal study ALSPAC cohort	United Kingdom (England)	n/a	n= 881, Infants and children 2-5 years	high energy intake at 4 months was a predictor of weight gain between birth and 1, 2, and 3 years of age (p=0.0005, p=0.0004, p=0.007) only in formula or mixed fed infants. Each 420kJ/day increase in energy associated with overweight at 3 years [OR, 95% CI: 1.46 (1.20-1.78)] and 5 years [OR, 95% CI: 1.25 (1.00-1.55)]. No association with breast feeding
(15)	Longitudinal study (ALSPAC study)	United Kingdom (England)	n/a	n= 8234 children, age 7 years	Parental obesity [OR, 95% CI: 10.44 (5.11-21.32)], very early (by 43 months) BMI or adiposity rebound [OR, 95% CI: 15.00 (15.32-42.30], >8 h watching television [OR, 95% CI: 1.55 (1.13-2.32)], catch-up growth [OR, 95% CI: 2.60 (1.09-6.60)], weight SDS at 8 months [OR, 95% CI: 3.13 (1.43-6.85)] and 18 months [OR, 95% CI: 2.65 (1.25-5.59)], birth weight per 100 g [OR, 95% CI: 1.05 (1.03-1.08)], weight gain per 100 g in first year [OR, 95% CI: 1.06 (1.02-1.10)], sleep deprivation (<10.5 h) at 3 years [OR, 95% CI: 1.45 (1.10-1.89)]
(29)	Crossectional survey CLASS survey	Canada	n/a	n=4298, Age range between 10 &11 years	Increased risk for; Lunch bought at school [OR, 95% CI: 1.39 (1.16-1.67)] Decreased risk; supper with family ≥3 times a week [OR, 95% CI: 0.68 (0.52-0.88)], attending physical education classes [OR, 95% CI: 0.63(0.43-0.87)], high income relative to lower income families [OR, 95% CI: 0.50 (0.25-0.70)]

(20)	Systematic review	n/a	39	n=181-1.1 million, Studies age range at recruitment; 2-19 years	Unadjusted; Type-2 diabetes (OR range: 1.22-2.04), hypertension (OR range: 1.35-3.75), coronary heart disease (HR range: 1.53-5.43), all-cause mortality (40-60% increase in risk), stroke (HR range: 1.4-3.2), cancer (20-40% increased risk), colorectal cancer (RR range: 2.1-9.1), Kidney cancer [RR (%CI): 2.6 (1.5-4.7)] Adjusted; no real association
(30)	Systematic review and meta-analysis	n/a	>80 for systematic reviews, 20 for meta-analysis	n >4000, US nationally representative sample	Increased risk; Non-Hispanic blacks (≥10% increased risk), low education except in black women (≥10% increased risk), urban dwelling (obesity prevalence of up to 30% or higher in 2005),
(31)	Systematic review	n/a	21	N= 90-19,257 Age; 4 yrs. up to 32 yrs.	Weight gain in infancy and first 2 years of life [OR (95% CI: 5.7(4.5-7.1)]
(17)	Systematic review	n/a	141	n= up to 0.2 million, adolescents	Parental fatness, genetic factors, lower socioeconomic status (parents occupation, education, and income), birth weight, early and rapid maturation, physical inactivity (TV, total activity),

CLASS; Children's Lifestyle and School-performance Study, ALSPAC; Avon Longitudinal Study of Parents and Children, HR; hazard ratio, OR; Odds ratio, RR; risk ratio, n; number, n/a; not applicable.

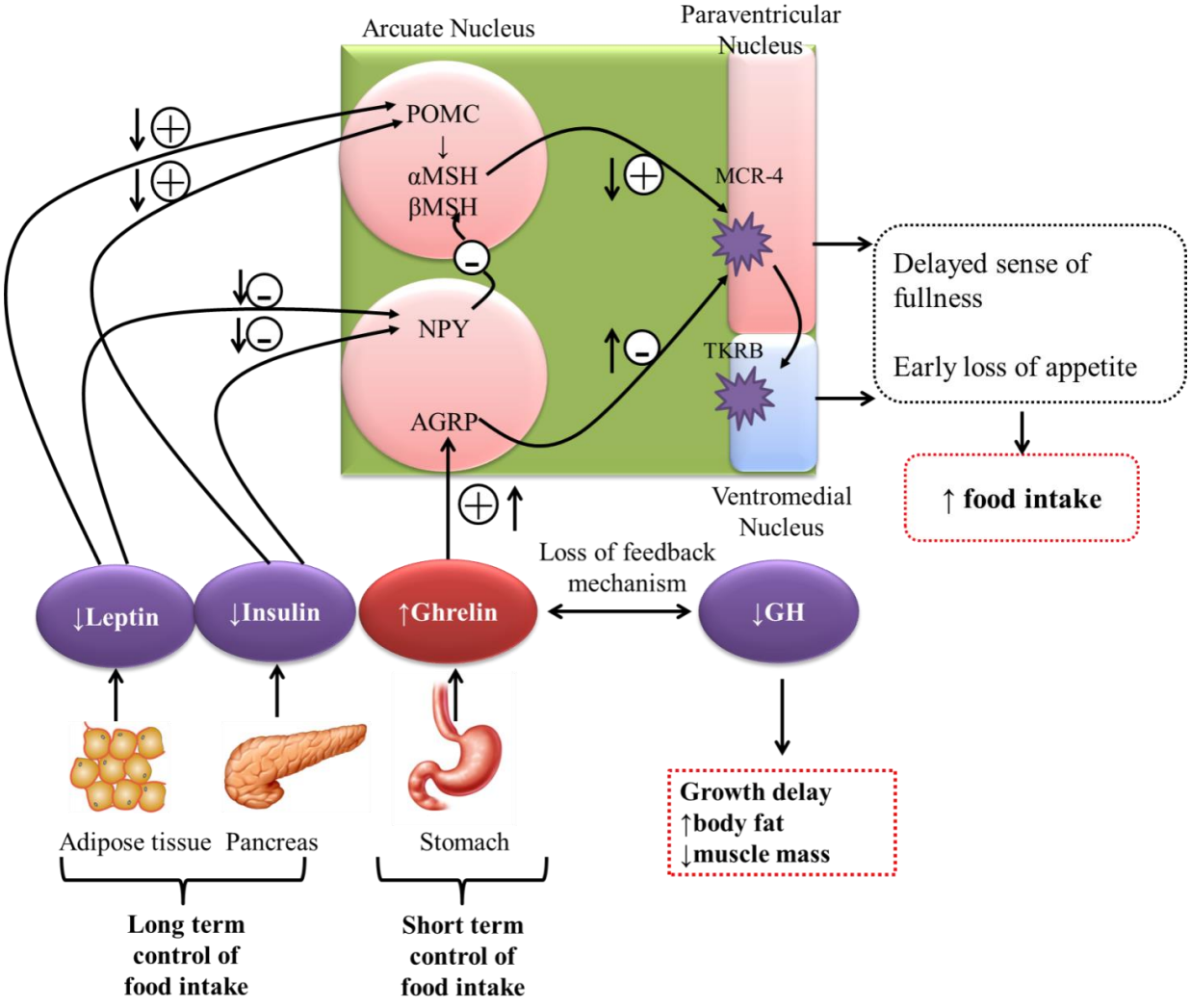
Nevertheless, the role of ghrelin in the aetiology of hyperphagia in PWS is still controversial. In a study by Erdie-Lalena *et al.* (2006), the authors found no significant difference in the fasting levels of total ghrelin (acylated & des-acylated ghrelin) in nine normal weight PWS patients less than 5 years age compared to eight healthy children matched for age, BMI, and gender (32). Levels of plasma insulin and glucose in PWS children were not significantly different than healthy controls. Moreover, levels of ghrelin were negatively associated with BMI in controls but not in PWS children (32). These findings indicate that levels of ghrelin in PWS patients might increase only in later childhood prior to the onset of obesity in the course of the disease which does not happen in healthy population. This was also suggested by Feigerlova *et al.* (2008) who found a significantly greater negative correlation of plasma total ghrelin levels in the children age <3 years, prior to the onset of obesity (33). However, there was a high inter-individual variation in the total plasma ghrelin levels which was even found when a more specific and active acylated form was used indicating small sample size. Additionally, plasma ghrelin levels were not assessed for other confounding factors such as difference in energy expenditure and requirements particularly at young age.

In healthy people, circulating acylated ghrelin stimulates the secretion of growth hormone by acting as a ligand for the growth hormone receptor, GH scretogogue receptor type 1a (GHS type 1a) (34). Moreover, high growth hormone levels down-regulate ghrelin levels via negative feedback (34). In contrast, children with PWS suffer from growth hormone deficiency despite high circulating levels of ghrelin. This might indicate the desensitization of GHS receptor type 1a. Growth hormone (GH) replacement therapy in PWS children have been shown to induce lipolysis, reduces fat mass, and increases lean mass in PWS children (35), but the levels of ghrelin are higher despite GH treatment and its beneficial effects indicating the failure of the negative feedback mechanisms to operate. Therapeutic use of acylated and un-acylated ghrelin in correcting this abnormality is still under extensive research (Figure 1.1).

Plasma insulin deficient states or insulin resistance cause diabetes mellitus, and up to 20% of PWS children develop type 2 diabetes in the course of the disease (36). However, the role of insulin in hypothalamic obesity is controversial. Some authors have suggested lower fasting plasma insulin and delayed insulin secretion during an oral glucose tolerance test (OGTT) in the presence of intact insulin sensitivity (37), while others have suggested increased plasma insulin depicting insulin resistance (38). Obesity in itself is a diabetogenic state, therefore it is unclear whether changes in insulin levels are a

consequence of severe obesity or the insulin secreting capability of PWS patients is abnormal (37). PWS patients have a higher fat to lean mass ratio and a lower bone mineral content suggesting a lower lean mass and a higher fat mass resulting in slow body and limb movements in PWS patients (39). Reduced physical activity due to poor co-ordination, eye abnormalities such as esotropia and myopia, and slow body movements due to poor muscle tone leads to reduced energy expenditure (40) which reduces the caloric requirement in these patients. This, in addition to lower lean body mass, favours the accumulation of excess body fat and hence obesity. Obstructive sleep apnoea leading to day time sleepiness is also a contributing factor in hypothalamic obesity in PWS (22).

Figure 1.1: Mechanism of obesity in Prader Willi Syndrome. Adapted from Mutch and Karine (2006) (41).



Decreased plasma leptin and insulin results in loss of stimulatory signals to the POMC neurons in the arcuate nucleus which fails to stimulate α and β-MSH to control satiety via activation of MCR4 receptor in the Paraventricular nucleus. On the other hand, persistent increase in plasma ghrelin results in stimulation of neurons expressing NPY and AGRP which inhibit MCR4 signaling and hence increase drive towards food intake. AGRP, agouti-related protein; α-MSH, alpha melanocyte stimulating hormone receptor; NPY, neuropeptide Y; POMC, pro-opiomelanocortin;TRKB, tyrosine kinase receptor.

1.3.5.2 Obesity in craniopharyngioma

Craniopharyngioma represents 1.2-4% of all brain tumours and is the leading cause of hypothalamic obesity. Obesity is one of the major causes of morbidity and reduced quality of life in children and adolescents with craniopharyngioma (42) leading to premature cardiovascular disease, psychosocial problems and failure to achieve academic potential, especially in children with hypothalamic involvement of the tumour (43).

The exact cause of hypothalamic obesity seen in craniopharyngioma is not well understood. The literature suggests that obesity may be related to the disruption of hormonal signals from leptin and insulin (44) to the arcuate nucleus. The neurons of the arcuate nucleus are responsible for secreting Pro-OpioMelanoCortin (POMC), Agouti-Related Peptide (AGRP), and neuro-peptide Y (45, 46), altered secretion of which result in abnormal appetite and hyperphagia. Data also suggest increased parasympathetic and reduced sympathetic tone, nocturnal insomnia and day time sleepiness (47, 48), and reduced functional capacity due to the higher BMI as factors leading to weight gain (49, 50). Pituitary hormone deficiencies as a result of both tumour and treatment modalities such as radiotherapy are additional factors which contribute to obesity in these patients. Hypothalamic involvement, tumour progression and tumour relapse affect long term quality of life (50, 51).

The known risk of obesity in patients with craniopharyngioma exists both at diagnosis and in the long term. Hypothalamic involvement has been established as a long term predictor of obesity and health related quality of life in these children. Evidence suggests that children who are obese at presentation generally have a higher incidence of hypothalamic involvement and hydrocephalus even though they have a normal BMI before diagnosis. Patients without hypothalamic involvement have better quality of life scores in long term follow up studies (52). Therapeutic interventions immediately after diagnosis in craniopharyngioma have therefore been recommended for the prevention of obesity given that a significant increase in BMI occurs in the postoperative period, especially during the first three years (53).

1.3.6 Complications of obesity

Both short and long term consequences of obesity are anticipated to increase in the population due to the recent surge of global overweight and obesity in childhood as well as

adults (Table 1.2). Complications of paediatric obesity encompass diverse aspects of health. Of particular concern are the rising psychosocial complications, cardiovascular diseases and metabolic disorders, long term morbidity and mortality, and economic burden (54). Although obesity in childhood is associated with consequences in the short and intermediate term, it is still unclear whether these complications are associated with childhood obesity are independent of adult BMI (17). A recent, extensive systematic review by Park *et al.* (2012) showed association between childhood BMI SDS and type 2 diabetes, cardiovascular disease and hypertension but the effect could not be seen when this was adjusted for adult BMI. There are many limitations in reports associating childhood obesity with short, intermediate, and long term complications. This is largely due to lack of longitudinal data, inadequate sample size, non-uniform measures of obesity, and reliance on retrospective cohorts such as from school and military records (21).

Table 1.2: Complications (consequences) of childhood obesity; adopted from Must and Strauss, 1999

System involved	Disease/signs and symptoms	Risk for obese
Immediate consequences (prior to adulthood)		
Orthopaedic	Slipped femoral epiphysis; leading to permanent femoral head damage	50-70%
	Blount's disease (tibia vara); bowing of the tibial bone due to excess weight bearing (80% children with tibia vara are obese)	80%
	Idiopathic intracranial hypertension (Pseudomotorcerebri); with headache, nausea, vomiting, blurred vision, and diplopia	30-80%
Pulmonary	Asthma	30%
	Decrease performance in exercise by at least 15% due to increased bronchial hyperactivity	80%
	Sleep apnoea with central hypoventilation	Up to 94%
	Memory and learning difficulties due to obstructive sleep apnoea	
	Pickwikian syndrome; severe obesity associated with hypoventilation, somnolence, polycythaemia, right ventricular hypertrophy and failure, Sudden death and pulmonary embolism with Pickwikian syndrome	
Gastrointestinal	Gall stones (cholelithiasis)	8-33%
	Liver steatosis due to insulin resistance and increased lipolysis	20-25%
	Fatty liver and Liver fibrosis with severe obesity	
	Steatohepatitis in severe obesity	40-50%
Endocrine	Insulin resistance with decreased glucose uptake by cells	20-45%
	Non-insulin dependent diabetes mellitus (BMI >90th centile)	90%
	Hyperandrogenemia	
	High total cholesterol, low density lipoproteins (LDL), and triglycerides (TG)	20-45%

Table 1.2 continued

	Polycystic ovarian syndrome; oligomenorrhea or amenorrhea with obesity, insulin resistance, hirsuitism, acnes, acanthosisnigricans, and	40-60%
Social and economic	Poor emotional development	
	Low self-esteem and concerns of body image, expectations of rejection and subsequent withdrawal	
	Fear of fatness; making an attempt to reduce weight while most (83%) of the adolescent girls are normal weight	50%
	Eating disorder (Bulimia) and smoking in girls; developed as an attempt to lose weight	Bulimia;40 % Smoking; 20%
	Downward social and academic mobility; Lower academic achievement and lesser income	10% higher than normal weight
Intermediate consequences (Relationship with CVD risk factor levels and persistence in adulthood)		
Cardiovascular risk factors	Elevated systolic and diastolic blood pressure in children age 7-11 years	20-30%
	Hypertension	8.5 fold
	Familial aggregation of hypertension	
	Deleterious effect on cholesterol, LDL, TG, and low high density lipoproteins (HDL) in adulthood	2.4-8 fold
Persistence of obesity	Adolescent obesity continuing to adulthood obesity (vary with gender)	25-50%
Long-term consequences (remote-aftereffects on morbidity and mortality)		
Adult morbidity	Risk of heart disease and atherosclerosis in both males and females	
	Risk of colon cancer for males	
	Risk of gout for males	
	Arthritis and hip fractures in females	
	Menstrual problems at age 33 years	
	subfertility at age 33 years	
	Gestational hypertension at age 33 years	
Adult mortality	All-cause mortality (independent of smoking, socioeconomic status, and adult weight status)	Relative risk (RR); 1.5
	Coronary heart disease mortality (independent of smoking, socioeconomic status, and adult weight status)	RR; 2.0

1.3.7 Management of childhood obesity

Obesity is a multifactorial disorder and therefore needs a multifaceted multi-environmental approach towards its management. For an obesity intervention to be successful, the intervention strategy should be aimed both at the child and the family and the risk factors or behaviours which have been found consistently associated with childhood obesity (54). Many randomised control trials and longitudinal studies suggest interventions in 5 different directions including lifestyle changes, dietary advice, physical activity, psychological therapy, and in some cases; pharmacologic therapy (Table 1.3) (55). However, there are

several potential barriers to successful management of obesity. These include lack of motivation of the child and their family, low compliance, socioeconomic status, and the general "obesogenic" environment (lacking recreation facilities and availability of healthy food choices).

Successful life style interventions result in the reduction of cardiovascular disease and diabetes risk. However, achieving weight loss and maintaining it over a longer period of time are challenging in obese paediatric and adult population. Furthermore, response to weight management across paediatric population is not uniform, as some patients respond to weight management while others not. Braet (2006) in her study suggested positive association of weight loss with the pre-management severity of overweight, age and initial weight loss with weight management and negative association with eating disorder especially in girls (56). Although 77.3% paediatric patients (n=122, age range 7-17 years) have been reported to lose 10% of their initial weight, only 24% of these tend to maintain weight loss over 2 years period while the rest (75%) tend to gain weight (56). Furthermore, a two-fold risk of weight regain still exists after weight loss due to continued sedentary behaviour. Avoiding sedentary behaviour is therefore recommended by lowering TV watching, computer, and use of other electronic equipment for leisure purpose to a maximum of 1-2 h per day by the European Society of Paediatric Endocrinology (ESPE) (55). In this context, making changes in the school dietary regimen, curriculum, and less use of own or public transport are also suggested to achieve weight loss in the long term (57). However, several established barriers to achieving these goals have been identified particularly lack of motivation of the patient and the lack of the parents' involvement in weight management (58). Moreover, despite the awareness and appreciation of the community and school based programmes, long term effectiveness is still rather less evident (59).

Successful weight maintenance also requires reduced caloric intake, reduced fat and food consumption, less snacking, regular meals especially breakfast, and less fast food consumption (55). It is debatable whether aerobic exercises contribute to weight loss, but a positive association of exercise with weight maintenance and weight loss in conjunction with dietary and lifestyle interventions have been shown (54). Parents' lack of obesity perception in their children has been documented. Education of parents along with the children is necessary to encourage healthy rearing patterns such as healthy dieting of the child, physical activity, eating behaviours and motivation and psychological support (60). Certain pharmacological agents have been suggested for adult obesity but evidence

regarding their efficacy and safety in children and adolescence is lacking. Only few drugs are approved by FDA, NICE and SIGN guidelines for use in childhood obesity such as orlistat in children more than 12 years, but always in conjunction with other diet, exercise and lifestyle measures. Although previous meta-analysis favoured the use of Sibutramine and orlistat with behavioural therapy as it was shown to reduce BMI by 2.2 kg/m^2 and 0.8 kg/m^2 respectively with some side effects (elevation of systolic and diastolic blood pressure with sibutramine and gastrointestinal side effects with orlistat) (61). However, sibutramine was withdrawn from the market in 2010 due to its cardiovascular complication and the use of orlistat is recommended with very careful consideration by a specialist physician and are indicated only in severe obesity where lifestyle, diet and exercise measures have failed to improve metabolic health. These drugs carry several detrimental effects on patient physiology and drug to drug interactions (such as anticonvulsants) and therefore are rarely used in clinical practice (62).

1.3.8 Summary of evidence on obesity, risk factors and management of obesity- "The knowledge gap"

In summary, several factors have been studied to explain the aetiology of obesity both in children and adults. These encompass genetic, environmental, dietary, and lifestyle factors. However, together they do not explain the current epidemic of obesity. Furthermore, obesity in childhood continues towards obesity in adulthood, however, the evidence for the link between childhood obesity and adult obesity related disease risk factors is missing. Different dietary, behavioural, lifestyle and pharmacologic interventions are suggested to tackle obesity but the success of these interventions is limited due to low compliance, lack of motivation and resistance to weight loss in some individuals.

Table 1.3: Management of obesity through prevention and treatment strategies (adapted from(54, 55, 62)).

Intervention	Components of intervention
Lifestyle	• Change in dietary habits • Family support and education • Encouraging weight loss • Change in school diet plans, curriculum, activities
Dietary	• Encouraging lower consumption of energy dense foods, fast foods, fats • Regular meal plans- not escaping breakfasts, less food at night • Discouraging continuous snacking • Encouraging dietary fibre intake
Physical activity	• Encouraging 60 minutes of vigorous activity most of the days in a week • Reducing sedentary activities especially TV watching, video games, computer entertainment limited to at least 1-2 hours
Psychosocial	• Parents education about healthy eating and activity • Motivation and behavioural change • Promoting good communication for support
Pharmacologic*	• Orlistat (for >12 years age) (SIGN, NICE & FDA) • Metformin (not approved by FDA for children and adolescents) • Octreotide (not approved by FDA for children and adolescents) • Leptin(not approved by FDA for children and adolescents) • Growth Hormone(recommended only for Prader Willi Syndrome) • Supplements (not recommended) • Dietary fibre supplements

SIGN; Scottish intercollegiate network, NICE; National institute for health and core excellence, FDA; Federal drug agency USA.* No pharmacologic therapeutic agents are approved by FDA, SIGN or NICE guidelines for sole use in paediatric populations. Orlistat is recommended in children above 12 years and should only be prescribed along with intensive lifestyle, dietary, and physical intervention and very careful consideration by a specialist physician.

1.4 Gut microbiota

Gut microbiota located at the interface of host and environment in the gut is a new area of research explored in an attempt to explain the excess accumulation of energy in obese population and is therefore a new potential target for therapeutic manipulation to reduce host energy storage.

Our gut is host to trillions of bacteria (approximately $1x10^{11}$ to $1x10^{14}$), predominantly residing in the proximal part of colon. The intestinal microbiota are thought to be composed of approximately 1000 different species, with a total mass of around 1.5

kg, constituting approximately 10^{11} bacteria per gram of colonic content. Up to 99% of the known cultivable species of gut microbiota are facultative anaerobes (63). With a total bacterial metagenome of 100 times more genes than the human metagenome, these bacteria contribute to various biochemical and metabolic functions such as breaking down indigestible dietary polysaccharides, conversion of conjugated bile acids into secondary bile acids, synthesis of vitamins, degradation of dietary oxalates and development of immunity against a variety of intrinsic and extrinsic allergens (64).

1.4.1 Diversity of gut microbiota; "the known amongst many unknown"

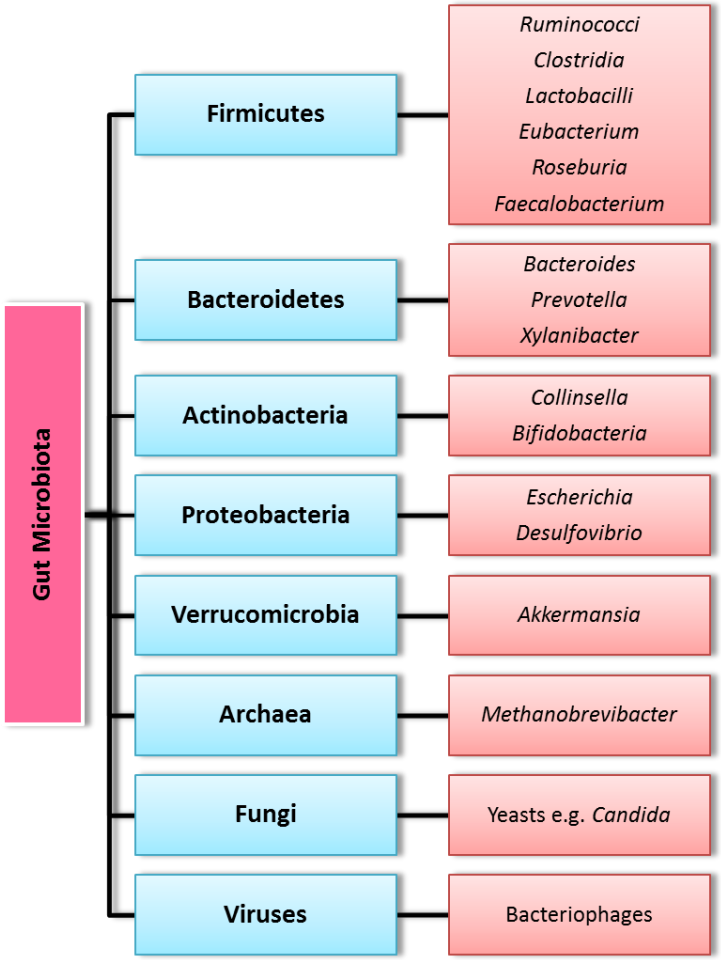
The advent of non-culture dependent techniques have made it possible to sequence the whole bacterial metagenome from different body sites including the gut (65). The majority of bacterial species residing in the gut are still uncharacterized. Two principal factors determining the composition of gut microbiota are substrate availability and gut transit time. Others include; competition for nutrients and binding sites in the gut and co-operation between different species and groups for the breakdown of dietary substrates (66).

Compositional differences between the gut microbiota are exhibited at various taxonomic levels between individuals even in the same family sharing the same food and environment. This was elegantly shown by Turnbaugh *et al.* (2009), who studied gut microbiota composition and function in faecal samples of 154 young adult female mono- and dizygotic twins concordant for leanness and obesity and their parents (n=46) (67). They found marked inter-individual variations between individuals' gut microbiota structure at different taxonomic levels even between members of the same family. However, there was a consistent similarity in the core gut microbiome (i.e. aggregate functional genes in gut microbiota) between related members of the family compared to unrelated participants. These inter-individual variations both in the structure and function of the gut microbiota may be determined by various innate host and environmental factors to which individual is exposed in his/her early life (65). These factors might include expression of cell surface receptors such as toll-like receptors 5 (TLR5) that recognize bacterial cell wall components to elicit immunologic response to determine the preferential colonization of certain species but not others. Furthermore, the expression of certain factors such as the nucleotide oligomerization domain (NOD) which is a ligand for bacterial lipopolysaccharide and activates proinflammatory signaling in the gut (68). Moreover, the expression of nucleotide-binding domain and leucine-rich-repeat-containing proteins (NLRPs) is associated with obesity and altered insulin signaling (69). Hence, the

population of the colonic microbiota depends on many environmental, dietary, innate host and immune response factors.

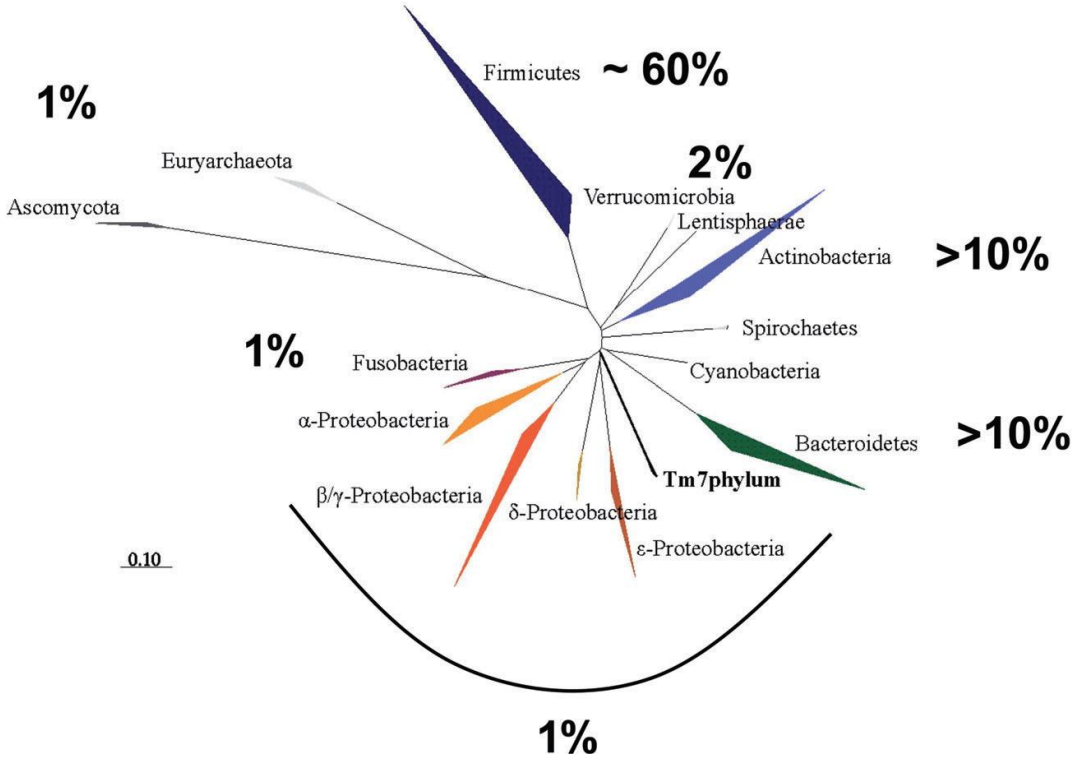
Although members of all three domains of life; Archaea, Eukarya and Bacteria reside in human gut, the majority of intestinal microbes (approximately 99%) are bacteria, the term microbiota is often therefore used synonymously with bacteria (64). Gut microbiota in the human gut are divided into 5 major phyla including Firmicutes, Bacteroidetes, Actinobacteria (e.g. *Bifidobacteria* and Actinomycetale), Proteobacteria (alpha-, beta-, gamma-, and zeta-Proteobacteria), and Verrucomicrobia (including *Akkermansia* spp.) (65) (Figure 1.2 andFigure 1.3). Firmicutes constitute up to 60% of the total bacteria and are represented by Bacilli (*Bacilli*, *Lactobacilli*, *Lactococci*, *Staphylococci*, *Streptococci*, *Leuconostoc*), Clostridia (Clostridial cluster, *Eubacteria*, *Roseburia* spp., *Peptococci*, *Petptostreptococci*), Erysipellotrichia (Erysipelotrichaceae), Negativicutes (such as *Veillonella*), Thermolithobacteria, and some unclassified Firmicutes. Phylum Bacteroidetes constitute approx. 10-20% of total bacteria and include Bacteroides (*Bacteroides*, *Prevotella*, *Porphyromonas*spp.*)*, Cytophagia, Flavobacteria, Sphingobacteria, and some unclassified Bacteroidetes (70). To date, approximately 64-70% of the detected sequences have not been assigned to any group and the functions associated to these sequences are still unknown (64).

Figure 1.2: Major gut bacterial phyla and their predominant sub-groups. Adapted from (66).



Methanogens, members of the Archaea, are other distinct microbes which have recently been discovered and are under extensive research. The structural and functional organization of gut microbiota varies between individuals despite similarities in the broad population of gut microbes in the gut. This is determined by various factors such as diet (type and amount of fibre), gut transit time, and other environmental and innate host immune response factors.

Figure 1.3: Summarised phylogenetic tree and proportion of major gut microbial phyla in the human gut. The proportion varies between individuals. Re-printed with permission from Diament *et al.* 2011 (71).



The size of the bar represents phylogenetic distance

1.4.2 Gut microbiota and human health

1.4.2.1 Production of SCFA

The gut microbiota degrade fermentable dietary carbohydrates (and some proteins) to 1-6 carbon organic compounds; the short chain fatty acids (SCFA). SCFA are the most abundant anions produced in the gut (72). The most important of these are acetate (C2), propionate (C3), and butyrate (C4) in a mean ratio of 60:20:20 respectively (73, 74). However, the proportion of SCFA may vary between individuals, type of diet, gut transit time, and gastrointestinal diseases such as inflammatory bowel disease. The amount and proportion of these SCFA may vary with the type of substrate available for fermentation.

The exact amount of SCFA produced by the gut microbiota in humans is unknown due to practical issues related to the lack of access to the proximal colon and therefore measurement of the whole fermentation process. Faecal SCFA which are often measured are the result of both production and absorption in the colon and do not reflect true production rates. Nevertheless, the rate of production of SCFA varies considerably with the amount and type of substrate available for fermentation and also with the gut transit

time (75). Although the extent of fermentation is quantified in the ruminants, the amount of SCFA produced as a result of fermentation in humans is derived from the equation for fermentation based on the known concentration of faecal SCFA, CO_2, and methane.

$$34.5C_6H_{12}O_6 \rightarrow 64 \text{ SCFA} + 23.75CH_4 + 34.23\ CO_2 + 10.5\ H_2O$$

Based on this equation, with daily average intake of 15-20 g fibre per day from a typical Western diet, the average production of SCFA varies between 100-200 mmol/day, of which approximately 15-20 mmol/day are excreted in faeces (76). However, this predictive equation does not take into account those people who do not produce methane. Additionally, it also underestimates SCFA production if the consumption of fibre is more than 20 g/day or if there are other carbohydrates also available for fermentation. The exact amount of SCFA produced per day is therefore still unknown.

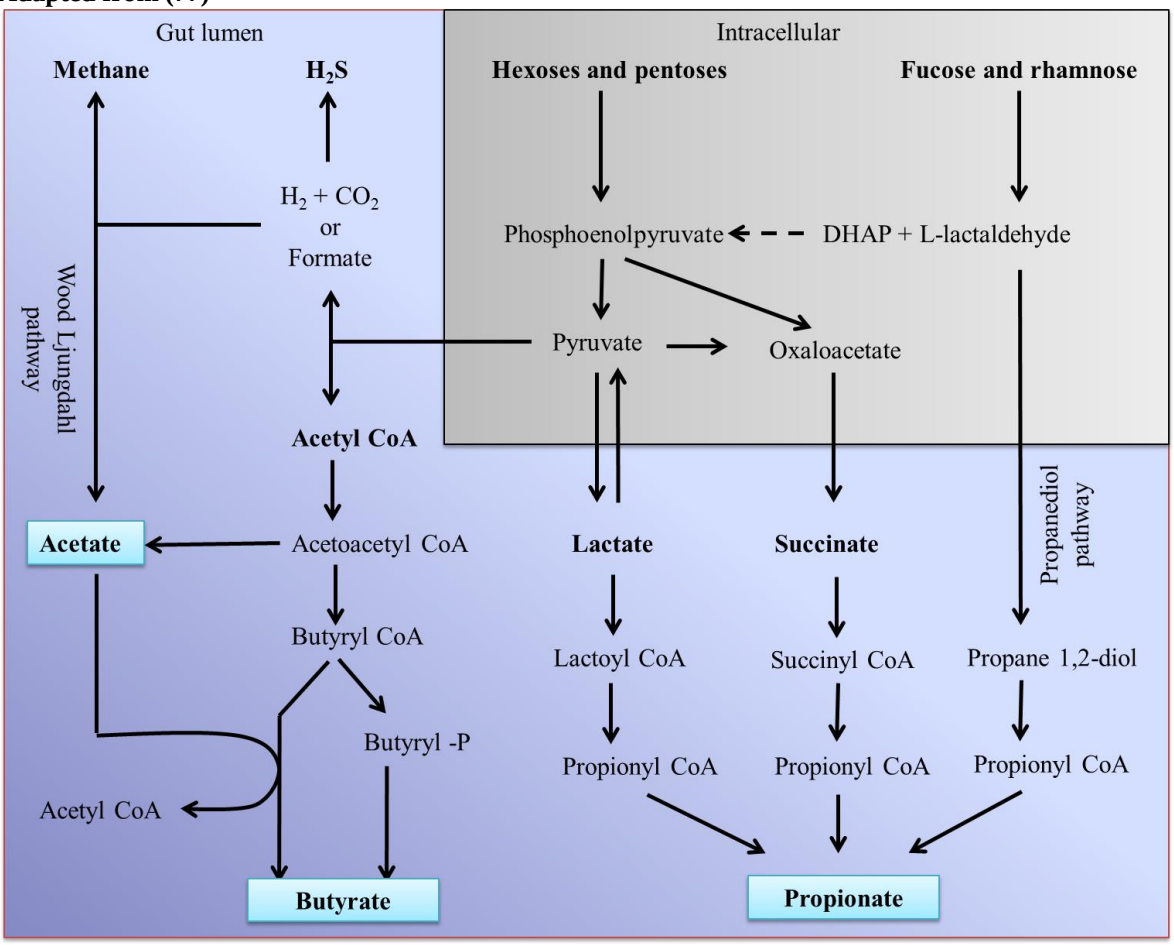
All hexose sugars are essentially converted to pyruvate in the Embden-Myeroff pathway (Figure 1.4). Acetate is formed by the oxidative de-carboxylation of pyruvate or by the conversion of formate to acetate with the help of formate lyase in Wood-Ljungdahl pathway catalysed by *Escherichia coli* and other Enterobacteriaceae (77). Propionate is formed by two major routes; the de-carboxylation pathway which involves carbon dioxide fixation to succinate, followed by the formation of propionate. The second pathway involves conversion of lactate and acrylate to propionate in acrylate pathway. The third and less common pathway is propanediol pathway for the conversion of fucose and manose to propionate (78). Butyrate is formed when acetoacetate is first formed from acetate and then reduced to butyrate (79). Additionally, butyrate can also be formed by the utilization of lactate by lactate-utilizing butyrate-producing bacteria such as *Eubacterium halii* and *Anaerostipes caccae* via acetyl CoA at a slightly lower pH (such as pH 5.9) (80). Although lactate (D & L form) is not a major faecal metabolite of the gut microbiota, it is formed in large quantities in the colon as an overflow in glycolytic pathways especially from readily fermentable carbohydrates. Lactate formation is also favoured when colonic pH is reduced after excessive fermentation. This occurs through the inhibition of metabolism of the gut microbiota that metabolise lactate. Colonic lactate production is also favoured in malabsorption states such as diarrhoea.

Belenguer *et al.* (2007) in their study of 4 volunteers reported that lactate is primarily produced by *Bifidobacterium* spp. and lactic acid bacteria such as *Lactobacilli* and *Enterococci* but it can be produced by other species in the gut. Propionate producing bacteria such as *Veillonella* and *Megasphaera elsdenii* convert lactate to propionate mainly by acrylate pathway at a higher pH (such as pH 6.4) while certain butyrate producing

bacteria such as *Eubacterium halii* and *Anaerostipes caccae* utilise lactate to produce butyrate via acetyl CoA at a slightly lower pH (such as pH 5.9) (80).

Other metabolites such as formic acid, ethanol, methane, and hydrogen are produced in small amounts. The production of these compounds varies between humans and animals and also between regions with low and high fibre intake (75-80% methane producers in Africa compared to 40-60% methane producers in the UK and USA) (79).

Figure 1.4: Simplified diagram of the different metabolic pathways of production of SCFA in the colon. Adapted from (77)



Most of the SCFA are produced by more than one pathway; the pathways represented here are the common routes of production. Acetate is produced by oxidative decarboxylation or from formate via Wood Ljungdhal pathway. Butyrate is formed from Butyryl CoA or through the conversion of acetate to butyrate. Propionate is formed mainly by succinate pathway, however it is also formed via acrylate pathway from lactate or via propanediol pathway from fucose and rhamnose.

1.4.3 Metabolism of SCFA

A substantial amount of the absorbed SCFA are metabolised in the mucosa. This ratio of metabolism increases with increase in chain length (Acetate<propionate<butyrate) and the amount available in blood decreases in the same order. About 30% of acetate is converted

to ketone bodies (acetoacetate and β-hydroxybutyrate) and 75% of butyrate is metabolised in the mucosal cells. It is suggested that 50% of propionate and 90% butyrate are absorbed by the colonic epithelial cells (81). The remaining SCFA absorbed into the portal blood are transported to the liver for further metabolism.

1.4.4 Acetate

Acetate is the major SCFA (approx. 60%) produced in the gut by a diverse range of gut microbiota belonging to phylum Firmicutes. Acetate is generated in the gut to feed the need to regenerate NAD^+ and co-enzyme A (CoASH) in the cells which are utilised in the process of glycolysis to generate pyruvate. Overall, acetate contributes 6-8% to the overall energy expenditure as reported in stable isotope study by Pouteau *et al.*(82). Acetate significantly contributes towards the formation of butyrate and propionate as it is consumed by the butyrate and propionate producing bacteria in the gut. Butyrate producing - acetate consumers primarily include *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, and *Eubacterium rectale* (83, 84). Schwiertz *et al.*(2002) identified *Anaerostipes caccae* as another acetate utilizing butyrate producing Clostridial bacteria in 2002 (85). The proportion of acetate utilization in an individual depends on the type of butyrate producers in the gut and the type of fibre available for fermentation (86). In their study, the amount of butyrate formed from acetate varied from 56% in pectin to 90% in xylan in continuous cultures and 72% to 91% in batch cultures (86).

Being the most abundant SCFA, acetate concentration is associated with several important metabolic functions critical for health of the host. First; it contributes to daily energy requirement as SCFA may make up to 10% of total energy used (87). This is however debated as the daily intake of dietary fibre in European diet is 15-20 g per day. The amount of energy gained from the fermentation of non-digestible carbohydrates (e.g. oligofructose) is about 25-35% of what would be gained if fructose was absorbed in the small intestine which is about 15.7 kJ/g. Assuming daily intake of 40 g fermentable carbohydrates (20 g of non-starch polysaccharides and 20 g of resistant starch), the net contribution of SCFA to the total energy requirements would be approximately 2.2% of the average 2000 Kcal intake (calculated as; 15.7 kJ/g x 40 g x 30%) (88). Secondly; acetate has been shown to increase hepatic de novo lipogenesis by stimulating acetyl co-enzyme A and fatty acid synthase which are key to lipogenesis (89). Thirdly; acetate is a substrate for hepatic cholesterol synthesis by stimulating carbohydrate response element binding proteins (ChREBP) (90) (Figure 1.5). Fourthly; acetate and propionate are thought to

reduce triglyceride hydrolysis by activation of GPR43 (89). Thus the overall effect of acetate is increased hepatic lipogenesis and systemic hypercholesterolemia which might suggest an inverse relationship with cardiovascular health. The role of acetate in hepatic lipogenesis is however controversial as oral administration of 5.2 mg/kg body weight acetate every day for 6 months in obese and diabetic rats were shown to improve glucose tolerance, reduce accumulation of fats in adipose tissue and liver by inhibiting the expression of genes for lipogenic enzymes such as acetyl CoA carboxylase, malic enzyme, fatty acid synthase, and glucose-6-phosphate dehydrogenase (91).

Acetate is a ligand for G protein coupled receptor 43 and 41 (GPR43 & 41), now called free-fatty acid receptor 2 and 3 (FFAR2 & 3), which have been shown to reduce the intake of food by GPR43 stimulation and stimulation of satiety hormones such as PYY and GLP-1 by GPR41 (92). However, Lin *et al.* (2012) have suggested that these effects are independent of GPR41 receptors (93). In their study, intake of high-fat diet supplemented with sodium salt of acetate (3.7%) in C57BL/6J mice reduced weight gain in lean high fat fed mice by up to 40% but did not improve glucose concentration and insulin sensitivity, while butyrate and propionate reduced food intake and weight gain independent of GPR41 and GLP-1 (93). Frost *et al.* (2014) have recently reported a possible direct effect of acetate on the suppression of appetite by inducing the expression of pro-opiomelanocortin via maloyl co-enzyme A and regulatory neuropeptides (glutamate-glutamine and GABA neuroglial cycles) favouring appetite suppression. Interestingly, ^{13}C stable isotope labelled acetate produced by carbohydrate fermentation was shown to be correlated with ^{13}C acetate in the hypothalamus (94).

1.4.5 Propionate

Gut microbiota involved in the production of propionate are taxonomically less diverse than butyrate producing bacteria. Reichardt *et al.*(78) have described 3 pathways for the production of propionate in the descending order of utilization; succinate > acrylate > propanediol pathway. Succinate pathway was the most common route of hexose conversion to propionate by the Gram-negative *Bacteroides* and many other Negativicutes as shown by an abundant expression of *mmdA* gene encoding methylmalonyl-CoA decarboxylase. Acrylate pathway was restricted to a few members of Lachnospiraceae such as *Coprococcus catus* and Negativicutes to convert lactate to propionate (as shown by expression of *IcdA* gene for lactoyl CoA dehydratase). However, butyrate, but not propionate, is the predominant route of utilisation of lactate by butyrate producing bacteria

(95). Expression of *PduP* gene encoding propionealdehyde dehydrogenase in propanediol pathway was shown to be used for the conversion of deoxysugars such as fucose and rhamnose by *Ruminococcus obeum* and *Roseburia inulinivorans*, members of Lachnospiraceae (78).

Higher levels of propionate have been associated with hypocholesterolemia, reverse cholesterol transport, anti-lipogenesis (96), improved satiety through hormone PYY, appetite suppression through leptin (97), and antiproliferative effect on colonic cancer cells (98) (Figure 1.5). SCFA especially propionate also act as a ligand for G protein coupled receptor GPR41. These receptors are expressed in adipose tissue, pancreas, spleen, liver, enteroendocrine L cells, and mononuclear cells. These receptors have been shown to improve insulin sensitivity and secretion via stimulation of GLP-1 from L-cells (99). However, other studies have found a reduction in insulin sensitivity with activation of GPR41 which indicate other mechanisms involved which are poorly known (99). Propionate and acetate added to a culture medium containing adipocytes and pre-adipocytes reduced lipolysis by 50% and the same effect was seen in C57BL/6 mice (100). This anti-lipolytic effect was abolished when GPR41deficient knock-out mice were used, suggesting that propionate and acetate exert this effect through GPR41 (100). Propionate has been shown to down-regulate hepatic de-novo lipogenesis through reduced expression of fatty acid synthase. It also inhibits hepatic cholesterol synthesis by inhibiting the rate limiting enzyme in cholesterol synthesis, 3-hydroxy, 3-mehtylglutaryl Co-enzyme A (HMG-CoA) (96).

1.4.6 Butyrate

Butyrate is one of the main SCFA whose production is increased by the fermentation of indigestible complex polysaccharides such as resistant starch from wheat bran and maize. Approximately 70% of the energy required by the colonocytes for cellular respiration is obtained from butyrate (87) (Figure 1.5). Although a diverse range of gut microbiota are attributed to the production of butyrate, *Faecalibacterium prausnitzii, Eubacterium rectale, and Roseburia intestinalis* are the main producers at species level corresponding to Clostridial cluster IV and XIV within the phylum Firmicutes (101). Recently, a new butyrate (and acetate) producing bacterium *Intestinimonas butyriciproducens* has been identified in mouse intestines by Klaring *et al.* (2013) in cultures with reduced agar medium containing yeast extract, rumen fluid and lactic acid (102). Several cross-feeding metabolic pathways are also suggested to be involved in the production of butyrate from

other substrates such as lactate and acetate which are produced by the *Lactobacilli* and *Bifidobacteria* (103).

Butyrate has been shown to have anti-inflammatory effects by regulating the chemotaxis and cellular adhesion processes (104). It increases the chemotactic response of neutrophils in the absence of a chemotactic stimulator and decreases the LPS induced migration of macrophages by reducing non-receptor tyrosine kinases (105) and monocyte chemoattractant protein 1 (MCP-1). Butyrate also increases Helper T-cell (TH2) response (106), reduces the levels of ICAM-1 adhesion molecules and lymphocyte function associated antigen-3 (104), reduces NFkB induced expression of proinflammatory cytokines, reduces LPS-induced IkB degradation (107). However, the response of butyrate is dose dependent and a lower response is seen when higher concentrations of butyrate are used. Butyrate also reduces the activation of mitogen activated protein kinase pathways which respond to stress and other extracellular stimuli. Some genes involved in these cascades are; ERK ½, JNK, and P38 (108).

Butyrate is a known histone deacetylase inhibitor. Histone deacetylase inhibits gene transcription by keeping the chromatin network in a compact form (109). Butyrate therefore regulates gene expression for cellular growth and proliferation by hyperacetylation. This effect may be important for the prevention of colorectal carcinoma. Butyrate has been shown to have an antiproliferative effect on colon cancer HT29 cells coupled with increased rate of differentiation and apoptosis (by activating caspases-3/7) (110). However, this effect is dependent on the type of cells used for experiments and the proliferative state of the cells (111). Other studies have shown that butyrate inhibits pro-inflammatory cyclooxygenase 2 (Cox-2) induced expression of TNF-α and Cox-2 induced angiogenesis in experimental colon cancer cells, which in turn may reduce cancer cell proliferation, survival, and growth (112). Interestingly, this antiproliferative and apoptotic effect is not seen in the normally differentiating cells (110). Butyrate had anti-obesity effect by maintaining β-cell function and reducing inflammatory response in pregnant obese mice without increasing risks of fetus toxicity (113).

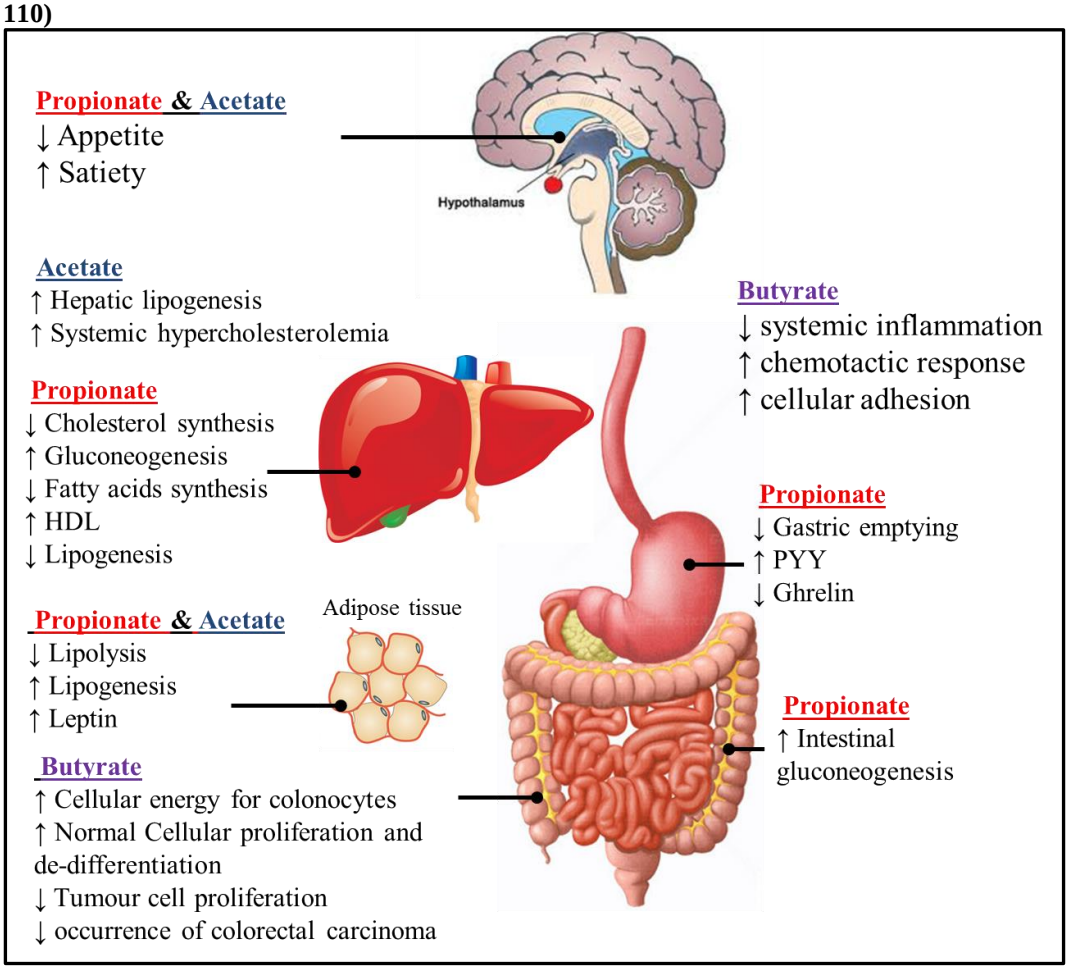
1.4.7 Branched chain fatty acids (BCFAs)

BCFA, iso-butyric acid and iso-valeric acid, are produced as a result of protein fermentation particularly from branched amino acids such as valine and leucine respectively (114) and also from endogenous sources of proteins in the form of sloughed cells. The concentration and proportion of BCFA are generally increased when fermentable

carbohydrate is limited in the colon. Although earlier studies by Croft and colleagues reported that approximately 300 g of sloughed cells per day from the mucosa of the entire gastrointestinal tract are available for degradation (115, 116). However, the calculations were based on human DNA from intestinal washings from a small length of human intestine and all samples were from patients with gastrointestinal disorders (such as inflammatory bowel disease and coeliac disease) which might have affected their results.

The production of BCFA seems to be correlated consistently in different diets and across different animal species. A study on faecal samples of 4 different species (humans, horses, rats, and pegs) have shown a consistently strong correlation between percentage of iso-butyric and iso-valeric acid in these species fed on different diets, irrespective of the amount of SCFA, age, diet, and living conditions (114). There is not much evidence of whether the production of BCFAs varies between obese and lean people. A crossectional study by Payne *et al.*(2012) found a higher concentration of iso-butyric acid in faeces of simple obese Swiss children than from lean children (95).

Figure 1.5: Functions of major SCFA in host metabolic homeostasis. Concept adapted from (91, 97, 98, 110)



HDL; high density lipoproteins, PYY; peptide YY

1.4.8 Lactate

Lactate is a metabolic intermediate during the production of butyrate, propionate, and acetate. As indicated above, lactate is primarily produced by *Bifidobacterium* spp. and lactic acid bacteria such as *Lactobacilli* and *Enterococci* but it can be produced by many other species such as propionate producing *Veillonella* and *Megasphaera elsdenii* which convert lactate to propionate mainly by acrylate pathway at a higher pH (such as pH 6.4) while butyrate producing *Eubacterium halii* and *Anaerostipes caccae* utilise lactate to produce butyrate via acetyl CoA at a slightly lower pH (such as pH 5.9) (80).

Levels of faecal lactate depend on a) the rate of production by lactate producing bacteria, b) absorption from the gut lumen, c) net utilization by lactate utilising-butyrate or propionate-producing bacteria, d) status of mal-absorption (faster transit), e) luminal pH, and f) dietary complex polysaccharides. Generally, a more acidic pH (<5.5) in the colon inhibits the utilization of lactate by lactate utilizing bacteria while production is still maintained (80). The levels of faecal lactate are therefore very low (<3 mmol/L) under physiological conditions as physiological gut transit and status of absorption does not allow the drop of pH below 5.5. However, concentrations of ≥80 mmol/L in faeces have been reported in an acidic gut luminal environment in conditions associated with mal-absorption and faster transit either due to inflammation such as ulcerative colitis (116) or due to short bowel (short-bowel syndrome) (117). The metabolism of lactate is also partly dependent on the presence or absence of polysaccharides and is also subject to inter-individual variations (80). Sato *et al.* (2008) showed that administration of galacto-oligosaccharide (GOS) to the Sprague-Dawley rats alone increased both butyrate and lactate in caeca of the rats. However, administration of GOS along with a lactate utilising bacterium *Anaerostipes caccae* isolated from human faeces resulted in further acceleration of butyrate but reduced the levels of lactate (118). Study by Mayeur *et al.* (2013) identified lactate accumulators and non-lactate accumulators in a group of patients with short bowel syndrome based on the presence of D and L lactate in the faecal samples. They found that lactate accumulators with higher D/L ratio and plasma bicarbonate ions were at a higher risk of developing D-lactate induced encephalopathy (117).

1.4.9 Hydrogen Sulphide

Faecal hydrogen sulphide is a metabolic degradation product of gut microbiota from sulphur related to dietary carbohydrates, proteins, excess supplemental sulphates, mucins,

and dead epithelial cells in the colon (119). It is usually difficult to measure the proportion of faecal sulphide contributed by the microbial production from the available luminal substrates (prokaryotic contribution) and that contributed by the host mucin peptidoglycans and peptides (eukaryotic contribution) (120).

Sulphate reducing bacteria (SRBs), particularly *Desulfovibrio* spp.(*D. desulfurican and D. piger*) utilise sulphate or sulphite as a terminal electron acceptors for the dissimilation of reduced SCFA or molecular hydrogen (121). This results in the generation of sulphide ion (HS^-) that is converted to free H_2S in the acidic distal gut lumen. SRBs compete with methanogenic bacteria for hydrogen as higher SRBs level is associated with a negligible or extremely low methanogens in the distal gut (122). *E.coli*, *Salmonella enterica*, *Clostridium*, and *Enterobacter aerogenes* in the colon can also produce hydrogen sulphide by the metabolism of cysteine(123). Whereas physiological concentrations of H_2S have beneficial effects on gut health as it promotes healing of gut ulcers and resolve mucosal inflammation through its angiogenic (cyclo-oxygenase-2 and nitric oxide) and anti-inflammatory properties (124), a number of potentially adverse effects of excess hydrogen sulphide have been suggested. It is thought to reversibly inhibit the β-oxidation of butyrate, increase intestinal permeability, induce abnormal cellular proliferation, goblet cell death, crypt cell loss, reduce opsonisation potential of polymorphonuclear leukocytes, and mucosal ulceration (119). However, the rise in hydrogen sulphide with tissue injury such as mucosal ulceration in experimental colitis is seen as a protective response to promote ulcer healing which suggests a protective role of hydrogen sulphide in a dose dependent manner as a protective effect against inflammation (125).

Although extensively studied in relation to inflammatory bowel disease (126); faecal hydrogen sulphide in obese humans , including children, has not been studied greatly. Most research has focussed on the differential colonisation of SRBs in the colon of obese children and adults as the presence of SRBs in faeces is a surrogate marker for the utilization of sulphate as an electron acceptor to maintain the redox potential in gut. Most studies have looked at the changes in sulphide with changes in diet in both animal and human studies. The association of excretion of sulphide in faecal samples with the presence of SRBs is debated as some studies have found no difference in the excretion of sulphide between SRBs positive and SRBs negative participants (127). Furthermore, evidence also suggests that the production of colonic tissue-produced hydrogen sulphide may be independent of the microbiota-produced faecal hydrogen sulphide since rise in

elevated colonic tissue-produced H_2S was observed in the absence of a rise in faecal H_2S in mouse models (125).

Levels of faecal sulphide differ between healthy lean people and people with altered function or physiology of the gut such as colonic motility. Chassard *et al.*(2012) reported significantly lower faecal sulphide, molecular hydrogen, and significantly higher methane in healthy lean women than women with constipated irritable bowel syndrome (IBS) (128). This was coupled with significantly higher lactate utilizing and methanogenic bacteria, and significantly lower sulphate reducing bacteria in healthy lean women than in constipated IBS women. Although total SCFA, acetate and propionate were similar between the two groups; levels of butyrate were significantly lower in the constipated IBS group signifying that H_2S may have inhibited oxidation of butyrate (128).

Faecal sulphide has also been shown to be associated with changes in faecal consistency and dietary interventions. A recent study using a gnotobiotic mouse model showed an increase in caecal sulphide, increase in a prominent SRB; *Desulfovibrio piger,* and a decrease in acetate and propionate on high fat and low complex polysaccharide diet. This was in addition to increased gene expression of sulfatase; an enzyme produced by Bacteroidetes (such as *B. thetaiotaomicron*) with capability to liberate sulphate moiety from sulphated oligosaccharide chains of the mucosal glycosaminoglycans(121). Liberated sulphate is then utilised by *D. piger* for the production of hydrogen sulphide (121). Preter *et al.*(2010) showed a significant reduction of sulphide in *in vitro* faecal incubations with oligofructose-inulin (129). In another study, volunteers who had SRBs in their faeces, showed a reduction in faecal sulphide with oligofructose along with an increase in total SCFA although the population of SRBs did not change after the intervention (130). However, reduction in faecal hydrogen sulphide with high intake of dietary fibre is controversial, as a recent study by Ou *et al.* (2013) reported higher sulphate reducing bacteria in African population consuming high fibre diet than in African-Americans consuming low fibre diet (131).

Systemic hydrogen sulphide is a gasotransmitter vasodilator and hence may mediate several beneficial cardiovascular and anti-obesity effects (120, 124). Little is known about the effect of microbiota produced hydrogen sulphide in the gut on the bioavailability of hydrogen sulphide in systemic circulation and different organs such as adipose tissue and lungs. To address this issue, Shen *et al.*(2008) have shown that gut microbiota may play a key role in regulating the bioavailability of hydrogen sulphide in the systemic circulation as they found significantly lower free and bound form of H_2S in plasma of germ free vs.

conventionally housed animals (132). However, the levels of tissue or organ sulphide was inconsistently correlated with caecal and colonic tissue free sulphide as they found lower amount of free H_2S in lungs and kidneys while a higher amount of free-H_2S in heart tissue in germ free mice compared to conventionally raised mice (132). The discrepancy between the colonic and caecal versus organs free H_2S might be due to the fact that the authors used caecal and colonic tissue H_2S as a marker of microbiota produced sulphide which may actually be the endogenously produced sulphide independent of the presence of gut microbiota. Furthermore, Flannigan *et al.*(2011) showed that faecal sulphide in Swiss Webster mice were not significantly different between germ free and conventional mice (120) which suggests that other as yet unknown mechanisms might be important in the production and bioavailability of sulphide. The differences seen between studies may be attributed to different mouse models, experimental conditions and sample size between the two studies. It will be interesting to correlate gut luminal H_2S, which is being considered as harmful metabolite, with H_2S in systemic circulation with its aforementioned beneficial actions and whether gut microbiota or obesity plays any role in determining this balance.

1.4.10 Ammonia

Ammonia in the colon is produced from endogenous and exogenous sources of proteins; however, the predominant route is from the endogenous mucin and epithelial cell proteins especially in the absence of carbohydrates as a substrate. This may lead to the formation of hazardous metabolic products that may be of particular relevance to colorectal cancer (133). In ruminants, gut microbiota producing ammonia are asaccharolytic hyper-ammonia-producing bacteria to retain nitrogen for nutrition, but in the hind gut fermenters like humans they are mostly potential pathogens such as *Clostridium* spp. (including *C. perfringens), Enterococcus, Shigella* and *Escherichia coli, Enterococcus faecalis, Staphylococcus* spp., *Eggerthellalenta.* and some Gram positive *cocci*(134).

Smith and McFarlane (135) showed that faecal pH and carbohydrate availability were the major determinants of peptides and amino acids fermentations in the large intestine; carbohydrate availability being stronger factor than faecal pH. Lower pH (pH 5.5 vs. 6.8) inhibited the fermentation of amino acid to produce ammonia while high starch content in the amino acid fermentation culture reduced the production of ammonia from peptides by routing the nitrogen towards the production of bacterial proteins independent of pH (135). Increased carbohydrate availability seems to divert the action of gut microbiota

from endogenous sources towards fermentation of dietary fibre (136). Birkett *et al.*(1996) showed a reduction in faecal ammonia and phenols with increased intake of resistant starch although there was no change in the urinary ammonia, phenol, urea, and total nitrogen (136).

The role of exogenous dietary proteins in the production of ammonia in the gut is controversial as endogenous sources and availability of carbohydrates are stronger determinants of faecal ammonia. Some authors have found an association of increased intake of meat protein with increased levels of faecal ammonia and production of SCFA in human volunteers (73). In contrast, Russell *et al.* (2011) found no difference in faecal ammonia concentration between obese human volunteers on diets with different protein concentration (such as high-protein medium-carbohydrate, high-protein low-carbohydrate, and weight-maintenance diets for 8 weeks (137).

1.5 Gut microbiota and early life

In utero, the fetus is dependent on maternal physiology and metabolism for its survival and growth. The impact of maternal physiology and the changing metabolism during pregnancy on "priming" the development of fetal gut microbiota is highly anticipated (138). However, the impact of maternal metabolic profiles and gut microbiota on the fetal gut microbiota development is scarcely studied in the literature. There are indications that certain maternal gut microbial metabolic signatures are associated with preterm labour and childhood obesity in later life (139). Pregnancy is associated with a decrease in maternal gut barrier integrity which may affect nutrient supply to the fetus. Children born by caesarean section harbour different gut microbiota than those born vaginally (140;141). Furthermore, data from large population based studies such as ALSPAC study (n=10,219) indicate that mode of delivery have a significant impact on the development of overweight and obesity in children starting as early as age 6 weeks even after the adjustment for parental weight, feeding patterns, parental socioeconomic status, gestational factors (such as parity, maternal age, and first trimester smoking, and gestational weight gain) (140). Additionally the differences in gut microbial population in gut of breast-fed infants versus formula fed infants due to maternal breast milk composition support the notion of significant impact of maternal physiology, gut microbiota, and metabolism on the development of gut microbiota in the infant. This may subsequently affect the development of lean and obese phenotype predisposition of infant in later life.

1.5.1 Gut microbiota before and at the time of birth

The concept of sterile fetal gut was presented more than 100 years ago in a French journal by Tissier (141) and has been considered the case since then. The idea that the preliminary gut microbiota are acquired during passage through maternal vaginal tract in case of normal delivery or from the immediate environment in case of caesarean section is generally accepted in the literature (142). However, Esther Gimenez and colleagues were able to isolate *Lactobacilli* from meconium of healthy new-born infants delivered by caesarean section (143). It is not clear if these bacteria were from maternal blood through the placental barrier or of vaginal origin. The same group tested for the presence of microbiota in the umbilical cord of healthy children born by elective caesarean section (144). Several bacterial genera commonly isolated from breast milk were detected in the umbilical cord blood by PCR after incubation and growth in selective media. All the isolates were Gram positive cocci and were the inhabitants of neonatal gut from day one of life. These included bacteria related to genus *Enterococcus, Streptococcus, Staphylococcus*, or *Propionibacterium.* Interestingly, bacteria related to oral mucosa such as *Staphylococcus epidermidis*, *Propionibacterium acnes*, *Enterococcus faecium*, and *Streptococcus sanguinis* were also detected in small quantities suggesting that oral microbiota could also enter the uterus through blood stream. Furthermore, oral inoculation of a genetically labelled *E. faecium*, a breast milk commensal, into pregnant mice was isolated from amniotic fluid of the same mice while no bacteria could be isolated from the non-inoculated group (144). This suggested that maternally derived bacteria from different body sites could cross placental barrier into the amniotic fluid.

Whether bacteria isolated from umbilical cord blood could also be seen in the meconium of the new-born was further investigated by the same group in 2008 (145). Bacteria belonging to the genus *Enterococcus (Enterococcus faecalis)* and *Staphylococci (Staphylococcus epidermidis)* were isolated from meconium of 17 out of 21 healthy neonates born by normal vaginal delivery or elective caesarean section, while *E.coli* and *Enterobacter* were isolated from some samples (six and five respectively). This transmissibility was confirmed in animal model in which labelled *E. faecium was* inoculated orally in pregnant mice. This inoculated *E. faecium* was then detected in the meconium of the new-born delivered one day before the expected date by caesarean section suggesting that the microbiota are potentially transferred from the mother to the fetal gut. (145). This study was however limited as similar but significantly higher counts of

bacterial colonies in agar plates were observed in samples incubated immediately compared to those which were incubated after four days of storage.

1.5.2 Gut microbiota in early infancy and childhood

Undoubtedly, neonatal gut maturation and colonisation by gut microbiota is affected by maternal gut health. These effects are mediated in pre and peri-natal period by 4 major routes 1) trans-placental transfer of maternal blood and growth factors, 2) ingestion of amniotic fluid by the fetus, 3) colonisation of the gut microbiota, and 4) maternal breast milk (146). The presence of gut microbiota in the amniotic fluids and the umbilical cord blood in the absence of any clinical infections or signs of inflammation suggests that maternal factors might play a crucial role to help in maturation of fetal immune system for the external world.

In a Norwegian cohort of 86 infants and their mothers, faecal samples were shown to exhibit all major components of the commensal gut microbiota within 3 days of life in full term infants represented by *Escherichia, Bacteroides, Bifidobacteria,* other Actinobacteria, *Faecalibacterium,* Lachnospiraceae, *Streptococcus,* and Lactobacillales. The population of gut microbiota shifted in relative abundance in subsequent months up to one year of life such as an increase in Bifidobacteria by up to 60% and a reduction in Lactobacillales and *Streptococci* at 4 months of age (147). This study also suggested that faecal microbiota of children at first year of life had some resemblance with maternal microbiota during early and late pregnancy except that infants at 1st year of life exhibited higher amounts of *Bifidobacteria* and Actinobacteria and lower *Faecalibacterium* and *Bacteroides* compared to their mothers (147). Although this study gave a global picture of the gut microbiota using next generation sequencing, the cohort used was only from Norway where feeding patterns are significantly different from the Southern Europe and the UK and hence these findings may not translate to the rest of the Europe.

In the INFABIO study, a large (n=606) multicentre European study, infants at 6 weeks of age were shown to harbour predominantly Bifidobacteria, followed by *Bacteroides,* Enterobacteria, *Clostridium coccoides, Clostridium perfringens,* and *Clostridium difficile* measured by FISH and flow-cytometry (148). The proportion of gut microbiota differed according to the geographic location (more *Bifidobacteria* in northern Europeans and *Bacteroides* in Southern Europeans), mode of feeding (more *Bifidobacteria* in breast fed while more *Bacteroides, Clostridium coccoides,* and *Lactobacilli* in formula fed), and mode of delivery and antibiotic treatment (More *Bacteroides* and *Atopobium*

cluster in caesarean section and antibiotic treated infants). The same infant cohort followed 4 weeks after the start of weaning (~17-23 weeks age) showed predominance of *Bifidobacterium* followed by *Clostridium coccoides*, and *Bacteroides.* Differences according to geography, feeding and delivery mode were observed similar to those observed during pre-weaning period (149). In the Dutch KOALA birth Cohort study, by Pender *et al.*(2006) reported detection of *Bifidobacteria* in all, *E. coli* and *Bacteroides fragilis* in most, and *Lactobacilli* and *C. difficile* in less infants at 1 month of age (150).

In contrast to findings of Avershina *et al.*(2013), qPCR based study by Johansson *et al.*(2012) on faecal samples of 13 full term infants at one week and 2 months of age suggested an increase in the frequency of Lactobacilli (*L. casei, L. paracasei, and L. rhamnosus*) from ~20% at 1st week to ~50% at two months, *Staphylococcus aureus* from 50% to 70%, *Bifidobacterium bifidum* increased to approximately 55%, while the *Bifidobacterium breve* and *B. adolescentis* remained stable throughout the 2 months period (151). Despite giving a narrow picture of the bacterial frequency, colonisation of *S. aureus* with peripheral blood mononuclear cells resulted in the increased expression of IL-10 and IFN-ɣ which are markers of allergic response. This was decreased in the presence of *Lactobacillus* alone or co-colonisation with *Staphylococcus aureus (151)*. Whether *Lactobacilli* could have a potential beneficial effect in the prevention of allergic disorders in earlier life is not known. However, there are reports that non-allergic 5 year old children acquired Lactobacilli (*Lactobacillus (L. rhamnosus, L. paracasei, L. casei*) and *Bifidobacterium (B. bifidum)* in first few weeks of life more often than allergic 5 year olds, whereas the opposite tendency was seen for *Staphylococcus aureus* colonization (152). The conclusions from these studies are limited due to differences in the methodology and technique of analysis (pyrosequencing vs. qPCR).

The gut microbiota of the child undergoes modifications to reach adult type at least until the age of 4 years with all major gut microbial phyla being represented. Gut microbiota communities of children age 4 years resemble those of their mothers at first trimester and are over represented by butyrate producing *Faecalibacterium* and *Eubacterium,* members of *Clostridiales* and *Lachnospiracea*e, *Ruminococci, Blautia*, and *Bifidobacteria* (138). Additionally, similarities have been observed between microbiome of older children and their families which suggest an established core gut microbiome in older children.

1.5.3 Factors determining the colonisation of gut microbiota in human colon

The structure and function of gut microbiota in early life is "plastic" i.e. subject to change with a variety of environmental stimuli. Additionally, the gut of new-born, although fully formed, is still in the process of maturation to develop immunity against external pathogenic stimuli and acquire gut microbiota population that suits the changing gut environment of the host (146). Since gut microbiota developed in early life become resilient in later life, it is essential to underline factors that affect its colonisation in early infancy. Several factors related to mother and the infant are therefore suggested that could affect the development of gut microbiota (148, 150) (Table 1.4). However, the evidence is inconsistent due to the use of different study designs, methodologies, differences in faecal sampling techniques and laboratory analyses which are not accounted for in many studies while comparing results with other studies. The actual contribution of these factors therefore remains unclear and requires further investigation (148).

Table 1.4: Factors affecting colonization of gut microbiota in early infancy and childhood

Factors	Changes in the gut microbiota induced in the infant	Reference
Maternal factors		
Pregnancy	Increased intestinal permeability, inflammatory markers in faeces, faecal energy loss, reduced insulin sensitivity, increased intestinal permeability	(138)
Maternal allergy	Allergic mother breast milk low in *Bifidobacteria*. Lower counts of *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* in maternal faeces at 35 weeks gestation associated with lower counts of *B. adolescentis* and *B. bifidum* in infant at one month age.	(153)
Caesarean section	Lower *Bifidobacteria* and *Bacteroides* and higher *C. difficile*	(150)
	Delayed detection of *Bacteroides fragilis*	(154)
	Higher *Clostridium coccoides* and *Streptococcus* group	(140;141)
	Delayed colonization of *Bifidobacteria* and *Lactobacilli*	(154)
Infant factors		
Gestational age	Higher rate of colonisation of *C. difficile* in premature babies	(150)
Country of birth	Higher Bacteroidetes and Actinobacteria in African while higher Firmicutes and Proteobacteria, *Xylanibacter*, *Butyrovibrio*, and Treponema in European children	(155)
Place of birth (Hospital/home)	Higher *Clostridium difficile* in hospital born	(155)
Nutritional status	Diminished OTUs in malnourished children, delayed maturity of gut microbiota in malnourished	(156)
Antibiotics exposure	Lower *Bifidobacteria* and *Bacteroides* with amoxicillin	(155)
	Higher Enterobacteria in antibiotic exposed pre-weaned infants	(148)
Breast or formula feeding	Lower *Clostridium difficile* and *E.coli* in breast fed	(157)
	Higher rate of colonisation of *E.coli*, *Clostridium difficile*, *Bacteroides*, and *Lactobacilli* in exclusive formula fed and higher *Bifidobacteria* in breast fed	(158)
	Higher *Bifidobacteria* and lower *Bacteroides*, *C.coccoides*, and *Lactobacilli* in breast fed	(148)

1.5.4 Gut microbiota in adulthood

Predominant gut microbiota in adults are discussed in section 1.4.1 above. Although gut microbiota of individuals are established by the age of 4 years, changes in the community structure are observed with variations in conditions such as change of diet, antibiotics, and pregnancy. The greatest variability observed in the phylogenetic analysis of gut microbial ecology is explained by the inter-individual variation and the sampling site (65).This adaptation of gut microbiota to the changing environment on individual basis suggests "awareness" and capability of gut microbiota to sustain its existence in their hosts in a new set threshold. However, changes in the metabolic pathways might affect the host physiology and metabolism in either direction (beneficial or harmful to the host). One such example is changes in the gut microbiota observed between 1st and 3rd trimester of pregnancy. Pregnancy is associated with decrease in richness and diversity of gut microbiota regardless of pre-pregnancy BMI and health status and an increase in relative abundance of Proteobacteria and Actinobacteria from 1st trimester to 3rd trimester by 69.5% and 57% respectively (138). This is coupled with significant variation in the relative abundance of Bacteroidetes and Firmicutes between individuals. Furthermore, gut microbiota in first trimester resemble that of normal healthy non-pregnant adults being overrepresented by butyrate producing bacteria such as *Faecalibacterium prausnitzii, Eubacterium, Blautia, Clostridia* and members of Lachnospiraceae. On the other hand, gut microbiota in 3rd trimester are aberrant; being overrepresented by *Enterococcus faecalis, Propionibacterium* and *Streptococcus* genus, *Streptococcus faecalis* and Tenericutes (138). Interestingly these changes in gut microbiota were shown to be driven by pregnancy and not by health status as no correlation was found between the relative abundance of specific groups with BMI, use of probiotics, antibiotics and diet in the pregnant mothers.

Whether changes in the gut microbiota are associated with changes in the functionality assessed by metagenomics is controversial as studies have shown no association of structural differences with changes in expression of genes for metabolism(138) while others have shown changes in functionality with differences in gut microbiota in aberrant health states such as obesity (159). A comprehensive metagenomic analysis of 39 individuals from 6 nationalities by Arumugam *et al.*(2010) revealed that gut microbiota of individuals formed three distinct clusters termed as "enterotypes"(160). Individual metagenome could be identified as one of the three enterotypes based on the variations in one of the three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2),

and *Ruminococcus* (enterotype 3). These three enterotypes strongly correlated with the presence or absence of many other genera suggesting that each enterotype is driven by groups of bacterial species to determine the predominant community structure in an individual (160). Additionally, this aggregation was more or less similar when the samples were clustered using a functional metric, as enterotype 1 was associated with overrepresentation of enzymes in carbohydrate and protein fermentation and biotin synthesis, enterotype 2 in mucus glycoprotein degradation and thiamine synthesis, and enterotype 3 in mucus degradation and haem synthesis. A further study by Wu *et al.* (2011) has linked these enterotypes with long term dietary patterns in individuals such that enterotype 1 (*Bacteroides*) were associated with diets rich in protein and fats while enterotype 2 (*Prevotella*) was associated with carbohydrate intake (161).

1.6 Gut microbiota and obesity

1.6.1 Initial evidence of the role of gut microbiota in obesity

The worldwide increase in obesity has prompted researchers to investigate the aetiology of obesity which is multifactorial, involving environmental, dietary, life style, genetic and pathological factors. Although the gut microbiota were already established as a metabolic organ that could ferment indigestible dietary components (particularly complex polysaccharides) to generate SCFA, their role as one of the environmental factors that could affect host adiposity through an integrated host signalling pathway was explored in 2004 by Backhed and colleagues (162).

Male C57BL/6J germ free mice (GF) were conventionalised with unfractionated gut microbiota from the caecum of conventionally raised mice (CONV). After 14 days of colonisation, conventionalised GF mice had a 57% increase in total body fat content, 61% increase in epididymal fat weight, elevated fasting plasma glucose and insulin levels and insulin resistance compared to non-conventionalised GF mice. This was in the presence of a reduced chow intake, reduced lean mass, and normal fasting triglyceride levels in the blood (162). The gut microbiota were shown to stimulate hepatic *de novo* lipogenesis by activating acetyl Co-enzyme A carboxylase and fatty acid synthase via expression of carbohydrate response element binding protein (ChREBP) and sterol response element binding protein 1 (SREBP1). Lipoprotein lipase is the key enzyme involved in triglycerides storage in adipose tissue, liver, and muscles. Conventionalisation of GF mice also resulted in an increase in triglycerides storage in the liver by suppressing fasting induced adipocyte

factor (*fiaf*) which is an inhibitor of adipocyte lipoprotein lipase. This inhibition of fiaf was not dependent on lymphocytes and PPARγ which are one of the key regulators of peripheral triglyceride storage. Together, this break-through evidence suggested that the gut microbiota induced adiposity by stimulating hepatic *de novo* lipogenesis and triglyceride storage. Further reviews from the same group proposed that this intestinal "high-efficiency bioreactor" in certain individuals might promote energy storage (obesity), whereas a low-efficiency reactor would promote leanness due to lesser energy harvest from complex polysaccharides (64). Differences in the gut microbiota between obese and lean people could therefore be one of the aspects for further exploration.

Differences in the gut microbial ecology and host energy homeostasis between obese and lean animals were reported by the same group in another study. Ley *et al.* (2005) analysed 5,088 bacterial 16S rRNA gene sequences in genetically obese leptin-deficient C57BL/6J *ob/ob* mice and lean mice (*ob/+* and +/+ wild-type siblings) fed similar polysaccharide rich diets (163). Intake of a polysaccharide rich diet was significantly higher (40% - 44% higher) in obese *ob/ob* mice resulting in a significantly greater weight gain. Although the gut microbiota communities were shared between mothers and offspring regardless of the *ob* genotype, obese *ob/ob* mice had a reduction in the relative abundance of Bacteroidetes by 50% and a proportional increase in Firmicutes regardless of the kinship (163). A higher Firmicutes to Bacteroidetes ratio was therefore suggested to be associated with increased energy harvest from food facilitated by the microbiota. However, no evidence was presented to show an increased expression of genes related to bacterial metabolic activity and how this could be affected by the diverse environmental factors such as diet and lifestyle nor whether these changes could also be seen in humans. These questions were explored first in an animal model of obesity (159) followed by an interventional study for the first time in 12 obese humans subjected to a low- caloric diet (164).

The first part of the question as to whether changes in gut bacterial diversity are associated with changes in metabolic potential were addressed by Turnbaugh *et al*. (2006) by whole metagenome shotgun metagenomic and microbiota transplantation studies (159). They observed a high Firmicutes rich microbiome in *ob/ob* mice clustered together (in non-metric multidimensional scale plot) compared to low a Firmicutes to Bacteroidetes in lean mice. The microbiome of *ob/ob* mice was richer in enzymes for degradation of complex polysaccharides including starch compared to lean mice. *Ob/ob* mice exhibited higher faecal concentrations of fermentation end products (high acetate and butyrate) and less

stool energy loss as measured by bomb calorimetry. These traits were transmissible, as the transplantation of gut microbiota from *ob/ob* mice or lean mice to germ free mice resulted in obese (high Firmicutes) or lean (high Bacteroidetes) gut microbiome in the recipients. Obese-microbiome recipients had higher percentage body fat despite similar quantity of chow consumption.

The second; whether these changes could also be seen in humans was investigated in another study (164). Obese adults were randomised into fat restricted or carbohydrate restricted diet and were followed for a period of one year. Despite the presence of marked interpersonal variations at species level diversity of gut microbiota, Firmicutes and Bacteroidetes were the predominant divisions representing 92.6% of the known 16S rRNA sequences (164). Obese people had a lower relative abundance of Bacteroidetes and a higher relative abundance of Firmicutes before the onset of diet therapy. However, over the period of follow-up, the relative abundance of Bacteroidetes significantly increased while those of Firmicutes significantly reduced. Increased Bacteroidetes was significantly positively correlated with percentage loss of body weight and not with the caloric content of diet over time (164). This suggested that the gut microbiota restructured by changing their metabolic priorities to support co-existence in a changed environment. However, this study did not explore the same relationship in a parallel lean group to see whether lean phenotype exhibit the same response to dietary intervention.

Further evidence suggested that the presence of gut microbiota was necessary to develop obesity as germ free mice were resistant to obesity even though these animals consumed more calories from a normal chow or after the consumption of high fat Western-type diet as compared to CONV mice (89). Compared to wild-type littermates, high-fat fed conventionalised GF mice showed a higher phosphorylated AMP kinase (AMPK-P), phosphorylated acetyl coenzyme-A carboxylase and carnitine palmitoyltransferase 1 activity, all of which are involved in muscle fatty acid oxidation. Increased peripheral fatty acid oxidation results in reduction in triglycerides levels in the tissues protecting GF mice against obesity. Additionally, GF mice exhibited higher expression of *fiaf* in the intestine and low serum level of leptin and insulin compared to wild type littermates which is a strong inhibitor of lipoprotein lipase activity thus preventing fat storage in adipose tissue (89).

However, the idea of GF mice being resistant to obesity and that gut microbiota were a causative factor in obesity was challenged in a later study by Fleissner *et al.*(2010) (165). GF and CONV mice were fed with low fat (LF), high fat (HF) or high-fat Western

(WD) ad-libitum diet. Over the period of follow-up, germ free mice on high fat diet gained significantly more weight and body fat and had less energy expenditure compared to lean CONV mice. Additionally, intestinal *fiaf* increased in HF and WD fed GF mice compared to CONV mice but not in systemic circulation (165). Importantly, both HF and WD diets increased the proportions of Firmicutes (especially Erysipelotrichaceae) at the expense of Bacteroidetes in CONV mice. This was one of the important evidence to suggest diet as one of the factor in affecting gut microbiota diversity.

In summary, this initial evidence spanning a period of 3 years indicated the role of gut microbiota in shaping host energy balance. Several possible mechanisms were proposed to explain the impact of structural and functional differences in gut microbiota between lean and obese that may contribute to host adiposity and whether an obese phenotype is transmissible by transplantation of gut microbiota. However, most of these studies were conducted in experimental animals which exhibit anatomical, physiological and bacterial colonisation pattern differences in the gut from that of humans. Several human and animal based studies have now revealed controversial evidence attributing differences in gut microbiota to the differences in diet while others suggested no such association. This is discussed further in proceeding sections1.7 and 1.8.

1.6.2 Proposed mechanisms for the role of gut microbiota in obesity

The gut microbiota can be regarded as a "microbial organ" contributing to a variety of host metabolic processes from digestion to modulation of gene expression. The differences in gut microbiota between lean and obese animals or human subjects suggest a role of gut microbiota in energy homeostasis. Evidence regarding various mechanisms linking gut microbiota to the pathogenesis of obesity and metabolic disorders mainly stem from experimental animal studies and some human studies (71). Various mechanisms have been suggested to link gut microbiota with obesity-genesis and other metabolic disorders (Table 1.5). However, it is still unclear how these mechanisms interact with each other to influence the overall metabolic status of an individual.

1.6.2.1 Short chain fatty acid production

As discussed earlier, complex dietary polysaccharides and proteins that escape digestion in small intestine are fermented in the colon through glycosyl hydrolases produced by the gut microbiota into short chain fatty acids (SCFA) mainly acetate propionate, and butyrate. The

amount of energy harvested from this important portion of our diet is hypothesised to be determined by the type of microbiota in the gut (64). Up to 10% of our energy needs and up to 70% of energy for cellular respiration for the colonic epithelium may be derived from short chain fatty acids. A chronic excess energy harvest may therefore impact the long term increase in energy accumulation in the body (95).

By and large there is a general agreement from many studies that the obese phenotype is associated with excess SCFA in the caecal and faecal samples in animal and human studies compared with the non-obese (Table 1.6). Furthermore, there is a considerable disagreement and controversy over the population of the gut microbiota that may be associated with increased SCFA measured in the caecum or faeces (Table 1.6). Whether increased SCFA result in increased energy harvest from the diet in obese phenotype depends on several factors such as substrate availability, gut transit, mucosal absorption, general gut health, production by the gut microbiota and symbiotic relationships between different groups of gut microbiota (166).

As mentioned above, the obese phenotype is associated with higher total caecal SCFA, acetate, and butyrate and higher expression of genes responsible for polysaccharide metabolism (159). Increased efficiency in the production of SCFA in obesity might also result from cross-talk between different species and genera to maintain their growth and population. A classic example of these cross-feeding pathways is between methanogenic archaea (hydrogen consuming bacteria) and members of Prevotellaceae (hydrogen gas producing bacteria) harboured in excess by obese subjects (167). Hydrogen is constantly generated as a result of fermentation process and the excess of residual hydrogen in the colon inhibits the fermentation process if accumulated in excess. This excess residual hydrogen is removed by three routes; mainly by the reduction of CO_2 to generate acetate, or in some cases utilization by the sulphate reducing bacteria (SRBs) to generate sulphide, or utilization by methanogenic archaea to generate methane. Methanogenic bacteria in some obese individuals have been shown to hydrolyse this residual hydrogen produced by the action of Prevotellaceae to accelerate the fermentation of plant polysaccharides causing increased energy harvest from the diet (167). Absorption of these excess SCFA, coupled with other lifestyle and environmental factors may result from excess energy storage and obesity. It is not clear whether this is an effect of substrate (i.e. carbohydrates) or the population of specific gut microbiota that is associated with increased SCFA production, absorption, and storage in adipose tissues and liver. The results are largely confounded by the study settings, lifestyle, and environmental factors of the study subjects.

Table 1.5: Suggested mechanisms for the role of gut microbiota in the aetiology of obesity.

	Proposed mechanism	Agents involved	Site of Production	Site of action	Outcomes for the host
Metabolic (162)	Increased production of Short chain fatty acids	Bacterial glycosyl hydrolases	Colon, distal ileum, rectum	Colonic enterocytes	↑energy harvest Energy for colonocytes. Alteration in cholesterol metabolism
	Muscle fatty acid oxidation	↓AMP kinase	Small intestine	Muscle, liver	↓Muscle fatty acid oxidation
	Bile acid circulation	Secondary bile acid production	Colon	Colon	Reverse cholesterol transport
	Expression of liver ChREBP/SREBP-1	↑glucose absorption	Liver	Liver	↑Hepatic lipogenesis
Inflammatory (168)	Chronic low grade inflammation	LPS, NF-kappaB and TNFα mRNA	Colon, ileum	Endothelium, hypothalamus?	Metabolic endotoxemia and hyperphagia
	↑Endocannabinoid (eCB) system tone	Bacterial LPS	Ileum, colon	Stomach, small and large intestine	↑ Gut permeability and ↓Apelin and APJ mRNA expression
Hormonal (162)	Suppression of Fiaf	Colonic L-cells	Colon	Adipose tissue	↑Lipolysis, ↓ muscle fatty acids oxidation
	↑PYY	Satiety centre	Ileum, colon	Hypothalamus	↓ Appetite, ↓ gastric motility, ↓ gut emptying
	Expression of G-Protein coupled receptors 41 & 43(GPRs 41 &43)	SCFA (acting as a ligand)	Colon, distal ileum, rectum	Liver, brain	↑Peptide YY (PYY), ↑ de-novo hepatic lipogenesis

AMP; Adinosine monophosphate, ChREBP; carbohydrate response element binding protein, SREBP; Sterol response element binding protein, PYY; peptide YY

Table 1.6: Studies looking at differences in SCFA in faecal or caecal samples in obese versus lean phenotypes in animal and human studies

Reference	Technique Used	SCFA Differences	Gut microbiota differences
Turnbaugh *et al* 2006 (159)	GC-MS, Pyrosequencing	↑caecal acetate and ↑ butyrate in obese ob/ob mice compared to lean	↑ Firmicutes and lower Bacteroidetes in obese than lean mice. No differences in genera level diversity
Duncan SH *et al* 2007 (166)	GC and FISH	↓total SCFA and↓ butyrate in obese adults on high protein medium CHO or high protein-low CHO diet	↓ in close relatives of *Roseburia intestinalis* and *Eubacterium rectale*.
Zhang *et al* 2009 (167)	GC, qPCR, and Pyrosequencing	↑ acetate in obese than lean and gastric bypass group	↑ *M. smithi* and Prevotellaceae in obese than lean and gastric bypass
Schwiertz *et al* 2010 (175)	GC and qPCR with SYBR Green	↑ total SCFA and Propionate (conc. & %) in obese than lean	↑ Bacteroides and ↓ Firmicutes,↓ *Ruminococcus flavifaciens*, ↓*Bifidobacterium*, ↓*Methanobrevibacter* in obese than lean
De Fillipo *et al* 2010 (155)	454 FLX pyrosequencing and SPME-GCMS	↑ total SCFA and ↑ Propionate and ↑ butyrate in African children than EU children. Acetic and valeric acids were comparable (conc. & %) in obese than lean	↑Actinobacteria and ↑ Bacteroidetes in high fibre African children. ↑ Firmicutes and ↑ Proteobacteria in EU children. Prevotella, *Xylanibacter*, *Butyrovibrio*, and Treponema exclusively found in African children
Payne *et al* 2011 (169)	qPCR, TGGE, and HPLC	↑ Butyrate, propionate, and iso-butyrate in obese than lean. ↑ Lactate and valerate in Lean than obese No difference in acetate and total SCFA	No difference in Firmicutes and Bacteroidetes, Firmicutes/Bacteroides ratio, Bifidobacteria, Enterobacteriaceae, Sulphate reducing bacteria between lean and obese children ↑ *Roseburia/E.rectale* in obese Highly variable banding pattern on TGGE for both obese and healthy
Kim *et al* 2013 (170)	SPME-GCMS and 454 pyrosequencing of v1-v2 regions	↓total SCFA, ↓ acetate, ↓ propionate, ↓ butyrate in obese adults on strict vegetarian diet for 28 days	After 28 days; ↓Firmicutes, ↑ Bacteroidetes, ↓Firmicutes/Bacteroidetes ratio, ↓*Escherichia*, ↓*Klebsiella*, ↓ *Veillonella parvula*, ↓ *C. Clostredioforme*, ↓*Lactobacillus ruminis*, ↓ *L. mucosae*, ↓ *Strept. lutetiensis*
Larsen *et al* 2013 (171)	qPCR and capillary gas chromatography	No difference in SCFA between placebo group and lactobacillus salivarius intervention group (12 weeks)	No significant difference in bacterial cell numbers with intervention. ↑ ratio of Bacteroides-Prevotella-Porphyromonas vs. *Clostridium cluster XIV-C.coccoides-E.rectale* group

Yang *et al* 2013 (172)	GC	↑ ratio of molar propionate: total SCFA and ↓ actate:SCFA ratio in obese vs. lean	Not measured
Taxairia *et al* 2013 (173)	GC	↑ acetate, propionate, and butyrate in obese vs. lean women SCFA correlated with body fat, blood pressure, waist circumference, insulin and HOMA index	Not studied
Belobradjic *et al* 2013 (174)	GC	Increase in total SCFA pool and stool energy irrespective of obese or lean phenotype	Not studied
Rahat *et al* 2014 (175)	GC	↑ total SCFA, acetate, butyrate in obese than lean No differences in iso-butyrate, iso-valerate, and valerate	↑ Firmicutes: Bacteroidetes ratio in obese. Firmicutes correlated with SCFA in obese
Fernandes *et al* 2014 (176)	GC, qPCR	Significantly ↑ propionate and valerate Marginally ↑ acetate and butyrate	*Escherichia coli* higher in lean than obese No difference in *Bacteroides/Prevotella, Clostridium coccoides and C. leptum* group, Bifidobacteria and total bacteria, F/B ratio
Li *et al* 2013 (113)	GC	Higher SCFA in obese than lean	↑ Firmicutes and lower Bacteroidetes in obese

GC; gas chromatography, GC-MS; gas chromatography-mass spectrometry, SPME-GCMS; solid phase microextraction-gas chromatography mass spectrometry, v1-v2; variable region 1 and 2, HPLC; high performance liquid chromatography, TGGE; temperature gradient gel electrophoresis, CHO; carbohydrate, EU; European Union, qPCR; quantitative polymerase chain reaction, F/B ratio; Firmicutes to Bacteroidetes ratio

1.6.2.2 Gut microbiota and G protein coupled receptors

SCFAs including acetate, propionate, and butyrate act as ligands for the expression of G protein coupled receptors 43 and 41 (GPR41 &43). GPR41 &43 are expressed by the gut epithelial cells, endocrine cells, and adipocytes. GPR43 receptors in white adipose tissue have been shown to act as sensors of post-prandial energy excess and regulate energy expenditure and hence, body energy homeostasis. GPR43 and GPR41 enhance insulin sensitivity and activate the sympathetic nervous system at the level of ganglion to prevent excess energy deposition in adipose tissue and enhance energy expenditure in other tissues such as liver and muscles (177). GPR43 deficient mice have metabolic abnormalities including excess fat accumulation. When treated with antibiotics or under germ free conditions, these metabolic abnormalities have been shown to reverse which suggests that the gut microbiota are key players in the expression of these receptors (177). Samuel *et al.*(2008) demonstrated that GF mice deficient in GPR-41 genes remain lean compared with their wild type counterparts, although their body composition was not different(178).

SCFAs bind to GPRs to stimulate leptin expression in mouse-cultured adipocytes. Propionate and butyrate act specifically on GPR41. In a mouse model of GPR41-/- and GPR41+/+ mice compared with wild type conventionalised and wild type germ free mice, Samuel *et al.* (2008) showed that GPR-41 stimulates the expression of the gut anorexigenic hormone, Peptide YY (PYY), which in turn causes inhibition of gastric emptying, reduced intestinal transit time (measured by flourescin isothiocynate labelled dextran), increased energy harvest (in the form of caecal acetate and propionate), and increased hepatic lipogenesis (178). The gut microbiota also convert primary bile acids into secondary bile acids which stimulate the secretion of another incretin hormone from intestinal K cells, called glucagon like peptide-1 (GLP-1), which regulate satiety through gastric vagal afferent signals to the satiety centre, reduce gastric emptying, and also acts on the pancreas and stimulates secretion of insulin (179).

However, *in vivo* studies have reported several controversies regarding the role of GPR43 in energy homeostasis. Some studies showed GPR43 mediated increased expression of GLP-1 from intestinal L-cells enhancing insulin sensitivity (180), while others suggested enhanced insulin sensitivity and reduction in metabolic dysfunction associated with GPR43 deficiency through unknown mechanisms (181). The differences between studies might be attributed to the differences in the colonisation of gut microbiota influenced by the genetic background of the mouse models, different environmental conditions (conventional versus specific) and strains of animals used in the study (177).

Overall, this evidence suggests that a selective increase in the gut microbiota may affect hormonal status of individual via G-protein coupled receptors. These hormonal changes bring a change in satiety, food intake, metabolic inflammatory markers, and ultimately metabolic endotoxemia that are a hallmark of the metabolic syndrome including obesity.

1.6.2.3 Gut microbiota and fasting induced adipocyte factor

Fasting induced adipocyte factor or angiopoitein-like protein 4 (Fiaf/ANGPTL4) is a target gene for peroxisome receptor activated proteins (PPARs) and is produced by large intestinal epithelial cells and liver. Fiaf/ANGPTL 4 inhibits lipoprotein lipase (LPL) which causes the accumulation of fat in peripheral tissues. Inhibition of fiaf by the gut microbiota with a resultant increase in LPL may be one of the mechanisms of gut bacterial induced host adiposity (162). This is further supported by studies on GF mice, genetically deficient in Fiaf genes (fiaf -/-). Lack of the *fiaf* gene causes dis-inhibition of LPL which leads to the deposition of up to 60% higher epididymal fat compared to germ free wild type littermates expressing fiaf genes (fiaf +/+). Fiaf/ANGPTL4 is therefore involved in the regulation of fat storage mediated by the gut microbiota. Controlled manipulation of the gut microbiota may alter the expression of this hormone as shown in a study by Aaronson *et al.* (2010) (182). Normal weight SPF C57B/6J mice were fed either with high fat (20%) diet or high-fat diet supplemented with probiotic *Lactobacillus paracasei* F19 for 10 weeks. Compared to non-supplemented group, plasma fiaf/ANGPTL4 was found to be up-regulated in *Lactobacillus paracasei* F19 supplemented group with significantly elevated plasma VLDL lipoprotein and no change in cholesterol levels (TG, VLDL, LDL and HDL). In another study, *Lactobacillus paracasei* F19 and *Bifidobacterium lactis* BB12 was found to up-regulate ANGPTL4 in colon carcinoma HCT116 cell line in a dose and time dependent manner while *Bacteroides thetaiotaomicron* did not have any effect (182). In another experiment in the same study, the authors fed germ free (GF) NMRI mice with normal chow and exposed them to F19. They found an increasing trend of ANGPTL4 in the serum after 2 weeks of colonization, while the effect could not be observed with heat killed F19 (182). This study suggested that manipulation in the expression of fiaf/ANGPTL4 is dependent on the presence of gut microbiota and that future interventional studies on weight management can be based on modification of ANGPTL4 by manipulating gut microbiota.

Whether the increase in levels of fiaf in systemic circulation and the subsequent suppression of LPL and fat storage is associated with a change in gut microbiota has been questioned in some studies as the authors found no difference in fiaf in serum of both GF

and conventionally-raised mice (165). Germ-free (GF) and conventional (CV) mice were fed on low fat diet (LFD), high fat diet (HFD), and commercial high-fat Western diet (WD). GF mice gained more weight and body fat than CV mice on HFD and vice-versa on WD. Although Intestinal Fiaf/ANGPTL4 was high both in GF mice on HFD and WD, circulating levels of fiaf did not change significantly as compared to CV mice. Gut microbiota changed differently with HFD and WD in CV mice. These observations suggested that diet affects the type of gut microbiota population in the gut, and that fiaf does not play a major role in peripheral fat storage as mentioned by other studies.

In summary gut microbiota-dependent-production of fiaf/ANGPTL4 inhibits lipoprotein lipase which is responsible for storage of lipids in peripheral tissues. The evidence however remains controversial.

1.6.2.4 Gut microbiota and fatty acid oxidation

Gut microbiota are thought to reduce muscle and liver fatty acid oxidation by suppressing adenosine monophosphate kinase (AMPk), an enzyme in the liver and muscle cells, that acts as a fuel gauge monitoring cellular energy status. Inhibition of AMPk results in a reduction in muscle and liver fatty acid oxidation ultimately leading to excess fatty acids storage in these tissues (162).

Phosphorylated AMPk inhibits the formation of malonyl Co-A via acetyl Co-A carboxylase. Inhibition of malonyl Co-A causes dis-inhibition of Carnitine Palmitoyl transferase-1 (Cpt-1) which in turn catalyses the rate limiting step in the entry of long chain fatty acyl-CoA into the mitochondria for fatty acid oxidation(183). Increased fatty acid oxidation is associated with enhanced cellular energy status coupled with glycogen level reduction, and increase insulin sensitivity (183).

Germ free mice lacking gut microbiota have a consistently raised level of phosphorylated Acetyl Co-A carboxylase (Acc) and carnitine palmitoyl transferase-1 (Cpt-1) activity in gastrocnemius muscles and raised AMPk in liver and skeletal tissue compared to CONV mice (89, 184). This effect has also been observed with high calorie diet suggesting that enhanced or suppressed muscle fatty acid oxidation is dependent on the presence or absence of gut microbiota. Gut microbiota may therefore impact the storage of peripheral adipose tissue and hence affect host adiposity by inhibiting fatty acid oxidation.

1.6.2.5 Gut microbiota and bile acids circulation

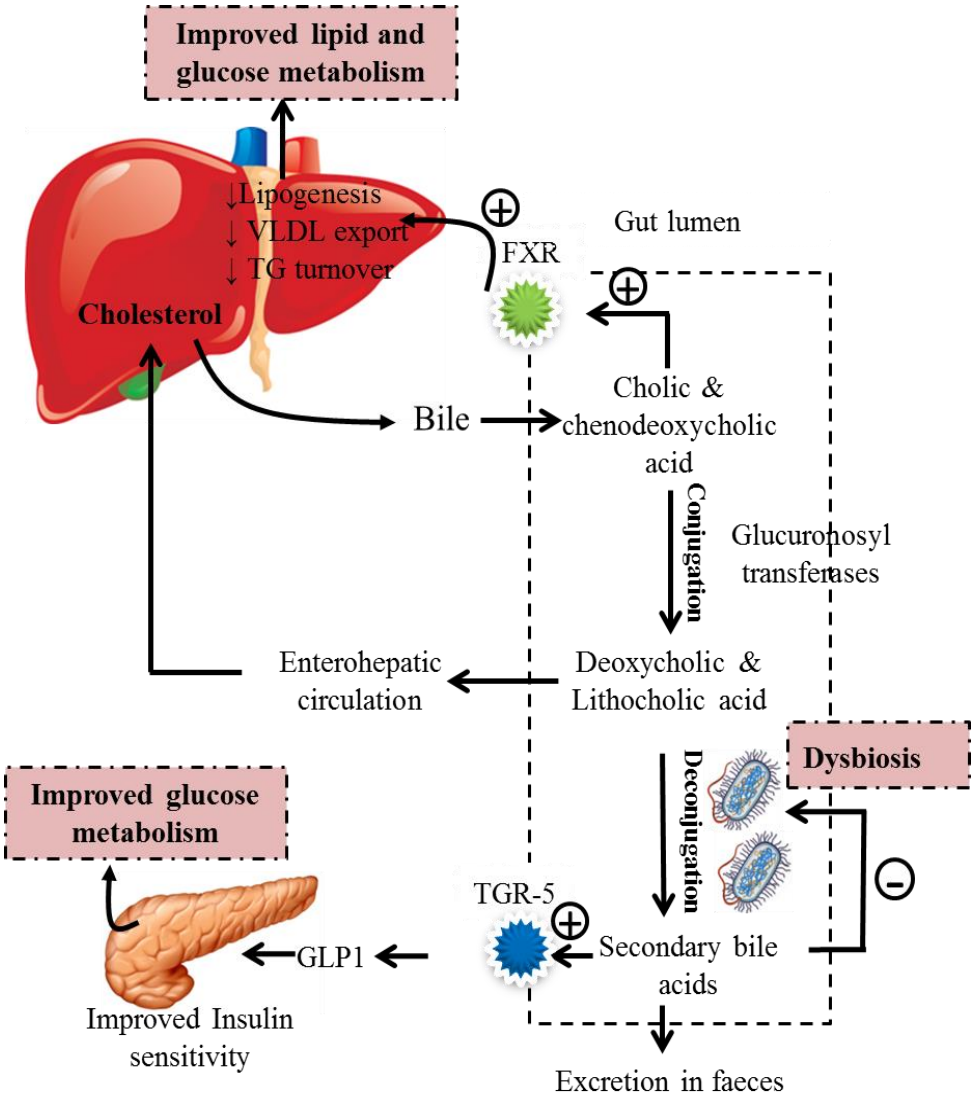
Bile acids secreted by the liver are one of the important contributors to the digestion and absorption of fats as they cause emulsification of dietary fat consumed. Bile acids,

cholicand chenodeoxycholic acids are conjugated by glucuronosyl-hydrolases in the small intestine to deoxycholic and lithocholic acids respectively. Primary bile acids (cholic and chenodeoxycholic acids) are ligands for Fernesoid x receptor (FXR) which has been shown to play a key role in the control of hepatic de novo lipogenesis, very-low-density lipoprotein-triglyceride (VLDL) export and plasma triglyceride turnover leading to improved lipid and glucose metabolism(185). By binding to FXR in ileal cells, bile acids are able to stimulate the expression of genes (Asbt, IBABP, and Ost α/β) which help in absorption, intracellular transport, and systemic transport of bile acids into the liver by enterohepatic circulation (Figure 1.6). Studies on germ-free and FXR deficient mice have shown that the expression of genes responsible for the uptake, transport and export of bile acids into circulation after ileo-caecal resection is dependent on gut microbiota (186). Primary bile acids are converted to secondary bile acids in the distal ileum and large gut by gut microbiota into secondary bile acids. Secondary bile acids are ligands for G protein coupled receptor-5 (TGR5) which helps in glucose homeostasis by stimulating the expression of Glucagon Like pettide-1 (GLP-1) (187, 188). Studies in animal models using TGR5 agonist have shown a reduction in serum and hepatic triglyceride levels. Gut microbiota may therefore affect host hepatic adiposity by altering bile acid circulation via FXR and TGR5 mechanisms. However, it is also suggested that bile acids may reciprocally affect the gut microbiota through its bactericidal activity as it can damage the microbial cell membrane phospholipid (189). High fat diet induced stimulation of bile acids into the gut may therefore alter the normal gut microbial population causing dysbiosis (190).

1.6.2.6 Gut microbiota and changes in satiety (gut-neural axis)

Gut microbiota, through the production of SCFA, may affect host energy metabolism and the development of obesity by changing the hormonal milieu in the intestine and other visceral organs (Figure 1.7). Glucagon like peptide-1 (GLP-1) plays a key role in regulating communication between the nutritional load in the gut lumen and peripheral organs such as brain, liver, muscle and adipose tissue by post-prandial increases in satiety, increasing gut transit time, and incretin induced insulin secretion (191). Secretion of GLP 1 is decreased in obesity secondary to weight gain which causes insulin resistance independent of circulating level of fatty acids (191). Gut microbiota regulate GLP-1 by influencing the expression of its precursor, pro-glucagon and increasing GLP-1 positive entero-endocrine L-cell in the gut (192). GF mice in this study expressed a higher GLP-1 secretion in the presence of high pro-glucagon and L-cells in the caecum and ascending colon than in the CONV mice.

Figure 1.6: Modulation of bile acid circulation by gut microbiota and its effect on glucose metabolism. Concept adapted from (185, 187, 188).



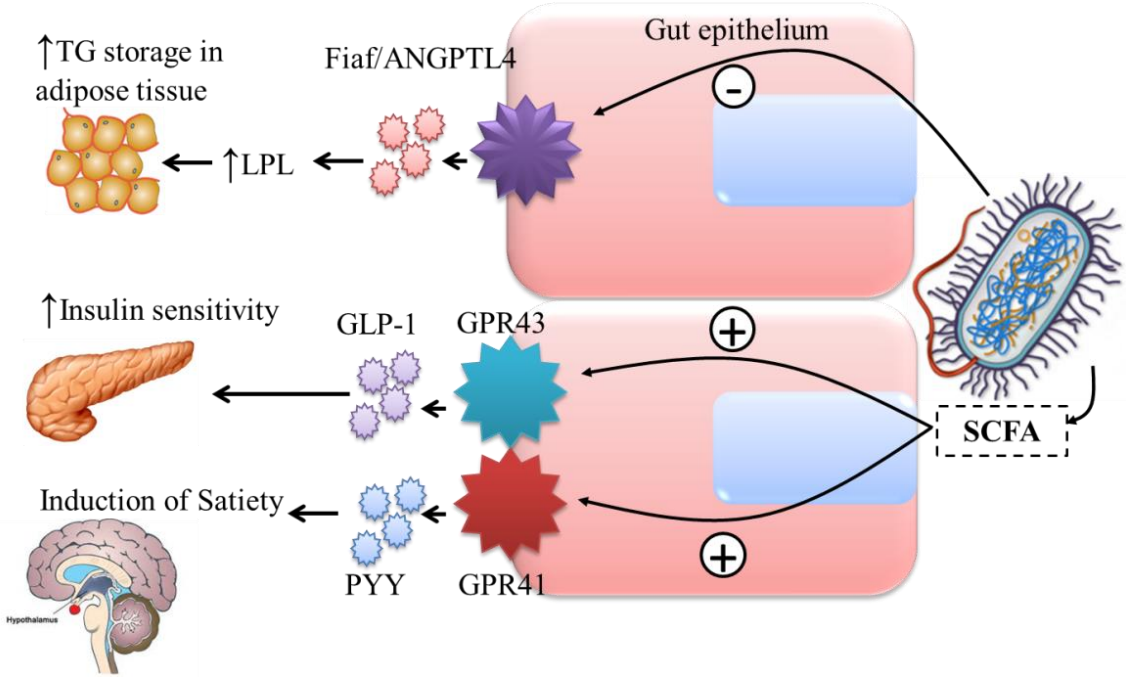
GPCR- TGR5; G protein coupled receptor TGR5, VLDL; very low density lipoprotein, TG; triglycerides, GLP-1; glucogon like peptide 1, FXR; fernesoid x receptor

Conventionalization of the GF mice with gut microbiota resulted in a significant decrease in both pro-glucagon and L-cell number after 24 to 72 h. These effects were induced by SCFA (independent of the type of SCFA) which are fermentation products of the gut microbiota(192).

Certain groups of gut bacteria such as *Bifidobacteria* have been reported to be inversely related to the development of fat mass, glucose intolerance, and bacterial lipopolysaccharide (LPS) level in the blood. Intervention with prebiotics such as dietary fructans stimulates the growth of Bifidobacteria species (184). These effects are mediated

by the stimulation of hormones PYY and ghrelin. High Intake of prebiotics oligofructose (21 g/day) has been shown to reduce weight accompanied by increased PYY and reduced Ghrelin consistent with a reduced food intake in the prebiotics group (193). A randomized double-blind placebo-controlled trial on 10 healthy adults (5 male, and 5 females) randomized into 16 g fructose/day or 16 g dextrin maltose/day group for 2 weeks, showed an increase in breath hydrogen (a marker of colonic bacterial fermentation) and increased production of satiety hormones PYY and GLP-1 (179). Similar results were observed in experimental studies where feeding ob/ob mice with oligofructose resulted in increased *Lactobacillus* species, *Bifidobacteria* species and *Clostridium coccoides-Eubacterium rectale* cluster in association with reduced intestinal permeability as shown by increased Zonula Occludin-1 expression and reduced inflammatory markers (194).

Figure 1.7: Proposed mechanism of the changes in gut hormonal axis by gut microbiota.



TG; Triglycerides, LPL; lipoprotein lipase, Fiaf; fasting induced adipocyte factor, ANGPTL-4; angiopoitein like protein-4, GLP-1; glucagon like peptide 1, GPR43 & 41; G-protein coupled receptor 43 & 41, PYY; peptide YY, SCFA; short chain fatty acids. Minus sign indicate inhibitory effect, plus sign indicate stimulatory effect.

1.6.2.7 Gut microbiota and intestinal permeability –chronic low grade inflammation

Emerging evidence suggests close ties between metabolic and immune systems (195).Obesity contributes to immune dysfunction by secretion of inflammatory adipokines from adipose tissues such as TNFα, IL-6, and leptin (196). The inflammatory adipokines induce carcinogenic mechanisms such as increased cellular proliferation and/or dedifferentiation that are potential risk factors for various cancers such as colonic,

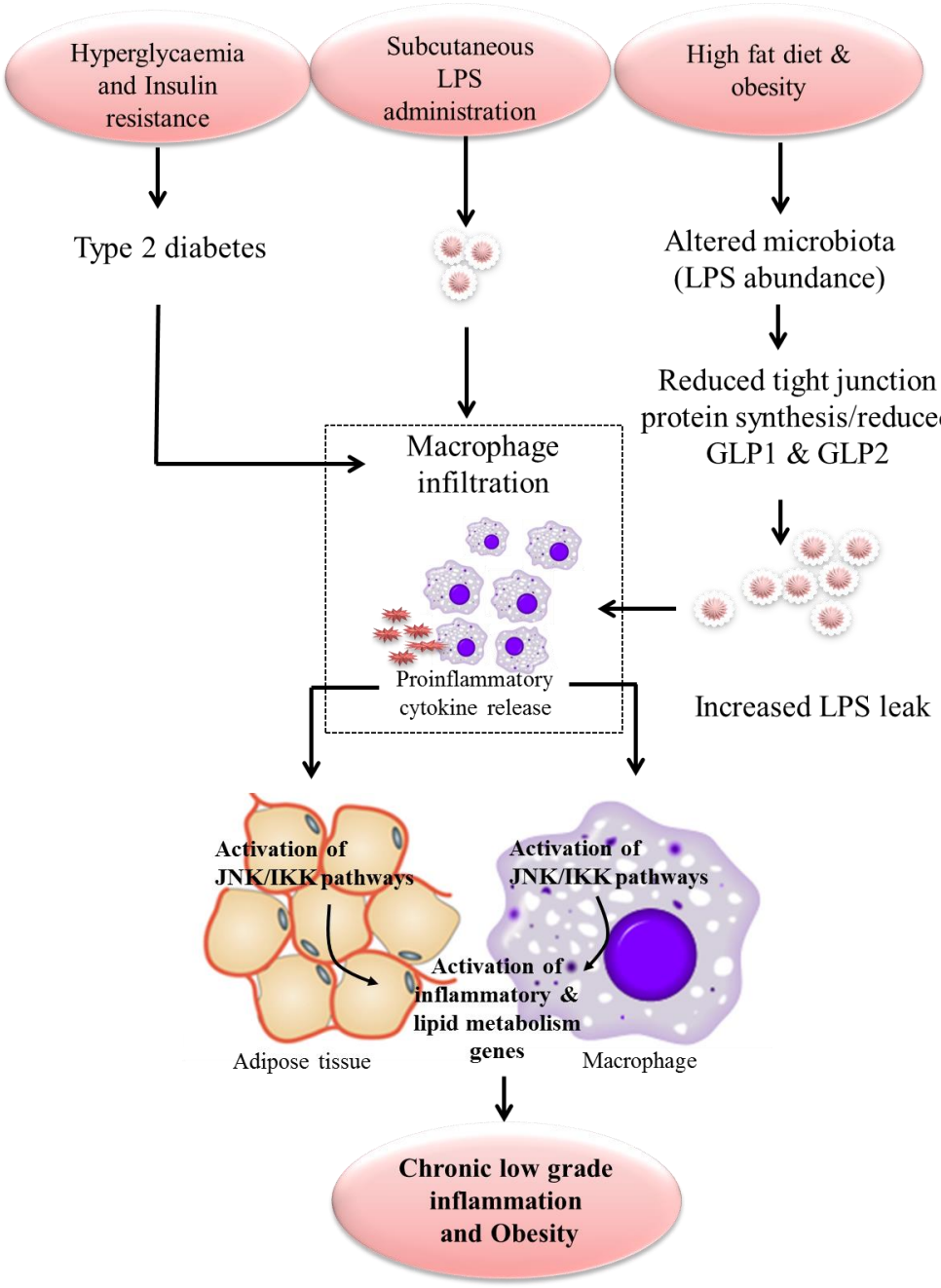
oesophageal and hepatocellular cancers. An example of this is the association of high levels of leptin with hepatocellular carcinoma (196). Intra-abdominal adipose tissue secretes adipokines with atherogenic properties (IL-1, IL-6, TNF-α, IFN-α) which increase the risk of cardiovascular diseases (197). These proinflammatory cytokines also activate certain kinases, which in turn initiate the expression of inflammatory and lipogenic genes, ultimately increasing inflammation and adipogenesis in a loop fashion (Figure 1.8).

Bacterial lipopolysaccharide (LPS) and inflammation

Gut microbiota have been suggested to contribute to chronic low grade inflammation and obesity via the absorption of bacterial lipopolysaccharide (LPS). LPS is an outer membrane protein of Gram negative bacteria, and is increasingly recognized as a player in chronic low grade inflammation, a hallmark of obesity.

Cani *et al.* (2007) demonstrated the link between LPS and metabolic disease by infusing bacterial LPS subcutaneously into germ free mice for 4 weeks which produced the same level of metabolic endotoxemia as by high fat diet (168). Furthermore, mice lacking functional LPS receptors were resistant to these changes. Feeding high fat diet to mice with mucosal immune dysfunction (Toll-Like Receptor-4 knockout mice) for 4 weeks resulted in two to three times increased systemic LPS levels in liver, adipose tissue and muscles, and higher body fat mass, termed as "metabolic endotoxemia" (168). This inflammatory status was associated with lower *Bacteroides, Bifidobacterium* species, *Eubacterium rectale-Clostridium coccoides* group (168). Additionally, LPS stimulated markers of inflammation (e.g. plasminogen activator inhibitor 1 & tumour necrosis factor alpha) and oxidative stress (e.g. lipid peroxidation) in visceral adipose tissue via CD14 receptor. Absence of CD14 in CD14 deficient ob/ob (CD14 -/-) mice has been shown to protect against diet induced obesity and inflammation in mouse models (194).

Figure 1.8: Proposed model for the role of LPS in generating inflammation and its relationship with obesity. Concept adapted from(168, 194, 195, 198).



Altered mucosal barrier function due to reduced expression of Glucagon like peptide- 1 &2 (GLP- 1&2) leads to altered mucosal function and reduced synthesis of tight junction proteins, Zonula Occludin-1&2 (ZO-1,ZO-2) increasing gut permeability. This allows LPS to enter the systemic circulation inducing the release of pro-inflammatory cytokines. Proinflammatory cytokines result in activation of a family of kinases JNK and IKK (Inhibitor of NFkB Kinase) that increase the expression of inflammatory and lipid metabolism genes. Subcutaneous administration of LPS, hyperglycaemia and insulin resistance induces the same pathway by increasing the Endoplasmic reticulum and mitochondrial stress. Type-2 diabetes, hyperglycaemia, and insulin resistance also cause macrophage infiltration and inflammatory cytokine release leading to the same process. HF; high-fat diet (168, 194, 195, 198).

Gut Barrier integrity and inflammation

The gut lumen is separated from the blood by a gut epithelial barrier that is composed of the series of layers starting from mucosal epithelium to basement membrane, submucosal tissue, lamina propria, muscularis propria, and the endothelium of blood vessels or central lacteals. Tight junctions between epithelial cells act as a barrier to the paracellular transport of nutrients and other biomolecules including the microbes into the circulatory system, limiting the transport based on charge and size of the molecules. These tight junctions are formed by complex network of proteins including Occludin and Zonula Occludin-1 (ZO-1).

Alteration in gut microbiota is linked to an altered gut barrier function (194) and may promote the release of bacterial endotoxins through the damaged and leaky gut. Cani *et al.*(2007) showed a significant reduction in the population of Bifidobacteria with high fat diet in male C57BL/6J mice (184). Supplementation with prebiotic oligofructose was shown to restore the *Bifidobacteria* population with improvement in the gut barrier function evidenced by the expression of precursors of GLP1, proglucagon mRNA and decrease in endotoxemia (184). No correlation was found between endotoxemia and other bacteria (*Lactobacilli/Enterococci*, *E. rectale/C.coccoides*, *Bacteroides*, and *sulphate reducing bacteria*) measured in their study (184). GLP-1 helps in the differentiation of mucosal cells into enteroendocrine L-cells, while GLP-2 helps in increased expression of mRNA for synthesis of tight junction proteins. These changes are associated with lower LPS levels in the blood suggesting increased integrity of the gut barrier function. In contrast treatment with antibiotics has been shown to reduce inflammation by reducing the LPS-producing gut microbiota population, further elucidating the relationship between gut microbiota, LPS levels, and inflammation (194).

High fat diet and inflammation

As mentioned earlier, obesity is a state of chronic low-grade inflammation and generally obese people consume a high-fat diet. The association of high fat-diet with sub-clinical or clinical inflammation in obesity has been investigated in several studies and there is a clear evidence to suggest that consumption of high fat diet is associated with metabolic endotoxemia and 2-3 fold increase in bacterial LPS levels in the blood. This was shown in experimental animals by Cani *et al.*(2007) (168). However, it is controversial whether this chronic low-grade inflammation is dependent on the gut microbiota. Cani *et al.*(2007) found a dramatic change in gut microbiota (reduced *Lactobacillus*, *Bacteroides Prevotella* and *Bifidobacteria* species) of obese *ob/ob* mice fed high-fat diet (184). This was

associated with an increase in gut permeability indicated by a reduced expression of Occludin and ZO-1 tight junction proteins.

In contrast, de La Serre *et al.*(2010) suggested that high fat diet induced intestinal inflammation in obese Sprague- Dawley rats may cause hyperphagia and obesity by impairing the regulation of food intake. However, changes observed in the gut microbiota were independent of lean and obese phenotype (199). On ingestion of a high-fat diet for 8 or 12 weeks, Sprague-Dawley rats emerged in two genetically distinct groups, diet induced obesity resistant rats (DIO-R) which were resistant to diet induced obesity, and diet induced obesity prone (DIO-P) rats, which were prone to diet induced obesity on feeding high fat diet. DIO-P rats had significantly increased gut permeability, increased LPS levels, lower intestinal alkaline phosphatase (iAP) levels (which detoxifies LPS),and systemic inflammation (high Toll-Like Receptor-4/Mitogen Detector-2 protein immunoreactivity) compared to DIO-R rats(199). Activation of TLR4 by LPS via MD-2 results in the production of an inflammatory cascade (IL6 and TNF alpha) (200)ensuing metabolic endotoxemia. Mice with genetic deficiency of TLR4 do not develop diet induced obesity(201). This series of changes associated with high fat diet inducing inflammation may alter food intake regulation and trigger hyperphagia, the mechanism of which is yet to be fully understood (212).

In summary, changes in the gut microbiota are associated with changes in the expression of GLP-2 which in turn leads to altered permeability of the gut epithelium, higher level of LPS in the blood, chronic low-grade inflammation, and metabolic endotoxemia. The association of obese or lean gut microbiota with inflammation is however controversial (168, 198, 202-209) (Figure 1.8).

1.6.2.8 Gut microbiota and endocannabinoid receptor system

Cannabinoid receptor 1 and 2 (CB1 & CB2) are G proteins activated by the endocannabinoid (eCB) system. The eCB system is composed of endogenous lipids and plays an important role in adipogenesis, as studied in genetically obese mouse models. Two of the most widely studied lipids in the eCB system are; N-arachidonoylethanolamine, and 2-Arachidonoylglycerol. Obesity and type-2 diabetes are associated with a higher tone of eCB system. Furthermore, the expression of CB1 and CB2 degrading enzymes (Fatty acid amide hydrolase) is increased in adipose tissue of obese *ob/ob* mice as compared to lean mice (194).

Bacterial LPS regulates the expression of cannabinoid receptors via the LPS receptor signalling system shown in both *in vitro* and *in vivo* studies (209). This increased

tone is represented by increased levels of the precursor enzymes N-acylphosphatidylethanolamine-selective phospholipase-D, CB1 mRNA and increased eCB components in plasma or adipose tissue(209). Using CB1 receptor antagonists in *ob/ob* obese mice with disrupted gut barrier and metabolic endotoxemia has been shown to improve gut permeability and reduce body weight, compared with lean littermates(209). The gut microbiota therefore regulate the activity of the eCB system and play an important role in host energy regulation.

A study by Geurts *et al.* (2011) in obese leptin resistant *db/db* mice suggested that the abundance of several taxa of Gram negative bacteria, higher Firmicutes, higher Proteobacteria, and lower Bacteroidetes were correlated with up-regulation of apelin and APJ expression. This was shown to be the result of direct action of bacterial LPS on the expression of apelin and APJ mRNA in obese diabetic mice through chronic low-grade inflammation (204).These newly discovered adipokines are widely expressed in mammalian tissues. Apelin is a ligand for APJ, a G-protein coupled receptor. Apelin/APJ system plays a key role in the cardiovascular system by acting on heart contractility, blood pressure, fluid homeostasis, vessel formation, and cell proliferation. Apelin also affects glucose homeostasis by acting through AMP kinase and nitric oxide (NO) dependant mechanisms (210). Endocannabinoid system down-regulates the expression of apelin and APJ mRNA in physiological conditions. In contrast, higher levels of apelin and APJ mRNA have been found in pathological conditions such as obesity and diabetes (204).

In summary, bacterial LPS increase the tone of eCB system, and increase the expression of Apelin/RPJ system in adipose tissue. However, how far gut microbiota population contribute to the actions of eCB and apelin/APJ and eCB in obesity is unknown. This has opened yet another area of interest about the role of gut microbiota in obesity.

1.7 Review of animal studies relating gut microbiota with obesity

The evidence from animal studies has thus far concentrated on studies which looked at the interplay of diet, gut microbiota and metabolic changes (changes in energy balance, lipoproteins, cholesterol etc.) in different animal models such as wild type mice, leptin deficient *ob/ob* mice, and Sprague-Dawley rats. These studies have concentrated on how changes in diet and differences in gut microbiota composition affect the efficacy of gut bacteria to extract energy from various carbohydrates, changes in hormonal status, changes in the levels of hepatic enzymes, expression of obesity-related genes and local and systemic

levels of inflammatory mediators. Some of the proposed mechanisms were discussed in the section above. However, several animal and human studies found conflicting evidence for the causative role of the gut microbiota in obesity.

1.7.1 Evidence from animal studies; Gut microbiota as a cause of obesity

Initial evidence suggesting the role of gut microbiota in the aetiology of obesity was explored in a series of studies using germ free and CONV mice. This was discussed in detail in section 1.6.1. The role of the gut microbiota in the aetiology of obesity was also explained through mechanisms that are only partly understood (Table 1.5). Differences in gut microbiota are therefore anticipated between lean and obese phenotype at three distinct levels as suggested by several animals models of obesity. These include 1) compositional differences, 2) functional differences in the expression of genes for metabolic pathways, and 3) the property of transmissibility of the phenotype.

Phylum level compositional differences in the relative proportions of the gut microbiota were seen in initial studies suggesting higher Firmicutes and lower Bacteroidetes in obese vs. lean mice (Table 1.7) (159, 162, 163).Although differences between lean and obese animals at species and genera level vary between studies, there is a general agreement on reduced diversity and richness of gut microbiome in obese vs. lean animals.

TLR5 knockout mice have been shown to develop hyperphagia and features of metabolic syndrome due to lack of an immune response to the bacterial flagellin. TLR5 is a ligand for bacterial flagellin which then activates an immune response to these bacteria. This lack of immune response in TLR5 knockout mice allows bacterial endotoxin to destroy tight junction proteins which may lead to metabolic endotoxemia, hyperphagia, and metabolic syndrome. Germ free mice did not exhibit these changes due to lack of gut bacteria but developed the same features after they were transplanted with faecal material from the TLR-5 deficient mice. Interestingly, 16SrRNA sequencing of bacterial diversity in TLR5 knock-out mice revealed a significantly reduced ratio of Bacteroidetes vs. Firmicutes against wild-type littermates (217). These observations show that components of gut microbiota may act as triggering factors to the development of metabolic syndromes including obesity. Diet-induced obesity-prone mice (DIO-P) develop obesity on a high fat diet and have also been shown to harbour higher Firmicutes and lower Bacteroidetes than mice which are resistant to developing obesity (DIO-R) on high-fat diets. This is coupled with capability of DIO-P mice to absorb carbohydrates more efficiently than lean DIO-R

mice (211) suggesting that the peculiar compositional differences alter the host response to prioritise its metabolism towards increased energy harvest.

Compositional differences in the gut microbiota between obese and lean phenotypes extend to the functional differences in the expression of genes capable of maintaining an obese or lean state. For example, gut microbiota of obese twins transplanted to lean mice not only caused obesity but also exhibited higher expression of genes involved in detoxification and stress response, biosynthesis of cobalamin, essential and non-essential amino acids and gluconeogenic pathways. In contrast, animals with lean- transplanted microbiota exhibited genes capable of fermenting plant polysaccharides and producing butyrate, and propionate (212). Additionally, the mere presence of the gut microbiota in conventionally raised mice compared to the amicrobiotic environment in germ-free mice has been shown to result in higher levels of energy metabolites such as pyruvic acid, citric acid, fumaric acid, malic acid, and higher rate of clearance of cholesterol and triglycerides (213). These studies suggest that the presence of the gut microbiota is essential for the characteristic metabolic outlook of a species. Won *et al.* (2013) found significant differences in urinary and serum metabolites between lean and obese C57BL/6J mice (214). Of many metabolites measured by H-NMR spectroscopy, 48 urinary and 22 serum metabolites were significantly up-regulated in obese compared with lean mice. These metabolites were involved in amino acid metabolism (particularly branched amino acids), tricarbolic acid and glucose metabolism (pyruvate, citrate, acetoacetate, glycolate, and acetone), lipid metabolism (cholesterol and creatinine), creatine metabolism (creatine and creatinine), and gut microbiome-derived metabolism (choline, trimethylamine N-oxide, hippurate, p-cresol, isobutyrate, 2-hydroxybutyrate, methylamine, and trigonelline). However, these differences were influenced by gender as obese male mice were associated with insulin signalling while female obese mice were associated with lipid metabolism (214).

Transmissibility of these characteristic compositional and functional differences has been observed (215). Colonisation of GF mice with the gut microbiota from obese animals favoured the development of compositional and metabolic features of the obese microbiome. Similarly, gastric bypass surgery in mouse models has been shown to affect the composition of the gut microbiota (increase in Proteobacteria, decrease in Firmicutes and Bacteroidetes compared to sham operated mice) for reasons partially explained; post-surgical dietary modification being the most important (215). However, the causative role of the gut microbiota in affecting the host physiology is supported by the finding that gut

microbiota transplanted from a post-gastric bypass animals who lost weight after surgery to other obese mice which had no surgery experienced reduction in weight and other metabolic aberrations in the recipient animals (215). However, the response of lean animals (by developing adiposity) to co-housing with obese cage mates is controversial. Some authors report the development of obesity and obesity related microbiota and metabolism in the lean animals (170). In contrast, others report successful acquisition of lean microbiota by obese animals characterised by the invasion of species from Bacteroidetes (including *B. cellulocyliticus, B. uniformis, B. vulgatus, B. thetaiotaomicron, B. caccae*), *Parabacteroides merdae*, and *Alistipes putridinis*. Lean animals on the other hand were not colonised by the members of the obese microbiome when they were caged together. As a consequence of invasion of lean microbiota, the metatranscriptome of the transformed-obese phenotype became similar to the lean phenotype (increased expression of genes related to branched chain amino acid degradation) suggesting a "functional transformation" (212).

In summary, despite similar caloric intake and amount of food ingested by lean and obese animals, characteristic differences in composition, function, and transmissibility of microbiome between obese and lean phenotype suggests that gut microbiota and its components might play a causative role in the aetiology of obesity. How far the obese vs. lean differences are dependent on the genetic make-up of the animals, physiology of GF and CONV animals, environment, gender, and experimental methodology is scarcely studied and needs further investigation.

1.7.2 Evidence from animal studies: Diet as a cause of obesity

Gut microbiota are located at the interface of the environment (from the luminal side of the gut) and host (from the epithelial side of the gut). The effect of environmental factors particularly diet is therefore highly significant and may contribute to the changes in the gut microbiota composition and function and ultimately their phenotype (obese or lean microbiome) (216).

Ingestion of high fat Western diet may play an important role in modifying the gut bacterial population which in turn alters the energy harvesting capability. This has been studied in various animal models such as GF/CONV mice and Sprague Dawley rats(199, 217), leptin deficient ob/ob mouse models (218), and immune deficient mouse models (Toll-Like Receptor proteins deficient mice) (203) showing a tendency towards an increase

in population of Firmicutes and reduction in Bacteroidetes after feeding with high fat Western diet.

Ingestion of high fat diet correlates with changes in the level of inflammatory markers and oxidative stress (194) such as Tumour Necrosis Factor alpha (TNF-α) and Nuclear Factor-kappaB (NF-kappaB), which play a major role in promoting inflammation(219), immune response, cellular proliferation and apoptosis. In CONV mice, but not in germ free mice, changes in the expression of these inflammatory markers in the intestine preceded weight changes and carried a strong positive correlation with high fat diet induced adiposity and markers of insulin resistance (202). This suggests an interaction of high fat diet and enteric bacteria promoting intestinal inflammation and insulin resistance prior to weight gain which is driven by the high fat diet.

In a study using GF and CONV mice, a significant surge in the Mollicutes class of Firmicutes was seen with reduced bacterial diversity and reduced number of Gram positive Bacteroides as a result of switch over from standard chow diet to a high fat Western diet (217). In contrast, reduction of fat and carbohydrates in the diet significantly decreased the consumption of calories, weight gain, and body fat than those on high fat Western diet (217). In another study, Sprague Dawley rats fed with milk-based high fat diet in addition to standard chow diet, had increased adiposity, lower jejunal Bacteroides, higher Firmicutes, higher Firmicutes to Bacteroidetes ratio, and higher jejunal Alkaline Phosphatase activity than those fed with standard chow diet (218). Observations from these studies suggest that a high fat diet, especially HF Western diet, is associated with a relative increase in the gut microbiota population in favour of Firmicutes. Increase in Firmicutes may in turn contribute to the chronic low-grade inflammation by production of LPS, and increased energy salvage by increased SCFA production.

Studies in mice indicated that although the obese phenotype is characterised by a particular set of gut microbiota, change in caloric load and type of diet changes and redistributes the equilibrium that may be independent of the genotype or phenotype (obese or lean) of the animal. Leptin deficient ob/ob mice are genetically prone to obesity due to the absence of the appetite regulating hormone leptin. Murphy and colleagues showed that leptin deficient *ob/ob* mice when fed low fat diet for 7 weeks show increased weight, increased fat mass and reduced muscle mass in association with significantly increased Firmicutes and reduced Bacteroides. However, similar changes were also observed in wild type lean mice fed the same high fat diet, although the caloric intake was similar amongst the two groups (220). These changes were however dissociated from markers of increased

energy harvest i.e. caecal SCFA and energy excreted in faeces. Caecal SCFAs were increased (acetate by ~16 µmol/g, propionate by ~4 µmol/g, and butyrate by ~3 µmol/g caecal content) and faecal energy excretion decreased (by ~0.4 kJ/g faeces) in ob/ob mice but the effect diminished after 7 weeks in both HF-fed CONV mice and ob/ob mice. These data suggest that the association of the gut microbiota with energy harvest from the diet is complex. Changes in gut microbiota composition may be attributed to the high fat diet rather than the genetic propensity of the animals to obesity. Furthermore, shift in microbiota towards higher Firmicutes to Bacteroidetes, or the absence of gut microbiota may not be associated with the development of obesity (165). The assertion that germ free mice having no gut microbiota are protected from obesity was contradicted in a study by Fleissner *et al.* (2010) where they found a significantly higher body weight gain in GF than CONV mice on high fat diet although the composition of gut microbiota in CONV mice increased in favour of Firmicutes (specifically, Erysipelotrichaceae) at the expense of Bacteroidetes on a high fat diet and Western diet (165).

As discussed above, the functional association of metabolic endotoxemia with gut microbiota was dependent on the intake of high fat diet in the obese ob/ob animal model (184, 194). However, these effects were later shown to be independent of obesity phenotype, as a high energy intake in lean C57BL/6J mice fed a high fat diet showed a 2-3 fold increase in plasma LPS compared to normal chow diet. Furthermore, the increase was blunted when the percentage intake of energy contributed by fat was reduced (221). Additionally, capability to harvest increased energy from the diet has also been shown to be independent of the phenotype or the peculiar pattern of gut microbiota. Murphy *et al.* (2010) reported that both wild type and leptin deficient *ob/ob* mice fed HF diet had a significant increase in Firmicutes and Bacteroidetes compared to lean controls after eight weeks but these changes were not associated with markers of increased energy harvest. Although energy harvesting capability initially increased (higher caecal SCFA and lower stool energy) with change in gut microbiota, this effect diminished over the period of follow up from 7 weeks to 15 weeks of age (222).

In a study by de Wit *et al.*(2012) a high fat diet composed of palm oil (with more saturated fat) was shown to distinctly increase the Firmicutes (particularly *Bacilli, Clostridial cluster* XI, XVII,XVIII) to Bacteroidetes ratio in the gut compared to a diet high in fat-olive oil, high fat-safflower oil, and low fat-palm oil (223). Additionally, high fat palm oil also stimulated the expression of 69 genes related to lipid metabolism in the

distal intestine which suggested that an overflow of lipids to the distal small intestine resulted in enhanced lipid metabolism and changes in gut microbiota.

Several other recent studies suggested similar changes in gut microbiota and the expression of genes for the metabolism of lipids in animal models using different dietary regimens (224-226) (table 1.6). Daniel *et al.*(2013) investigated composition and function of gut microbial ecology after 12 weeks dietary intervention with high fat (HF) (60% fat, 21% carbohydrates) or high carbohydrate (CARB) diet (with 66% carbohydrate and 11% fats) in male C57 BL6/N mice (n=6 per group) (227). Diets, and not the gut microbiota, were shown to affect not only the distribution of the gut microbiota communities (decrease in *Ruminococcaceae* and increase in *Rikenellaceae* with HF compared to CARB) but also the metabolome and proteome of the individual groups. CARB group had proteome related to energy production, carbohydrate metabolism, post translational modifications, and protein turnover while HF group had proteome related to amino acid and simple sugars metabolism, translation and other unknown functions (227). Although this study used two functional approaches (LC-MS/MS for metaproteome and Fourier-transform ion cyclotron resonance mass spectrometry for metabolome) to explore gut microbiota function, the study numbers available for these techniques were very low (n=3) which might have contributed to the variation within the groups.

1.7.3 Conclusion from animal studies

In conclusion, the relationship of gut microbiota with diet and metabolic disorders has been studied in a variety of animal models. Various mechanisms have been suggested in an attempt to probe the relationship between the gut microbiota and energy harvest and subsequent development of obesity. Some of these mechanisms include the production of short chain fatty acids, regulation of liver lipogenic enzymes, regulation of metabolic and satiety hormones, and inflammation. With the onset of increase in weight, there is good evidence of a reduction in overall diversity of the gut microbiota, reduction in Bacteroidetes, and an increase in Firmicutes. Increase in the endotoxin-producing gut microbiota may increase gut permeability that helps in the systemic absorption of bacterial LPS. LPS in turn induces inflammatory cytokines, metabolic endotoxemia, and metabolic syndrome. However, there is controversy as to whether these changes are attributable to the diet itself or that they are caused by the gut microbiota (199, 224) as the increase in Firmicutes is not always associated with increased weight. Some authors suggest changes in the gut microbiota occur due to the change in diet and they observed similar changes of

gut microbiota and energy harvesting capability in genetically obese low-fat fed and wild type CONV high-fat fed mice (224). On the other hand, studies in GF mice suggest the gut microbiota is the critical player in inflammation, development of immunity, and host metabolic regulation (194). Diet is also considered as a confounding factor that determines a change in gut microbiota and obesity because the diversity of gut microbiota has not been found to be different between wild-type and certain genetic models of obese mice (224).

Discrepancies between and within studies could be attributed to the selection of animals (rats vs. mice) each study using different strains for reasons partially explained. A recent study by Walker *et al.* (2014) observed a distinct microbiome and metabolome in two strains of C57BL/6J and C57BL/6N mice (228) . Some differences in the metabolome might also be attributed to the gender of the experimental animals as suggested by Won *et al.* (2013) (214) and described above. This, in addition to other methodological, host, and environmental differences in experimental conditions add to the complexity of the relationship. The exact mechanism of how these changes lead to an obesity phenotype is still not known. Large humans based interventional studies are therefore required to establish the true association between diet and gut microbiota and obesity.

Table 1.7: Evidence from animal studies about the role of gut microbiota in obesity

Reference	Study model	Aim of the study	Study design and outcomes measures	Results	Conclusion
Fleissner CK *et al.* 2010 (165)	Male adult C3H Germ free (GF) and conventionally raised (CV) mice	To study the influence of different diets on the body composition of GF and CV mice.	Ad libitum intake of low fat (LF), high fat (HF) and commercial Western diet (WD) for GF and CV mice. Real time PCR, FISH and fiaf/angplt4 in gut and blood	GF mice gained more weight and body fat and had less energy expenditure than CV mice on HFD. Higher Firmicutes (especially Erysipellobacteriacae) and lower Bacteroides in CV mice on HF and WD. Intestinal Fiaf increased in GF mice but no change in plasma fiaf levels as compared to CV mice.	GF mice are not protected from diet induced obesity. Diet affects gut microbiota composition and fiaf does not play a role in fat storage mediated by gut microbiota.
Ajslev TA *et al.* 2011 (229)	28 354 mother-child dyads, age 7 years	To assess the influence of delivery mode, maternal pre-pregnancy BMI, and child's early exposure to antibiotics on the child's risk of overweight	Maternal pre-pregnancy BMI, delivery mode and antibiotic administration in infancy. Children followed at 7 years of age	No significant association of delivery mode with overweight. Increased risk of overweight and obese in children, born to normal weight mothers, given antibiotics in first 6 months of life and decreased risk in children born to overweight mothers.	Antibiotics use in early infancy and pre-pregnancy weight of mother affect tendency of child to become overweight and obese.
Sefcikova Z *et al.* 2010 (218)	8-10 pups per nest, Sprague-Dawley rats, from day 21-40.	To evaluate the effect of normal and over-nutrition on the development of gut microbiota, intestinal Alkaline Phosphatase and occurrence of obesity	Standard laboratory diet for control group and additional milk based liquid diet for study group. Epididymal and peri-renal fat pads and bacterial enumeration via FISH, Alkaline phosphatase activity via immunocytochemistry	Obese rats gained more energy (25%) and higher body fat (27%) than lean rats. Alkaline phosphatase increased in obese rats. Lactobacilli increased while Bacteroides decreased in obese rats significantly.	Due to early changes in diet and the intestinal environment, this study may provide a baseline for further insight into the ways of involvement in programming of a sustained intake and digestion
Ding SL *et al*. 2010 (202)	GF/CONV mice and KFκB knockin mice (GF/CONV)	To test the hypothesis that Intestinal inflammation is promoted by the interaction of gut bacteria and high fat diet, which contributes to the progression of insulin resistance and obesity.	High and low fat diets for 2, 6 or 16 weeks.GF mice fed with diet after exposure to faecal slurries of CONV mice. Blood glucose and ELISA for insulin. TNF- α mRNA expression by qPCR. Intestinal EGFP expression of NF kB mice by fluorescent light microscopy.	CONV mice gained more weight than GF. Increased expression of TNF-α mRNA and NFkB in CONV HF diet mice. TNF- α changes precedes weight changes. Enhanced NFkB in GF NFkB mice on feeding CONV NFkB faecal slurry.	HF diet and enteric bacteria interact to promote inflammation and insulin resistance prior to the development of weight gain, adiposity and insulin resistance.
Turnbaugh *et al.* 2008 (217)	8-9 weeks old GF/CONV mice	To study the inter-relationship between diet, energy balance and gut microbiota using mouse model of obesity.	Conventionalization of GF mice with HF Western diet followed by introduction of Western or CHO diet in CONV mice. CARB-Reduced or FAT-Reduced diets in another subset. qPCR, DEXA scan and weight measurements done	Western diet-associated caecal community had a significantly higher relative abundance of the Firmicutes (specifically Mollicutes) and lower Bacteroidetes. Mice on the Western diet gained more weight than mice maintained on the CHO diet and had significantly more epididymal fat. Mice on CARB-R and FAT-R diet consumed fewer calories, gained less weight and had less fat.	There is restructuring of gut microbiota with Western diet, specifically reduction of Bacteroides and surge in Mollicutes class of Firmicutes with increased capacity to harvest energy from diet.

de La Serre CB *et al.* 2010 (199)	Male Sprague Dawley rats	To evaluate whether changes in gut bacteria and gut epithelial function are diet or obese associated.	Measurement of intestinal permeability, intestinal alkaline phosphatase, plasma LPS, tissue Myeloperoxidase activity, Immunochemical localization of TLR4/MD2 complex and Occludin, Sequence analysis of the microbial 16S rRNA gene. body fat composition and measures of adiposity from fat tissue samples	Appearance of two distinct groups; Diet induced obesity Prone and Resistant groups. DIO-P rats had more features of adiposity, higher MPO activity, TLR4 MD2 immunoreactivity and higher plasma LPS levels, increased gut permeability, immunoreactivity of Occludin and lower alkaline Phosphatase levels than LF and DIO-R group. HF diet was associated with an increase in Clostridiales regardless of propensity for obesity. A marked difference in Enterobacteriales in DIO-P animals compared with either DIO-R or LF-fed animals.	Changes in gut bacteria are independent of obese status. Gut inflammation marked by increased LPS may be a triggering mechanism for hyperphagia and obesity
Daniel *et al.*2013 (227)	Male C57BL/6NCrl mice (n=6, per group)	To investigate changes in function and activity of the gut ecosystem in response to dietary change	LC-MS/MS for metaproteome, Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) for metabolome, Miseq illumina pyrosequencing for gut microbial composition. Intervention with high fat (HF) and control (carbohydrate) diet for 12 weeks	HF diet did not affect caecal taxa richness. Bacterial communities clustered according to diet. Significantly lower *Ruminococcaceae (*Firmicutes*)* and higher *Rikenellaceae (*phylum Bacteroidetes*), Lactobacilli,* and *Erysipelotrichiales* in HF fed vs. carbohydrate fed diet. 19 OTUs affected by HF diet. Carbohydrate group had proteome related to energy production, carbohydrate and post translational modifications, protein turnover while HF group had proteome related to amino acid and simple sugars metabolism, translation and other unknown functions. Caecal metabolome clustered distinctly based on diet.	High fat diet affects gut microbial ecology both in terms of composition and function
Backhed F *et al.* 2004 (162)	Adult germ-free (GF) C57BL/6 mice	To evaluate the effect of gut microbiota on the host energy metabolism using animal model	Conventionalization of GF mice with murine gut microbiota or β-thetaiotaomicron, intestinal fiaf, liver metabolism, total body fat, LPL activity in adipose tissue, faecal microbiota composition by Sybr-Green qPCR	Conventionalized GF mice showed 57% increase in body fat, increased energy expenditure, suppressed intestinal fiaf, increased LPL activity and increased expression of ChREBP and SREBP-1 in liver. Firmicutes to Bacteroides ratio similar in GF and CONV.	Gut microbiota alters host energy storage by affecting fiaf and LPL activity. It may be regarded as an environmental factor that affects host energy storage
Backhed F *et al.* 2007 (89)	Adult germ-free (GF) C57BL/6 mice (n=5) and CONV mice (n=5)	To assess whether GF mice are protected against obesity on high fat Western diet	Dietary intervention with low fat followed by high fat Western diet for 8 weeks	CONV mice gained more weight on HF diet while Conventionalised GF mice didn't. Stool energy was similar to the LF fed GF mice. Persistent elevated TG in HF fed GF mice. GF mice had high Acc-p, AMPK-P and Cpt-1 activity depicting increased fatty acid oxidation. GF mice had reduced hepatic glycogen and glycogen-synthase activity. High fiaf in HF fed GF mice	GF mice are protected against diet induced obesity by two mechanisms; 1. Increased phosphorylated AMPK 2. Increased *fiaf*
Vijay-Kumar M *et al.* 2010 (203)	TLR5 Knock out mice(T5KO), Wild type mice (WT)	To show that mice deficient in TLR-5 exhibit hyperphagia, which is a principal factor in the development of obesity and metabolic syndrome?	Introduction of broad spectrum antibiotics to assess the role of altered microbiota resulting from loss of TLR5. Pyrosequencing of 16S rRNA genes in the caecum to find out the extent of alterations in microbiota due to loss of TLR5. Transplantation of T5KO mice microbiota into WT germ free hosts	Antibiotic treatment lowered the bacterial load by 90%, correction of metabolic syndrome similar to the wild type mice. Relative abundance of bacterial phyla was similar in both; with 54% Firmicutes, 39.8% Bacteroides. 116 phyla observed to be enriched or reduced in R5KO relative to WT mice. Microbiota of WT mice transplanted to the R5KO mice resulted in all features of metabolic syndrome in the R5KO group	Loss of TLR-5 results in metabolic syndrome and alteration in gut microbiota. Acquired microbiota from mother can be an important means by which environmental factors can exert long lasting influence on metabolic phenotype

Ley *et al.* 2005 (163)	Leptin deficient C57BL/6J *ob/ob* mice, lean *ob/+*, and +/+ mice (n=19)	To study differences in bacterial diversity between obese genetic model of obesity and its relationship with kinship	16S rRNA gene amplification of caecal bacteria followed by analysis using PHRED and PHRAP software. All mice fed the same polysaccharide rich chow.	*ob/ob* mice consumed 42% more chow, gained significantly higher weight. Mothers and offspring shared bacterial community. Obese ob/ob had 50% reduction in Bacteroidetes and a proportional increase in Firmicutes as compared to lean regardless of the kinship and gender	Obesity is associated with altered bacterial ecology. This however needs to be correlated with the metabolic attributes of gut microbial diversity in obese and lean
Turnbaugh *et al.* 2006 (159)	Leptin deficient C57BL/6J ob/ob mice (n=13) and lean ob/+, and +/+ mice (n=10)	Whether gut microbial gene content correlates with characteristic distal gut microbiome of leptin deficient ob/ob mice and their lean counterparts and whether this trait is transmissible	1S rRNA whole metagenome Shotgun Metagenomics, GC-MS for SCFA analysis, bomb calorimetry for stool energy, gut microbiota transplantation, DEXA for fat mass	Firmicutes-enriched obese microbiome clustered together while lean phenotype with Low Firmicutes to Bacteroidetes ratio clustered together. Obese microbiome was rich in enzymes involved in the breakdown of dietary polysaccharides particularly glycoside hydrolases. Ob/ob had higher acetate and butyrate and significantly less stool energy	Obese microbiome is associated with increased energy harvest
Cani *et al.* 2007 (168)	C57bl6/J Mice and CD14-/- mutant strain	To evaluate the influence of gut microbiota on the development of metabolic endotoxemia	Metabolic, inflammatory and microbiological differences (FISH) between high-fat-fed obese or rodent lean chow-fed mice	High-fat feeding and obesity decimates intestinal microbiota– *Bacteroides*-mouse intestinal bacteria, *Bifidobacterium*, and *Eubacterium rectale–Clostridium coccoides* groups all significantly lower than in control animals	High-fat diet-induce changes in gut microbiota that leads to elevated plasma LPS leading to metabolic endotoxemia, by altering the gut barrier function
Cani *et al.* 2008 (201)	C57bl6/J ob/ob mice	Manipulating gut microbes through antibiotics to demonstrate whether changes in gut microbiota control the occurrence of metabolic syndromes	Caecal microbiota of mice under High-fat low-fibre diet and antibiotics. qPCR and DGGE	Antibiotic reduced LPS caecal content and metabolic endotoxemia in both ob/ob and high fat. High-fat diet increased intestinal permeability and LPS uptake leading to metabolic endotoxemia. Absence of CD14 mimicked the metabolic and inflammatory effects of antibiotics	High fat diet modifies gut microbiota which induce inflammation and metabolic endotoxemia. Antibiotics can reverse these changes.
Caeser *et al.*2012 *(198)*	Swiss-Webster mice (GF, CONV and *E.coli* monocolonised mice)	Whether gut microbiota especially LPS promote inflammation in white adipose tissue (WAT) and impair glucose metabolism	DEXA, insulin and glucose tolerance, Macrophage isolation, immunohistochemistry, and flowcytometry and immunoblot in WAT, LPS analysis, RT-qPCR	Monocolonisation of GF mice with *E.coli* W3110 or isogenic strain MLK1067 with low immunogenic LPS had impaired glucose tolerance. However, only GF mice with *E.coli* W3110, and not MLK1067, showed increased pro-inflammatory macrophage infiltration in WAT	Macrophage accumulation is microbiota dependent but impaired glucose tolerance is not.
Caricilli *et al. 2012* (230)	TLR2 Knockout mice (TLR2 -/-) and wild-type mice (n=8 per group)	Influence of gut microbiota on metabolic parameters, glucose intolerance, insulin sensitivity, and insulin signaling in TLR2 Knockout mice	454 pyrosequencing	Higher Firmicutes (47.92% vs. 13.95%), Bacteroidetes (47.92% vs. 42.63%), and lower Proteobacteria (1.04% vs. 39.53%) in TLR2 -/-. Higher LPS absorption, insulin resistance, impaired insulin signalling and glucose intolerance in TLR2 -/-compared to controls.	Alteration in gut microbiota in non-germ free conditions links genotype to phenotype
Everard *et al. 2013* (231)	C57BL/6 mice (genetically obese, HF fed, type 2 diabetic)	To ascertain the role of *Akkermansia muciniphila* in obesity and type 2 diabetes	Real time qPCR, MITChip analysis, LTO- Orbitrap mass spectrometer, ELISA for insulin and faecal IgA	*Akkermansia muciniphila* decreased obesity and type-2 diabetes which was normalised by oligofructose. Administration of *A. muciniphila* reversed markers of metabolic disorders. These effects needed viable *A. muciniphila*	This microbe could be used as part of a potential strategy for the treatment of obesity

Fei & Zhao 2013 (232)	C57BL/6J GF mice	Endotoxin producing *Enterobacter cloacae* B29 isolated from obese human gut could induce obesity and insulin resistance in GF mice	16S rRNA gene sequencing for bacteria and Limulus amebocyte lysate test for endotoxin measurement	Mono-colonisation of GF mice with *E.cloacae* induced obesity and insulin resistance on HF diet while GF control mice only on HF diet didn't. *Enterobacter*-colonised GF obese mice had higher plasma endotoxin levels and inflammatory markers	Gut microbiota-produced endotoxin may be causatively related to obesity in human hosts
Geurts *et al.*2011 (204)	Leptin resistant *db/db* mice	To investigate the gut microbiota composition in obese and diabetic leptin resistant mice vs. lean mice	Combined pyrosequencing and phylogenetic microarray analysis of 16S rRNA gene	Higher Firmicutes, Proteobacteria, and Fibrobacters phyla in *db/db* mice compared to lean mice. Odoribacter, Prevotella, and Rikenella were exclusively present in db/db mice while enterorhabdus was identified exclusively in lean mice. Db/db mice had a higher tone of eCB, and higher Apelin and APJ mRNA levels. Gut microbiota were significantly correlated with Apelin/mRNA tone	Gut microbiota varies with genotype and play a significant role in the regulation of eCB and Apelin/APJ mRNA system
Murphy *et al. 2010* (220)	HF fed wild-type mice, and leptin deficient *ob/ob* mice (n=8 per group)	To investigate the effect of high fat diet and genetically determined obesity for changes in gut microbiota and energy harvesting capability over time.	GC, metagenomic pyrosequencing High fat or normal chow diet fed to ob/ob mice and wild type mice for 8 weeks.	Increase in Firmicutes and Bacteroidetes in HF fed and obese mice but not in lean. Changes in microbiota were not associated with markers of energy harvest initial increase in caecal SCFA (acetate) and decrease in stool energy with HF diet did not remain significant over time. No correlation of bacterial phyla with energy harvest	Changes in bacterial phyla is a function of high fat diet and these changes are not related to the markers of energy harvest
de Wit *et al.*2012 (223)	Male C57BL/6J mice	To study the effect of dietary fat type (polyunsaturated and saturated fatty acids ratio) on the development of obesity	Phylogenetic microarray (MITChip) analysis, bomb calorimetry, measurement of triglycerides, plasma insulin	HF diet with high saturated fatty acids (palm oil) induced higher weight gain and liver triglycerides compared to high fat diet with olive oil and safflower oil. HF diet with palm oil reduced microbial diversity and increased Firmicutes (Bacilli, *Clostridium cluster* XI, XVII, and XVIII) Bacteroidetes ratio. Up-regulation of 69 lipid metabolism genes in distal small intestine and increase fat in stool suggest overflow of fats to distal small intestine	Type of dietary fat influences the weight gain and hepatic lipid metabolism
Faith *et al.*2011 (216)	Male C57BL/6J mice (n=10 per group)	Changes in 10 model gut communities species abundance and microbial genes with changes in peculiar diet ingredient were studied	Shotgun sequencing of faecal DNA Diets used for each community; casein (for protein), corn oil (for fat), starch (for polysaccharides), and sucrose (for simple sugars)	61% variance in abundance of the community members was explained by diet particularly casein. Absolute abundance of *E.rectale*, *Desulphovibrio piger* and *M. formatixegens* decrease by 25-50% while *Bacteroides caccae* increase with increase in casein, although the total community biomass increases.	Host diet explains configuration of gut microbiota both for refined diets and complex polysaccharides
Hildebrandt *et al.* 2009 (224)	RELM-B knock out female mice and wild type mice	To assess the influence of host phenotype, genotype, immune function, and diet on gut microbiota	16S rDNA 454 FLX pyrosequencing, metagenomic sequencing	Switching to high fat diet resulted in decreased Bacteroidetes and increased Firmicutes and Proteobacteria in both wild type and RELM B knockout mice irrespective of the genotype. Genetic makeup (RELM B KO) only modestly influenced the gut microbiome composition. Changes in gene content were seen with high fat diet	Diet determines the gut microbiota composition

Huang *et al.*2013 (225)	Adult male C57BL/6	To assess the relationship of diet content and source on gut microbiota, and adiposity	16S rRNA analysis vi terminal restriction fragment length polymorphism and V3-V4 sequence tag analysis via next generation sequencing. Mesenteric fat and gonadal fat tissue analysis. Milk, lard, or safflower based diets for 4 weeks.	Higher weight gain and caloric intake with high fat than low fat diet. Milk based and PUFA based diets animals had higher adipose tissue inflammation than lard based or low fat diet. Milk based and PUFA diet had significantly higher *Proteobacteria* and lower *Tenericutes.* PUFA based fed animals had higher expression of adipose tissue inflammation genes (MCP1, CD192, resistin)	Dietary fat components reshape gut microbiota and alter adiposity and inflammatory status of the host
Jacobstadir *et al.*2013 (233)	Male Wister rats	To investigate the effect of dietary fibre on metabolic risk markers in low and high fat diets at 2, 4, and 6 weeks	Gas liquid chromatography, liver fat content, cholesterol and triglycerides analysis, terminal fragment length polymorphism. Diets were supplemented with guar gum, or a mixture	Decrease in weight gain, liver fat, cholesterol, triglycerides with fibre. Change in formation of SCFA. Reduction in serum SCFA with high fat diet followed by recovery after 4 weeks. Succinic acid increased with high fat consumption. Dietary fibre reduced this effect and also reduced inflammation. *Bacteroides* were higher with guar gum and *Akkermansia* was higher with fibre-free diet.	High fat diet increase metabolic risk factors which are partly reversed by high fibre diet.

1.8 Review of human studies relating gut microbiota with obesity

1.8.1 Gut microbiota as a cause of obesity

Evidence linking the gut microbiota with obesity in humans is thus far inconclusive and controversial. This may be partly due to marked inter-individual variations in the gut microbiota and metabolic activity in humans with age, diet, use of antibiotics, genetics and other environmental factors (234). Apart from the inter-individual variation in faecal microbiome and diversity, re-analysis of large datasets such as human microbiome project (HMP) and MetaHIT has shown large inter-study variability which was far greater than the actual differences between the lean and obese phenotypes (235). A refined statistical modelling therefore led to the loss of some correlations previously found, such as correlation of BMI with Firmicutes to Bacteroides ratio (235). Bridging these gaps in analysis and accounting for these technical and clinical factors is therefore important to elucidate differences between normal and altered host microbiome and metagenome.

Although 16S-rRNA sequencing based studies revealed differences in the gut microbiota between and within individuals, the general concept about predominant phyla is emerging. Firmicutes, Bacteroidetes and Actinobacteria in descending order of abundance, form the major groups of gut bacteria constituting about 92.6% of all the known gut microbes (164). Differences in these groups may drive the difference of energy harvest from food in individuals.

The first evidence regarding aberrant relative abundance of the gut microbiota in human faeces was presented by Ley *et al.*(2006) who found higher Firmicutes and lower Bacteroidetes in obese vs. lean adults before the onset of dietary intervention (164). This was followed by a number of studies reviewed in Table 1.10. Moreover, several gut microbes have been associated with obesity (such as *Lactobacillus reuteri)* or leanness (such as *Bifidobacteria*) (236, 237) (Table 1.8). The type of gut microbiota and their exact hierarchy at which they exhibit differences is still under investigation.

The energy harvesting capability of the gut microbiota in obese subjects is thought to be set at a higher threshold than in lean phenotype with or without differences in the relative abundance of the gut microbiota.

Table 1.8: Association of gut microbial species/genera with obesity or leanness in human studies

Bacteria	Association with obesity	Group	Level	Other associations	Reference
Lactobacillus reuteri	+ve	Firmicutes	Species	-	(236), (238)
***Clostridium cluster* XIVa**	+ve	Firmicutes	group	Anti-inflammatory	(170)
E. coli	+ve	Proteobacteria	Species	Non-alcoholic steatohepatitis (NASH)	(170)
***Staphylococcus* spp.**	+ve	Firmicutes	Genus	Energy intake	(239)
Bacteroides	-ve/+ve	Bacteroidetes	Genus	Controversial	(164)
Akkermansia muciniphila	-ve	Verrucomicrobia	Species	Mucus degradation	(231)
Methanobrevibacter smithi	-ve	Archaea	Species	Increase in anorexia	(240)
Clostridium cluster* IV*; F. prausnitzii	-ve	Firmicutes	Species	Anti-inflammatory	(241)
Bifidobacteria	-ve	Actinobacteria	Genus	-ve association with allergy	(236)

+ve: positive association, -ve; negative association, +ve/-ve; controversial.

In a crossectional study by Fernandes *et al*. (2014), significantly higher faecal acetate, propionate, butyrate, valerate, and total SCFA were found in obese vs. lean adults(176). Although relative abundance of Firmicutes, Bacteroides/Prevotella, and Firmicutes to Bacteroides ratio were not significantly different between the lean and obese phenotype, faecal total and major individual SCFA (acetate, propionate, butyrate) were negatively correlated with Bacteroides and positively correlated with Firmicutes/Bacteroides ratio (176). Additionally, the anti-obesity effect of polyunsaturated fatty acid (PUFA) derived conjugated linoleic and linolenic acid in obese women has been shown to be produced by the action of *Bifidobacteria, F. prausnitzii,* and *Lactobacilli*. The production of these compounds from dietary PUFA is unaffected by intervention with prebiotics which suggest that indigenous gut microbiota might be determining this potential (242). A recent study by Bergstorm *et al.*(2014) found no significant correlation of gut microbiota at 9, 18, or 36 months of age with dietary intake (measured by 7 days food diary), body composition (measured by DEXA), gender, and mode of delivery (243). Furthermore, positive correlation of BMI SDS with Firmicutes particularly butyrate producing *C. leptum, E.halii*, and *Roseburia* indicated that changes in the gut microbiota at these developmental stages may not depend on the dietary factors. Bervoets *et al.* (2013) also found a higher Firmicutes to Bacteroides ratio, lower *Bacteroides fragilis*, higher *Lactobacillus* spp. and

no correlation of major colonic bacterial groups with dietary intake in obese and healthy children (age 6-16 years) (239). The only exception was *S. aureus* which was positively correlated with energy intake (239). However, this study was crossectional and the dietary intake of obese and lean children was similar (~2200 Kcal/day in obese vs. ~2100 Kcal/day in lean) which might indicate underreporting by the obese children. Karlsson *et al.* (2012) found significantly higher *Enterobacteriaceae* and significantly lower *Desulfovibrio* and *Faecalibacterium prausnitzii* in obese vs. lean children (n=20 each, age 4-6 years)(244). However, no differences in the counts of *Lactobacillus*, *Bacteroides fragilis,* and *Bifidobacterium* were found between lean and obese (244). Moreover, concentration of Bifidobacterium was negatively correlated with serum alanine aminotransferase in obese and overweight children while faecal calprotectin was not different between lean and obese indicating the absence of inflammation at the early age of overweight and obese (244).

In this context, differences in gut microbiota have been observed distinctly in obese compared to lean phenotype before pregnancy (such as higher counts of Bacteroides in obese vs. lean women) and during pregnancy in obese and lean women (such as increase in Bacteroides and *S.aureus* from 1st to 3rd trimester in obese vs. lean women). However, these have not been studied in relation to the changes in diet that occur over the period of pregnancy thereby limiting its implications.

Modulation of gut microbiota with the use of antibiotics have been shown to affect the gut microbiota development in early infancy and childhood and hence bacterial metabolic activity (150, 245). In the context of obesity, population based studies suggest that the use of antibiotics may affect weight gain in early childhood (Table 2.3) and the maturation/ stabilisation of gut microbiota in the long term. Long term changes in gut microbiota composition (such as lower counts of *Bifidobacteria* and higher *Bacteroides*) have been observed in children who were exposed to antibiotics in early childhood (150, 245). Modulation of gut microbiota with antibiotics (e.g. norfloxacin and ampicillin) have been shown to alter the expression of hepatic and intestinal genes involved in inflammation and metabolism thereby changing the hormonal, inflammatory, and metabolic milieu of the host (246). These antibiotic-induced changes may predispose children to overweight and obesity by a selective "obesogenic-bacterial-growth" promoting effect. This is confirmed in a large population based study of the Danish National Birth Cohort (229). This study found a higher tendency of overweight at 7 years in children who had a history of antibiotics use in their early infancy born to normal weight mothers. Interestingly, mode of delivery (vaginal vs. caesarean section) had no impact on the rate of antibiotic use in first 6 months

of life of infant (Odds ratio 1.02, 95% CI; 0.88-1.19) suggesting that the effect of antibiotics on overweight is independent of this confounder (264).

The development of gut microbiota in infants and their tendency towards overweight and obesity in later childhood are linked to mother's pre-pregnancy BMI and gut microbiota. In a study by Santacruz *et al*. (2010), obese pregnant women revealed significantly lower numbers of faecal *Bifidobacteria* and *Bacteroides* and significantly higher *E coli* and *S.aureus* compared to normal weight pregnant women (247). Furthermore, *Staphylococcus aureus* was positively correlated with high cholesterol levels in obese women (247). These gut microbiota were transferred to the infants as infants born to mothers who gained excessive weight during initial periods of pregnancy or were overweight and obese prior to pregnancy were found to have lower number of *Bacteroides* and higher number of *Clostridia* in first month of birth and the relationship reduced at 6 months of age (248). Interestingly, higher *Staphylococcus* and lower number of *Bifidobacteria* have been associated with the presence of obesity in later childhood.

In addition to the relative higher abundance of Bacteroides than Firmicutes in lean vs. obese subjects (164), functional differences in the metabolome of the obese and lean phenotype may be more important. Calvani *et al*. (2010) in their preliminary study of 15 morbidly obese and 10 age matched controls found distinct gut microbial co-metabolites in urine of obese versus lean participants, including lower levels of hippuric acid (benzoic acid derivative), trigonelline (niacin metabolite), and xanthine (purine metabolism) and a higher levels of 2-hydroxybutyrate (metabolite of dietary protein) (241). The metabolic or functional representation of gut microbiota might be proportional to each other despite differences in the relative abundance of gut microbiota in the gut. Disturbance of this equilibrium is a hallmark of the obese phenotype as suggested by Ferrer *et al.* (2013) in a comparative metagenomic and metatranscriptomic analysis of faecal samples from obese and lean adolescents (249). Faecal samples of obese adolescents had higher relative abundance of Firmicutes (~95%) and lower Bacteroidetes (~4%) compared to lean (Firmicutes ~79%, Bacteroidetes ~18%). However, up to 81% of the expressed proteins were contributed by Bacteroidetes despite a low compositional representation.

Table 1.9: Population based studies to investigate the risk of obesity and overweight in children who were given antibiotics for treatment of infections in early infancy

Study reference	Design & Population	Age group	Tools	Primary outcome	Factors considered	Findings
ISAAC study (International Study of Asthma and Allergies in Childhood)(250)	n=74,946 crossectional	5-8 years	Questionnaires/interviews, measurements	Antibiotics use in first 12 months of life	Ht., Wt., BMI, age, gender, antibiotics, paracetamol, breast feeding, Maternal smoking, gross national income, Asthma	Association of antibiotics use and BMI in Boys (+0.107 kg/m^2 $p<0.0001$), not in girls even after adjustment for the other variables
DNBC study (Danish National Birth Cohort) (229)	n=28,354	Up to 7years	Questionnaires/ telephonic interviews based	Antibiotics use in <6 months of life	Socioeconomic status, maternal age and smoking, gestational weight gain, parity, delivery mode, breastfeeding, paternal BMI, birth weight and age at 7-year follow-up.	Increased risk of overweight in children born to normal weight mothers (adjusted OR: 1.54, 95% CI: 1.09–2.17). and especially in boys when adjusted for maternal age, smoking, SE status, birth weight and breast feeding
ALSPAC study (Avon Longitudinal Study of Parents and Children) (251)	n= 11, 532 Longitudinal	7 years	Questionnaires based, hospital records, objective measurements	Antibiotic exposure at <6 months, 6-14 months, and 15-23 months and BMI at 6 weeks, 10 months, 20 months, 38 months and 7 years	Maternal parity, social class, education, parental BMI, parental smoking, breastfeeding, lifestyle and dietary patterns	Increased risk of overweight at 38 months (OR 1.22, P=0.029) but not at 7 years in children exposed to antibiotics <6 months

Furthermore, obese metagenome showed higher aerobic and anaerobic vitamin B12 and 1, 2- propanediol metabolism genes compared to lean which expressed genes related to vitamin B6 metabolism (249). A recent study by Cottilard *et al.* (2013) has shown a reduced bacterial richness, reduced diversity and higher dys-metabolism and low-grade inflammation in obese vs. lean humans (252). Although dietary intervention partially improved gene richness, reduced measures of adiposity such as waist circumference and fat mas and reduced plasma cholesterol, it was less efficient in improving low grade inflammation (levels of hsCRP) (252). Furthermore, the tendency of the changes in gene clusters to return to the pre-dietary restriction phase suggests that gut microbiota tend to remain stable in individuals after the dietary stimulus is removed.

1.8.2 Gut microbiota as a consequence of dietary differences in obesity

Diet seems to play an important role in altering the proportion of gut microbiota in individuals because the amount and type of bacteria change significantly with change of diet (247, 253). This change varies between individuals and may be due to the distinct microbiota colonising the colon during early life, altering the capacity for energy harvest from the diet. Composition and caloric content of the diet significantly alters the relative abundance of the gut microbiota (253). An increased intake of resistant starch was shown to be associated with an increase in population boom of *Eubacterium rectale* (a butyrate producing bacteria) to ~10% and *Ruminococcus bromii* (an acetate producer) to ~17% compared to ~4% in volunteers consuming non-starch polysaccharides (253). These changes were seen to be reversed with weight loss diets along with a decrease in *Collinsella aerofaciens*, a member of Actinobacteria. This shows the substantial effect of the nature of diet on gut microbiota population and its energy harvesting capability in the form of short chain fatty acids (247, 253).

Compositional differences in the gut microbiota have been a subject of controversy driven by dietary and environmental factors. Zhang *et al.*(2009) showed an association between methanogenic archaea and obesity in lean, obese, and post-gastric bypass patients as they found more Prevotellaceae (hydrogen producing bacteria) and methanogenic archaea (hydrogen consuming bacteria) in obese people than lean subjects (167). In contrast, Schwiertz *et al.* (2010) (254) found low methanogenic bacteria in obese and overweight individuals along with a low Bacteroides and Firmicutes/Bacteroides ratio. SCFA production is affected by the nutrient load and amount of dietary carbohydrates available for fermentation. Weight loss diets usually have low carbohydrate and high protein content and have been shown to reduce the population of butyrate producing *Roseburia* and *Eubacterium rectale* (166). Human subjects on high protein/medium

carbohydrate (HP/MC) and high protein/low carbohydrate (HP/LC) diet had lower total SCFA while faecal butyrate was significantly lower on HP/LC diet than the HP/MC and maintenance diet. These carbohydrate-reduced diets may therefore help in reducing weight by reducing the substrate availability for the amount of energy extracted from the diet available for fermentation in the colon (166). In another study, faecal propionate was found to be significantly higher in obese than lean volunteers from the general population (254).

Changes in the anatomy (such as removal of part of the stomach in Roux-n-Y gastric bypass surgery) and as a consequence physiology (changes in pH, nutrient availability) of the gut have been shown to have an impact on gut microbiota composition (such as increase in Proteobacteria, *Bacteroidetes* and *Alistipes* and a decrease in *Blautia*, *Dorea*, *Lactobacilli*, and *Bifidobacteria*) and its relationship with change in the expression of genes related to host metabolism (such as those expressed in white adipose tissue) within 3 months after surgery (238). Whereas these changes are associated with post-surgical modifications in the dietary behaviour, about 50% of the associations were found to be independent of caloric intake. However, post-surgical changes in gut microbiota and the expression of genes between 0-3 months did not change in the long term between 3 months and 6 months which suggested restructuring of the gut microbiota and plateau of the response to changes in gut physiology.

It is argued that probiotics (such as *Lactobacillus paracasei* species *paracasei* strain F19) may beneficially affect host metabolic parameters when used as probiotics, which have been shown to beneficially affect energy homeostasis in weaning infants (4-13 months age) by reducing mono-unsaturated fatty acids (palmitoleic acid) associated with triglyceridemia in the short term (255). However, no differences in the serum lipids, glucose, insulin, and anthropometric measurements were seen in F19 intervention group compared to placebo group when the same cohort of children were followed at age 8-9 years (256). Another study by Weickert *et al.* (2011) found no significant difference in the total and individual faecal SCFA and gut microbiota relative abundance after 18 weeks intervention with diet supplemented with either high (43 g/day), moderate (26 g/day), or low (14 g/day) in cereal fibre (257). This indicates that several other factors including diet play their role in determining the overall health and energy homeostasis rather than gut microbiota and that the nature of the gut microbiota already established in the colon may be resisting "foreign microbiota" even though declared as beneficial to the energy homeostasis of the host.

In this context, factors affecting colonisation of the gut microbiota in the new-born from before birth to early and late childhood might play an important role. These were discussed in section 1.5.3. However, the role of these factors in determining the

colonisation of gut microbiota with tendency towards obesity or allergic disorders in later life is controversial. For example; higher numbers of *Bifidobacteria* and lower numbers of *Staphylococci* in breast fed children age 6 and 12 months had a negative correlation with overweight and obesity at 7 years (258). Similarly, the mode of delivery through caesarean section has been studied in relation to the development of overweight and obesity in male children in a population based cohort study of a Danish National birth Cohort(229). However, despite a larger cohort involving more than 10,000 children, the data was not adjusted for other confounding factors such as socioeconomic status, anthropometric and behavioural factors. In contrast, a recent Brazilian study following children born by caesarean section (n=5914) at age 4, 7, 15, and 23 years showed that although children born with caesarean section had ~50% higher prevalence ratio of obesity, this effect was no more significant when mode of delivery was adjusted for socioeconomic, demographic, maternal, anthropometric, and behavioural factors (259). This suggests that other factors play role in the genesis of obesity in children born with caesarean section.

In summary, an overall increase in the metabolic potential of the gut microbiota may be due to a change in the amount and nature of the diet which results in changes in the structural and functional distribution of the gut microbiota. The association is however complex as it is affected by many other factors such as colonisation of the gut microbiota in early infancy, maternal factors, and use of antibiotics.

1.8.3 Conclusion from human studies

Controversies exist as to whether or not obese and non-obese individuals differ in hosting a particular type of bacterial phyla or enterotype and whether the response of the gut microbiota to the nature of diet or a change in dietary habits differs between obese and non-obese individuals. Correlation of BMI with Bacteroides in obese and non-obese subjects on different dietary regimens (260) is unclear as an inverse relationship has also been observed (254), adding to the complexity of the relationship of diet, gut microbiota, and obesity. The population of gut microbes in the human intestines is affected by a variety of factors from birth till adulthood; of which some are known and others are largely unknown. Additionally, inter-individual variations have been observed almost universally in all human studies suggesting peculiar host-diet interaction at individual level that may affect the metabolic activities to contribute towards obesity, diabetes, and cardiovascular disease risks.

Table 1.10: Evidence from human studies about the role of gut microbiota in obesity

Reference	Study model	Aim of the study	Study design and outcomes measures	Results	Conclusion
Santacruz A *et al.* 2010 (247)	16 Overweight and 34 normal weight Pregnant women	To investigate the relationship between gut microbes, body weight, weight gain, and various biochemical parameters in pregnancy	Faeces by qPCR and blood samples for glucose, total cholesterol, HDL, TAG, LDL, urea, creatinine, uric acid, bilirubin, Iron, Ferritin, transferrin, folate, food 24-72 h food diaries for caloric intake. Weight of the babies at birth.	*Bifidobacteria* and *Bacteroides* significantly higher while *E coli* and *S.aureus* lower in normal weight. Total bacteria especially *S.aureus* positively correlated with cholesterol. *Lactobacillus* group negatively correlated with infant birth weight in women with excessive weight gain. Normal weight women had higher HDL, iron and folate	*Bifidobacteria* and *Bacteroides* may play a positive role in weight management of pregnant women and in their metabolic regulation.
Kalliomaki M. *et al.* 2008(258)	Children, 25 obese and 24 normal weight at 7 years age	To evaluate whether differences in gut microbiota at an early age precedes the development of atopy.	Subjects examined at 3, 6, 12, 24 months and 7 years. Gut microbiota composition at age 6 and 12 months by FISH, FISH with flowcytometry and qPCR.	Higher *Bifidobacteria* numbers and lower *S. aureus* at 6 and 12 months age in children remaining normal weight. More Bacteroides in obese and overweight children during 6 and 12 months than in normal weight children. Bifidobacteria constitute an internal link between breastfeeding and weight development	Higher numbers of *Bifidobacteria* and low numbers of *S. aureus* in infancy may provide protection against overweight and obesity development.
Zhang HS *et al.* 2009 (167)	3 Obese (OB), 3 normal weight (NW) and 3 post-gastric bypass (GB) patients	To compare the gut microbial community of normal weight, morbidly obese and post-gastric bypass surgery patients	DNA pyrosequencing and amplification by real time PCR.	GB group had a marked increase in Gammaproteobacteria, Enterobacteriaceae, and Fusobacteriaceae and fewer Clostridia. Prevotellaceae (H2 producing) enriched in the OB group compared with the NW group. Methanobacteria (H2 consuming bacteria of the group Archaea) were found more in obese group.	Confirm an association between methanogenic Archaea and obesity. H_2 uptake by methanogens accelerates fermentation by H_2-producing Prevotellaceae, which leads to increased production of acetate.
Duncan SH *et al.* 2008 (260)	33 obese and 24 non-obese subjects	To examine the relationships between BMI, weight loss and the major gut microbial groups	16S rRNA sequencing using FISH, quantitative PCR. Dietary intervention with high protein low carbohydrate ketogenic diet and high protein moderate carbohydrate non-ketogenic diet	No difference in total bacteria and Bacteroides between obese and non-obese. No significant relation between BMI and Bacteroides. *Bacteroides* were not affected either by diet or by diet order, and no significant relationship between number of Bacteroides and weight loss. Reduction in *Roseburia-Eubacterium rectale.* Reduction in *Bifidobacteria* after 4 weeks of low carbohydrate weight loss diets.	No relationship of Bacteroides and Firmicutes ratio at phylum level with obesity. Low carbohydrate weight loss diets results in reduction in butyrate producing bacteria *Roseburia-Eubacterium rectale,* together with *Bifidobacteria*
Duncan SH. *et al.* 2007 (166)	20 obese healthy volunteers	To evaluate the effect of high protein and low fermentable carbohydrate diet on gut microbiota activity and population.	Dietary intervention with maintenance, high protein medium carbohydrate and high protein low carbohydrate diets. Bacterial enumeration with FISH and butyrate with GC	Total SCFA were lower during consumption of the HPMC and HPLC diets. Butyrate was lower for the HPLC than for the HPMC diet. Butyrate proportion decreased as carbohydrate supply was lowered. Most abundant bacterial group was *Cytophaga-Flavibacterium-Bacteroides* group	butyrate production and counts of certain bacteria are largely determined by the content of fermentable carbohydrate in the diet

				and *Clostridial cluster* IV. Bacterial count reduced significantly with reduction in carbohydrate including close relatives of *Roseburia intestinalis* and *Eubacterium rectale*. *Faecalibacterium prausnitzii* had fewer roles in fermentation.	
Nadal *et al.* 2009 (261)	39 obese adolescents	Effect of weight loss intervention on the faecal gut microbial composition and immunoglobulin coating bacteria and its relationship to weight loss.	Restricted calories diet and increased physical activity for 10 weeks. BMI, BMI z-scores before and after intervention. Microbiota by FISH and immunoglobulin coating bacteria by fluorescent-labelled F(ab')2 antihuman IgA, IgG and IgM	*Clostridium histolyticum, Eubacterium rectale-Clostridium coccoides* groups' decreased count with weight loss. *Bacteroides Prevotella* increased and total faecal energy decreased upon weight loss of >4 kg. IgA coating bacteria decreased with weight loss of >6 kg.	Changes in adolescents' body weight is linked to specific gut microbiota and an associated IgA response in obesity after lifestyle interventions
Walker AW *et al.* 2010 (253)	16 obese stable weight subjects	To examine the influence of the precisely controlled diet on the human colonic microbiota population and composition	Intervention with maintenance diet, resistant starch, non-starch polysaccharide, low carbohydrate diet, and wheat bran. Chemical analysis of diet composition and digestibility. Real time qPCR, denaturing gradient gel electrophoresis (DGGE).	Marked Inter-individual variation was noted. *Ruminococcus bromii* increased with RS diet. Oscillibacter group increased on the RS and WL diets. Relatives of *Eubacterium rectale* increased on but decreased, along with *Collinsella aerofaciens*, on WL.	Depending on the initial composition of gut microbiota of an individual, different dietary carbohydrates can produce substantial changes in gut bacterial diversity.
Tihonen K *et al.* 2010	40 obese and non-obese adults	To compare obese and lean individuals' gut bacterial and immunological biomarkers with blood glucose, lipids, satiety related hormones and inflammatory markers.	Interview for dietary fibre, anthropometry, faecal sample for microbiota diversity using PCR and inflammatory markers. Blood biochemistry for hormones and inflammatory markers	IL6, CRP, Insulin, TAG and leptin rose in obese. BCFA and phenolics increase in obese faecal samples indicate increased bacterial fermentation due to protein rather than carbohydrates. waist circumference and Bacteroides were inversely correlated while positively correlated with IL6	Increased phenolics and lactic acid in intestine of obese subjects most probably have an effect on the physiology of systemic inflammatory condition.
Larsen N *et al.* 2010 (262)	36 adults; diabetic (n=18) and non-diabetic controls (n=18).	To assess the differences between gut microbiota of diabetic and non-diabetic persons	Bacterial composition of faecal samples by real time PCR and by tag-encoded amplicon pyrosequencing of V4 region of 16S rRNA gene	Bacteroides, Proteobacteria and lactobacilli more in diabetics, Firmicutes (clostridium group) were higher in non-diabetics. Ratio of *Bacteroides Prevotella* group to *C.coccoides-E. rectale* group positively correlated with glucose level and negatively correlated with BMI. B-Proteobacteria highly enriched in diabetics and positively correlated with plasma glucose levels.	Reverse Firmicutes to Bacteroides ratio in diabetic patients indicate a different Bacterial composition in this group. Increased number of Gram negative bacteria may explain the chronic low grade inflammation in diabetic patients.

Santacruz A *et al.* 2009 (263)	18 male and 18 female overweight and obese adolescents	The evaluate the influence of weight loss intervention on the gut microbiota and body weight of overweight adolescents	Energy restricted diet and increased physical activity to all participants. Anthropometric measurements, food diaries and faecal sample for qPCR	Two distinct groups emerged with high (>4 kg) and low (<2 kg) weight loss groups. In Overall groups and in high weight loss group; increase in *Bacteroides fragilis*, *Lactobacillus* group and decrease in *C.coccoides*, *Bifidobacterium longum* and *Bifidobacterium adolescentis*. In high vs. low weight loss groups. Total bacteria, *B. fragilis* group and *Clostridium leptum* group, and *Bifidobacterium catenulatum* group counts significantly higher while levels of *C. coccoides group*, *Lactobacillus* group, *Bifidobacterium*, *Bifidobacterium breve*, and *Bifidobacterium bifidum* significantly lower in high vs. low weight loss groups	Correlation of gut microbiota with body weight may be sensitive to the life style intervention such as weight loss to a different extent depending on the composition of gut microbiota of an individual
Schwiertz A *et al.* 2010 (254)	30 normal weight, 35 overweight and 33 obese adults	To evaluate the differences in gut bacteria and faecal Short chain fatty acids between lean and obese individuals	Faecal samples for quantitative PCR and SCFA analysis	>20% higher SCFA in stools of obese than lean, with higher propionate and butyrate. Significantly higher Bacteroides in overweight than lean but not obese. Overweight and obese had higher Bacteroides and lower *Ruminococcus flavifaciens* and *Methanobrevibacter*. *C.leptum* and *Bifidobacteria* significantly lower in obese only. Positive correlation between BMI and propionate, % propionate, Bifidobacteria and Methanobrevibacter even after correction for the influence of age and gender.	Because of controversial results, no specific bacterial group can be attributed to obesity at this stage.
Armougom F *et al.* 2009 (240)	Obese (n=20), normal weight (n=20) and anorexia nervosa(n=9)	To determine the role of *Methanobrevibacter smithii* and *Lactobacilli* in patients with abnormal weights using real time PCR	Real time PCR	Reduction in the *Bacteroidetes* community and higher *Lactobacillus species* in obese patients than in lean controls or anorexic patients. *M. smithii* much higher in anorexic patients than in the lean population.	*Lactobacilli* used as probiotics may be linked to obesity. *M. smithii* in Anorexia Nervosa patients may represent an adaptive response to the disease.
Turnbaugh PJ *et al.* 2009 (264)	31 Adult Mono- and 23 Di-zygotic (MZ & DZ) female twins and their mothers (n=46)	To assess how gut microbiome is influenced by the host genotype, external environment, and the extent of host adiposity	UniFrac analysis, and gut microbiota assessed by 16SrRNA pyrosequencing	No significant difference in the degree of similarity in the gut microbiota of adult MZ versus DZ twin-pairs. Decreased Bacteroides and increased Actinobacteria in obese. Difference in Firmicutes was not significant. Glucosyltranferases were similar in all individuals while glycoside hydrolases were variable. Phosphotransferases involved in microbial processing of carbohydrates were rich in obese.	Genomic profile of microbiota exists at a level of metabolic function and not by a definite set of microbiota.

Collado MC *et al.* 2010 (248)	Overweight and obese mothers (n=16) with their infants and non-obese mothers (n=26) with their infants	To evaluate the faecal microbiota of infant born to overweight and normal weight mothers and to find out their relationship with the weight and weight gain of mothers during pregnancy.	Faecal sampling of infants, weight of mothers before and during pregnancy. Real time PCR and FISH with flowcytometry for bacterial composition	Bacteroides and *S.aureus* higher in infants of overweight mothers. Higher weights and maternal BMI related to higher concentrations of *Bacteroides*, *Clostridium*, and *Staphylococcus* and lower concentrations of the Bifidobacterium group. Lower counts of *Akkermansia muciniphila*, *Staphylococcus*, and *Clostridium dificile* groups and higher no. of *Bifidobacteria* in infants of normal weight mothers and those with normal pregnancy weight gains.	Lower *Bifidobacteria* and higher *S.aureus* associated with obesity in children. BMI, weight and weight gain of mothers before and during pregnancy affects the gut microbiota composition in infants
Ley *et al.* 2006 (164)	12 obese Human adults, followed over a period of 1 year	To show that the relative proportion of Bacteroidetes group of bacteria in the gut of obese people are more as compared to lean individuals	16S rRNA gene sequence library of gut microbiota in obese subjects on weight reduction diets (low carbohydrate or low fat, n = 12)	Gut bacteria are remarkably constant in individuals. Relative proportion of Bacteroidetes increased compared with Firmicutes and correlated with percentage of weight loss	The gut in obesity exerts ecological pressure promoting a higher relative abundance of Firmicutes
Jumpertz R *et al.* 2011 (265)	12 lean and 9 obese adults	To assess the influence of change in nutrient load on the gut microbiota of lean and obese individuals and correlation of microbiota with energy harvest from the diet	Stool and urine energy content with change in caloric content of diet, culture independent metagenomic studies of microbiota	Nutrient load caused 20% increase in Firmicutes and corresponding decrease in Bacteroides in lean subjects with approximately 150 kcal increase in energy harvest from diet	Nutrient load affects gut microbiota composition which is also associated with increased energy harvest from the diet
Bergstrom *et al*.2014 (243)	Healthy Danish infants (n=330) at 9, 18, and 36 months of age	Characterization of gut microbiota of infants at different ages	qPCR, DEXA and bio-electrical impedance analysis for body composition, barcoded food diary for 7 days for dietary analysis	At 9 months: higher *Lactobacilli*, *Bifidobacteria*, and *Enterobacteria*. At 18 months; Firmicutes (particularly *C.leptum, E.halii, and Roseburia*) and Bacteroidetes increase while *Bifidobacteria, lactobacilli*, and *Enterobacteria* decrease except *B. adolescentis. At 36 months;* high Firmicutes, Bacteroidetes, and small fraction of Actinobacteria, Proteobacteria, and Verrucomicrobia. No correlation of specific bacteria with mode of delivery, dietary or anthropometric parameters. Increase in BMI between 9-18 months was associated with higher Firmicutes	Significant differences occur between 9 and 18 months, and changes at 36 months are independent of breast feeding at early age. Butyrate producers positively correlated with BMI might indicate increased capability of energy harvest

Bervoets *et al.* 2013 (239)	Overweight and obese chidlren (n=26), healthy lean children (n=27) age 6-16 years,	To assess differences in gut microbiota between lean and obese children	Selective plating and qPCR, MALDI-TOF-MS for detailed study of *Bacteroides fragilis* group. Dietary records for dietary intake	High Firmicutes to Bacteroides ratio in obese vs. lean. Low *B. vulgatus* and high *Lactobacillus* spp. in obese vs. lean. In all groups, *S. aureus* positively associated with energy intake. *Lactobacilli* in obese children positively associated with plasma CRP.	Obese microbiota are different from lean.
Calvani *et al.*2010 (241)	Morbidly obese (n=15), and healthy lean (n=10) adults, age	To assess differences in gut microbiota-associated urinary metabolites between obese and lean and how these differences are affected by biliopancreatic or Roux-en-Y surgery	high-resolution proton NMR (1H NMR) spectroscopy	Baseline: Lower levels of hippurate, xanthine, and trigonelline and higher levels of 2-hydroxybutyrate in obese vs. lean. Inverse relationship of xanthine with plasma uric acids levels 3 months after surgery: reversal of the above metabolites with weight loss	Obese phenotype is associated with a peculiar metabotype compared to lean. These metabolic changes are reversed with bariatric surgery
Cotillard *et al.* 2013 (252)	Obese (n=38) and overweight (n=11) adults	To investigate temporal relationship between food intake, gut microbiota, and metabolic and inflammatory phenotype	6 week energy restricted, high protein diet followed by 8 weeks weight maintenance period, food diaries, quantitative metagenomics	Gene counts showed bimodal distribution. Patients with low gene count (<480,000 genes) had a tendency towards higher LDL, dys-metabolism, insulin resistance, inflammation and obesity and vice versa for high gene count. Weight loss diet partially reduce inflammation and improve dys-metabolism but not to full extent	Obesity is associated with lower gene richness which is partially corrected by dietary intervention
Druart *et al.*2014 (242)	Obese women (n=15)	To investigate the effect of prebiotic induced gut microbiota modulation on PUFA derived bacterial metabolites production	Inulin type fructans (oligofructose 50/50) supplementation (16g/day) for 3 months, qPCR, Human intestinal tract chip analysis, circulating fatty acids levels (UFA derived bacterial metabolites)	Treatment with prebiotics did not affect levels of PUFA derived conjugated linoleic and linolenic acids. PUFA derived bacterial metabolites were negatively correlated with total cholesterol, LDL, and HDL, while positively correlated with *Bifidobacterium* spp., *Eubacterium ventriosum*, and *Lactobacillus* spp.	
Fernandes *et al.* 2014 (176)	Overweight and obese adults (m=37, age 21-60 years), lean adults (n=52, age 18-67 years)	To investigate dietary intakes, faecal SCFA, gut microbiota composition and physical activity levels in simple obese vs. healthy lean adults	3 day food diary, breath methane and hydrogen, faecal SCFA, qPCR	Higher acetate, propionate, butyrate, valerate, and total SCFA in obese vs. Lean. No difference in Firmicutes to Bacteroides/Prevotella ratio between lean and obese. Higher *E.coli* in lean compared to obese. Irrespective of the group, total faecal SCFA were negatively correlated with Bacteroides/Prevotella and positively correlated with Firmicutes/Bacteroides ratio	Obese phenotype carries distinct energy harvesting capability than lean. However, the evidence is not conclusive due to limitations such as small differences, age differences between participants, methodology and sample processing procedure.

Ferrer *et al.*2013 (249)	Obese adolescent (n=1), lean adolescent (n=1)	To perform a holistic phylogenetic and functional analysis of the gut microbial communities of the lean and obese microbiome	454 FLX pyrosequencing, Orbitrap MS/MS	Lean microbiome more diverse than obese. High Firmicutes (~95% vs. 78%) and low Bacteroidetes (~4% vs. ~18%) in obese vs. lean. Obese metagenome associated with vitamin B12 and 1, 2-propandiol metabolism while lean metagenome with B6 metabolism. High butyrate production in obese than lean	Lean and obese metagenome and microbiome differ from each other however; both shows functional redundancies in terms of proteins expression
Karlsson *et al.* 2012 (244)	Overweight and obese (n=20), lean (n=20) children	To investigate differences in faecal gut microbiota between lean and obese children	qPCR and RFLP, liver function tests	Significantly higher *Enterobacteriaceae* and significantly lower *Desulfovibrio* and *Akkermansia muciniphila i*n obese than lean. No difference in *Lactobacillus*, *Bifidobacterium*, and *Bacteroides fragilis* between lean and obese. Serum alanine aminotransferase negatively correlated with *Bifidobacterium.* No difference in faecal calprotectin between lean and obese.	Differences in gut microbiota composition exist at an early age between lean and obese. The study is however crossectional. Not controlled for diet, and based on qPCR
Kong *et al.*2013 (238)	Morbidly obese women (n=30)	To assess the impact of Roux en Y gastric bypass surgery (RYGB) on the gut microbial population and its effect on the genes expression in white adipose tissue (WAT)	454 GS-FLX Pyrosequencing of faecal samples at 0, 3, and 6 months after RYGB, dietary assessment,	Increase in Proteobacteria after RYGB by 37%, increase in association between 102 genera and 562 WAT genes. Bifidobacteria and Firmicutes such as *Dorea*, *Lactobacilli*, and *Blautia* decreased while Bacteroides such as *Bacteroidetes* and *Alistipes* and Proteobacteria such as *E.coli* increased after 3 months. About 50% of changes in genes expression were independent of caloric intake. No difference seen between 3 and 6 months	Gut microbiota richness increase after RYGB with changes in associations with genes expression in WAT. Further exploration of gut microbiota with weight loss is needed
Weickert *et al.* 2011 (257)	Overweight and obese adults (n=69, age 24-70 years)	To investigate mechanisms for the effect of high cereal fibre on insulin sensitivity by exploring gut microbiota composition and colonic fermentation	18 weeks intervention with cereals. Gas chromatography for SCFA. *In-vitro* fermentation on healthy volunteer faeces with fibres, FISH and flowcytometry. Euglycemic clamp for insulin sensitivity	No difference in faecal SCFA at 0, 6, and 18 weeks. No differences in SCFA with *In vitro* fermentation. Roseburia tended to decrease, Clostridium cluster IX decreased after 6 weeks but not at 18 weeks, Atopobium increased after 18 weeks. Insulin sensitivity improved after 18 weeks	Improvement in insulin sensitivity is not associated with colonic microbiota metabolism and fermentation
Brignardello J*et al.* 2010 (266)	13 obese and 11 normal weight adults	Evaluation of gut permeability in asymptomatic obese and its relationship with plasma and faecal markers of inflammation and alteration in gut microbiota	Lactulose- mannitol sucralose test for intestinal permeability, blood CRP and fatty acids. Faecal G+C profiling, calprotectin and leptin	CRP levels were significantly higher in obese than non-obese individuals. Faecal fat, calprotectin and leptin and ARA/EPA were not significantly different in both groups. Obese subjects had significant increase in relative abundance bacteria with 23-37% G+C contents in their DNA and significant decrease in the relative abundance of those with 40–47% and 57–61% of G+C content. G+C peak values negatively correlated with CRP values.	Gut microbiota differ between obese asymptomatic and non-obese. High CRP levels in asymptomatic obese individuals do not have signs of gut inflammation

1.9 Conclusion and proposed area of research

Recent decades have witnessed an increase in prevalence of obesity in pandemic proportions both in adults as well as children. Several factors have been identified in the literature to explain the aetiology and pathogenesis of obesity such as factors related to diet, life style, environmental factors, and host genetic factors. However, as discussed in detail in this chapter, none of these factors fully explain the aetiology of obesity and the search for possible causes of obesity is still under exploration. The gut microbiota have recently been advocated as one of the factors affecting host energy homeostasis through several putative mechanisms investigated in mouse models and some human studies (Table 1.5). However, several studies in animal and humans have suggested a profound effect of diet on the changes in the gut microbiota to influence host metabolism towards a lean and obese phenotype.

When taken together, evidence linking gut microbiota to the increasing epidemic of obesity in animal and human studies is inconclusive and controversial to suggest a "cause or effect" relationship. There are limitations in exploring this relationship partly due to differences in methodology, study designs, lack of control over the diet, genetic propensity of individuals to obesity, and other life-style factors. Moreover, faecal samples are the usual source of gut microbiota used in human studies which may not represent the true picture of the gut microbial population. Access to the full length of the gut is restricted for medical or ethical reasons. In addition; differences between animals and human beings such as differences in intestinal microbiota, metabolic rate, and length of intestine, caecal fermentation, coprophagy (habit of rodents eating their own faecal matter) and genetic variability limit the extrapolation of results from animal studies to humans.

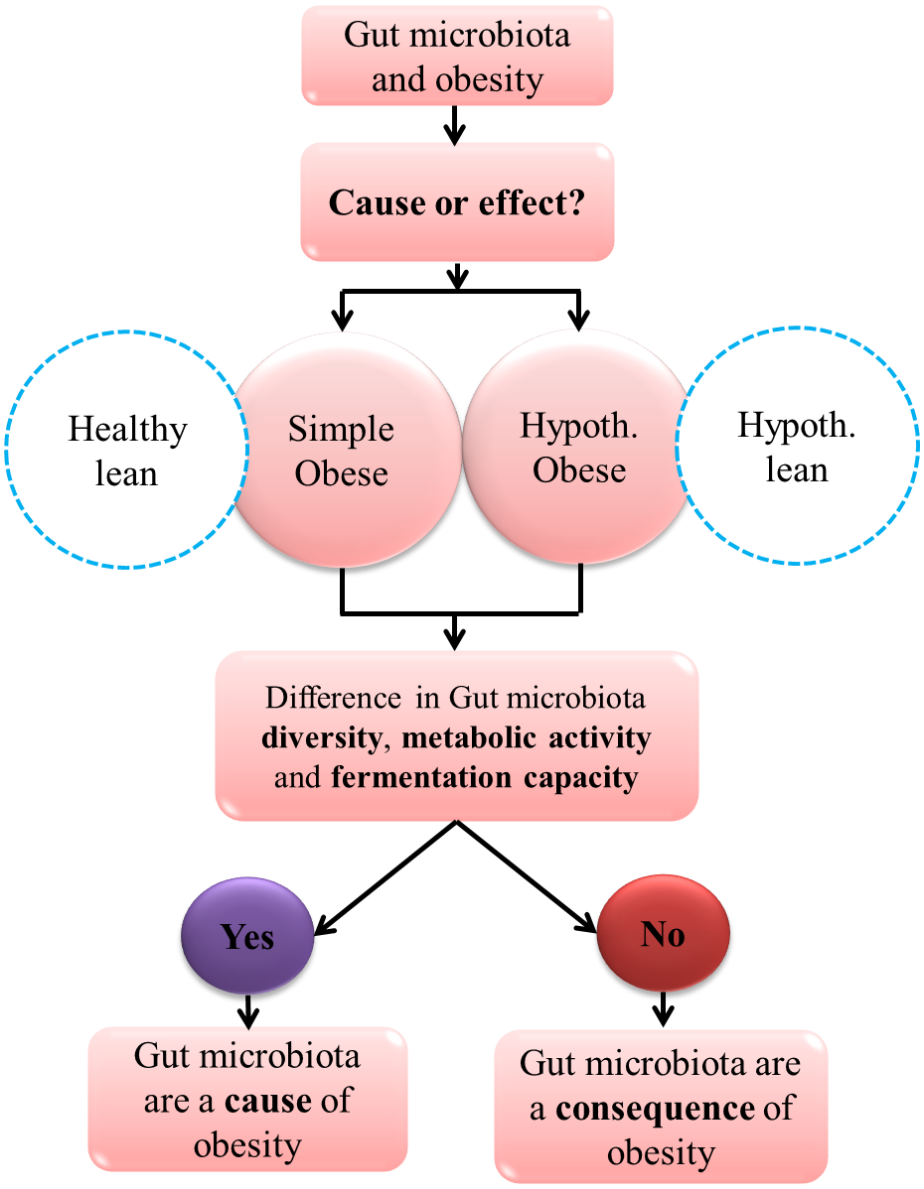
Whether gut microbiota are causally related to the aetiology of obesity or it is an effect of differences in dietary intakes between lean and obese people is a question unanswered and less well explained. Most of the studies thus far have studied obesity only in relation to lean phenotype and have therefore been unable to establish reverse causality. Furthermore, obesity in childhood is linked to obesity in adulthood and gut microbiota metabolic activity and composition is a subject of great controversy in this age group similar to that in adults. To help unravel this conundrum, we designed this observational study comparing obesity of known versus unknown aetiology to investigate differences in the gut microbiota metabolic activity and composition using high throughput sequencing technology. A difference in the gut microbial composition and metabolic activity between known and unknown cause of obesity would implicate a causal relationship of gut microbiota with obesity.

Out of thousands of bacterial species in the gut, very few have been cultured so far. Therefore new culture independent molecular microbiology techniques have been developed (267) of which most high throughput analysis techniques used are barcoded pyrosequencing and phylogenetic microarrays (268, 269). Most recent advances in molecular microbiology focus on functional studies with suffix "-omics": including metabolomics, proteomics, and metatranscriptomics. This has resulted in an extensive catalogue of 3 million non-redundant bacterial genes which has been derived from 124 European subjects and 178 intestinal bacterial species metagenome (270, 271) including healthy volunteers, obese subjects and patients with Inflammatory Bowel Disease (IBD). Development of these techniques has further enhanced our understanding of the diet-host-gut microbiota relationship.

Many studies report faecal SCFA in relation to specific gut microbiota as a surrogate marker of colonic fermentation capacity (table 1.5). Since, faecal gut bacterial metabolites (such as SCFAs) might be the result of either an increased production in the colon or malabsorption in the gut, the actual colonic fermentation capacity might be over or under-estimated. Incubating faecal samples in batch cultures with a range of dietary fibres mimicking gastrointestinal condition might therefore be a practical and reliable way to establish the energy harvesting capability of the gut microbiota. To date only 4 studies have measured fermentation capacity of gut microbiota of obese and lean individuals and none in children. To establish the energy harvesting capability of the gut microbiota in obesity of different aetiology, we therefore incubated faecal samples in *in-vitro* batch culture fermentation using 5 dietary fibres.

By and large the evidence to prove reverse causality is still missing. The aim of this PhD journey was therefore to investigate the relationship of gut microbiota diversity, metabolic activity, and fermentation capacity in a set of patients with known of cause of obesity (pathological or hypothalamic obesity) and compared them with an unknown cause of obesity (simple or classical obesity) (Figure 1.9).

Figure 1.9: Schematic representation of the study question for this PhD



Chapter 2: Subjects and Methods

This chapter describes the study design, eligible participant characteristics, research methodology, data handling, statistical analysis and process of ethics approval.

2.1 Study design

This was a prospective observational study, carried out as a joint collaboration of the Unit of Human Nutrition, University of Glasgow and the Departments of Paediatric Endocrinology and Paediatric Dietetics, Royal Hospital for Sick Children Yorkhill, Glasgow. The study was approved by the West of Scotland research Ethics committee (WoREC) and Research and Development department of National Health Service (R&D NHS) Greater Glasgow and Clyde on 14th of September 2011 for a period of 4 years under the study reference number WS/11/032 and title "Diet, gut microbiota, and energy from colonic fermentation of dietary carbohydrates in children with simple and pathological obesity; cause or effect?" (appendix 1).

2.2 Recruitment

2.2.1 Definition of obesity and study participants

For the purposes of this study "obesity" was defined in those under 24 years as a BMI SDS more than or equal to 2 standard deviations (≥2 SD). Those with a BMI SDS less than 2 SD were classed as lean. Participants aged more than or equal to 24 years were classed as obese if their body mass index was ≥30 kg/m^2 based on the International Obesity Task Force criteria for obesity in adults (4). Eligible participants for this study were:

A. *Children and young adults with classical/simple obesity:* This group included children and young adults who were classified as obese in the presence of unknown aetiology of obesity. This group of children were recruited from the weekly dietetic outpatient clinics in Royal Hospital for Sick Children Yorkhill Glasgow. The dietetic clinics manage obese patients who are either referrals from GP surgeries or the community weight management programs for the dietetic management of obesity.

B. *Hypothalamic/pathological obesity:* These were those whose obesity was related to a known medical cause, usually related to malfunctioning hypothalamic control of

dietary intake (e.g. craniopharyngioma or due to a genetic syndrome such as Prader-Willi Syndrome (PWS)).

C. *Lean hypothalamic group:* As not all of the PWS children were obese, those with normal BMI were assigned to a separate subgroup (lean hypothalamic group) who would act as a "control" for the obese children with the same pathology.

D. *Healthy lean children and young adults* with normal weight were recruited as a control group (healthy participants with no chronic disease or other illness: Normal weight participants had a BMI within 2 standard deviations of the mean for that particular age and sex or between 18-25 kg/m^2 in the case of adults).

E. *Parents of the above four groups* (simple obese, hypothalamic obese, hypothalamic lean and healthy lean control): The aim of their optional recruitment was to analyse their gut microbiota for assessment of similarity and differences of bacterial community structure and metabolic activity with their children. This group included adult parents only of the recruited participants. However, data generated as a result of parents' recruitment, body composition, and collected faecal samples were not included in this thesis.

2.2.2 Exclusion criteria

Each patient was screened using a health check questionnaire to rule out conditions related to changes in physiology of the gut which can alter gut microbiota composition and metabolic activity such as gut surgeries, immune disorders of the gut, systemic use of probiotics and prebiotics, or medications. Specifically exclusion criteria were:

A. Participants who had an active gastrointestinal condition or any surgical procedure involving removal of part or the whole gut.
B. Use of antibiotics in the past 4 weeks.
C. Systemic use of pre- or probiotics, defined as a daily use of prebiotics or probiotics. The use of certain complex polysaccharides (prebiotics) or live cultures of bacteria (probiotics) have also been shown to alter the metabolic activity and gut microbial diversity. Therefore children who were prescribed with or using probiotic drinks as daily food component were excluded from the study.

2.2.3 Recruitment of subjects

2.2.3.1 Recruitment of hypothalamic obese subjects and their parents

Participants in this group were recruited from the 4-monthly endocrine PWS or craniopharyngioma clinics. This clinic manages patients from the West of Scotland and some parts of Northern England. Potential participants were first identified by the consultant endocrinologist from the hospital database. The age range of these participants varied between 2-25 years. Information sheets about the study were sent to all identified patients under the age of 25 years, 7 days prior to their regular clinical appointment. These information sheets aimed to inform the eligible participants and their parents about the study, and that a researcher would approach them to ask if they were willing to participate.

On the day of visit to the hospital, these patients were introduced to the study by the clinician. If the participants were willing to know more about the study, the researcher would discuss the study with them. The participants were then given enough time to ask any questions about the project. If the participants and their parent/carer agreed to participate, the researcher would ask them to sign a consent form in triplicate; one for the patient, the researcher, and the hospital records. They were then given instructions by the researcher about sample collection, a food frequency questionnaire and 24 hour food diary for dietary records. They were also given contact information for mail, email or phone if they had any queries in future.

2.2.3.2 Recruitment of simple obese subjects and their parents

Participants in this group were recruited through weekly dietetic clinics. Participants attending these clinics were recruited in the same way as hypothalamic obese patients.

2.2.3.3 Recruitment of control group and their parents

Participants in the control group and their siblings were recruited through advertisements and by word of mouth. We tried to match their age (2-16 years and above) and demographic characteristics with those of simple obese and pathological obese groups; however, this was not successful. The advertisements were posted in and around the hospital, the University, and different parts of Glasgow. Interested individuals were requested to contact the researcher through e-mails or phone. All interested responders were sent information sheets through the post or electronically via email. If they were happy to participate in the study, they were requested to arrange a meeting with the researcher either at the hospital, or a place of their own convenience, or at their home if

they were happy for the researcher to come. To check for their eligibility for the study, the participant were asked questions regarding any chronic disease, use of medication, use of antibiotics over the last 4 weeks, and regular use of probiotics. Participants who met the eligibility criteria and were happy to participate gave written informed consent. Dietary information was collected as described below. They were instructed by the researcher about collection of faecal samples. Contact information was given to the participants for queries they might have.

2.2.3.4 Follow-up assessments

The process of health check, sample collection, and 24 hour food diary was repeated after 2-3 months for the participants only. Parents were asked to give only one sample, if they were happy to participate.

2.2.4 Collection of dietary information

Dietary information was collected by a 24 hour food diary. The participants or their parents on the participant's behalf were asked to record all food intakes throughout 24 h starting from one time of the day chosen by the participants at their own convenience. Questions were asked about the time when food was taken (e.g. 8 am), details of the food (e.g. cornflakes, semi-skimmed milk and sugar), quantity of the food consumed (e.g. a bowl of cereal in one cup of semi-skimmed milk and half teaspoon white sugar), method of preparation (e.g. un-cooked, cooked, boiled, grilled etc.), and method of food serving (e.g. cold or hot, refrigerated, frozen etc.)

Participants were asked to fill in the food diary prior to the day they were intending to provide a faecal sample to match the faecal metabolites with the diet being consumed. They were also encouraged to fill the food diary prospectively, starting from early morning, to reduce the chance of memory-recall bias or insufficient (<24 h) food entry. However, some participants filled the diary in retrospect (n=10).

The food diaries were collected at the same time as faecal samples or were handed in by the participants in the clinic at the time of recruitment or in some cases, were posted after the sample was collected.

2.2.5 Sample collection and processing

At the time of signing the consent, every participant was given a faecal sample collection kit. Each kit had a pre-weighed stool collection pot, a plastic bag to enclose the pot along

with an anaerobic gas production kit(Anaerocult® A Merck KGaA 62471 Darmstadt, Germany or Anaerogen™ Compact Oxoid limited Hampshire, England), a cool pack (to keep the sample cold), a pair of gloves (for sample handling), a paper bed pan (for use on the toilet seat to facilitate sample collection), and an instruction sheet (for sample collection and handling at home and contact details of the researcher). A "possible" sample collection day was agreed between the parents and the researcher. Courtesy calls and reminders were made every two weeks in cases where the sample was not available on the agreed day. When the sample was ready for collection, the participant or parent would call the researcher immediately. The participants in most cases asked the researcher to come and collect the sample or brought the sample themselves, or rarely, sent it via a taxi. Any expenses incurred by the participant in relation to the study participation were reimbursed. The radius of the distance from which the samples were collected is shown in Figure 2.1.

Figure 2.1: Approximate radius for sample collection.

Outer circle represent approximately 120 miles radius. About 15% participants fall in the outer circle. The public transport time from the farthest point on this radius would take at least 5 h to and from the lab. This has been a major limitation in processing the sample within 4h period

2.2.6 Progress of recruitment

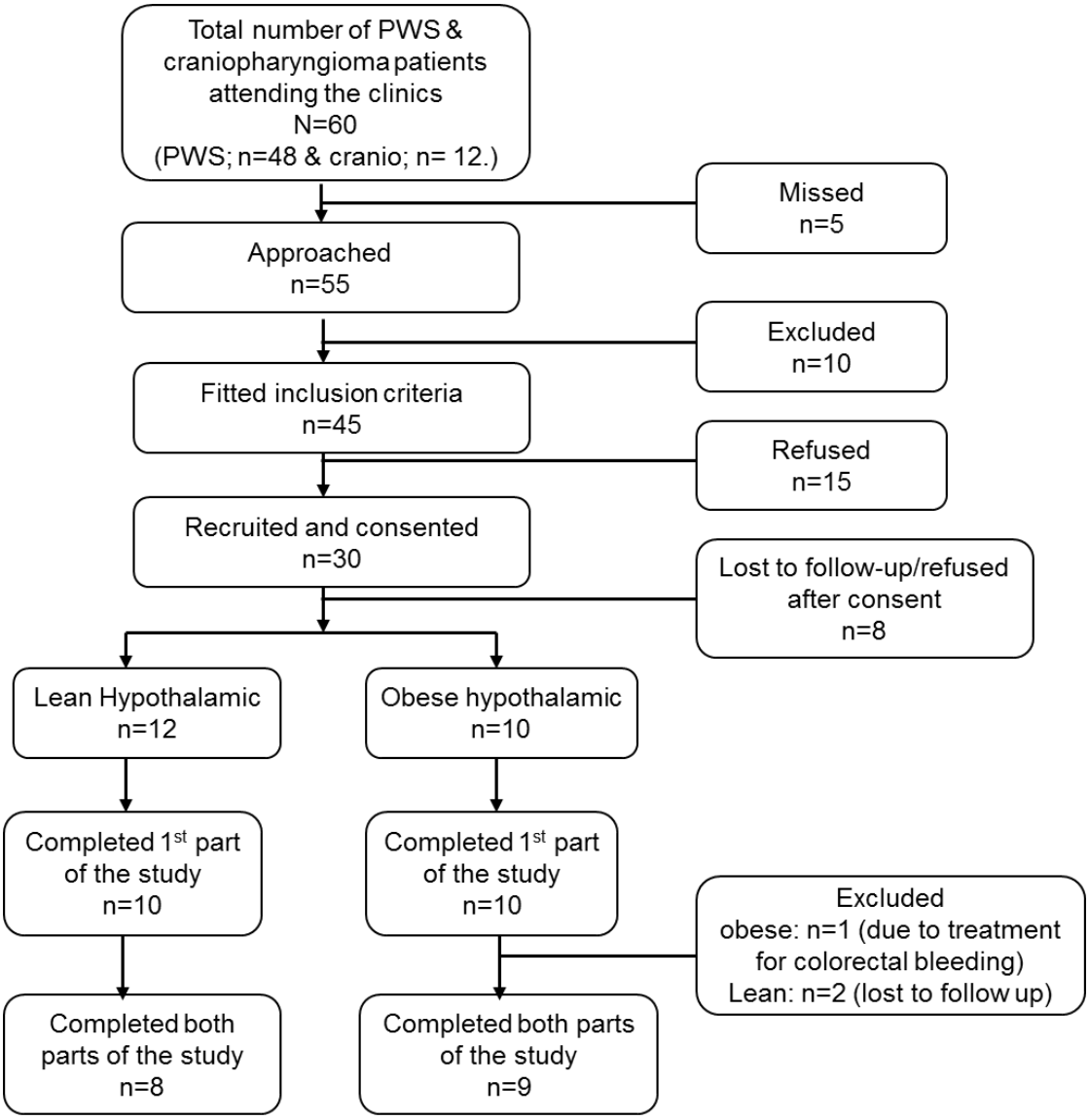
2.2.6.1 Recruitment of PWS and craniopharyngioma patients

Recruitment of this group began from the first available clinic on the 28th of October 2011 and ended at the January 2013 clinic. In total 5 clinics (each after every 4 months) were

attended during this period for recruitment purposes. Forty eight PWS patients including adults more than 25 years attending the Yorkhill endocrine clinics were approached. Participants with age >25 years (n=7) or having severe co-morbidities (n=2) or using regular probiotic drinks (n=1) were excluded from the study. Amongst patients attending craniopharyngioma clinics (n=12), one patient age >25 years was excluded from the study, 5 patients declined to participate, while one patient did not respond after giving written informed consent. Response rate of the participants in this group after consent was 63% (Figure 2.2).

Parents/carers of the recruited participant in this group were also requested to take part in the study by giving single faecal sample. Thirteen of the 25 parents managed to give a faecal sample while others refused after giving consent (n=10) or did not respond after consent (lost to follow up) (n=2).

Figure 2.2: Progress of recruitment for PWS & Craniopharyngioma group

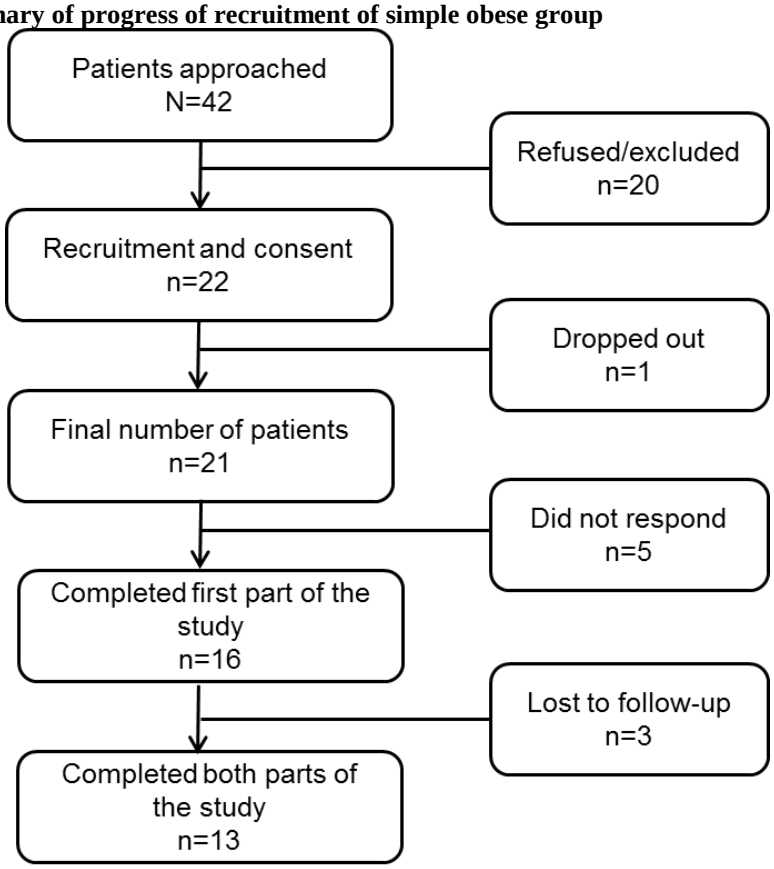


Missed; those participants who did not attend the clinics during this period and were not available on phone or did not reply to the information sheets sent at the address, n; number of participants

2.2.6.2 Recruitment of simple obese group

Despite more regular clinics than the PWS and craniopharyngioma clinics, this group was the least successful among all the three groups to recruit due to a high refusal and a low attendance rate. A summary of the participants recruited in this group is given in Figure 2.3. On average, weekly clinics were scheduled for 2-3 simple obese patients who were eligible for participating in the study, in addition to other obese or non-obese children with other diseases and who were not eligible for the study. Response rate of the participants in this group after consent was 72.7%. Of 17 parents of children in this group who initially consented to give a faecal sample; 9 were able to give one sample, 1 parent refused after giving consent, and 7 parents did not respond after consent.

Figure 2.3: Summary of progress of recruitment of simple obese group

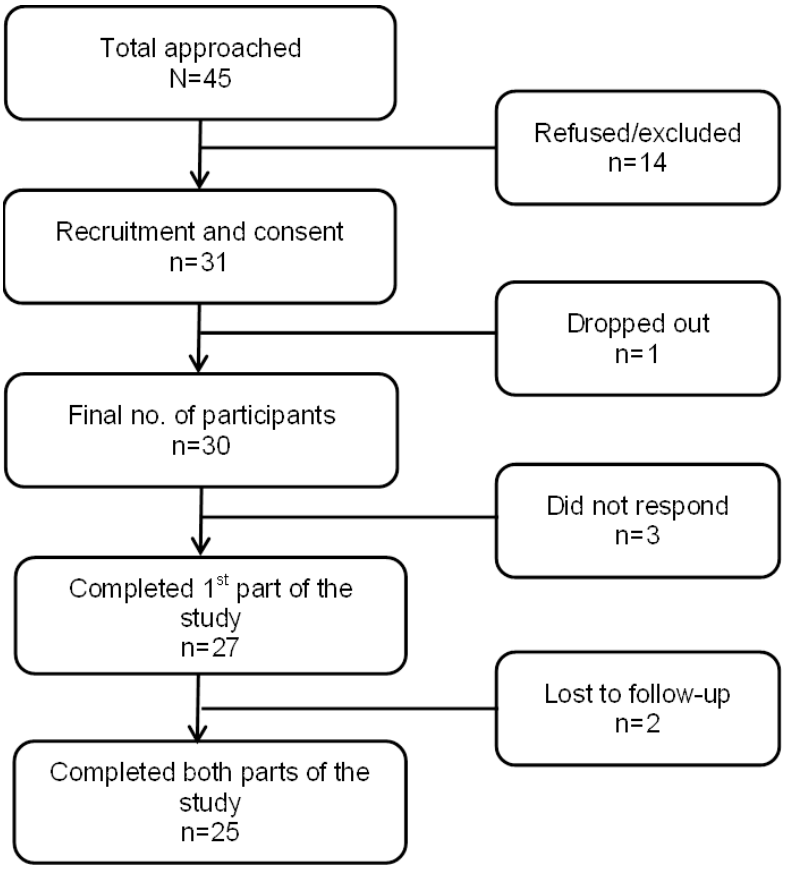


2.2.6.3 Recruitment of control group

A summary of recruitment of this group is given in Figure 2.4. A total of 45 potential participants were approached, of which 25 participants completed the study. Response rate of participants after consent was 80.6%. Although attempts were made to match participants in this group by age, ethnicity, demography, and gender with the other two groups, this was not fully achieved.

Fourteen parents agreed to take part along with their children in this group; only 8 of whom gave one faecal sample, one parent dropped out after consent, and 5 parents did not respond after consent.

Figure 2.4: Summary of recruitment progress for lean healthy participants



N; total number of participants approached, n; number of participants

2.3 Laboratory Methods

2.3.1 Sample analysis time

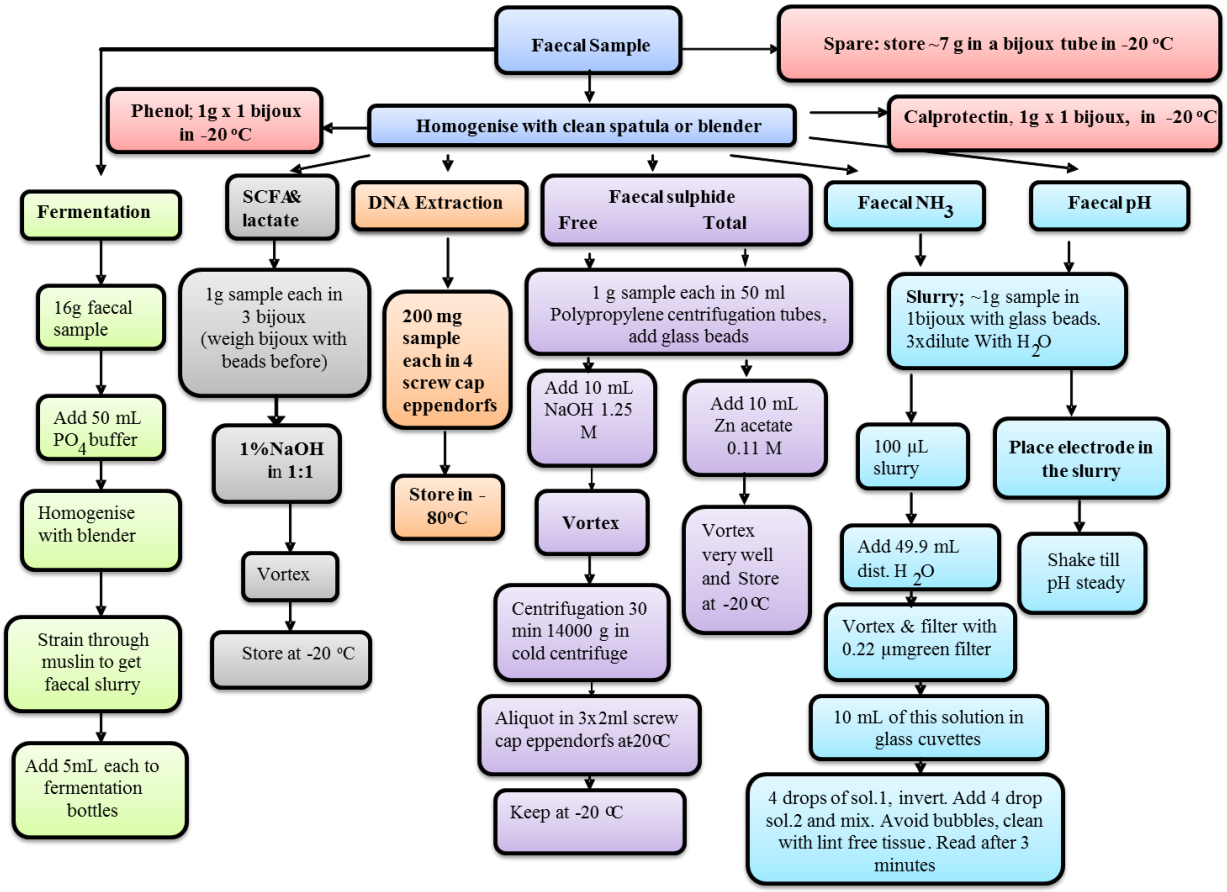
It was aimed to process every faecal sample within 4 h after collection. The median (IQR) time elapsed between sample production (telephone call for sample collection taken as the time of sample production) and storage of the sample (after being processed) was 4 (1.1) h for healthy lean group, 5.1 (3.75) h for hypothalamic lean group, 4 (1.9) h for hypothalamic obese group, and 3.7 (2.9) h for simple obese group. It should be noted that some samples took longer than 4 h to be transported to the laboratory for processing either due to the longer distance for sample collection or because more than 3 faecal samples were unexpectedly received from different participants at the same time which made it difficult to be processed all at once. Some of these faecal samples (n=4, one from hypoth.

Lean, 2 from simple obese, one from hypoth. obese) were immediately frozen at -20°C for few hours to stop any bacterial activity, and then processed as soon as possible. However, analysis of faecal short chain fatty acids (SCFA) and SCFA from the incubated samples from *in vitro* fermentation did not reveal any significant difference from other samples of the same group.

2.3.2 Initial processing of the sample

Each sample collected in cold anaerobic conditions was weighed along with the stool collection pot and then manually homogenized with sterilized wooden spatulas under a laminar flow cabinet. Any large undigested food pieces were removed whenever possible. A summary of the initial processing of the sample is given in Figure 2.5.

Figure 2.5: Flow chart for initial sample processing in the lab



Approximately 200 mg faecal sample was weighed in 2mlscrew-cap tubes in quadruplicate and immediately stored in -80°C. About 1 g faecal sample was weighed in 5 ml bijoux

tubes containing 3-5 glass beads in triplicate. To this was added equal volumes (1:1 w/v) of 1 M NaOH to help retain the volatile fatty acids and stored in -20^{o}C.

To store sample for free and total faecal sulphide, about 1-1.5 g sample was weighed in 2 universal 25 ml tubes containing 3-5 glass beads. For free sulphide; 10 ml of 1.25 M NaOH (sonicated on a sonicator (Sonomatic® Langford, Jencons Scientific ltd) for 15 minutes) was added to one of these tubes and vortexed well. The diluted sample was then centrifuged at 15,000 g for 30 minutes in a centrifuge (Sorvall legend RT+, Thermo Scientific®, UK) in quadruplicate. The supernatant was then aliquoted in separate 2 ml safe-lock eppendorf tubes and stored in -20 ^{o}C until for further analysis. For total sulphide, 10 ml of 0.11 M zinc acetate was added to one of the universal tube and vortexed to mix. The tube was then stored in -20 ^{o}C until analysed. Approximately 1 gram faecal sample was stored in 5 ml bijoux tubes for faecal calprotectin and faecal phenol and cresol and stored in -20 ^{o}C. Any spare sample was stored in a 5 ml bijoux and/or 25 ml universal tube.

2.3.3 Faecal pH

Approximately 1 g of well homogenized fresh faecal sample was weighed on a digital scale. To this was added equal volumes of sterilized distilled water (1:3 v/v), vortexed thoroughly, and the pH measured on a portable bench-top pH meter (HANNAH®).

2.3.4 Faecal Ammonia

Faecal ammonia was measured using the same 1:3 v/v faecal slurry used for the measurement of pH. This slurry was diluted down by 1:500 by mixing 100 µl 1:3 v/v faecal slurry in 49.9 ml distilled water in a 50 ml universal tube. Ten millilitres of this 1:500 slurry was filtered through 0.22 µl green Millipore filter (Millex®GP, Millipore, USA catalogue number; SLGP033RB) into a clear glass bottle. After adjusting the ammonia meter to zero with this clear filtrate, 4 drops each of the proprietary solution 1 and 2 were added, gently inverted to mix, and then measured on the automated ammonia analyser (Hannah Electrical HI93715).

2.3.5 In-Vitro Batch Culture Fermentation

2.3.5.1 In vitro batch culture fermentation

In vitro batch culture fermentation is a commonly applied method to determine the fermentation capacity for dietary fibres. This system does not remove fermentation products from the incubated inoculum, in contrast to the continuous and semi continuous *in vitro* systems that remove the fermentation products either continuously or intermittently. Many methods have been developed in different studies (Table 2.3). Most, if not all of them, have used different methods for reasons partially explained. Also, few of these studies have been validated by inter-laboratory validation studies and there is lack of *in vivo* validation data (272). Two of the validated methods are those of Barry *et al.* (1989) (273) and Edwards *et al.* (1996) (274). The method of Barry *et al.* (1989) is different in some aspects from that of Edwards *et al.* (1996) (Table 2.1). However, Barry *et al.* (1989) found different results in different laboratories for the same individuals after fermentation, despite similar conditions provided.

Table 2.1: Salient features of method from Barry *et al.* (1989) that makes it different from methods by other authors

Features of *in vitro* batch culture fermentation model used by Barry *et al.* (1989)
• Different carbohydrates in addition to the resistant starch • Low inoculum size • Faecal samples from three volunteers whose samples were used in all the labs to maintain uniformity • Use of trace elements and urea in the medium for nutrition • Complementary experiments for investigating their ring test

2.3.5.2 Rationale for fermentation method used in this chapter

The *in vitro* batch culture fermentation technique used in this thesis is adopted from the study of Edwards *et al.* (1996) (274). This method differs from other methods in various aspects. It considers the physiology of the colon; therefore Edwards *et al.* have attempted to mimic human colon by using shaking water bath at a rate of 50 strokes per minute to mimic peristalsis. The medium is not required to be bubbled continuously with CO_2 because phosphate buffer is used to prepare the faecal slurry. A higher concentration of the faecal slurry is used that has been shown to provide suitable nutritive environment for the faecal microbiota thus reducing the need for excess fermentation medium. The use of carbonate and phosphate buffer does not allow a drop of pH due to SCFA production before the sample is being incubated in the fermentation bottles. The method has been validated in 8 laboratories on 40 healthy volunteers from different geographical regions, all

with similar laboratory settings. Although the method has only been validated for starch, it has been used for other fibres in later studies based on the same method for different types of carbohydrates (295;296).

2.3.5.3 Protocol for *in vitro* batch culture fermentation used in this thesis (Figure 2.6)

Faecal samples were collected from study participants of all three groups, and brought to the laboratory as soon as possible [time between sample collection and taking the 0 h fermentation sample; median (IQR) 4 (2.0) h minimum 1.6 h and maximum 13.6 h]. The composition and preparation protocol for the solutions used are shown in Table 2.2 and the entire procedure is summarised in Figure 2.6. Sodium phosphate buffer [0.06 M KH_2PO_4 (9.078 g/L) and 0.06 M Na_2HPO_4 (11.876 g/L mixed in a ratio of 1:4] was boiled on a hot plate and then cooled to 37 ^oC under Oxygen-Free nitrogen (OFN). A 32% w/v faecal slurry was prepared by adding sodium phosphate buffer (pH 7) and homogenizing it with the help of blender (Braun™) to maintain a constant pH before the samples are incubated in the fermentation bottles. Fermentation medium was freshly prepared using tryptone, carbonate buffer (ammonium bicarbonate and sodium bicarbonate buffer), macromineral solution (di-sodium hydrogen sulphate, potassium dihydrogenortho-phosphate, and magnesium sulphate), micromineral solution (containing cobalt chloride , manganese chloride, calcium chloride, and iron chloride), and a colouring reagent (0.1 M resazurin).

The pH of the medium was adjusted before boiling on a hot plate for 5 minutes. Boiled medium was then cooled under Oxygen-Free Nitrogen (OFN) to 37 ^oC, and the pH was adjusted to pH 7 using 6 M HCl. Forty-two ml fermentation medium was added to each 100 ml pre-sterilized fermentation vessels. One gram each of five different substrates; pectin, Raw potato Starch, Maize starch, raftilose, and wheat bran were added to the bottles, except for the blank. Raw potato starch (starch from potato, cat. No. S2004) and Pectin (pectin from apple) were obtained from Sigma Aldrich (catalogue number; 76282), wheat bran from Infinity foods Co.(Product no.; BRAN3), Raftilose (Orafti® P95, BENEO, Tienen, Belgium) was kindly provided by BeneoOrafti® Belgium, and maize starch (Hi-maize® 260) was kindly provided by the National Starch Food Innovation Manchester. Two ml of freshly prepared reducing solution (cysteine hydrochloride, sodium sulphide and 1 M sodium hydroxide) was then added to the bottles. All fermentation vessels were crimped and sealed with self-sealing silicon rubber-crimp tops to provide airtight anaerobic environment. The contents were mixed by shaking manually and then purged with OFN for two minutes each. The faecal slurry was strained through a nylon mesh to remove any particulate material and 5 ml of this final slurry was added into each

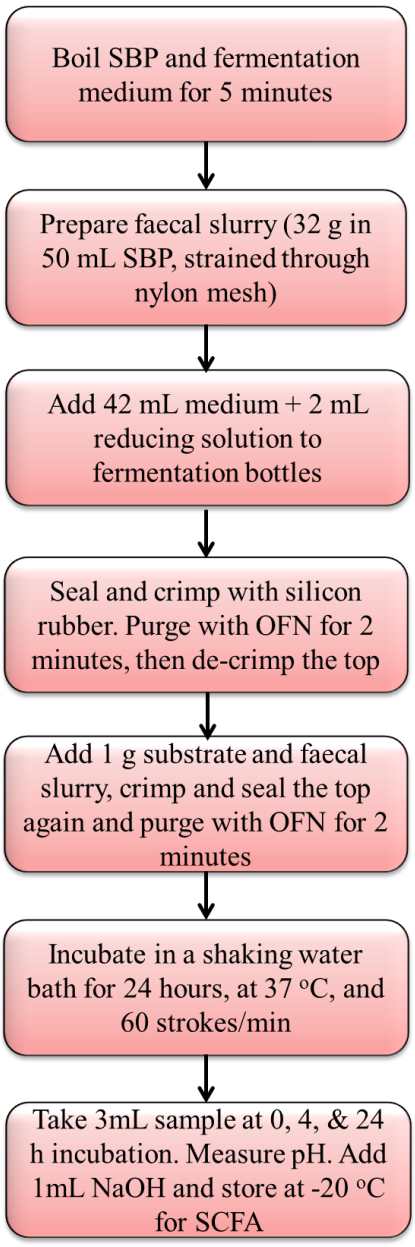
bottle with the help of a 10 ml syringe. The final concentration of the faecal slurry in the 42 ml medium and 1 g substrate obtained was 8.04%. All faecal samples from participants with initial weight sufficient to give the required amount of slurry for fermentation were incubated in duplicate for substrate as well as the blank.

The bottles were incubated in a shaking water bath for 24 h at a speed of 60 strokes per minute. Three ml of fermentation supernatant was taken in 5 ml bijoux tubes from each incubated sample at 0, 4, and 24 h of incubation, their pH measured, and 1ml of 1 M NaOH added to stabilise faecal short chain fatty acids. These samples obtained were immediately stored at a temperature of -20 ^{o}C.

Table 2.2: composition and preparation of the solutions used for *in vitro*fermentation studies

Solution	Volume	Composition	Preparation
Buffer Solution	500 ml	NH_4HCO_3	2 g
		$NaHCO_3$	17.5 g
		Distilled water	up to 500 ml
		Store at 4 ^{o}C	
Macromineral Solution	500 ml	Na_2HPO_4 or $Na_2HPO_4.2H_2O$	2.85 g or 3.57 g
		KH_2PO_4 or $KH_2PO_4.2H_2O$	3.1 g or 3.92 g
		$MgSO_4.7H_2O$	0.3 g
		Distilled water	up to 500 ml
		Store at 4 ^{o}C	
Micromineral Solution	100 ml	$CaCl_2.2H_2O$	13.2 g
		$MnCl_2.4H_2O$	10 g
		$CoCl_2.6H_2O$	1 g
		$FeCl_3.6H_2O$	8 g
		Distilled water	up to 100 ml
Fermentation Medium	1000 ml approx.	Tryptone in 450 ml of distilled water	2.25 g
		Micromineral solution	112.5 µl
		Agitate solution to dissolve the chemicals	225 ml
		Buffer solution	225 ml
		Macromineral solution	1.125 ml
		0.1%resazurin solution	
		Adjust the medium pH to 7, using 6 M HCl	
		Store at 4 ^{o}C	
Sodium Phosphate Buffer (66 mmol)	200 ml	KH_2PO_4 0.06 M (9.078 g/L): 1.82 g/200ml	78 ml Of KH_2PO_4 0.06 M to 122 ml Of Na_2HPO_4 0.06 M
		Na_2HPO_4 0.06 M (11.876 g/L): 2.38 g/200ml	
		Maintain pH of 7 & at 20^{o}C by adding 1 M NaOH	
		Store at 4^{o}C up to 1 week max.	
Reducing Solution	50 ml	Cysteine hydrochloride	312.5 mg
		NaOH 1 M	2 ml
		$Na_2S.9H_2O$	312.5 mg
		Distilled water	up to 50 ml

Figure 2.6: Flow chart summarizing the *in-vitro* fermentation procedure (adopted from Edwards *et al.* 1996)



SBP; Sodium phosphate buffer, OFN; Oxygen-free nitrogen,

2.3.5.4 Considerations

The procedure was performed in aseptic environment, using sterilized containers, bottles, pipette tips etc. to avoid any external contamination. The medium was prepared fresh using sterilized distilled water and stock solutions were stored at an appropriate temperature up to a maximum of one week.

Keeping in view the distance to travel and the variable timings of habits of the participating children, there was a difficulty in managing early processing of some faecal samples in the laboratory. However, the participants and their parents were provided with stool collection kits which would keep the samples cold with the help of ice packs and in an anaerobic environment with anaerocult® A, which produces gas that removes all the oxygen around the sample.

Raw potato starch and maize starch would partly settle down in the medium even in shaking water bath at 60 strokes per minute, which might have affected the fermentibility of the dietary fibre due to insufficient exposure to the faecal slurry. This was minimized by constant monitoring and manual shaking during incubation and that the incubation period of 24 h was considered sufficient to reduce the overall effect of insufficient contact. Evidence from the literature suggests that the rate of fermentation is not affected by the mixing rate (272). Also many researchers did not use a shaking water bath during their fermentation experiments (275).

The pattern of fermentation of the true fibre fraction of certain polymeric substrates (e.g. wheat bran) is masked by some starch content. Enzymatic pre-digestion is therefore applied in some studies to facilitate fermentation of the substrates (276, 277). Raw potato starch and wheat bran had no enzymatic pre-digestion in our study and no overnight hydration which might have affected the overall *in vitro* fermentibility of dietary fibre and the concentration of colonic SCFA because of protein contents of the cells. This however was thought unlikely in our experiments.

Table 2.3: Various *in vitro* models of fermentation used in studies.

Reference	Subjects	Substrate(s) used	Inoculum	Medium	Buffer for inoculum	Incubation period	Mixing rate
(274)	40 healthy individuals in total, 4-7 in each of the 8 laboratories	Pregelatinized potato starch (RS1), raw potato starch (RS2), semi purified retrogradated amylase (RS3), Glassy pea starch (RS3)	160 g/L Fresh faecal inocula. processed within one hour	Carbonate buffer, mineral salts, at pH 6.5	Phosphate buffer	24 h	50 strokes/min or 50 rev/min, in a shaking water bath for 24 h,
(273)	3 Healthy volunteers for all the labs	Solca-floc Cellulose, sugar beet, soybean fibre, maize bran, apple pectin, 100 mg each	10 g/L Human faecal inoculum	Carbonate-phosphate buffer solution, Trace elements, urea, medium bubbled with CO_2 at 100 ml/sec	Carbonate-phosphate buffercomplex	24 h	Shaking Water bath (speed not given) at 37 ^oC,
(278)	Healthy adults	0.5 g each of Guar, pectin, gum tragacanth, gum Arabic, Karaya, course wheat bran, carboxymethylcellulose, Xanthan, Gellan	200 g/L fresh human faecal inoculum. 10 ml into each bottle	Trypticase (pancreatic digest of casein), minerals	Phosphate buffer	24 h	Shaking water bath at 37 ^oC
(279)	6 Healthy human volunteers	Sugar beet arabino-oligosaccharides in 5 g/L concentration	10 g/L faecal inoculum in degassed PBS(prepared from stored faeces in 50% glycerol)	Minimal Basal Medium (containing peptone water, yeast, salts, bicarbonates, vitamins, reducing agents)	Phosphate buffer	24 h	Anaerobic cabinet at 37 ^oC
(276)	3 healthy volunteers	100 mg each of commercial rye, wheat, and oat bran,commercial inulin, raftilin P95. Starch and protein content removed after enzymatic digestion process	208 g/kg of faecal inoculum;	Culture medium with carbonate phosphate buffer and trace elements.	Culture medium	24 h	Anaerobic chamber at 30 ^oC for 2 h before adding the slurry, and then in the shaking water bath at 37 ^oC
(275)	6 healthy volunteers (24-45 years age)	Oligofructose, inulin, other carbohydrates (glucose, arabinose, galactose, fructose, lactose, sucrose, lactulose, cellobiose, sorbitol, lactitol, Linter's starch, polydextrose, pectin, maltitol, arabinogalactan)	5% w/v faecal slurry in anaerobic Sodium Phosphate buffer 0.1 M/L, pH 7.0	Batch culture fermenters (70 ml working volume) details not mentioned	Anaerobic sodium phosphate buffer 0.1M/L, pH 7.0	48 h	Incubation at 37^{o}C. (No details given). Medium was flushed with high purity argon for 10 minutes

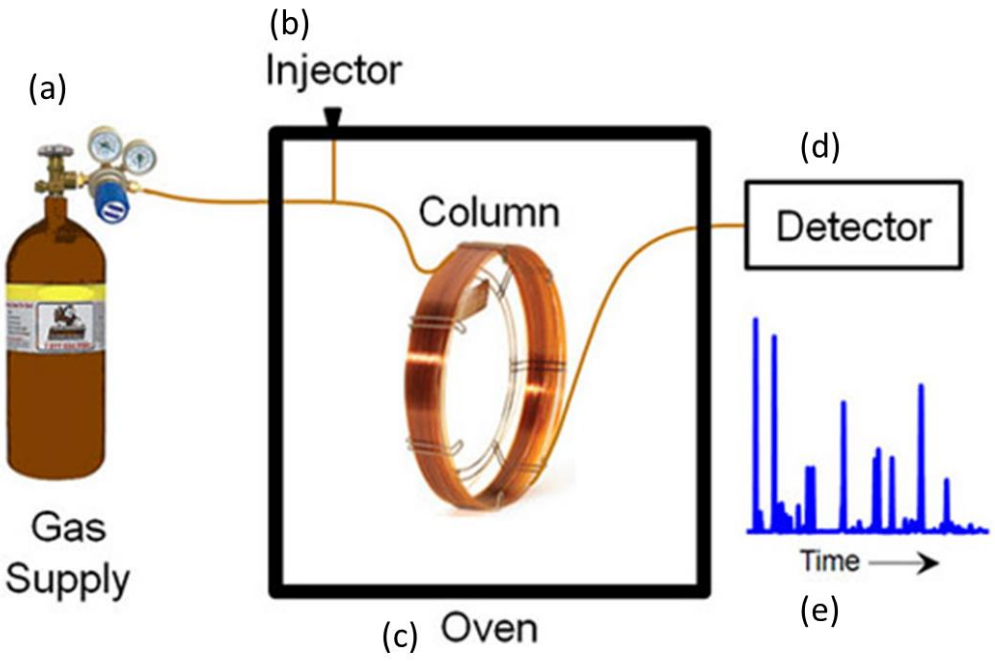
2.3.6 Short chain fatty acids (SCFA) analysis

Short chain fatty acids, including short, medium, and branched chain fatty acids, were extracted using diethyl ether and then analysed using Gas Chromatography TRACE GC 2000 series Gas Chromatograph (GC) (Thermo Quest CE Instruments, Manchester, UK). The method is adopted from Laurentin and Edwards (126, 280). The methods have been used in the same research facility and elsewhere with or without modifications.

2.3.6.1 Principles of gas chromatography

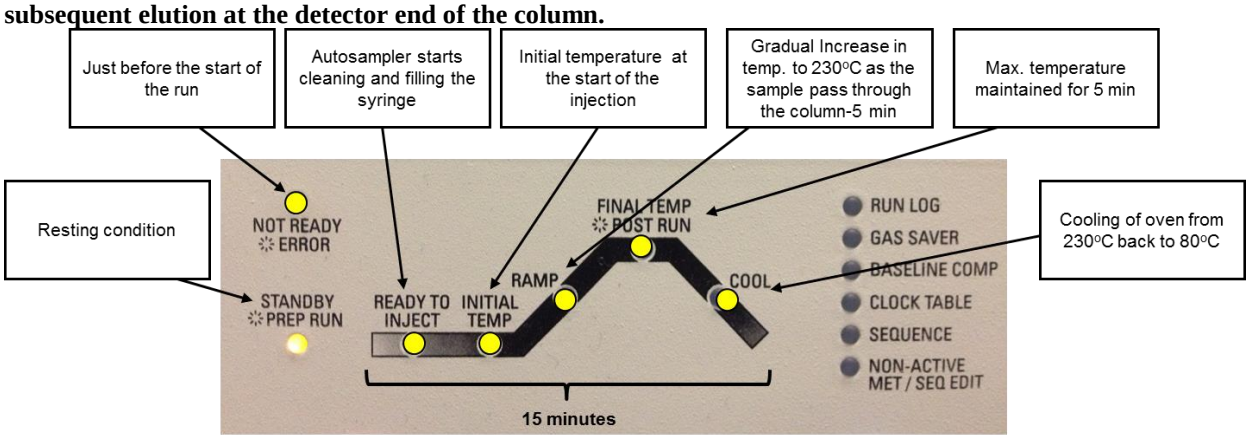
Gas chromatography is a technique for separation of compounds in a sample mixture (Figure 2.7, Figure 2.8). The samples are injected into the "gas chromatograph" through an injection port, where the sample is separated at a high temperature and pressure and converted into a gaseous phase. The vaporized sample is then allowed to flow through a column by a carrier gas also called as "mobile" phase. The carrier gas is an inert gas, usually helium, nitrogen, or sometimes hydrogen. In the column; the samples are separated into its components with the help of a liquid or "static" phase which has a high partition coefficient and a high solubility for the samples. Through the process of repeated absorption and desorption; the vaporized sample is separated at different speed and time interval and elute at the distal end of the column where the molecules are detected by a flame ionization detector. Electrical signals are generated by the detector, which are recorded in the form of peaks, "the chromatogram", at intervals depending on their molecular weight. The time from the point of injection to the appearance of the peak is called as the "retention time" which identifies the peak. The area that is covered by the peak is called as the "area under the curve", which is proportional to the concentration of the SCFA. Gas chromatography is a useful technique for SCFA extractions as it is a sensitive, accurate, speedy technique, relatively cheap and has a high resolution. However, it is limited in certain aspects such as the samples must be volatile and clean, as dirty samples can destroy the columns.

Figure 2.7: Schematic diagram of a gas chromatograph.



The sample is injected into the injection port (b) and is vaporised by a high temperature (about 250^{o}C) and pressure, the vaporised sample then flows through the column (c) with the help of a carrier gas (a). The sample separates into its components through absorption and desorption by mobile and static phase. The eluted components are detected by a detector (d) and peaks are generated on a chromatogram (e) which is used to identify and quantify the fatty acids.

Figure 2.8: Series of temperature changes occurring in the oven to facilitate sample disintegration and subsequent elution at the detector end of the column.



Note that the temperature of the injection chamber reaches 250 ^{o}C at the "initial temperature" stage when the column temperature is still 80 ^{o}C (the baseline temperature of the oven). This corresponds to the initial solvent peak visible on the chromatogram. As the temperature in the oven starts increasing at "ramp" stage, the peaks for SCFAs start to appear on the chromatogram. The "ramp", "final temp post run", and "cool" stage lasts for 5 minutes each. The vertical indicators with their labels are part of the self-check system that goes through every time the GC is switched on.

2.3.6.2 Preparation of samples for gas chromatography

All collected samples were homogenised thoroughly with a wooden disposable spatula under laminar flow in a biological cabinet. Approximately 1 gram homogenised faecal sample was weighed in 5 ml bijoux tubes with glass beads in triplicate, and equal volumes of 1M NaOH was added to preserve and stabilize short chain fatty acids. A high alkaline environment allows the substitution of free carboxylic hydroxyl group by divalent ions which decrease their volatility and further metabolic activity of the faecal microbiota. The sample was then vortexed thoroughly and saved in -20 ^{o}C until analysed. As mentioned earlier, samples for fermentation were also stored with 1 ml 1M NaOH and stored at -20 ^{o}C immediately after collection from the fermentation vessels and pH measurement.

2.3.6.3 Freeze drying

Faecal samples for SCFA measurement stored in -20 ^{o}C were taken out, holes were created in the lids of the sample tubes through syringe needles, then placed in -80 ^{o}C overnight and finally freeze dried (Edwards apparatus Micro Modulyo, Thermo Scientific®) for 24 h. The freeze dried samples were then homogenized thoroughly with sterile wooden spatulas, and kept in sealed tubes away from moisture at room temperature until analysed.

2.3.6.4 Extraction of short chain fatty acids from the faecal samples using diethyl ether

Various methods have been used to extract faecal SCFA. Some of these include; ultrafiltration with a membrane (281), vacuum distillation (282), derivatization using propylchloroformate (283), and steam distillation (284). However, extraction using organic solvents such as diethyl ether is widely used (284-287). This method takes into account the property of diethyl ether to form two phases in a mixture of the extract after a strong acid (such as ortho-phosphoric acid) is added to increase the dissociation constant (pKa value) of SCFA in the sample. These two phases include; (a) an upper ether phase which has diethyl ether and SCFA released out of the (b) lower faecal sample phase which includes faecal sample and orthophosphoric acid.

For freeze dried faecal material; 100 mg of freeze dried faecal samples was weighed in duplicates in 15 ml polypropylene centrifugation tubes (Corning®, Mexico, USA). The sample was mixed with 300 µl of distilled water and vortexed to homogenise. To this mixture was added 100 µl orthophosphoric acid and 100 µl 2-ethyl butyric acid (as internal standard). Di-ethyl ether (1.5 ml) was added to the tube and vortexed on a shaker (IKA® VIBRAX VXR basic) at 1200 shakes/min for 1 minute. The upper clear phase was

aliquoted in separate 15 ml polypropylene tubes and the process repeated 3 times (1.5 ml diethyl ether + vortex for 1 min). The clear supernatant was then immediately transferred to 1.5 ml glass vials (Agilent technologies®, USA, cat. No. 5181-3375) and crimped with silicone rubber seal crimp tops (Fisher scientific®, UK, cat. No. 11588150) for GC analysis to avoid evaporation.

For samples from *in vitro* batch culture fermentation, the same procedure was used to extract SCFA except that 800 µl of supernatant from fermentation was used instead of freeze dried faecal sample and 3 ml diethyl ether was used instead of 1.5 ml diethyl ether.

To ensure correct calibration of the machine, external standard was run after every 12th sample measured and two quality controls at the beginning and the end of the whole. Some samples were re-extracted and re-analysed after about 1 year of storage to see the repeatability of the procedure and it showed no difference in the calculated concentrations of SCFA.

2.3.6.5 Preparation and extraction of external standards

Since the gas chromatograph does not give an equimolar response to the sample, we used external standard to quantify SCFA in our samples. An external standard with a total of 11 short, medium and branched chain fatty acids (Table 2.4) was extracted with 6 dilutions (10, 25, 50,100, 200, and 300) using the same protocol as for the unknown samples to obtain the retention times and draw quantification calibration curves. The molarities of the individual acids to be used in the standard were optimized previously by Laurentin and Edwards (2004).

Table 2.4: Concentration of external standard used in the experiments

No. of carbons	Acid Name	Molarity used (g/L)	Calculated Molarity (mmol)
2	Acetic acid	60.05	183.50
3	Propionic acid	74.08	134.52
4	Butyric acid	88.11	111.74
5	Valeric acid	102.13	89.92
6	Caproic acid	116.16	80.12
7	Enanthic acid	130.18	68.53
8	Caprylic acid	144.21	57.59
9	Iso-butyric acid	88.11	104.22
10	Iso-valeric acid	102.13	85.51
11	Iso-Caproic acid	116.16	52.41

All chemicals were supplied by Sigma Aldrich (Dorset, UK) except acetic acid which was supplied by Fisher scientific (Loughborough, UK)

To account for the losses of the short chain fatty acids due to handling and processing, 2-ethyl butyric acid (73.6 mmol) was used as an internal standard with each extraction. On the gas chromatogram, internal standard gives a peak that can be related to the mass of an analyte, so it is used to find the area ratios (see below in calculation). Internal standard extracted alone (with water, orthophosphoric acid and ether) was analysed after every 12 sample to estimate the consistency of the analysis.

2.3.6.6 Conditions of the column and gas chromatograph machine for sample analysis;

All the extracted samples along with standards were loaded onto the AS2000 Autosampler (Thermo Quest CE® instruments, Manchester, UK) and were allowed to run on the GC in splitless mode, at base temperature of 250 ^{o}C, oven temperature of 260 ^{o}C (max) (Table 2.5). The samples analysed on the GC were using 32 bit Chrom-card data-system software (version 2.2 (April 2003) Thermo-Scientific®, Milan Italy). The parameters of the gas chromatograph method are summarised in Table 2.5. Individual peaks were identified based on the component table from the analysis of external standards. To ensure that the column is clean prior to the start of the analysis; two injections of ether (only) were allowed to run and this was shown by only one peak for solvent on the chromatogram. The needle was washed with ether and absolute methanol (100%) each time between two injections by the Autosampler.

2.3.6.7 Calculations;

Calculations for the measurement of concentration of sample were based on the area under the curve obtained from the peaks in chromatogram. For SCFA concentration measured in freeze dried faecal material, the concentration was expressed as µg/g of freeze dried faecal material, while in case of samples from fermentation, the same was expressed as µmol/ml.

First; the area ratio was calculated by the following formula:

Area ratio of individual SCFA= *Area under the curve for individual acid/Area under the curve for Internal Standard*

Relative response factor measures area ratios between two points. It was calculated for each short and branched chain fatty acid in external and internal standard by the formula;

Area ratio of individual SCFA in 100 µl ext. standard/ (conc. of that SCFA in the final vial/concentration of internal Standard in the final vial)

Finally the concentration of SCFA in µmol/ml was calculated by the formula

(Area ratio of individual acid/Relative Response factor) x Concentration of internal standard

Table 2.5: Parameters of the gas chromatograph for the analysis of samples

Program	Parameter	Value
Oven	Ramp rate	15 ^{o}C/min
	Initial temperature	80 ^{o}C
	Ramp temperature	210 ^{o}C
	Oven maximum temperature	260 ^{o}C
	Prep run time out	10 min
	Equilibration time	0.25 min
	Acquisition time	10.67 min
Right inlet	Inlet temperature	230^{o}C
	Mode	Splitless
	Purge	Constant septum purge
Right Carrier	Ramps; Flow	12 ml/min
	Flow mode	Constant flow
Autosampler	Sample volume	1µl
	Sample Speed	100 µl/sec
Column parameters	Length	15 M
	Internal diameter	0.53 mm
	Temperature	100 ^{o}C
	Inlet pressure gauge	70kPa
	Outlet absolute pressure	10kPa
Carrier gas parameters	Carrier gas	Nitrogen (N_2)
	Flow standard	1.89 cc/min
	Velocity	35.14 cm/sec
	Hold up time	85.38 sec.
Right detect	Base temperature	250 ^{o}C
	Hydrogen Pressure	25 PSi
	Nitrogen Pressure	30 kPa
	Air	350 kPa

2.3.6.8 Limitations

Extraction of SCFA using diethyl ether is a robust method and can measure SCFA concentration with a confident level of precision however; it has got several limitations which include:

Faecal sample used in the extraction may have freeze dried material that is not actual faecal sample but undigested food material. This was prevented by the through homogenization and separating the gross unfermented food material like seed coats etc.

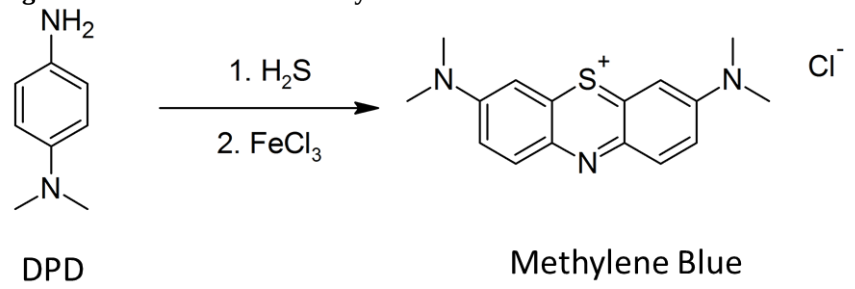
Diethyl ether used for extraction is a volatile compound and may facilitate the loss of the volatile SCFA during handling and analysis after they are extracted from the sample. However, this is accounted for by the internal standard to a greater extent.

The final concentration of SCFA may vary due to intrapersonal variation with extractions. This is true in both the fermentation and freeze dried samples. This has been overcome with meticulous practice and re-extraction of the samples that were analysed in the beginning of the project. To quote as an example; median (IQR) %CVs for major SCFAs such as acetate, propionate, butyrate, and for total SCFA compared were 2.08(2.91)%, 1.02(0.60)%, 1.40(1.04)%, and 1.48(1.65)% respectively.

2.3.7 Faecal hydrogen sulphide

Butyrate is the primary source of energy for colonic epithelium (81). Sulphur compounds have been shown to inhibit fatty acid oxidation especially, butyrate, in the distal and ascending colonic epithelium (119). This mechanism was thought to explain, at least in part, the aetiology of ulcerative colitis (119); an inflammatory bowel disease. Sulphate and sulphite ions are utilized by the sulphate reducing bacteria as electron acceptors for the dissimilation of reduced butyrate and molecular hydrogen. This causes the release of sulphide (HS^-) into the luminal mucosa which is converted to free hydrogen sulphide (H_2S) in the acidic environment (pKa 7.04). Total and free hydrogen sulphide from faecal samples in this study were measured by a modified methylene blue method (288). Under the oxidative effect of ferric chloride, H_2S reacts with n, n-dimethyl-p-phenylenediamine to form methylene blue (288), which absorbs visible light at 670 nm region of the electromagnetic spectrum (Figure 2.9). This method was originally developed for the measurement of hydrogen sulphide in water by Cline in 1969(289) and modified for hydrogen sulphide in faeces by Strocchi *et al.* (1992) (290) and Gerasimidis *et al.*(2014) (126).

Figure 2.9: Mechanism of methylene blue reaction.



DPD; n, n-dimethyl-p-phenylenediamine, H_2S; Hydrogen sulphide (total and free), $FeCl_3$ Ferric Chloride. Source; (314)

2.3.7.1 Reagents and samples preparation

Typically; 1-1.5 g of well homogenised fresh faecal sample was weighed in a pre-sterilized 25 or 50 ml polypropylene tubes for free and total sulphide. To prevent oxidation of sulphide in the faecal samples, 10 ml of NaOH 1.25 M (for free sulphide) or 10 ml of Zinc acetate 0.11 M (for total sulphide) were mixed with the faecal sample. Glass beads (3-5 in each tube) were added to the tubes to ensure thorough mixing of the sample with the solutions. To remove oxygen, both the solutions were ultra-sonicated in a sonicator bath (Jencons® Scientific ltd, Sonomatic® Longford) for 15 minutes before mixing with the sample. Preparation and use of all the reagents in this protocol are presented in Table 2.6. Zinc acetate-diluted sample for total sulphide was immediately stored in -20 ^{o}C until further analysis, while NaOH-diluted free sulphide sample was centrifuged at 15,000 g for 30 minutes. The supernatant, containing a rich free sulphide fraction was transferred to 2 ml eppendorf tubes and stored in -20 ^{o}C until further analysis.

2.3.7.2 Dilution

The already processed frozen faecal samples were thawed at room temperature and measured immediately to minimize sulphide losses due to oxidation. Samples for total sulphide were diluted by 1:20 (v/v) with zinc acetate 0.11 M by mixing 0.5 ml of total-H_2S faecal slurry with 9.5ml zinc acetate 0.11 M and stored at 4 ^{o}C or kept on ice racks until measured. The eppendorfs for free-H_2S were centrifuged at 15,000 g for 5 minutes and the clear supernatant recovered in new eppendorf. This supernatant was diluted by 1:5 (v/v) with distilled water. This step significantly reduces turbidity and increases sensitivity of the assay as was observed during optimization phase.

Table 2.6: Reagents and their composition used in the spectrophotometric assay for free and total sulphide

Chemical	Preparation	Use
NaOH 1.25 M	10 g NaOH mixed with 200 ml distilled water Stored in glass McCartney tubes with metallic rubber stopper caps. Sonicated for 15 minutes to remove oxygen. Stable in room temperature. This was done prior to measurement.	Prevent hydrogen sulphide oxidation in initial processing of free sulphide samples.
Zn-acetate 0.11 M	3.62 g of zinc acetate dihydrate (Sigma Aldrich, 383058-500G) mixed with 150 ml distilled water Stored under the same conditions as NaOH 1.25 M.	Stabilize sulphide (free and total) by forming insoluble zinc sulphide salts.
HCl 6 M	1:1 (v/v) dilution of HCl 12 M (Sigma Aldrich, 07102) with distilled water	Part of the reaction reagents and to drive off all the hydrogen sulphide from blank samples.
Reaction Reagent	Concentrated Reagent: 500 ml ice cold HCl 6 M mixed with 2 g n, n-dimethyl-p-phenylenediamine (DPD, Sigma Aldrich, Fluka, 0775025G) and 3 g iron chloride hexahydrate ($FeCl_3$, Sigma Aldrich). Diluted Reagent: 1:1 (v/v) concentrated reagent mixed with ice cold HCl 6 M. Both reagents are stable for a week when kept in dark and at -20 ^{o}C.	Reaction components for the methylene blue reaction
Sulphide standard 2 mmol	48 mg crystal sodium sulphide nonahydrate (Na_2S $9H_2O$), Sigma Aldrich, 208043-100G); rinsed and dried with lint-free cellulose tissue and then mixed with 100ml zinc-acetate 0.11 M. Storage at 4 ^{o}C in glass McCartney tubes with metallic rubber stopper caps after flashing with nitrogen for 10 minutes.	Used for the preparation of spike sulphide and standard working dilution used in calibration curve.
Spike sulphide standard 0.5 mmol	Sulphide standard 2 mmol, 200 µl mixed with 600 µl of zinc acetate 0.11 M. Prepared fresh every day	Measure the recovery of this assay in spiked samples and Corrected absorbance in all samples

NaOH; Sodium hydroxide, HCl; hydrogen chloride, M; Moles

2.3.7.3 Colorimetric reaction

Each sample for free and total hydrogen sulphide was measured in duplicate and different samples from the same participant were measured on the same day, using the same calibration curve, to reduce the intra-assay variability. As a standard practice, equal

number of samples from all the groups were analysed on the same day to reduce inter-assay variability.

For the measurement of each sample in duplicate, 6 eppendorf tubes and cuvettes were prepared; 2 each for the sample, the blank, and the spiked sample (Table 2.7). Equal amount of diluted faecal sample was added to each of the 6 tubes. Hydrogen sulphide was driven off the blank sample using 80 µl concentrated HCl, while spiked sample had 15µl of spike-standard sulphide 0.5 mmol in addition to the other reaction components. Concentrated hydrochloric acid was used to drive off sulphide out of the blanks, while spiked samples were used to estimate the recovery of the method and to correct the sample's absorbance.

Table 2.7: composition of the colorimetric reaction of the methylene blue protocol.

Reagents/sample (µl)	Blank	Sample	Spiked sample	Blank standard	Standard
Diluted sample	900	900	900	-	-
Zinc acetate	-	-	-	900	900
Spiked standard	-	-	15	15	15
HCl Concentrated	80	-	-	80	-
Reaction reagent (concentrated)	80	-	-	80	-
Reaction reagent (Diluted)	-	160	160	-	160

All values are expressed in µl.

The reaction reagent (both diluted and concentrated) was always added cold, and was followed by vortex and 45 minutes incubation in dark (15 minutes at room temperature and 30 minutes at 37 ^{o}C) which allows the colorimetric reaction to take place. Subsequently, all samples were centrifuged at 14,000 g for 6 minutes and the supernatant was measured in a spectrophotometer (Biomate 3, Thermo Electron Corporation) at 670 nm.

2.3.7.4 Calibration Curve and calculations

Standards were prepared and a calibration curve plotted each day with the sample measurements. Eleven serial dilutions (Table 2.8) of working standard sulphide solution 0.05 M (200 µl 2 mmol sulphide standard mixed with 7.8 ml of zinc-acetate 0.11 M) were measured.

Table 2.8: Composition of the calibration curve used in the methylene blue reaction.

Chemical	1	2	3	4	5	6	7	8	9	10	11
Sulphide (µmol)	0	0.7	1.4	2.3	3.7	4.7	7	9.3	18.7	28	37.4
Zn- Acetate	910	895	880	860	830	810	760	710	510	310	110
Standard	0	15	30	50	80	100	150	200	400	600	800
Reaction reagent	160	160	160	160	160	160	160	160	160	160	160

All values are expressed in µl

Absorbance at 670 nm was plotted against sulphide concentrations to obtain a linear curve. An equation was drawn based on the linear curve to calculate the faecal sulphide in the samples. The values of free and total sulphide, expressed in μmol/g of wet and dry faeces, were adjusted for the recovery of the assay and the corrected absorbance.

$$\%\ Recovery = (\frac{Abs(spiked\ sample) - Abs(sample)}{Abs(standard)} \times 100$$

$$Corrected\ abs(CorAbs) = \frac{Abs(sample)}{Recovery} \times 100$$

$$Free\ sulphide\left(\frac{\mu mol}{g} wet\ stool\right) = \frac{5.97 \times (CorAbs - b)}{100 \times a \times W}$$

$$Total\ Sulphide\left(\frac{\mu mol}{g} wet\ stool\right) = \frac{23.9 \times (CorAbs - b)}{100 \times a \times W}$$

$$Total\ or\ Free\left(\frac{\mu mol}{g} dried\ stool\right) = \frac{\mu mol\ per\ g\ wetstool}{(100 - \%WC)/100}$$

Where: Abs: absorbance, CorAbs: corrected absorbance, a: slope of the calibration curve, b: intercept of the calibration curve, W: weight of the stool sample, %WC: percentage of sample's water content

2.3.7.5 Optimization of the assay; testing the sensitivity and specificity

The protocol used for these measurements was previously established in the laboratory by M.Sc. student (Svolos V, masters' dissertation). The same student also measured the samples for hydrogen sulphide concentrations in faeces from our participants. However, the dilutions were further investigated to achieve a higher recovery values. The dilutions eventually decided to be used were 1:20 (dilution with zinc acetate) for total sulphide and 1:5 (dilution with distilled water) for free sulphide with a recovery varying between 70-120%. As the absorption values for these dilution rates were relatively low despite a satisfactory recovery; we used both less (1:10 and 1:3, or 1:5 and 1:1 respectively) and high diluted slurries to achieve a higher absorbance values. However, the absorbance did not improve and the recovery values decreased even further. This might indicate that more diluted or concentrated faecal samples inhibit methylene blue reaction. In order to increase the method's sensitivity around the low absorptions area, three more standard sulphide points of low concentration were plotted in the calibration curve.

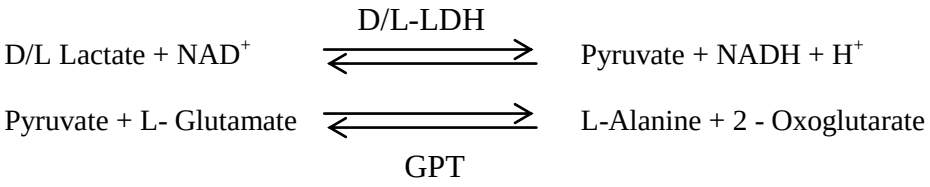
Dilutions of various inorganic sulphate salts including ferrous sulphate, calcium sulphate, ammonium peroxodisulphate, ammonium iron sulphate, potassium persulphate, magnesium sulphate were used to test the specificity of the assay and to measure the interference of non-bacterial sulphate compounds. This was found to be negligible.

2.3.8 Measurement of D- & L- Lactate

Lactate is an important fermentation product of gut microbiota, especially, the lactic acid bacteria. Although total faecal lactate can be measured by gas chromatography (291); measurement of the isomers, D & L Lactate, can effectively be measured by an enzymatic colorimetric assay. Different brands of kits for measurement of lactate in faecal samples based on enzyme action have been used in the literature (117, 292, 293). The kit used for the measurement of faecal D and L lactate in this study was D & L-lactic acid, UV method, R-Boehringer/Biopharm AG, Roche (Cat. No. 11112821025), actually designed for the measurement of lactate in food material. However, the method was previously optimized in our laboratory for faecal samples, after applying a series of different lactate extractions and enzymatic reaction conditions (126).

This method is based on the oxidation of D & L lactate (in the freeze dried faecal sample) to pyruvate and NADH (Nicotinamide Adenine Dinucleotide Hydrate) by D & L lactate dehydrogenases. Since the reaction is reversible in favour of lactate; pyruvate is trapped in another reaction catalysed by glutamate pyruvate transaminase (GPT) in the presence of L-glutamate to displace the equilibrium in favour of pyruvate and NADH (figure 2.10). The amount of NADH thus generated is stoichiometrically related to the concentration of D or L lactate in the sample. NADH absorbs light at 340 nm which is measured spectrophotometrically on a 96 well plate.

Figure 2.10:Principle of enzymatic determination of D & L-lactate in freeze dried faecal samples



NAD; nicotinamide Adenine Dinucleotide, LDH; lactate Dehydrogenase, NADH; nicotinamide Adenine Dinucleotide Hydrate, H^+; Hydrogen ion, GPT; Glutamate Pyruvate Transaminase

Measurement of lactate from freeze dried faecal material involves extraction of lactate from the freeze dried samples and spectrophotometric determination of lactate in the extract. Different chemicals used in this procedure, and their function is given in Table 2.9.

Table 2.9: Components of lactate extraction.

Chemical	Preparation	Function
Carrez 1 (potassium hexacyanoferrate(II) trihydrate ($K_4[Fe(CN)_6]$ x $3H_2O$)	Ready-made 15 mg/100ml	Together with Carrez 2; Precipitate proteins, eliminate turbidity, break emulsions
Carrez 2 (zinc sulphate heptahydrate ($ZnSO_4$x $7H_2O$)	Readymade 30 g/100ml	Together with Carrez 1; Precipitate proteins, eliminate turbidity, break emulsions
NAD (Nicotinamide Adenine Dinucleotide) 46.4 mg/ml	232 mg NAD in 5 ml double distilled H_2O	Required for oxidation of lactate to pyruvate by accepting H+
GPT (Glutamate Pyruvate Transaminase) 207.36 units/ml	0.8 ml GPT in 4.2 ml double distilled H_2O	Catalyse reaction of L-glutamate with pyruvate
Glycylglycine buffer	4.75 g Glycylglycine and 0.88 g L-glutamic acid in 50 ml double distilled H_2O. Adjust pH to 10 with 10 M NaOH, make up to 60 ml.	Buffer to facilitate reaction
L-LDH (L- Lactate Dehydrogenase) 107.92 units/ml	38 mg L-lactase in 5 ml double distilled H_2O	Catalyse the oxidation of L-lactate to pyruvate
D-LDH (D- Lactate Dehydrogenase) 107.879 units/ml	4.63 mg D-lactase in 5 ml double distilled H_2O	Catalyse the oxidation of D-lactate to pyruvate

Glycylglycine buffer is stable for 12 weeks at 4^{o}C, can be stored in bottles after filtration with 0.22µl filter.

2.3.8.1 Extraction of D & L-lactate from the faecal sample

Faecal samples (with 1:1 NaOH 1 M) stored at -20 ^{o}C were freeze dried and 60 mg of this material was weighed in pre-sterilized 2 ml eppendorf tubes in duplicate. To this was added 800 µl sterilized distilled water and vortexed for 1 minute. The samples were incubated at 65 ^{o}C for 20 minutes in a water bath (Grant GLS400, Grant®); vortexed and inverted after every 10 minutes. To precipitate proteins, eliminate turbidity, and to break emulsions (which can interfere with the analysis in subsequent steps), 100 µl each of Carrez 1 (potassium hexacyanoferrate(II) trihydrate ($K_4[Fe(CN)_6]$ x $3H_2O$) 15 g/100 ml) and Carrez 2 (zinc sulphate heptahydrate ($ZnSO_4$x $7H_2O$) 30 g/100 ml) were added, and the samples vortexed each time after adding the solutions. Carrez 1 and 2 (BIOQUANT Carrez clarification reagent kit for sample preparation in food analysis, Merck) form a sparingly soluble precipitate that adsorbs and also binds high molecular weight substances.

To separate this precipitate from the aqueous portion, the samples were centrifuged for 8 minutes at 14,000 g and then 500 µl of this supernatant recovered in separate 2 ml safe-lock eppendorf tubes. For further purification, 50 µl each of Carrez 1 and 2 were

added and then centrifuged for 8 minutes at 14,000 g. The supernatant thus obtained was aliquoted into a separate set of 2 ml eppendorf tubes and centrifuged again for 8 minutes at 14000 g before being stored in the freezer at -20 ^{o}C for further analysis.

2.3.8.2 Spectrophotometric determination of D & L lactate

The clear supernatant obtained from extraction was centrifuged after being defrosted at room temperature. D and L- Lactate were measured on separate 96 well reaction plates (Sero-Wel, Sterilin, UK). To carry out enzymatic reaction, 30µlsample was added to each well followed by 15 µl NAD, 15 µl GPT, 100 µl glycyl-glycine buffer, and 45 µl water, as given in table 2.10. Equal volumes of standard, quality control, and water were added instead of sample in their respective allocated wells (Table 2.10).

Table 2.10: Proportions of different components in the blank, samples, and the quality control

	GPT	Buffer	NAD	H_2O	Sample	QC	D-LDH	L-LDH
Blank (µl)	15	100	15	75	0	0	10	10
Control (µl)	15	100	15	45	0	30	10	10
Sample (µl)	15	100	15	45	30	0	10	10

GPT; Glutamate Pyruvate Transaminase, NAD; Nicotinamide Adenine Dinucleotide, QC; quality control, D-LDH; D- Lactate Dehydrogenase, L-LDH; L- Lactate Dehydrogenase, Sample; faecal sample.

Nine standard dilutions (Table 2.11) prepared from working solution of 0.454 g/L D-Lactate (Lithium D-lactate, Sigma-Aldrich, cat. no. L1000-100MG) & 0.63 g/L L-lactate (Lithium L-lactate, Sigma-Aldrich, L2250-10G) were analysed with each 96 well plate (Sero-Wel, Sterilin, UK) to create a calibration curve which was then used for calculation of D & L-lactate in a polynomial equation. The standard dilutions and the time of incubation (2 h) were optimized prior to the start of the sample extractions.

Each plate was sealed with a membrane sealer (Thermo® scientific, USA). The plates were gently vortexed on orbital shaker (IKA® VIBRAX VXR basic) for 10 minutes and the absorbance was measured at 340 nm. The membrane was gently removed from the plate and the enzymes D and L- LDH were added to their respective plates to initiate the enzymatic reaction. Each plate was incubated for 2 h with continuous shaking on the shaker and the absorbance was measured at 340 nm at the end of 2 h. Each sample was extracted in duplicate and each of this duplicate was analysed in triplicate. The triplicates were compared at the end of the analysis, averaged, and any value with a higher variance was excluded from calculation.

Table 2.11: Standard dilutions of D & L Lactate used for the calibration curve

Standard	D-lactate (0.454 g/L)		L-Lactate (0.63 g/L)	
	D-Lactate (ml)	ddH_2O(ml)	L-Lactate (ml)	ddH2O (ml)
1	0.025	1.975	0.03	1.97
2	0.05	1.950	0.06	1.94
3	0.10	1.90	0.09	1.91
4	0.15	1.85	0.15	1.85
5	0.20	1.80	0.30	1.70
6	0.30	1.70	0.45	1.55
7	0.50	1.50	0.65	1.25
8	0.70	1.30	1.00	0.00
9	1.16	0.90	1.30	0.70

ddH_2O; double distilled water

2.3.9 DNA extraction by chaotropic method

Increasing knowledge about the role of the gut microbiota in host energy metabolism and the advent of various culture independent techniques necessitates good quality and yield of genomic bacterial DNA to ensure reliability of the results obtained. For DNA to be extracted from the faecal samples; there are two major obstructions to achieve a good quality and quantity. Firstly; faecal samples are a mixture of undigested food material along with sloughed mucosa, dead cells, mucus secretions, bile, enzymes, bacteria, and other secreted and excreted substances. Secondly; bacterial DNA is intra-cellular and needs to be recovered by removing these physical barriers to access. Choice of an efficient DNA extraction method is therefore of utmost importance in extracting genomic bacterial DNA. There are a variety of commercially available kits used for extraction of faecal bacterial DNA (Table 2.12), and although they are considered equally efficient in extracting DNA of bacterial species, studies have found variation in the quantity and purity of DNA between the kits which might partially explain the differences in the relative abundance of gut microbiota between lean and obese individuals in the literature (294). In addition, there is a controversy whether freezing and various storage conditions may (295, 296) or may not (297, 298) affect the population of gut bacteria.

Table 2.12: Some kits commercially available for extracting bacterial DNA

Full name of the kit	Manufacturer details
QIAsymphony® Virus/Bacteria Midi Kit	Qiagen, Hilden Germany
ZR Faecal DNA MiniPrep	Zymo Research Corp. Irvine USA
QIAamp® DNA Stool Mini Kit	Qiagen, Valencia CA USA
Ultraclean® Faecal DNA Isolation Kit	MoBio Laboratories Inc. Carlsbad, USA
PowerSoil® DNA Isolation Kit	MoBio Laboratories Inc. Carlsbad, USA

The chaotropic DNA extraction method used in this dissertation was adapted from Godon 1997 (299) and has already been used by previous researchers in the same lab(126). This method was chosen because it involves extensive cleaning and purification steps in an attempt to remove the impurities and inhibitors present in the faecal samples while keeping the gut microbiota composition and the quality of the obtained DNA intact. Previous work in the same department has shown that this method gives the highest yield and purity of genomic bacterial DNA (on spectrophotometry and agarose gel electrophoresis) as compared to other methods such as the QIAamp® DNA Stool Mini Kit, and phenol chloroform method. A summary of different chemicals/materials used in the bacterial DNA extraction process along with their concentrations and functions are given in Table 2.13.

Faecal samples were collected and homogenised as previously described. Approximately 200 mg faecal sample was stored in 1.5 ml screw cap tubes in quadruplicate and immediately stored in -80 ^{o}C. Before starting DNA extraction, each sample was thawed at room temperature. To avoid variability of extraction between the samples and to maintain efficiency of the researcher, DNA was extracted from a set of 12 samples each time with all samples from the same patient included in the same run. Each set of DNA extractions needed 2 days to complete. A summary of the steps involved in the DNA extractions using chaotropic method is given in the flowchart (Figure 2.11).

To lyse cells and virus particles in the samples, 250 µl of 4M Guanidine Thiocyanate 0.1 M Tris-Cl (pH7.5) (Sigma Aldrich®) and 40 µl of 10% N-Lauroylsarcosine (Sigma Aldrich®) was added to each sample and homogenised by vortexing followed by centrifugation for three seconds at 15,000 g in Thermoscientific ultracentrifuge (Thermo Electron corporation, UK). The sample was then incubated at room temperature for 10 minutes.

Anionic surfactant, N-Lauroylsarcosine (5%) and 500 µL, already prepared in 0.1 M Phosphate buffer (pH8.0), was added to the sample. The sample was then homogenised thoroughly by vortexing and centrifuged for three seconds before being incubated for one hour at 70 °C in a dry bath (Dri-Block Teche, UK). The sample was vortexed at 20 minute intervals, then centrifuged for three seconds before adding 750 mg of sterile 0.1 mm zirconia glass beads (Biospec Products. USA) to disrupt cells.

After vortexing briefly; further cell disruption was achieved using an MP FastPrep®-24 benchtop homogenizer for 3x30 seconds at 6 m/s, resting between each burst for 15 seconds to allow cells to cool down. Samples were then placed on ice for 5 minutes before homogenizing again for 3x30 sec at 6 m/s. The samples were again placed in ice for five minutes; centrifuged for three seconds before adding 15 mg of PVPP powder (Sigma Aldrich® Co) and vortexed upside down to dissolve the pellet.

Table 2.13: Chemicals used in bacterial DNA extraction with their method of preparation and respective functions

Chemicals	Conc.	Preparation	Function
Guanidine Thiocyanate (GTC)	4 M	GTC [Mw = 118.16] 12.37 g Double distilled water (ddH_2O) 13.5 ml Tris-Cl 1 M (pH 7.5) 2.6 ml Filter-sterilise *(Heat to dissolve)* Filter into 5 ml bijoux tubes Cover with foil (light sensitive) Store in fridge	Lyse cells and virus particles in RNA and DNA extractions Prevent activity of RNase enzymes and DNase enzymes by denaturation
N-Lauroylsarcosine	5 %	N-Lauroylsarcosine 1 g Fill up to 20 ml with Phosphate buffer 0.1 M (pH 8) Store in fridge	Anionic detergent
N-Lauroylsarcosine	10 %	NLS 1.1g + 8 ml sterile H_2O Fill up to 11 ml with ddH_2O Store in fridge	Anionic detergent
NaCl	5 M	NaCl [Mw = 58.44 g/mol] 14.61 g Fill up to 50 ml with ddH_2O Autoclave	Used in TENP buffer
Phosphate buffer (pH 8.0)	0.1 M	Na_2HPO_4 1M 9.32 ml mix with NaH_2PO_4 1M 0.68 ml sterile H_2O 90 ml Autoclave Adjust pH with 37% HCl	Act a buffer
Potassium Acetate	5 M	Potassium Acetate (Mw = 98.15 g/mol) 4.9075 g ddH2O fill up to 10 ml Filter-sterilise	Used as a salt for the ethanol/isopropanol precipitation of DNA
Ethanol	70 & 100%	For 70%; Absolute ethanol mixed with dH2O in 70:30 ratio For 100%; Absolute ethanol used as such	Precipitation and recovery of DNA
PVPP (Polyvinylpyrrolidone)	NA	PVPP 15mg in a cheap 1.5 ml microcentrifuge tube (1 tube = 1 sample) Centrifuge for 10 seconds at high speed	dispersion enhancing agent and ensure removal of polyphenol contamination that could inhibit subsequent qPCR reactions
Tris-Cl (pH 7.5 and pH 8)	1 M	Trizma base [Mw = 121.1 g/mol] 12.11 g ddH_2O Fill up to 100ml Autoclave Split into 2x 50ml and adjust pH with concentrated HCl	Mixed with EDTA, NaCl, and PVPP as dispersion agent to clean DNA
RNAase	10 mg/ml	RNAase 10 mg Tris-Cl 1 M (pH 7.5) (= 10 mM) 10 µL NaCl 5 M (= 15 mM) 3 µL ddH_2O fill up to 1 ml Keep in freezer	Removes RNA from DNA preparations by cleaving phosphodiester bond between any two ribonucleotides
Sodium Acetate	3 M	Sodium acetate [Mw = 82.03 g/mol] 2.4609 g ddH_2O Fill up to 10 ml filter-sterilise	Acts as a buffer and added before ethanolic precipitation of DNA as a source of monovalent cations.
TENP buffer	-	Tris-Cl 1M (pH 8) 1 ml EDTA 0.5 M (pH 8) 0.8 ml NaCl 5 M, 0.4 ml ddH_2O Fill up to 20 ml PVPP (= 1 %) 0.2 g (just before using)	Mixture used as dispersion agent to clean DNA

Proteinase K	-	-	serine protease Activated by calcium and digests proteins preferentially after hydrophobic amino acids (aliphatic, aromatic and other hydrophobic amino acids).
Sodium Dodecyl Sulphate (SDS)	10%	SDS 10 mg dissolved in 90 ml of ddH_2O	strong anionic detergent. Works by disrupting non-covalent bonds in proteins, denaturing them, and causing the molecules to lose their native shape

Samples were then placed on an orbital shaker (IKA® VIBRAX VXR basic) for 5 minutes, at low speed to avoid damaging DNA and then centrifuged for 3 minutes at 15000 g and 4 °C. The supernatant was carefully recovered in sterile 2ml eppendorf tubes and the pellet washed with 500 µl TENP buffer which had been vigorously shaken immediately before use to disperse the PPVP. The sample was vortexed upside down until the pellet dissolved and then centrifuged for 3 minutes at 15000 g and 4 °C. The supernatant was recovered in the same 2 ml eppendorf and this step of washing, centrifuging and recovery repeated twice.

The final 2 ml supernatant was centrifuged for 10 minutes at 15,000 g (4 °C) and then split into 3 eppendorf tubes (2 x 750 µl and 1 x 500 µl) .Proportionate amount of isopropanol (1:1, v/v) was added to each sample to precipitate nucleic acids and gently mixed by hand before being incubated for 10 minutes on the bench.

Each sample was centrifuged for 5 minutes at 15000 g and 4 °C and the supernatant was discarded. Each inverted eppendorf was tapped onto absorbent paper until the paper was dry and then left to air dry with the lid open for 15 minutes. To precipitate DNA, 225 µl of phosphate buffer 0.1 M (pH 8) was added to each sample before adding 25 µl potassium acetate 5 M. The 2 ml eppendorf tubes were vortexed shortly and put on an orbital shaker for 5 minutes to break down the pellet. All the samples were then left overnight in the fridge at 4 ^{o}C.

The following day (Figure 2.11); samples were shaken on an orbital shaker for 10 minutes. Each sample split previously into three eppendorf tubes were combined together in one of the 2 ml tubes. Samples were then centrifuged for 30 minutes at 15000 g (4 °C) and the supernatant was recovered in a new 2ml eppendorf tube. To remove any potentially present RNA in the sample, 5 µl of RNAase (RNAse ONE, M426A, Promega®) 10 mg/ml added to each sample. Samples were vortexed shortly thereafter, centrifuged for 3 seconds

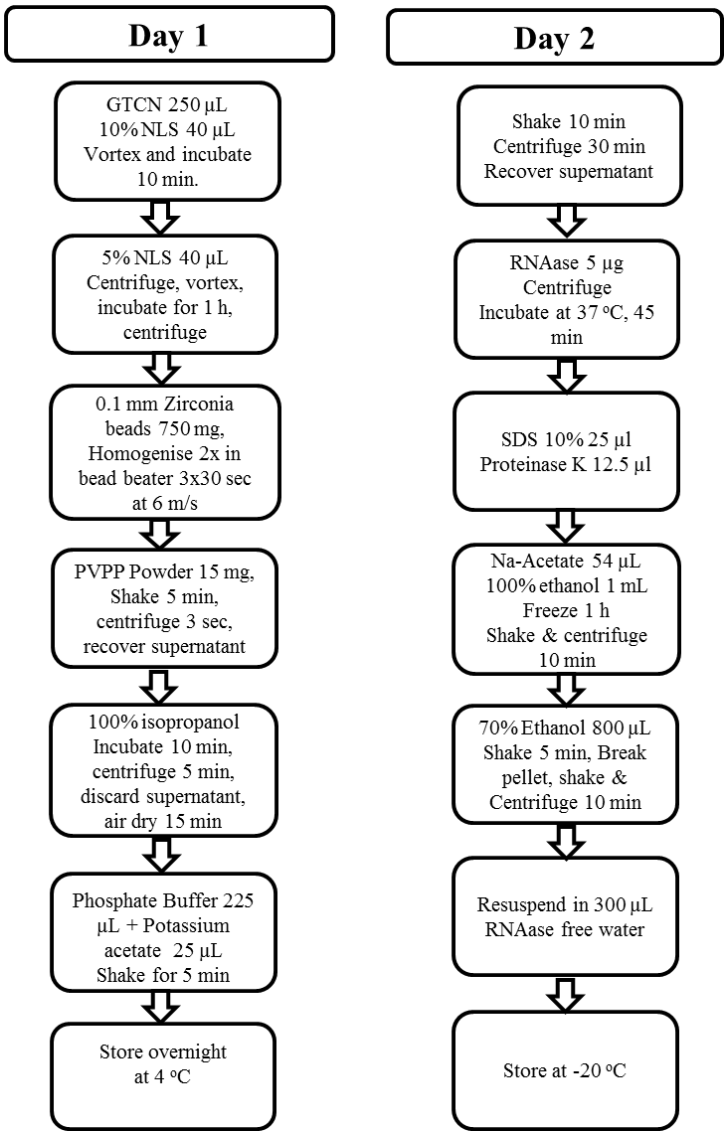
to bring the contents down, and then incubated for 45 minutes in dry bath at 37 °C. Each sample was intermittently vortexed after every 15 minutes to homogenise the contents.

An additional step involving the addition of 25 µl of SDS 10% (Sigma Aldrich® Co), and 12.5 µl of Proteinase K 800 units/ml (Sigma Aldrich® Co) to the sample was used to break down proteins in order to reduce potential inhibition of the PCR reaction. The samples were vortexed for 3 seconds and then incubated at 45 °C for 2 h. Each sample was vortexed briefly after every 30 minutes to mix the components.

After incubation; each sample was centrifuged for 3 seconds and 54 µl of 3 M sodium acetate buffer (S2889, Sigma Aldrich®) was added to precipitate the DNA. This was followed by 1 ml 100% ethanol (-20°C) which was mixed by inverting. The sample was then frozen at -20 °C for 1 h, shaken on an orbital shaker for 10 minutes and then centrifuged for 10 minutes at 15000 g and 4 °C.

The supernatant was discarded and 800 µl of ice cold 70% ethanol (Fisherscientific®, UK) (-20 °C) was added to the pellet, vortexed, and then shaken at medium speed on an orbital shaker for 5 minutes. The pellet was then broken up by pipetting with a wide tipped 1ml pipette and shaken on an orbital shaker at medium speed for 10 minutes followed by centrifugation for 10 minutes at 15000 g and 4 °C. The supernatant was discarded and these steps (washing-breaking the pellet - vortexing for 10 min - centrifugation for 10 min - discarding the supernatant) were repeated 3 times in total. The pellet was then dried on a lint-free paper and then air dried under the biological cabinet (name) for one hour. Each sample was then re-suspended in 300 µl of RNAase-free water (Fisherscientific®, UK). Each sample was aliquoted equally in 5 flat cap thin-walled 0.2 ml PCR tubes and stored at -20 °C until further analysis.

Figure 2.11: Day 1 (2.11a) and day 2 (2.11b) of the chaotropic method used for genomic bacterial DNA extraction.

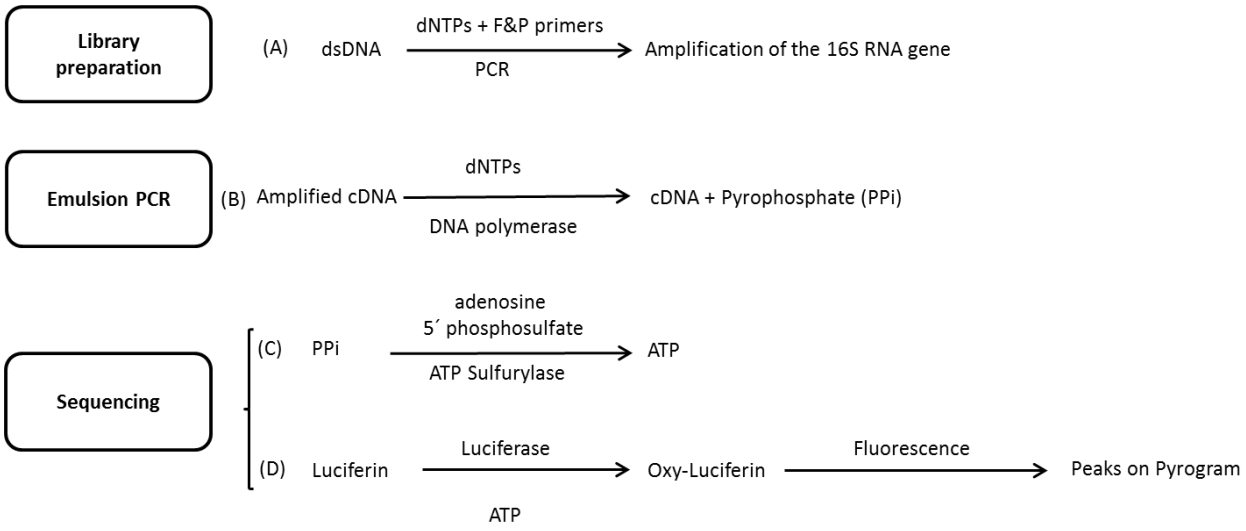


GTCN; Guanidine thiocynate, NLS; N-LauroylSarcosin, PVPP; Poly vinyl Pyrolidine, SDS; Sodium Dodecyl Sulphate, Na-acetate; Sodium acetate

2.3.10 Preparing amplicon pools for pyrosequencing

Genome wide sequencing is becoming more popular in current research on gut microbiota due to its ability to generate sequences for a large number of bacterial groups applying an untargeted approach. This enables the researcher to have a global view of the data in terms of relative abundance of gut microbiota rather than absolute quantities of a specific set of gut microbiota. Pyrosequencing involves sequencing of nucleotide bases along the length of DNA by the release of pyrophosphate after the incorporation of each nucleotide base along the complementary DNA strand (Figure 2.12). This is "sequencing by synthesis" unlike Sanger sequencing; which is "sequencing by chain termination" with di-deoxynucleotides.

Figure 2.12: Summary of reactions involved in Pyrosequencing.



16S RNA gene of the double stranded DNA (dsDNA) is amplified by PCR reactions in the presence of degenerate forward primers (F), bar coded reverse primers (R) specific for each amplicon, and deoxynucleotides triphosphate (dNTPs). This library of Amplicons is then resized into small fragments. Each amplicon is then attached to a bead in a water-oil emulsion with solutions for emulsion PCR to give several millions copy numbers per bead. DNA polymerase attaches the nucleotide into the template. The pyrophosphate (PPi) released with each amplification process reacts with adenosine 5' phosphosulphate to generate Adenosine triphosphate (ATP) with the help of enzyme ATP Sulfurylase. This ATP is used for converting Luciferin to oxy-Luciferin with the help of enzyme luciferase. Oxyluciferin gives visible light that is detected by the detector to give a pyrogram. All unreacted nucleotides and ATPs in this reaction are degraded by the enzyme apyrase into adenosine monophosphates (AMP) in a wash cycle. This process is repeated with the attachment of every nucleotide and subsequent release of PPi.

As shown in Figure 2.12; the process of pyrosequencing involves preparation of amplicon library of 16S RNA using degenerate forward, and bar coded reverse primers prior to an emulsion PCR and pyrosequencing. This preliminary step was done in our laboratory and is therefore included in this dissertation.

The process of preparing 16S RNA gene amplicons involves a) PCR; using forward primers, reverse primers (Golay, barcoded), hotstart PCR mastermix and di-methylsulphoxide (DMSO), b) agarose gel electrophoresis to extract the bands and c) band extraction d) measuring the concentration using flourometer e) adjustment of the amplicons concentration to the desired concentration, and pooling of the amplicons.

2.3.10.1 PCR amplification;

A day before PCR amplification of the double stranded DNA, bar coded fusion reverse primers (Golay barcoded primers) specific for each sample were diluted to 1 ng/µl from their original concentration and aliquoted into separate thin walled 200 µl PCR tubes from the 96 well plate. The list of reverse primers used for all 150 samples is given in appendix (appendix-3). Sequence of nucleotides used in the forward primer obtained from Eurofins

MWG Operon (Ebersberg, Germany) was 5´-GTGNCAGCMGCCGCGGTAA-3´, where "N" represents "any base" (A, C, T, G) and "M" represents "amino (A & C)". This is an example of a degenerate forward primer, which are often used if the same gene (such as 16S rRNA) is to be amplified from different microorganisms. It is commonly used in molecular microbiology because it allows the amplification of genes from organisms that have not yet been cultivated, thus increasing the recovery of more and more genetic information.

Although this combination reduces the specificity of the PCR reaction by mishybridizations and primer dimers but this can be reduced by using modified PCR cycle conditions. In addition to dilution of the primers, a set of 4 PCR 200 µl tubes were sterilized for each sample (3 tubes for PCR in triplicate and one tube for non-template control labelled as "N"). Molecular biology grade, nuclease free water (Fisher scientific) was aliquoted into eppendorf tubes (Eppendorf®, USA, cat. No. 022363352) and UV sterilized under the biological laminar flow cabinet.

On the day of PCR; the biological cabinet was cleaned with 70% ethanol. All the pipettes to be used were given a clean wipe with alcohol. UV light was switched on for 15 minutes to ensure clean non-contaminated working space. Components for PCR reaction were mixed in the order given in Table 2.14. Nuclease free water was obtained from fisher scientific (Fisher Scientific® UK), PCR mastermix used was KAPA HiFi HotStart Ready Mix (KR-03070-v4.13) from KAPABIOSYSTEMS®, USA, and DMSO (cat. No. D1970-5VL) from SIGMA-Aldrich to improve the efficiency of the reaction. The PCR kit contained a hotstart DNA polymerase (which has the ability to amplify long and GC & AT rich targets), KAPA HiFi buffer, magnesium chloride 2.5nM (1x), and dNTPs (specifically treated for each kit). Additionally the enzyme is inactivated by a proprietary antibody until first denaturation step to reduce non-specific amplification.

All components for 4 reactions (3 for sample, 1 for non-template control) except template DNA were mixed in the tube "N", vortexed, and then added to the PCR tubes in triplicate (23 µl in each tube). DNA template (2 µl) was added to the 3 PCR tubes for the sample, and same volume of water was added to the tube "N" instead of the template DNA. A set of 10 samples were amplified at one time as a standard practice.

Table 2.14: Components of PCR reaction

Component of PCR reaction	Volume per reaction
Nuclease Free water	7.51 µl
KAPA hifireadymix (2x)	12.5 µl
forward Primer (same for all samples)	0.87 µl (0.35 µM)
Reverse Primer (different for each sample)	0.87 µl (0.35 µM)
DMSO(Di-Methyl Sulfo-Oxide)	1.25 µl
Template DNA	2 µl
Total volume of one reaction	25 µl

Within each set; equal number of samples from all the groups (lean, obese, hypothalamic obese/lean) were used to eliminate a possible variation with time and conditions. All the tubes were vortexed again and then placed in the PCR thermal cycler machine (PCR Engine, MJ Research USA). The samples were amplified in the following cycle temperatures to aim for the amplicon band size of 378-400 bp;

95 ^{o}Cfor 5 min

98 ^{o}Cfor 20 sec

60 ^{o}Cfor 15 sec

72 ^{o}Cfor 1 min

Repeat step b to step d for 25 cycles.

4 ^{o}C (until the samples were taken out of the PCR machine).

2.3.10.2 Agarose gel electrophoresis

While the PCR reaction was in progress, the casting base of the agarose gel tank was cleaned and prepared for agarose gel electrophoresis. 2 g agarose was weighed out and mixed with 200 ml 1X TAE buffer. 1X TAE buffer was prepared by diluting 1 part 50X TAE buffer in 49 parts dH_2O. This solution was then heated in the microwave to boil until the solution was clear. The solution was cooled for 2-3 minutes at 60 ^{o}C in a water bath. SYBR® safe DNA gel stain 10,000X in DMSO (Invitrogen®, USA) in a ratio of 1 µl/10ml was mixed in this solution. This DNA staining dye is safe and less toxic as compared to the

more popular ethidium bromide. The dye is light sensitive; hence, it was always stored in the dark and wrapped in aluminium foil. The 1% agarose gel in solution was emptied into the gel casting base and allowed to cool for 50 minutes in the dark (by covering the gel with aluminium foil). The tank was filled with 1X TAE buffer. This buffer was replaced after every three gels to avoid contamination of the samples.

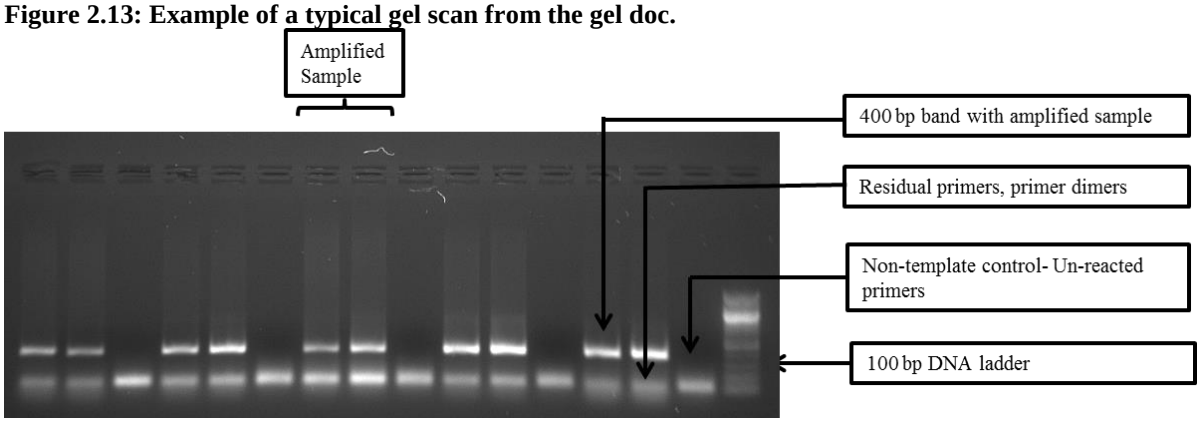
After 50 minutes, the combs were vertically lifted and carefully removed from the gel. The gel was then placed in the tank. One of the three amplified sample tube was equally distributed between the other two amplified samples. To each of the amplified sample and the non-template control was added 3 µl blue/orange loading dye 6X (Promega Corporation, USA). This dye has xylene cyanol, bromophenol blue, and orange G to facilitate optimization of the gel run time and to track the gel to prevent the smaller fragments migrating too far in the gel. A 100 bp DNA ladder was used to quantify the base-pair size of the DNA templates. The wells were loaded in the order; 2 samples followed by a non-template control. The total volume added in each well was 40.5 µl for the samples (37.5 µl PCR amplicons + 3 µl loading dye 6x) and 28 µl for the non-template control (25 µl un-reacted PCR amplicons + 3 µl Loading dye 6x). Tanks and gels of different sizes were tried before the actual samples were applied to ensure that the entire sample from the tube is electrophoresed without leakage and cross contamination. The samples were then allowed to run for 45 minutes at 125 volt. The gel was visualized using Gel Doc 2000 (Bio Rad) with ethidium bromide filter.

Each sample typically gave two bands (Figure 2.13); a proximal band with the amplified sample and a distal band with the residual primers, primer dimers, and other degraded nuclear material. The non-template control gave only one distal band at the end of the gel signifying that the reaction did not amplify any external DNA and was not contaminated. Samples which showed extensive smearing, indistinct bands, absent bands, or multiple bands were re-amplified.

2.3.10.3 Extraction of gel bands using QIAquick gel extraction kit

Samples that gave a distinct band were extracted using QIAquick gel extraction kit (Cat. no. 28705 QIAGEN® Germany) (Table 2.15). This is a silica membrane assembly based on the binding of DNA to a high salt buffer and eluting DNA with a low-salt buffer or water. The membrane allows the elution of the DNA free of enzymes, primers, salts, nucleotides, mineral oils, agarose, staining dyes, and other impurities while ensuring approximately 80% DNA recovery.

Figure 2.13: Example of a typical gel scan from the gel doc.



The amplified sample (in duplicate) gives two bands with proximal amplified sample band of about 400 bp and a distal more diffuse residual primers, primer dimers. The non-template control (in singlicate) with each duplicate sample gives only one band

Table 2.15: Components of the QIAquick gel extraction kit with their functions

No.	Component	Proportion used	Function
1	QIAquick spin column	One column for each sample	Elution of impurities-free DNA
2	Buffer QG (guanidine isothiocynate with a pH indicator)	3 volumes(3x w/v in µl) the weight of the band excised and 500 µl in another step	High salt buffer to bind DNA
3	Isopropanol (100%)	One volume(1x w/v in µl) the weight of the excised band	For precipitation of amplicons
4	Buffer PE	300 µl	Washing the spin column
5	Buffer EB(10 M Tris.Cl, pH 8.5)	30 µl	Low salt buffer to elute amplicons
6	Gel Loading dye	3 µl in each PCR amplified sample	Stains the amplicons and holds DNA in the wells

Amplicons were carefully excised from the gel in square shape bands into a 2 ml DNA low-bind Eppendorf tube (Eppendorf®, USA) under the UV light and then weighed on electronic scale. Care was taken not to expose bare skin to the UV light by using a UV protection board, full body cover, and UV gargles. The maximum weight of excised band was kept below 400 mg as the subsequent process of purification involves addition of proportionate volumes of buffers (given below) that would not accommodate the entire sample in a single eppendorf tube. Three volumes (3x w/v) of buffer QG were added to the eppendorf tubes having DNA fragment. Yellow colour of Buffer QG indicates a pH $\leq$7.5 and DNA adsorption to the membrane in spin column is only efficient at this range of pH.

The mixture was then incubated at 50 ^{o}C for 10 min in a dry bath and vortexed every 2-3 minutes to dissolve the gel slice. The colour of the mixture was observed after the gel slice had dissolved completely (it should be similar to the buffer QG). A change in colour to orange or violet shows an increase in pH. If so, 10 µl, 3 M sodium acetate, pH 5.0, was added and mixed. The mixture would turn yellow. Equal volume of 100% isopropanol (equal to the weight of DNA fragment excised) was then added to the sample and mixed shortly.

QIAquick spin columns labelled for each sample were placed in the provided 2 ml tubes. The sample was applied to the QIAquick column and centrifuged for 1 min at 17,900 g. The flow-through in the 2 ml tube was discarded, and the column placed back into the same tube. For sample volumes of >800 µl, the sample was loaded onto the column, spun again, and the flow-through discarded. 500 µl Buffer QG was added to the QIAquick column and centrifuged for 1 minute at 17900 g and the effluent was discarded again. To wash the sample, 300 µl Buffer PE was added to the QIAquick column, allowed to stand for 2-5 minutes, and centrifuged for 1 min at 17,900 g. The flow-through was discarded and the QIAquick column was placed back into the same tube. The remaining sample in the QIAquick column was centrifuged again for 1 min at 17,900 g to remove any residual wash buffer.

The column was then placed into a clean 1.5 ml DNA Lowbind eppendorf tube. To elute DNA, 30 µl Buffer EB (10M Tris.Cl, pH 8.5) was carefully pipetted on the middle of the membrane so that amplicons up to 30 µl can elute. The buffer was allowed to stand for up to 8 minutes. The column was centrifuged for 1 min at 17,900 g to precipitate DNA into the DNA Lowbind eppendorf tube and then aliquoted into 200 µl thin walled PCR tubes and stored in -20 ^{o}C.

2.3.10.4 Measuring the concentration of amplicons using Qubit® 2.0 flourometer (life® technologies, USA)

This is a 2nd generation flourometer to measure the concentration of DNA by high sensitivity fluorometric probe that emits light only when it specifically attach to the target molecules, thus giving more accurate results. It has the additional advantage of measuring of broad range DNA quantities using separate broad-range assay reagents.

To measure the concentration of amplicons in each samples, working solution was prepared by adding 1 µl dsDNA HS assay reagent (life® technologies, USA) in 199 µl buffer (life® technologies, USA). One µl of amplicon was mixed with 199 µl of this working solution in special 500 µl PCR tubes (life® technologies, USA) supplied with this equipment. The flourometer was calibrated before these measurements every day, using

two standard reagents S1 and S2 (Cat. No. life® technologies, USA) supplied with the kit. Working solution was prepared as mentioned above, but instead of adding 1 µl sample, 10 µl of Standard S1 and S2 were added to 190 µl of working solution in separate tubes, vortexed briefly, and then measured on the Qubit®.

Samples with concentration less than 2.5 ng/µl were re-amplified (using the same reverse primers) and then purified after gel electrophoresis until this minimum concentration was achieved.

2.3.10.5 Adjusting the concentration of the amplicons and preparing amplicons pool

The final concentration of the amplicons for each sample was standardised to 2.5 ng/µl so as to standardise the quantity of the DNA used for emulsion PCR and sequencing. Proportionate amount of sample and nuclease free water were mixed in separate 200 µl thin walled PCR tubes to make a final volume of 30 µl.

From each of these samples (n=150); 2 µl sample was pipetted into a single 0.5 ml DNA lowbind eppendorf tube (Eppendorf®, USA) to make a total of 300 µl amplicon pool and stored in -20 ^{o}C. DNA concentration of 5 samples measured by Qubit®, selected in random, showed a median concentration of 2.45 ng/µl. From this 300 µl pool, 100 µl was aliquoted into another 0.5 ml DNA lowbind tube and transported to the laboratory for pyrosequencing, in dry ice.

Chapter 3: Subject Characteristics

3.1 Chapter Outline

This chapter discusses the basic anthropometric, demographic, and dietary characteristics of the participants recruited in this study. It further explores changes in weight, body composition and dietary intake during the observational period of the study. These results will further be related to the gut microbial metabolic activity and diversity in chapters 4 and 6.

3.2 Patients and methods

Study subjects

Detailed description of the recruitment process is given in chapter 2 (subjects and methods). Briefly, simple obese (total n=16, young adults, n=2, children & adolescents, n=14), hypothalamic obese (total n=10, young adults, n=3, children & adolescents, n=7), and hypothalamic lean children/young adults (total n=12, young adult, n=1, children & adolescents, n=11), age 2-25 years, were recruited from the endocrine and dietetic clinics at the Royal Hospital for Sick Children Yorkhill Glasgow. Healthy lean children and young adults (total n=27, young adults, n=4, children & adolescents, n=23) were recruited from the local community.

Methods

Height was measured using a Seca® Leicester stadiometer (Seca213, Birmingham, United Kingdom) to the nearest 1 mm by the researcher. Body weight and body composition were measured with a TANITA® (TBF300, TANITA, Japan) body composition weighing scale. This scale measures single frequency foot-to-foot bioelectrical impedance and two compartment body composition analyses. Since the 1980's, impedance analysis has been widely accepted as a valid but mostly practical method to measure body fat and lean mass (300). Height measured in centimetres, age (in years), and gender was entered into the analyser prior to the measurement of body composition. The device uses 50 kHz, 500µA insensible current to measure impedance. The analysis of body composition, calculated by the in-built equations in the machine, was printed. Each of the body composition measurements was taken at baseline and after 2-3 months. Body composition with this method was not measured for some participants (n=19) because the feet of the children were too small to fit on both anterior and posterior electrodes in order to allow the measurement of impedance analysis (n=10/19), or the child was unwilling to have their

weight measured (n=3/19), or we were unable to arrange time with the participants (n=6/19).

The body composition data (including fat mass, fat free mass, and %fat) of children less than 6 years of age were excluded from the analysis due to lack of equations to calculate body fatness for this age. Bioelectrical impedance was adjusted for the height of each participant by dividing height squared in centimetres over resistance in ohms (Ht^2 cm/Ω) and then used for analysis as this is a measure of the resistance of the body to electrical current corrected for height(301). Fat index was expressed as fat mass in kg divided by height squared in metres. Lean index was expressed as fat-free mass in kg divided by height squared in metres. Weight velocity was expressed as weight gained in grams per kg body weight in 24 h.

Since, physiological weight gain, growth pattern, and body composition varies with age and gender, the UK 1990 reference growth standards were used to classify children as obese or non-obese. Standard deviation scores (SDS) were calculated using LMS growth software which is also based on the UK 1990 reference growth standards data(302). Children with BMI at or above 2 SDS were classed as obese and those below 2 SDS as non-obese. Height, weight, and BMI were expressed as standard deviation scores. Change in BMI SDS per month (ΔBMI SDS/month= BMI SDS at recruitment - BMI SDS after 2-3 months/ period between the two assessments in months) was calculated. Change in weight (g/kg body weight/day) in all participants during the period of the study was expressed by calculating growth weight velocity.

$$\text{Weight velocity (g/kg/day)} = \frac{\text{Weight in kg after 2-3 months} - \text{Weight in kg at recruitment}}{\text{Weight in kg at baseline/No. of days between the two assessments}} \times 1000$$

To obtain an estimate of socioeconomic status, the Scottish Index of Multiple Deprivation (SIMD) score was calculated (http://www.scotland.gov.uk/Topics/Statistics/SIMD). SIMD scores is a system of identifying small areas of multiple deprivations across the whole of Scotland. All areas are identified based on 7 different criteria including current income, employment, housing, health, education, geographic access, and crime. Each area is ranked from the most deprived (SIMD rank of 1) to a least deprived (SIMD rank 6505) area. SIMD ranks are also represented as quintiles from 1 to 5. The first quintile includes population range between rank 1-1301, 2nd quintile from 1302-2602, 3rd quintile from 2603-3903, 4th quintile from 3904 to 5204, and 5th quintile ranges from 5205-6505.

The food diaries were analysed using Windiet® 2005 software (Robert Gordon University Aberdeen UK). All foods inserted in the software were recorded as total caloric intake and major macronutrients per day. The amounts of macronutrients were then

expressed as proportion of total energy intake. The intake as a proportion of the daily recommended nutritional intake of protein was calculated using Department of Healthy recommendations 1991 (DoH 1991) while intake of dietary fibre as a percentage of recommendations was calculated using the UK Scientific Advisory Committee on Nutrition (SACN) 2011 report (303). Portion sizes for all unspecified foods were estimated from the information based on published data from National Diet and Nutrition Survey (NDNS). Foods not mentioned in the Windiet® software were added as per 100 grams dietary values to the software from the information given by the manufacturer such as Tesco and ASDA stores in the UK. A total of 99 new foods were added.

Ethical considerations

The study was approved by the West of Scotland Research Ethics committee and Research and Development (R&D) NHS Greater Glasgow and Clyde under the study number WS/11/032 (appendix 1), for a period of 4 years. Each participant (when age appropriate) and their carer gave informed written consent.

3.3 Data handling and cleaning

All the data collected during the course of the study were organised in a single Microsoft Office, Excel spreadsheet. The dietary data was first analysed by two independent researchers using Windiet® 2005 software for the macronutrients (carbohydrates, fats, proteins), dietary fibre, percent estimated average requirements (%EAR) and recommended nutritional intake of proteins (%RNI). These data were then compared between the two researchers, and the coefficient of variation (%CV) calculated. All macronutrients and dietary fibre with %CV of more than 25% were re-analysed and compared again for any differences in the diet plans (in terms of their food amount, proportion, and composition). These data from two researchers were averaged before being used for further analysis.

All other data collected from the analysis of the faecal samples (discussed in chapter 4) were first organised in individual Excel spreadsheets and then pulled together in a large dataset. All data for each participant were individually checked for any random, user-specific, or systematic error. The data were then copied onto a statistical package for summarized descriptive statistics and distribution to identify any abnormal values.

3.4 Statistics

The Anderson-Darling test of normality was applied to evaluate the normal distribution of continuous variables (i.e. BMI SDS). Probability plots showed a highly significant deviation of the anthropometric and dietary variables from normality hence non-parametric

statistics were used. All data were expressed as median and inter-quartile range (IQR). Kruskal-Wallis test was used to explore the differences between more than two independent variables. Mann-Whitney U test was applied to measure the differences in independent variables between simple obese and hypothalamic obese, hypothalamic lean and hypothalamic obese, healthy lean and hypothalamic lean, and healthy lean and simple obese children/young adults. Spearman Rank correlation was used to determine rank correlations between different continuous variables. No adjustment for multiple testing or false discovery rate was done for these tests. All significant p-values mentioned in this chapter should therefore be considered as nominally significant.

Univariate analysis followed by multivariate regression analysis was used to assess the associations of BMI SDS and changes in BMI SDS with demographic variables (i.e. SIMD rank scores) and with dietary macronutrients (fats, carbohydrates, proteins as grams and percentage) and energy intake expressed as %EAR. P-values of 0.05 were considered significant. All the data were analysed by Minitab Version 16 (Minitab® V. 16 Inc. USA).

3.5 Results

3.5.1 Demographic characteristics

The hospital records showed 83 patients with Prader Willi Syndrome diagnosed between 1950 and 2013 (Table 3.1). Over the past few decades, increasing numbers of female patients were diagnosed as the number of female children diagnosed with PWS increased from 27.27% (n=6) between 1950 &1980 to 46.43% (n=13) between 2001 and January 2013. During our study period (2011-2013), 48 patients with PWS were attending the Yorkhill endocrine clinics. Patients aged more than 25 years (n=7, 14.5%), or having severe co-morbidities (n=2, 4.2%) or using a regular probiotic drink (n=1, 4.2%) were excluded. Of the participants eligible for this study (n=38), 22 (57.8%) PWS patients were recruited into the study. Of these 22 patients, 10 (45.45%) were hypothalamic obese patients while the remaining 54.54% (n=12) were hypothalamic lean patients.

Table 3.1: Distribution of PWS patients on the Royal Hospital for Sick Children database from 1950 till January 2013

Time Period	Total PWS patients (n)	Male (n)	Female (n)
1950-1980	22	16 (72.72%)	6 (27.27%)
1981-2000	33	21 (63.63%)	12 (36.37%)
2001-2013	28	15 (54%)	13 (46%)
1950-2013	83	52 (62.65%)	31 (37.35%)

n; number of patients

Although there were 26 craniopharyngioma patients on the hospital record since 1976, 12 patients were attending the clinic in Yorkhill hospital during the study period. Of these, patients age >25 years (8%, n=1) were excluded, five patients (41%) declined to participate, and one patient (8%) did not respond after giving written consent. Five craniopharyngioma patients (41 %) took part in our study.

Overall, we were able to recruit 65 participants (34 females and 31 males); 16 simple obese, 10 hypothalamic obese, 12 hypothalamic lean, and 27 healthy lean children/young adults. Although the number of participants attending the endocrine and dietetic clinics in hypothalamic and simple obese group was more than the number we aimed at, we were not able to recruit a number of them because of their age, disease related co-morbidities, distance of residence from the hospital, decline to participate, and multiple admissions in the hospital.

3.5.2 Anthropometric characteristics of participants at baseline

Height and weight SDS of simple obese patients were significantly higher than hypothalamic obese group (p=0.01) (Table 3.2, Figure 3.1); however, BMI SDS was not significantly different (simple vs. hypoth. obese, p=0.1). As expected, the two obese groups (hypothalamic and simple obese groups) had a significantly higher BMI SDS than the two lean groups (healthy lean and hypothalamic lean) (Table 3.2, Figure 3.1). Height SDS was similar between hypothalamic lean and hypothalamic obese participants, however, both were significantly shorter [median (IQR): hypoth. Lean; -0.88(1.47), hypoth. obese; -1.09(1.85)] than the healthy lean [median (IQR); 0.67(2.22)] and simple obese participants [median (IQR); 0.74(2.58)] (Table 3.2, Figure 3.1). This indicated a strong influence of pathology on height SDS. Therefore, when participants were grouped together based on the presence or absence of pathology, pathological group (hypothalamic lean and hypothalamic obese) showed a significantly lower height SDS than healthy group (healthy lean and simple obese) [Median (IQR), pathological group; -0.998(1.622) vs. healthy group; 0.704(2.458), p=0.0002] (Figure 3.2).

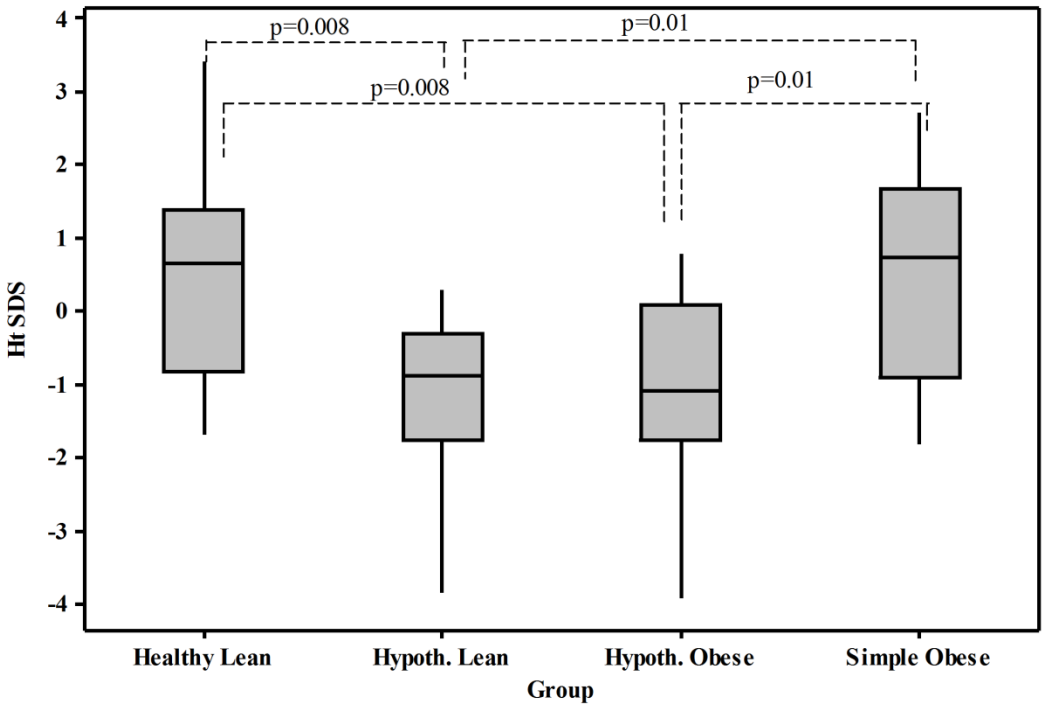
Table 3.2: Basic anthropometric characteristics of the study participants at baseline (at the time of recruitment).

	Hypoth Lean (n=12)		Hypoth. Obese (n=10)		Healthy Control (n=27)		Simple Obese (n=16)	
Variable	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Age (Years)	6.31	9.14	9.84	13.13	9.47	7.67	11.38	6.76
Ht.(cm)[a]	111.5	61.15	136.1	45.98	143	51	150.5	23.05
Wt.(kg)[a]	23.25	33.92	55.5	61.8	30.8	39	79.95	59.55
BMI (kg/m^2)[a]	18.17	3.34	29.04	15.27	16.28	5.30	36.15	12.63
Ht. SDS	**-0.88¥**	1.47	**-1.09‡**	1.85	**0.67¥**	2.22	**0.74‡**	2.58
Wt. SDS	**0.31†**	1.37	**2.21‡†**	1.713	**0.43***	1.70	**3.55‡***	1.27
BMI SDS	**1.01†**	0.68	**2.91†**	1.161	**-0.28***	1.114	**3.74***	0.91

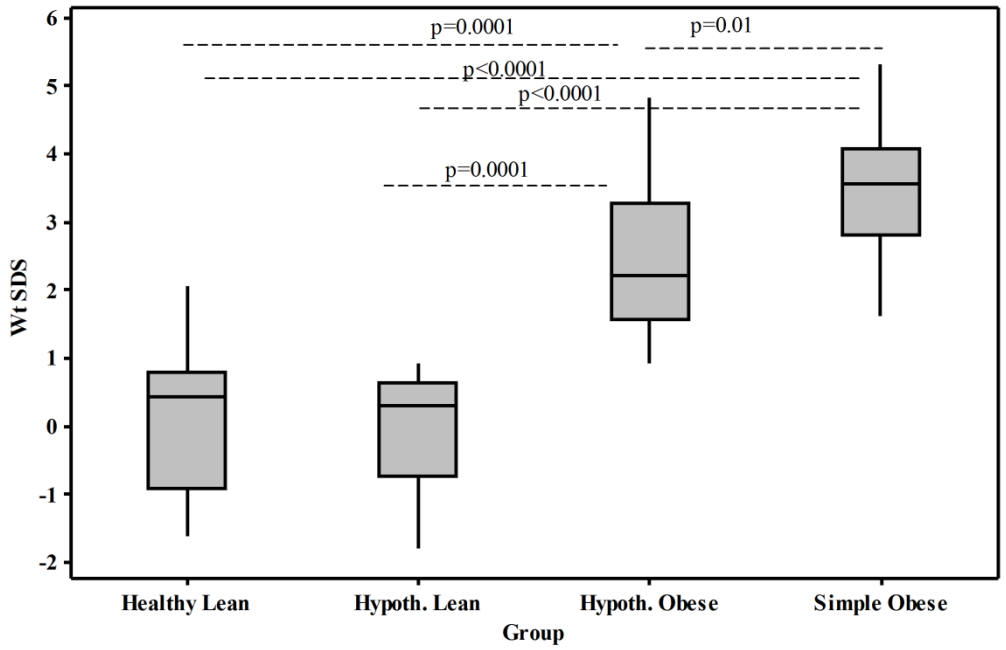
Hypoth.; hypothalamic (lean or obese), N; total number, SDS; Standard Deviation Scores.
All values are expressed as median (Interquartile range).
[a] Ht. (cm), Wt. (kg), and BMI (kg/m^2) were not used in the analysis. Age and gender adjusted SD scores were used instead.
† indicate significant differences between hypoth. lean and hypoth. obese
* indicate significant differences between healthy lean and simple obese
‡ indicate significant differences between hypothalamic obese and simple obese
¥ indicate significant differences between hypothalamic lean and healthy lean

Figure 3.1a, b, c: Boxplots showing height SDS (a), weight SDS (b), and BMI SDS (c) of all participants at the time of recruitment.

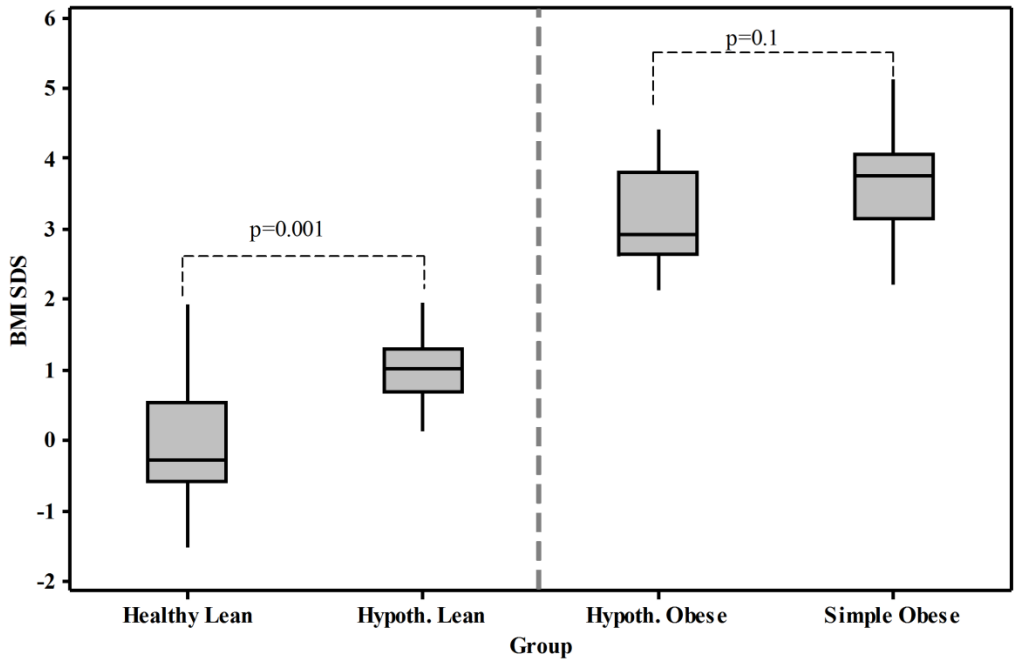
a)



b)

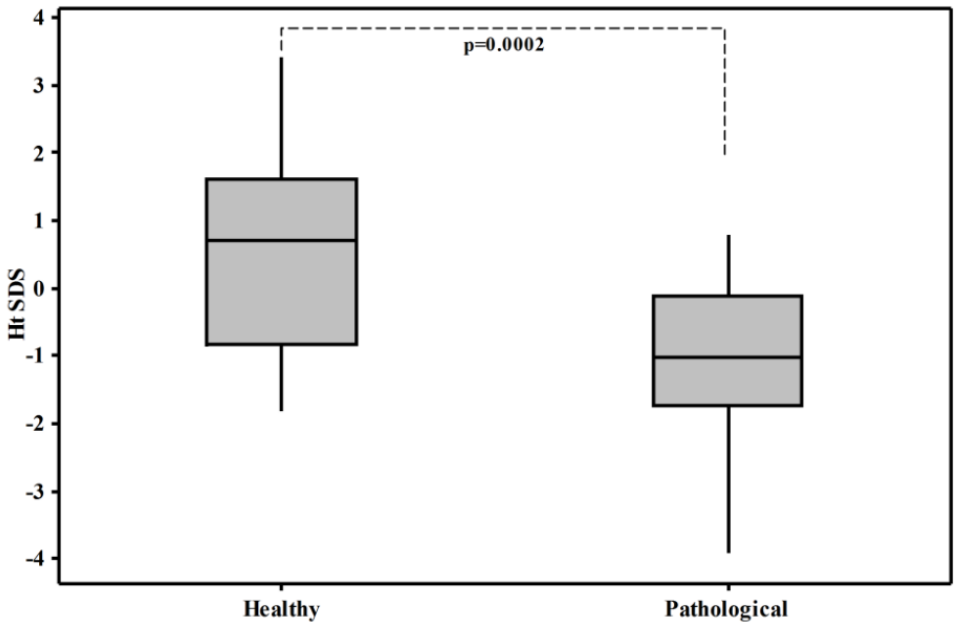


c)



Hypoth.; hypothalamic (lean or obese), Ht. height, Wt. Weight. Significant differences are based on Mann-Whitney U test.

Figure 3.2: Boxplot showing height SDS at the time of recruitment based on the presence of pathology.



Healthy; Healthy lean and simple obese, pathological; hypothalamic lean and obese. P-value based on Mann-Whitney U test

3.5.3 Social deprivation status of the study participants

Generally, participants in simple obese group were from a lower socioeconomic group with low SIMD rank score compared with all other three groups. However, this difference was only significant between simple obese and healthy lean group (p=0.01) (Table 3.3).

Considering all groups together, Spearman rank correlation showed a significant negative correlation of SIMD rank score with BMI SDS (p=0.006, R=-0.34) (Figure 3.3), height SDS (p=0.03, R=-0.26), and a trend towards weight SDS (p=0.08, R=0.28). Similarly, BMI SDS was significantly negatively associated with SIMD rank with the latter explaining 64.2% of the BMI SDS variation in general linear model analysis (p=0.005, R^2=64.16%), considering all groups together.

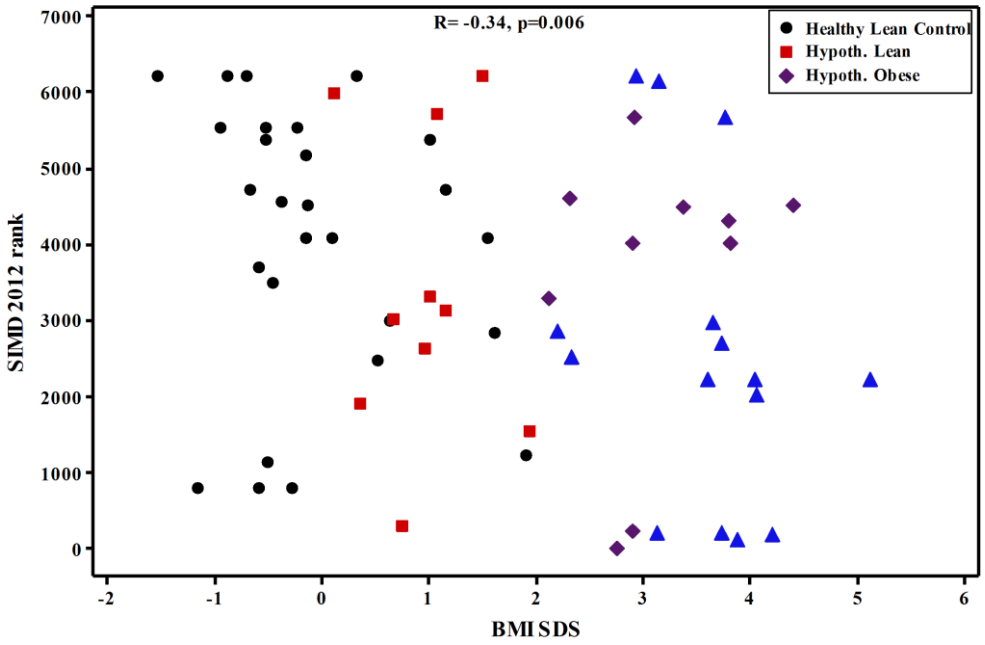
When participants were grouped based on the presence of pathology into healthy (healthy lean & simple obese) and pathological groups (hypoth. lean & obese), SIMD rank score was significantly negatively correlated with BMI SDS in healthy group (R= -0.47 p=0.002) and weight SDS (R=-0.32, p=0.039), but not in the pathological group (BMI SDS; R=0.92, p=0.701 & Wt. SDS; R=0.041, p=0.863) (figure 3.4). Height SDS was significantly positively correlated with SIMD rank scores in healthy group (R=0.400, p=0.008) but not in pathological group (R=0.02, p=0.935). Furthermore, general regression analysis adjusted for pathology showed a significant association of SIMD rank scores with height SDS (β-coefficient=5.625, R^2-adjusted=9.45%, p=0.005).

Table 3.3: SIMD rank scores and quintiles of all the study participants.

SIMD Quintile	Hypothalamic Lean† (N=12)	Hypothalamic Obese (N=10)	Healthy lean Control (N=27)	Simple Obese (N=16)
1n(%)	1(8.33)	2(20)	5(18.52)	5(31.25)
2n(%)	2(16.67)	0(00)	1(3.70)	5(31.25)
3n(%)	4(33.33)	1(10)	4(14.81)	3(18.75)
4n(%)	4(33.33)	6(60)	8(29.63)	0(00)
5n(%)	0(00)	1(10)	9(33.33)	3(18.75)
SIMD Rank	3127 (3806)	4160(2022)	4513 (2700)*	2193 (2732) *

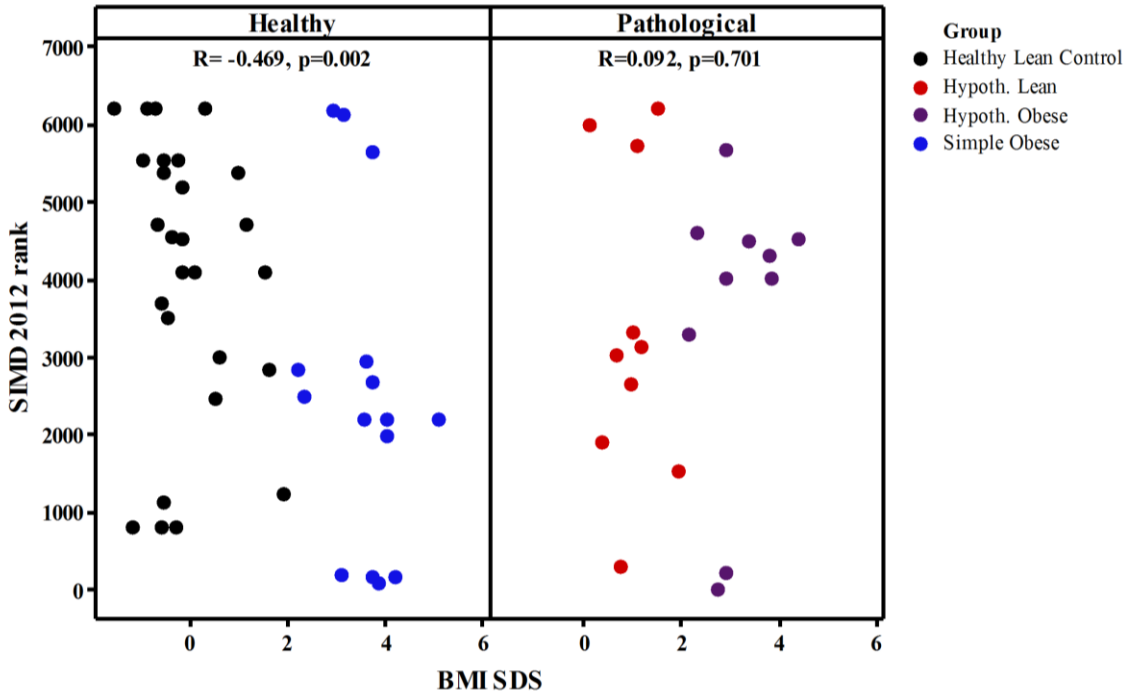
† One hypothalamic lean child was from Northern England. His deprivation score was therefore not included. * shows significant difference (p=0.01) between healthy lean and simple obese (Mann-Whitney U test). SIMD Quintiles are expressed as n(%) while SIMD Rank values are expressed as medians (Interquartile range).

Figure 3.3: Scatter-plot showing correlation of SIMD rank scores with BMI SDS of all participants at the time of recruitment.



R; Spearman rank correlation, Hypoth.; hypothalamic (lean or obese)

Figure 3.4: Scatter-plot showing correlation of SIMD rank scores with BMI SDS based on the presence or absence of pathology.



Healthy; healthy lean & simple obese, pathological; hypothalamic lean & obese, R; Spearman-rank correlation, hypoth.; hypothalamic (lean or obese)

3.5.4 Differences in body composition measurements

Body composition measurements were not available for all participants (especially <7 years) as explained above. Data for participants was available for 5/12 hypothalamic lean, 4/10 hypothalamic obese, 23/27 healthy lean, and 13/16 participants in simple obese groups at the time of recruitment. Body composition analysis of simple obese participants showed significantly higher fat mass (kg) compared with the hypothalamic obese group (median (IQR): simple obese; 40.7(25.28) kg vs. hypoth. obese; 26.85(20.25) kg, p=0.031) (Table 3.4, Figure 3.5b). However, no differences were seen between simple and hypothalamic obese participants when fat mass and fat-free mass was expressed as fat index (fat mass in kg/m^2) or lean index (FFM in kg/m^2) (Table 3.4).Both simple obese and hypothalamic obese groups had significantly higher fat mass, percentage body fat, and fat index (fat mass in kg/height^2 in meters) when separately compared with the two lean groups at the time of recruitment and after 2-3 months (Table 3.4, Figure 3.5a, b, c, and e). There was a striking similarity in the composition of fat-free mass between hypothalamic lean, hypothalamic obese and healthy lean participants (Table 3.4, Figure 3.5d).

Bioelectrical impedance expressed as height squared in centimetres divided by the resistance in Ohms (Ht^2/Ω) was not significantly different between any of the groups except between healthy lean and hypothalamic lean participants after 2-3 months (p=0.011, Figure

3.6) When participants were grouped either into a pathological group (hypoth. Lean & obese) or healthy group (simple obese & healthy lean), the former showed significantly lower impedance compared to healthy group only at the time of final assessment (p=0.0096, Figure 3.7b).

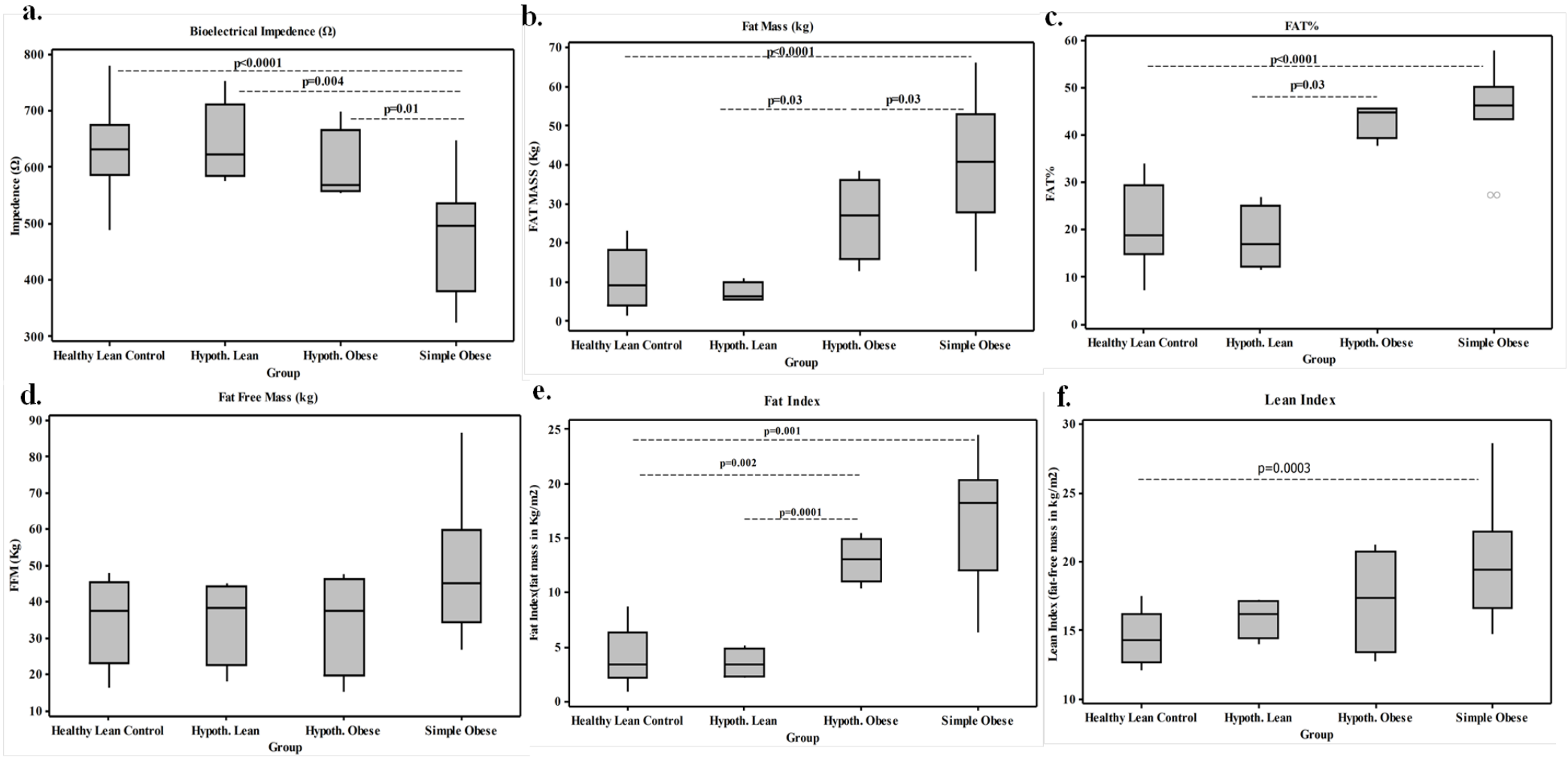
Additionally, fat mass (p<0.0001, R=0.87), percentage fat (p=0.0001, R=0.88), fat index (p=<0.0001, R=0.91) and fat-free mass (p=0.003, R=0.46) were positively and significantly correlated with BMI SDS.

Table 3.4: Body composition measurements of the participants at the time of recruitment and after 2-3 months.

	Hypoth. lean		**Hypoth. obese**		**Healthy lean**		**Simple obese**	
Variable	Median	IQR	Median	IQR	Median	IQR	Median	IQR
At recruitment	**n=5**		**n=4**		**n=23**		**n=13**	
Resistance (Ω)	623	126	568‡	108.8	631*	89	495*‡	156
Impedance Ht^2 / Ω	32.57	22.67	36.71	20.48	36.15	28.76	46.35	28.42
Fat%	16.85†	12.9	44.8†	6.25	18.7*	14.65	46.2*	6.8
Fat Mass (kg)	6.3†	4.33	26.85†‡	20.25	8.95*	14.23	40.7*‡	25.28
FFM (kg)	38.4	21.7	37.55	26.32	37.65	22.38	45.2	25.5
Fat Index (kg/m^2)	3.351†	2.628	13.02†	3.9	3.377*	4.085	18.19*	8.29
Lean Index(kg/m^2)	16.205	2.732	17.37	7.31	14.276	3.526	19.44	5.59
After 2-3 months	**n=7**		**n=7**		**n=20**		**n=12**	
Resistance (Ω)	718†¥	163	607†	159	600.5*¥	67.5	499*	139.5
Impedance Ht^2 / Ω	14.7¥	26.71	34.17	17.64	41.63¥	29.05	48.67	23.51
Fat%	24.3†	8.9	42.4†	11.2	14.8*	14.7	47.7*	10.1
Fat Mass (kg)	16.2†	6.3	28.5†‡	28.2	6.9*	13.8	43.6*‡	26
FFM (kg)	35.6	16.6	40.9	28.9	40.2	20.9	44.6	18.2
Fat Index (kg/m^2)	5.677†	2.767	13.37†	6.87	2.572*	3.617	18.47*	6.77
Lean Index (kg/m^2)	15.2	3.3	18.75	10.95	14.345	2.3	18.21	4.88

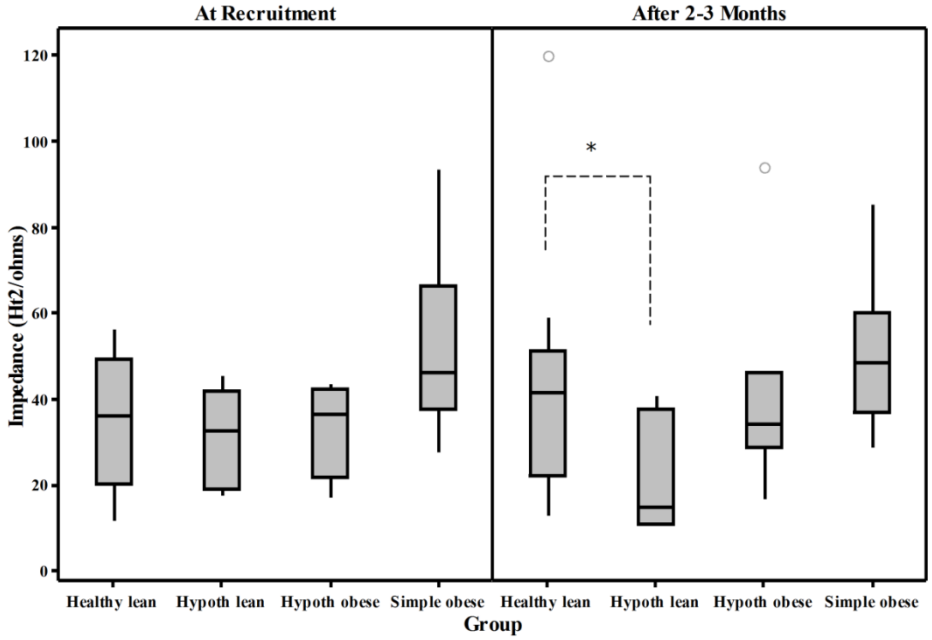
† indicate significant differences between hypoth. lean and hypoth. obese (p<0.01)
* indicate significant differences between healthy lean and simple obese (p<0.001)
‡ indicate significant differences between hypoth obese and simple obese (p<0.05)
¥ indicate significant differences between hypoth lean and healthy lean (p<0.01)

Figure 3.5: Boxplot showing bioelectrical impedance (a), fat mass (b), percentage body fat (c), fat-free mass (d), fat index (e), and lean index (f) of all groups at the time of recruitment.



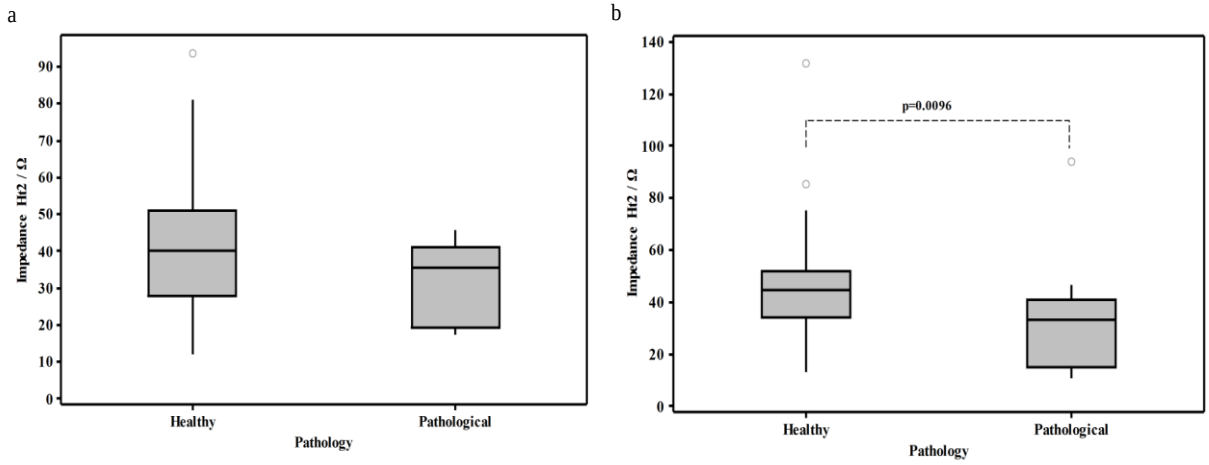
Significant differences shown in the figures are by Mann Whitney U test.

Figure 3.6: Boxplot showing bioelectrical impedance expressed as Height in cm^2/resistance in Ω at recruitment and after 2-3 months.



*indicate p=0.011 (Mann-Whitney U test)

Figure 3.7: Boxplot showing Bioelectrical impedance expressed as Height in cm^2/resistance in Ω in all groups according to pathology at recruitment (a) and after 2-3 months (b).



Healthy: Healthy lean and simple obese, Pathological: Hypothalamic lean and obese

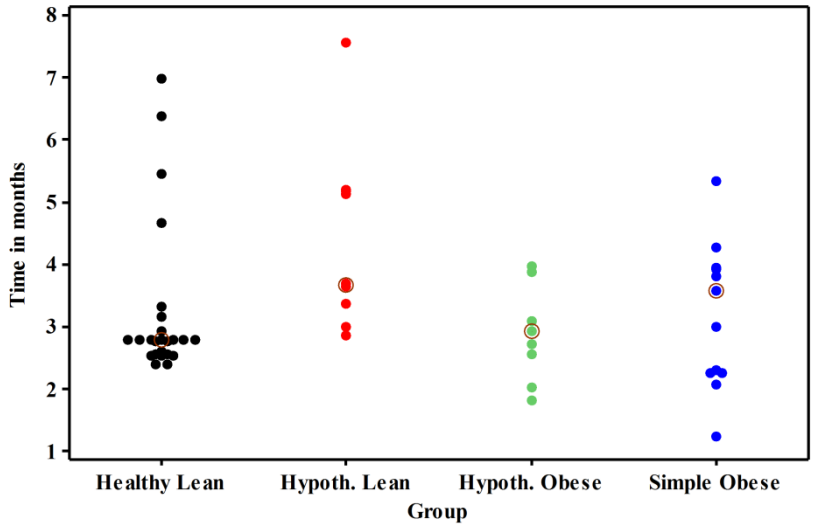
3.5.5 Changes in body composition and anthropometric parameters between baseline and after 2-3 months

The second assessment of participants from all the groups was carried out in median (IQR), 2.92(1.28) months (range: 1.25-7.56 months) and the time interval was not significantly different between any of the groups (Figure 3.8).

Changes in all other body composition measurements including impedance (Ht^2 cm/Ω), fat mass (kg), percentage body fat (%), fat-free mass (kg), fat index (fat mass as kg/m^2), and lean index (FFM expressed as kg/m^2) were not significantly different between the two obese groups and the two lean groups (Table 3.5).

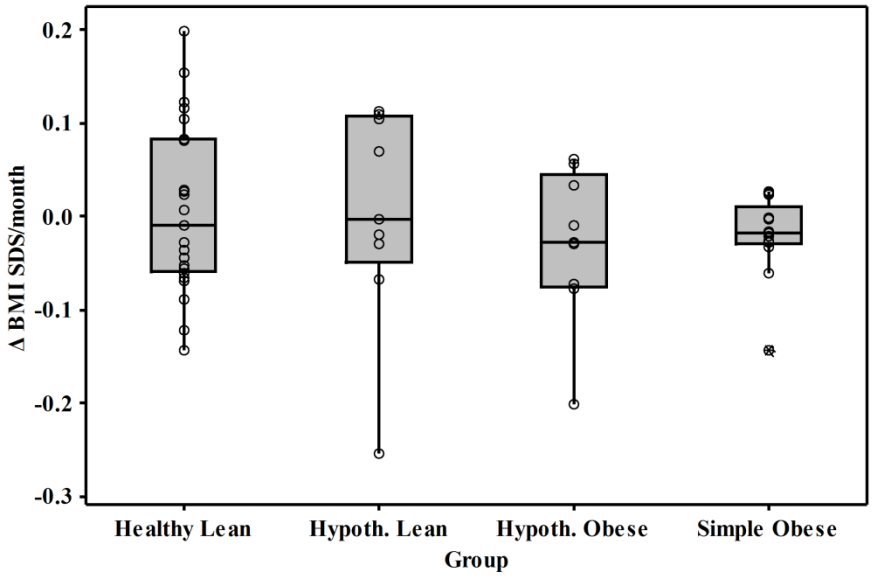
Change in weight SDS, height SDS, and BMI SDS (Δ) were not significantly different between any of the groups (Table 3.5). When expressed as change in BMI SDS per month (ΔBMI SDS/month), no significant differences were observed between any of the groups (Figure 3.8). Interestingly, the variation in Δ BMI SDS/month in simple obese participants was lower compared to all other groups. Δ BMI SDS/month increased in significantly higher proportion (%) of healthy lean than simple obese participants over the study period (n (%): healthy lean; 11(40.74) vs. simple obese; 3(18.75), $p<0.05$) (Figure 3.9, Table 3.6) while no differences were found between the hypothalamic lean and hypothalamic obese groups or hypothalamic obese and simple obese group.

Figure 3.8: Individual value plots of time (in months) elapsed between two body composition assessments.



Blank circle in each row represents median time in months.

Figure 3.9: Boxplot showing change in BMI SDS/month in all groups.



Blank Circles represent individual ΔBMISDS/month

Table 3.5: Changes in anthropometric and body composition measurements between baseline and after 2-3 months.

	Healthy Lean			Hypoth. Lean			Hypoth. Obese			Simple obese		
Variable	**Median**	**(Min**	**Max)**	**Median**	**(Min**	**Max)**	**Median**	**(Min**	**Max)**	**Median**	**(Min**	**Max)**
Changes in anthropometric measurements												
Number (n)		n=24			n=10			n=9			n=13	
Δ Ht (cm)	1.15	(0.00	4.00)	1.45	(0.00	7.10)	1.30	(0.00	8.30)	1.50	(0.00	3.10)
Δ Wt (kg)	0.60	(-2.20	2.40)	0.95	(-1.20	1.74)	0.90	(-4.00	6.00)	0.70	(-1.90	4.70)
Δ Ht SDS	0.03	(-0.36	0.60)	-0.15	(-0.35	2.63)	0.03	(-0.27	2.15)	0.04	(-0.41	0.50)
Δ Wt SDS	0.00	(-0.32	0.73)	0.26	(-0.43	1.99)	0.01	(-0.51	1.14)	-0.03	(-0.19	0.16)
Δ BMI SDS	-0.03	(-0.40	0.78)	-0.02*	(-1.30	0.57)	-0.06	(-0.62	0.22)	-0.07	(-0.18	0.10)
Changes in body composition												
Number (n)		n=17			n=1			n=3			n=9	
Δ Resistance (Ω)	**-26.0‡**	(-47.0	93.0)	-40.0	(40.0	40.0)	53.0	(-57.0	-43.0)	**2.50‡**	(-108.0	34.0)
Δ Impedance (Ht^2/Ω)	1.87	(-3.58	3.90)	1.92	(1.92	1.92)	**-2.97†**	(-3.55	-0.69)	**0.45†**	(-5.44	5.38)
Δ fat%	-1.00	(-4.10	1.60)	11.1	(11.1	11.1)	1.20	(-2.60	3.40)	0.00	(-6.50	4.10)
Δ fat mass (kg)	-0.30	(-2.30	1.40)	5.00	(5.00	5.00)	1.00	(-1.30	3.30)	-0.10	(-5.70	3.70)
Δ FFM (kg)	0.90	(-1.40	1.90)	-5.70	(-5.70	-5.70)	-0.70	(-1.10	0.00)	-0.60	(-2.70	5.40)
Δ fat index	-0.22	(-1.02	0.43)	2.03	(2.03	2.03)	0.36	(-1.42	1.89)	-0.09	(-3.24	1.26)
Δ lean index	0.15	(-0.65	1.01)	-2.36	(-2.36	-2.36)	-0.47	(-0.49	-0.06)	-0.45	(-1.36	1.54)

FFM; fat-free mass, Hypoth.; Hypothalamic (Lean or obese).
Delta represents the difference between the two measurements (follow-up – baseline).
All values are expressed as medians and (minimum-maximum).
Participants in each group above 6 years of age for whom body composition data was available at both time points were included.
Hypothalamus lean group is not compared in this table with healthy lean or hypothalamic obese due to small sample size
* Delta BMI SDS changes to 0.1(0.61) when one of the participant in this group, who lost weight, is excluded
‡ indicate significant difference between healthy lean and simple obese.
† indicate significant difference between hypoth obese and simple obese

Table 3.6: Change in BMI SDS/month in participants in all groups over the period of study.

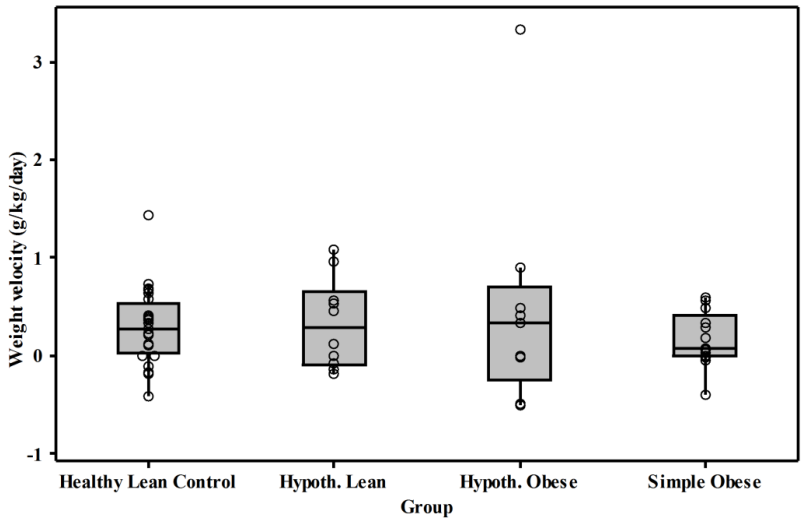
Group	Weight Gain (↑ΔBMISDS/month)				Weight loss (↓ΔBMISDS/month)			
	n (%)	Median	Q1	Q3	n (%)	Median	Q1	Q3
Healthy Lean (N=24)	11(40.74)*	0.08	0.03	0.12	13(48.15)	-0.05	-0.08	-0.03
Hypothalamic lean (N=9)	4(33.33)	0.11	0.08	0.11	5(41.67)	-0.03	-0.16	-0.01
Hypothalamic obese (N=9)	3(30.00)	0.06	0.03	0.06	6(60.00)	-0.05	-0.11	-0.02
Simple obese (N=13)	3(18.75)*	0.03	0.02	0.03	10(62.50)	-0.02	-0.04	-0.01

Data for 3 healthy lean, 3 hypothalamic lean, 1 hypothalamic obese, and 3 simple obese participant were not available at follow-up. * indicate significant difference between healthy lean and simple obese, $p<0.05$.

3.5.6 Differences in weight velocities observed over the period of follow up

Median weight velocities calculated as g/kg/day showed variations within the groups; however, the differences were not statistically significant between any of the groups (Figure 3.10).

Figure 3.10: Weight velocity (as g/kg/day) of study subjects expressed as median (IQR).



Blank circles represent individual values

3.5.7 Differences in dietary macronutrients and energy intake over the period of recruitment

To estimate the extent of under reporting, the reported energy intake was compared with the predicted energy requirement of each participant calculated using the Schofield equation (304) which is also used by the FAO (Food and Agricultural Organization) and WHO (World Health Organisation) in their expert reports (305). This equation estimates basal metabolic rate (BMR) in kilojoules/day considering body mass (in kg), age, and gender of an individual. Estimated BMR from the Schofield equation was multiplied by a physical activity level (PAL) value of 1.2 to calculate predicted or estimated energy

requirement per day. PAL value of 1.2 corresponds to the value to be multiplied with the BMR of a bed bound or chair bound person with no strenuous or leisure activity. Although our participants were not bed-bound, and Goldberg cut-offs (minimum suggested PAL=1.35) are the commonly used cut-offs (306), we applied this value as it was likely that some of our participants were on a weight loss diet. The actual energy intake reported by the participant was compared with the predicted energy requirement per day to estimate the proportion of actual energy intake (%AEI) of the participant. Based on these calculations, ~62% (40/65) participants under-reported, half (48%) of which was contributed by simple obese group.

The %AEI was significantly higher in healthy lean group compared to simple obese group, both at the time of recruitment [%median (IQR): healthy lean; 142.5(69.0) vs. simple obese; 67.4(39.1), p=0.0003] and after 2-3 months [%median (IQR): healthy lean; 157.7(71.0) vs. simple obese; 65.4(51.8) p=0.0005] (Table 3.7). Similarly, healthy lean participants also showed a significantly higher %EAR compared to hypothalamic lean group after 2-3 months (median (IQR) %: healthy lean; 97.88(43.89) vs. hypoth. lean; 64.78(27.39), p= 0.014 (Figure 3.11, Table 3.7).

In terms of the intake of individual macronutrients, no significant differences were observed between simple and hypothalamic obese participants (Figure 3.12&Figure 3.13). Compared to healthy lean participants, simple obese group reported significantly lower intake of carbohydrates in grams [median (IQR) g: simple obese; 136.8(57.6) vs. healthy lean; 235.7(85.0), p=0.0009] as well as proportion of carbohydrates [median (IQR) %: simple obese; 46.02(4.48) vs. healthy lean; 53.22(7.53), p=0.0013] only after 2-3 months (Figure 3.12, Table 3.7). On the contrary, the proportional intake of protein was significantly higher in simple obese than healthy lean participants [median (IQR) %: simple obese; 20.06(5.94) vs. healthy lean; 13.75(3.14), p=0.0013] only after 2-3 months (Figure 3.13, Table 3.7).

Although a general tendency of a lower dietary fibre intake was observed in obese groups compared to the lean groups (Figure 3.15), the intake of dietary fibre expressed as % DF recommended intake was significantly lower only in simple obese compared to healthy lean group after 2-3 months [median (IQR) %: healthy lean; 88.3(39.6 vs. simple obese; 40.8(33.5), p=0.0003] (Figure 3.16).

3.5.8 Changes in dietary macronutrients intake between two assessments within the groups

No significant differences in the dietary macronutrient intake were observed within the group over the period of study except that there was a significant increase in percentage

intake of daily protein in simple obese group [(median (IQR) %: 20.06 (5.94) vs. 18.91 (4.5), p=0.05] and in percentage intake of recommended dietary fibre in healthy lean group [median (IQR) %: 88.33 (39.5) vs. 77.78 (36.68), p=0.05].

Table 3.7: Intake of dietary macronutrients as measured by 24 hour food diary at the time of recruitment and after 2-3 months

Dietary variables	Hypoth. Lean	Hypoth. Obese	Healthy Lean	Simple Obese
Energy intake and macronutrients at recruitment				
Number (n)	(n=10)	(n=8)	(n=17)	(n=15)
Predicted energy req. (Kcal)	997(653)	1545(902)‡	1198.3(535.5)†	1917(684)†‡
% of actual Energy intake	138.6(71.5)	87(485)	142.5(69.0)†	67.4(39.1)†
Energy (kcal)	1369(509)	1064(1227)	1438.0(585.6)	1318(735)
Energy % EAR	86.36(40.05)	74.84(37.83)	86.98(29.50)	62.56(42.89)
Fat (g)	37.02(19.74)	32.6(47.5)	46.75(37.55)	50.00(29.95)
Prot (g)	47.38(40.49)	46.2(44.6)	61.65(36.30)	53.90(41.45)
CHO (g)	208.1(61.9)	177.8 (137.6)	194.4(88.9)	157.9(56.9)
DF (g)	12.52(11.16)	11.82 (10.65)	13.40(7.25)	10.40(3.20)
Fat %	25.25(8.92)	30.23(19.53)	32.08(11.37)	35.27(10.52)
Prot %	13.84(6.95)	17.23(4.14)	15.61(7.48)	17.02(4.50)
CHO %	62.67(8.96)	58.30(19.83)	54.70(17.63)	49.86 (8.49)
DF % intake	73.9(93.3)	67.2(47.9)	77.78(36.67)	59.44(16.12)
Prot %RNI	179.4(154.3)	192.1(142.0)	158.1(100.9)	167.8(106.3)
Energy intake and macronutrients after 2-3 months				
Number (n)	(n=9)	(n=9)	(n=22)	(n=12)
Predicted energy req. (Kcal)	936(560)	1514(1152)‡	1218.1(563.5)†	1864(557)‡†
% of actual Energy intake	105.00(34.32)*	77.4(270.8)	157.7(71.0)*†	65.4(51.8)†
Energy (kcal)	942(595)*	1014(573)	1734.9(514.9)*	1213(378)
Energy % EAR	64.78(27.39)*	73.42(46.44)	97.88(43.89)*	63.1(49.4)
Fat (g)	28.75(38.52)*	39.90(29.78)	65.90(34.05)*	50.05(33.96)
Prot (g)	38.95(27.05)*	42.65(27.45)	61.00(28.57)*	59.80(24.85)
CHO (g)	148.4(32.1)*	159.8(86.7)	235.7(85.0)*†	136.8(57.6†
DF (g)	12.10(12.27)	10.30(4.97)	16.00(7.16)	6.72(6.07)
Fat %	27.28(17.22)	29.66(15.93)	32.14(7.99)	37.13(8.34)
Prot %	15.28(2.39)	16.54(2.94)	13.75(3.14)†	20.06(5.94)†
CHO %	61.91(15.23)	57.38(14.28)	53.22(7.53)†	46.02(4.48)†
DF % intake	66.7(74.2)	53.89(37.78)	88.33(39.58)†	40.84(33.47)†
Prot %RNI	186.2(103.0)	147.9(132.1)	201.0(153.3)	179.2(131.6)

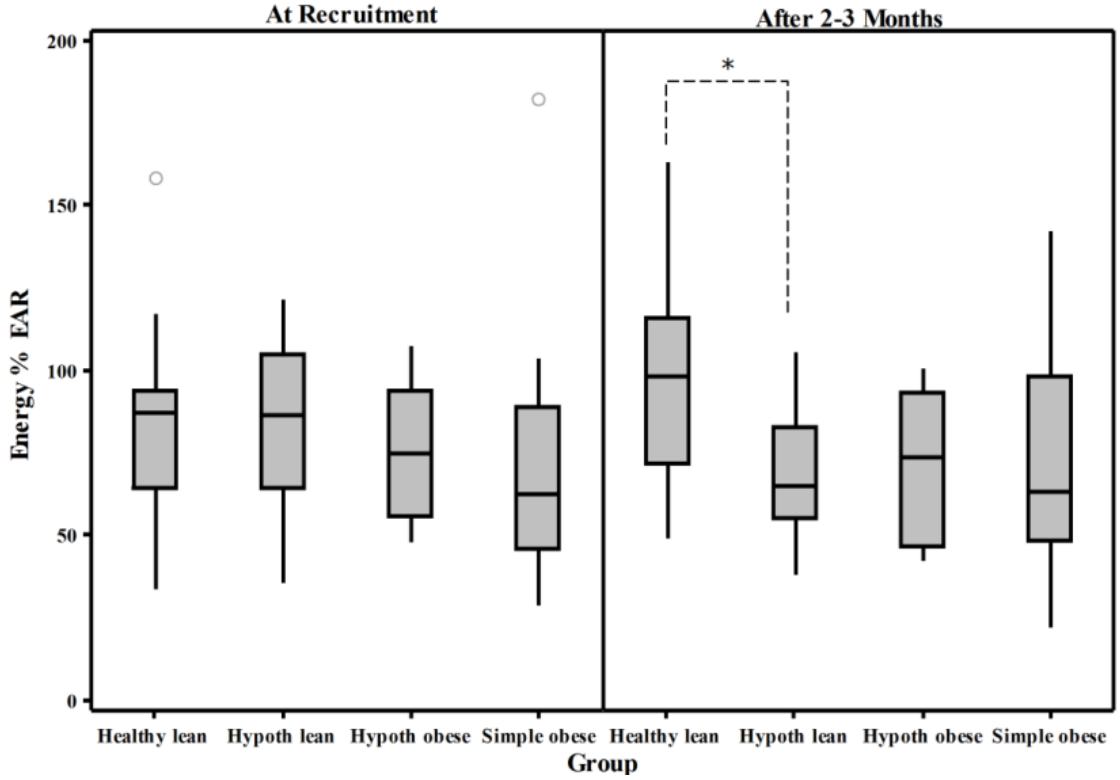
Kcal; kilocalories, Prot; proteins, CHO; carbohydrates, DF; dietary fibre, Prot. %RNI; Percentage recommended nutritional intake of proteins, Energy %EAR; percentage estimated average recommended intake of energy.

*Indicate significant differences between healthy lean and hypothalamic lean

† Indicate significant differences between healthy lean and simple obese

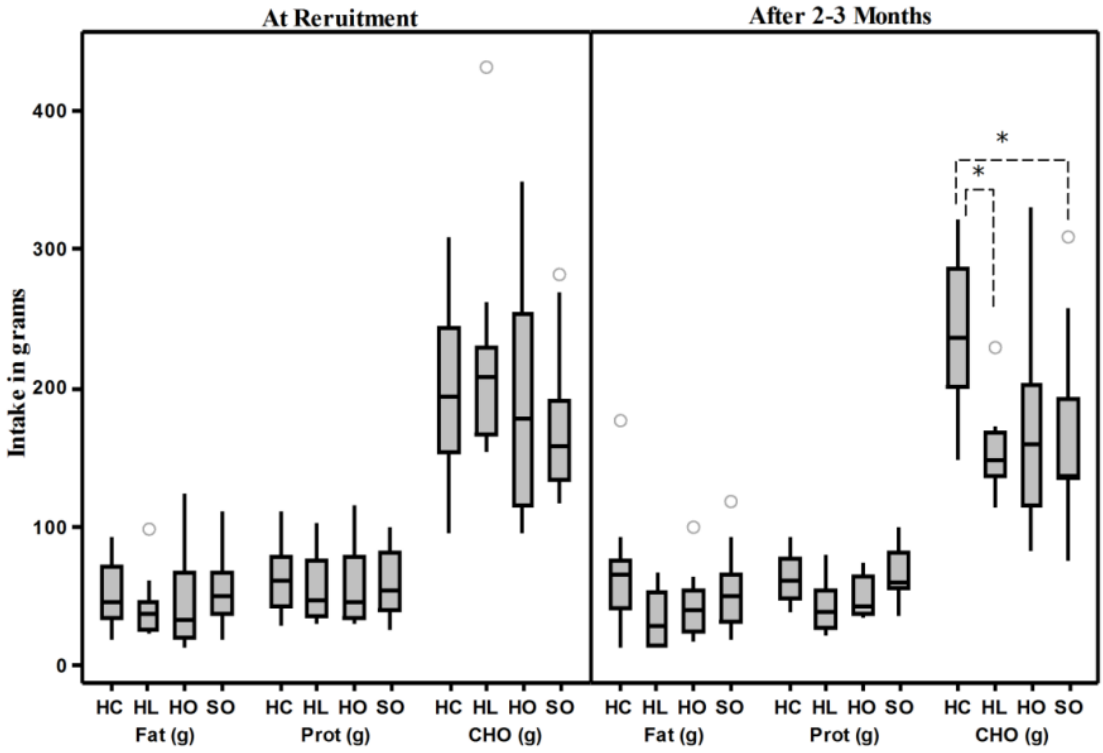
‡ Indicate significant differences between Simple obese and hypothalamic obese

Figure 3.11: Boxplots showing total caloric intake expressed as estimated average energy requirements (%EAR) in all groups at the time of recruitment and after 2-3 months.



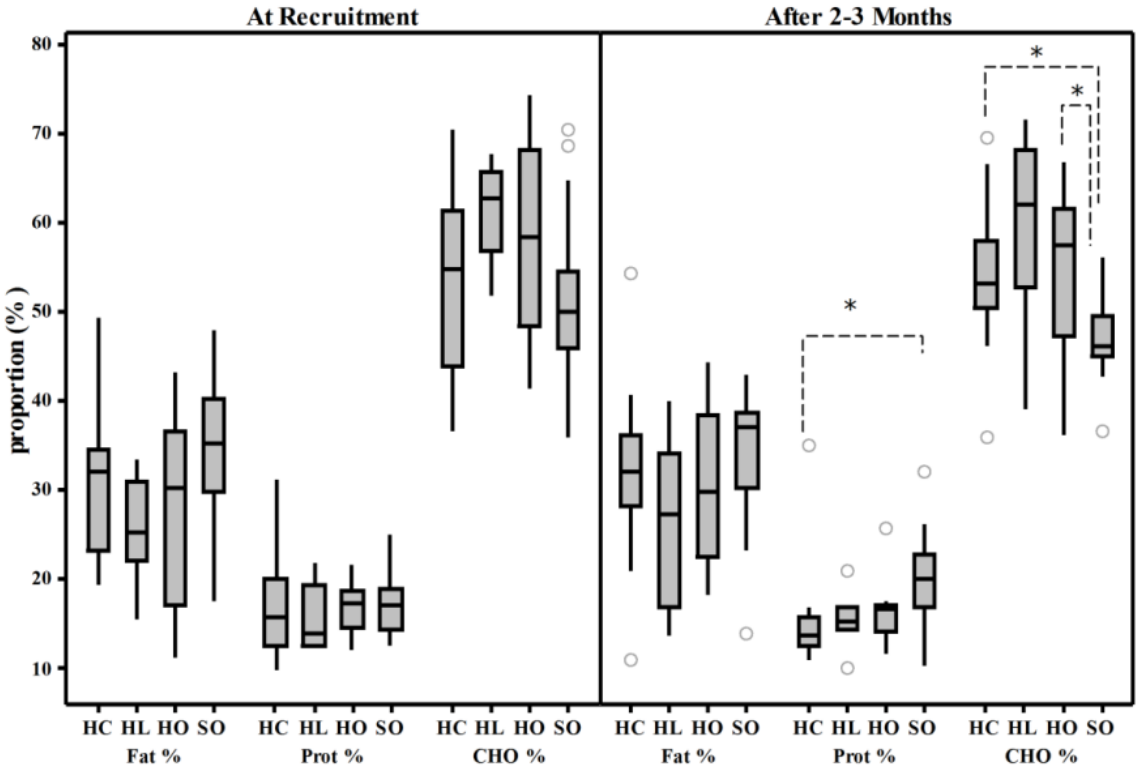
*indicate p<0.05, Hypoth; hypothalamic lean or obese

Figure 3.12: Boxplots showing intake of fats, carbohydrates, and proteins in all participants expressed in grams at the time of recruitment and after 2-3 months.



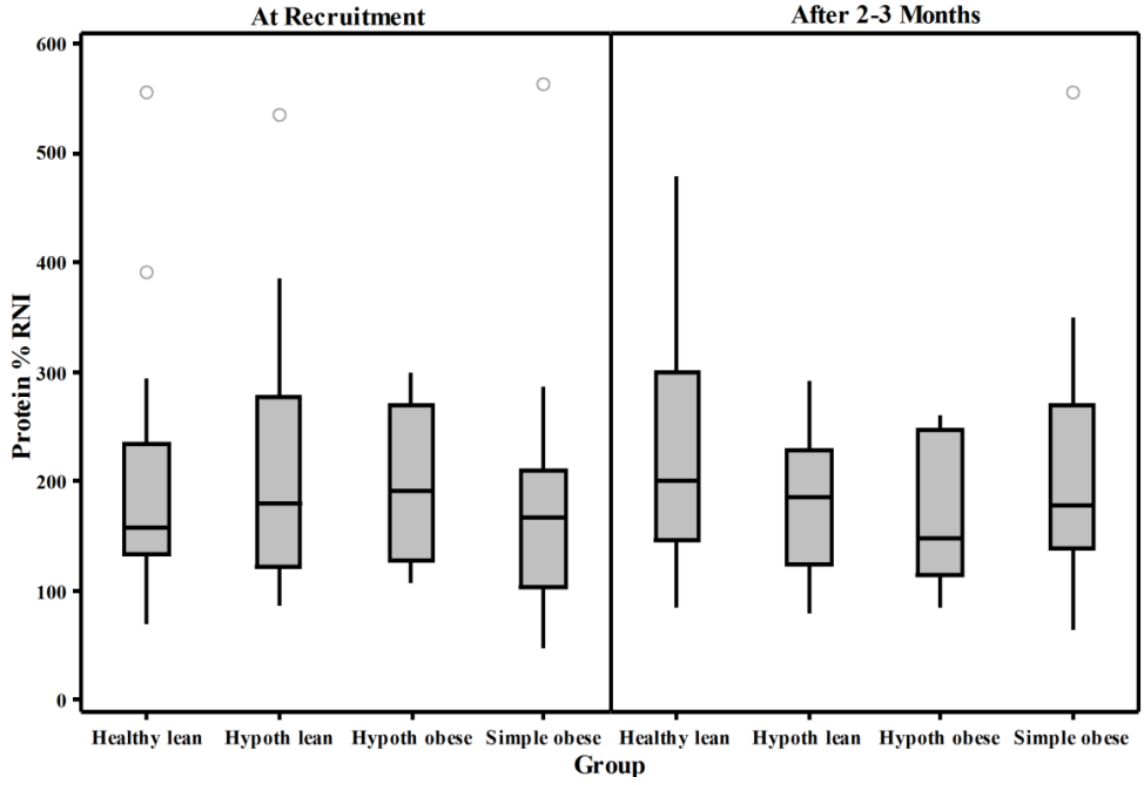
*indicate p<0.05, HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; Simple obese, CHO; carbohydrate, Prot; protein,

Figure 3.13: Boxplots showing proportional intake of fats, carbohydrates, and proteins in all participants expressed as percentage at the time of recruitment and after 2-3 months.



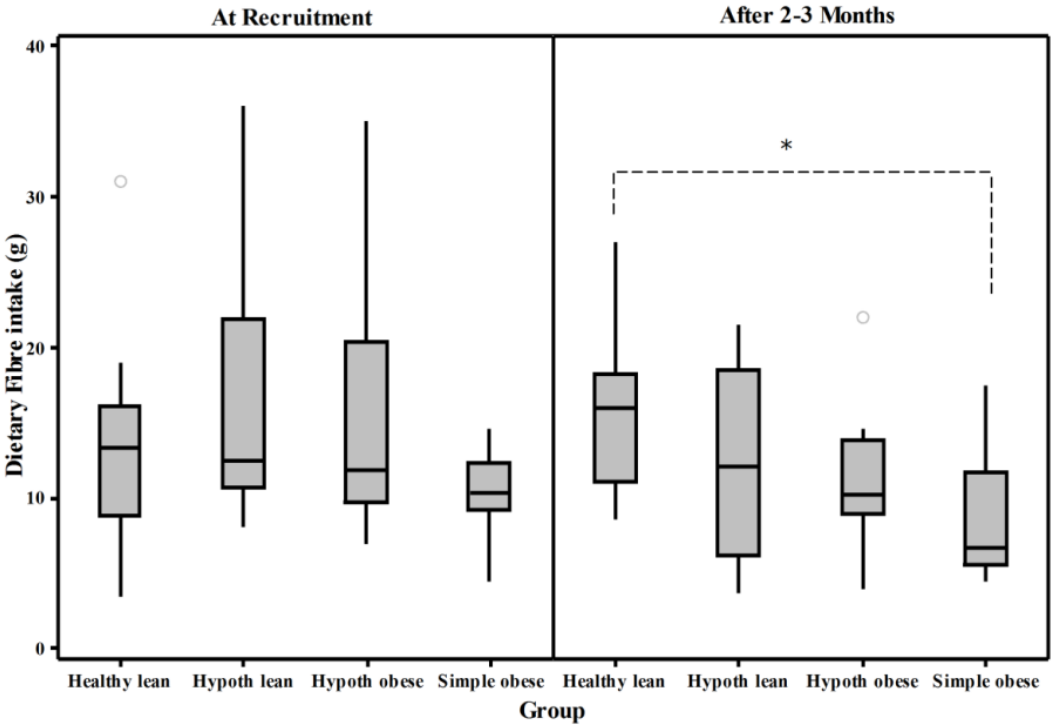
*indicate p<0.05, HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; Simple obese

Figure 3.14: Boxplots showing proportional intake of recommended nutritional intake of proteins (%RNI) in all participants at the time of recruitment and after 2-3 months.



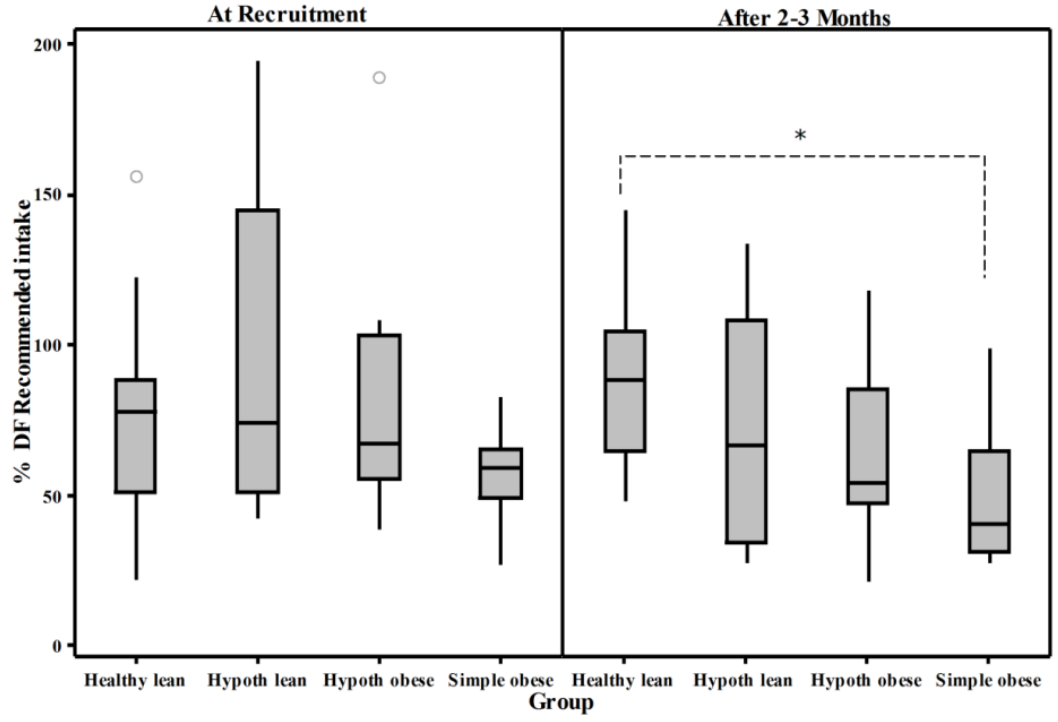
Hypoth; hypothalamic lean or obese

Figure 3.15: Boxplots showing dietary fibre intake (in grams) in all participants at the time of recruitment and after 2-3 months.



*indicate p<0.05, Hypoth; hypothalamic lean or obese

Figure 3.16: Boxplots showing SACN 2011 recommended proportional intake of dietary fibre (%) in all participants expressed as percentage at the time of recruitment and after 2-3 months.



*indicate p<0.05, Hypoth; hypothalamic lean or obese

3.5.9 Correlation of dietary macronutrients intake with adiposity

To assess the relationship of the degree of adiposity with the dietary intake, BMI SDS of individual groups were tested for any correlation with the amount and proportion of major macronutrients (fats, carbohydrates, proteins and dietary fibre) and energy intake (%EAR) both at the time of recruitment and after 2-3 months.

No characteristic patterns of positive or negative correlations between macronutrient intake and adiposity were observed in the groups. Inconsistent negative correlation of carbohydrate intake (in grams) ($R=-0.506$, $p=0.038$) and %EAR ($R=-0.532$, $p=0.028$) with BMI SDS were observed in healthy lean participants only at the time of recruitment (Table 3.8). Furthermore, inconsistent significant positive correlation of fats (g), carbohydrates (g), and protein intake (g) were observed in hypothalamic obese participants only after 2-3 months and not at the time of recruitment (fats (g): $R=0.483$, $p=0.013$, CHO (g): $R=0.792$, $p=0.013$, and proteins (g): $R=0.753$, $p=0.019$) (Table 3.8).

When participants were grouped according to their phenotype (lean vs. obese) or according to their pathology (pathological vs. healthy), we observed that lean or obese phenotypes were not significantly correlated with any of the dietary macronutrient (amount and proportion) or energy intake (as %EAR) both at the time of recruitment and also after 2-3 months (Table 3.9). However, when participants were grouped according to the presence or absence of a pathology (pathology vs. healthy), significant negative correlation of BMI SDS with energy intake expressed as %EAR and amount of dietary carbohydrate was observed in healthy group (healthy lean and simple obese) both at the time of recruitment and after 2-3 months (table 9). Moreover, dietary fibre in healthy group was significantly negatively correlated with BMI SDS in terms of its amount at recruitment (DF (g): $R=-0.564$, $p=0.001$) and proportion after 2-3 months (%DF: $R=-0.610$, $p<0.001$).None of these significant differences were seen in the group of patients with pathology (Table 3.9).

Table 3.8: Spearman rank correlation of dietary macronutrient intake with BMI SDS in each group at the time of recruitment and after 2-3 months.

	Healthy Lean		**Hypoth. lean**		**Hypoth. Obese**		**Simple Obese**	
	R	p	R	p	R	p	R	p
At recruitment								
Fat (g)	-0.227	0.381	-0.217	0.575	0.400	0.326	-0.074	0.794
CHO(g)	**-0.506**	**0.038**	-0.331	0.385	0.241	0.566	**-0.484**	**0.067**
Prot(g)	-0.058	0.825	-0.283	0.461	0.197	0.640	-0.159	0.572
DF(g)	-0.290	0.259	0.247	0.521	0.120	0.777	0.049	0.862
%Fat	-0.042	0.874	0.077	0.844	0.397	0.330	0.159	0.573
%CHO	-0.214	0.409	-0.047	0.905	-0.303	0.466	-0.207	0.460
%DF	-0.239	0.356	0.168	0.665	0.170	0.687	0.093	0.741
%RNI	-0.267	0.301	0.107	0.783	-0.546	0.161	0.083	0.768
%EAR	**-0.532**	**0.028**	0.128	0.743	-0.421	0.300	-0.091	0.747
After 2-3 months								
Fat (g)	0.318	0.160	0.432	0.286	**0.783**	**0.013**	-0.266	0.403
CHO(g)	0.279	0.221	0.435	0.281	**0.792**	**0.011**	-0.477	0.117
Prot(g)	0.065	0.781	0.160	0.704	**0.753**	**0.019**	-0.515	0.087
DF(g)	-0.036	0.878	0.059	0.890	0.204	0.599	-0.293	0.356
%Fat	0.105	0.650	0.300	0.471	0.251	0.514	0.100	0.758
%CHO	-0.066	0.775	-0.253	0.545	-0.066	0.866	-0.253	0.428
%DF	-0.002	0.993	0.006	0.989	0.090	0.817	-0.272	0.393
%RNI	-0.110	0.634	0.490	0.217	-0.206	0.594	-0.031	0.924
%EAR	0.044	0.849	**0.791**	**0.019**	0.259	0.500	-0.18	0.577

Significant differences or correlation with a tendency are highlighted. Prot; proteins, CHO; carbohydrates, DF; dietary fibre, Prot. %RNI; Percentage recommended nutritional intake of proteins, Energy %EAR; percentage estimated average recommended intake of energy.

Table 3.9: Spearman rank correlation of dietary macronutrient intake with BMI SDS based on phenotype (lean or obese) and pathology (pathological vs healthy) at the time of recruitment and after 2-3 months.

	Obese (simple & hypoth. obese)		**Lean** (healthy & hypoth. lean)		**Pathology** (Hypoth. Lean & obese)		**Healthy** (healthy lean & simple obese)	
	R	p	R	p	R	p	R	p
At recruitment								
Fat (g)	0.261	0.254	-0.098	0.611	0.474	0.055	-0.157	0.384
CHO(g)	0.027	0.906	-0.275	0.149	0.487	0.047	**-0.595**	**<0.001**
Proteins (g)	0.253	0.269	-0.281	0.140	0.428	0.087	-0.029	0.874
DF (g)	-0.163	0.480	-0.098	0.613	-0.095	0.716	**-0.564**	**0.001**
%Fat	0.308	0.153	-0.164	0.424	0.348	0.172	0.230	0.200
%CHO	-0.298	0.168	0.078	0.705	-0.320	0.210	-0.215	0.238
%DF	0.039	0.858	-0.105	0.611	0.104	0.691	-0.322	0.073
%RNI	-0.166	0.448	-0.079	0.700	-0.197	0.448	-0.133	0.467
%EAR	-0.231	0.289	-0.231	0.257	-0.379	0.133	**-0.405**	**0.022**

After 2-3 months								
Fat (g)	0.197	0.367	-0.292	0.147	0.003	0.990	-0.055	0.766
CHO(g)	-0.161	0.464	-0.279	0.168	-0.257	0.319	**-0.432**	**0.014**
Proteins (g)	0.014	0.948	-0.158	0.441	-0.025	0.924	-0.073	0.690
DF (g)	0.007	0.975	-0.113	0.582	0.049	0.851	-0.323	0.067
%Fat	0.267	0.243	-0.049	0.799	0.304	0.235	0.239	0.181
%CHO	-0.354	0.116	0.060	0.755	-0.232	0.370	**-0.526**	**0.002**
%DF	-0.185	0.423	-0.120	0.534	-0.168	0.520	**-0.610**	**<0.001**
%RNI	-0.006	0.980	-0.085	0.661	-0.122	0.640	-0.093	0.605
%EAR	-0.003	0.991	-0.139	0.471	0.201	0.438	**-0.371**	**0.034**

Significant differences or correlation with a tendency are highlighted. CHO; carbohydrates, DF; dietary fibre, Prot. %RNI; Percentage recommended nutritional intake of proteins, Energy %EAR; percentage estimated average recommended intake of energy.

3.5.10 Association of dietary macronutrients and energy intake with adiposity

Univariate regression analysis was done to assess the association of BMI SDS with dietary macronutrients (the amount and proportion of fats, carbohydrates, and proteins), dietary fibre, and energy intake (%EAR), both at the time of recruitment and after 2-3 months. Variables showing a significant association ($p<0.05$) or a tendency ($p<0.10$) in univariate analysis were further analyzed in multivariate regression analysis with and without adjustment for pathology.

Although BMI SDS of individuals was significantly associated with the intake of carbohydrates (g), proteins (%), dietary fibre (g and %), and energy (as %EAR) in univariate analysis, none of the dietary variables were strongly significantly associated with BMI SDS in multivariate regression analysis with and without adjustment for pathology except a significant association of proportional intake of fats only at the time of recruitment ($\beta=0.063$, $p=0.054$, R^2-adj=13.63%) (Table 3.10).

Table 3.10: Univariate and multivariate regression analysis of dietary macronutrients intake with BMI SDS with and without adjustment for pathology

Macronutrient		At recruitment		After 2-3 months	
		Adjusted for pathology	**Un-adjusted for pathology**	**Adjusted for pathology**	**Un-adjusted for pathology**
CHO(g)	**P**	0.032	0.047	0.025	0.007
	B-coef	-0.008	-0.007	-0.001	-0.010
	R^2-Adj	7.00	6.17	12.31	12.27
DF (g)	**P**			0.003	0.003
	B-coef			-0.014	-0.144
	R^2-Adj			19.6	14.39
%Fat	**P**	0.089			
	B-coef	0.055			
	R^2-Adj	3.43			
%Proteins	**P**			0.014	0.025
	B-coef			0.122	0.114
	R^2-Adj			14.19	8.16
%CHO	**P**				0.022
	B-coef				-0.065
	R^2-Adj				8.53
%DF	**P**			0.002	
	B-coef			-0.025	
	R^2-Adj			20.52	
%EAR	**P**	0.046	0.052	0.006	0.026
	B-coef	-0.017	-0.017	-0.017	-0.020
	R^2-Adj	5.71	5.82	9.48	8.06
		R^2=13.63	None	None	DF (g) R^2=18.87
Fat%	**P**	0.054			0.088
	B-coef	0.063			-0.093
EAR%	**P**	0.087			
	B-coef	-0.017			

B-coef; beta coefficient, R^2-adj; R^2 adjusted,CHO; carbohydrate intake, %EAR; % intake of recommended average requirements, DF; Dietary fibre,

3.6 Discussion

3.6.1 Anthropometric measurements

Children with PWS suffer from growth retardation partly due to growth hormone deficiency, feeding difficulties in early life, hypotonia and limited activity which contribute to short stature in these children (22, 307). This was particularly true in our group of PWS patients as their height SDS was significantly lower than the simple obese (p=0.01) and healthy lean group (p=0.008). As mentioned earlier, low height for age coupled with reduced physical activity due to poor co-ordination, low muscle mass, eye abnormalities such as esotropia and myopia, and slow body movements due to poor muscle tone leads to reduced energy expenditure which reduces the caloric expenditure in these patients. Low caloric requirement, in addition to lower lean body mass and increased

appetite favours the accumulation of excess body fat and hence obesity in hypothalamic obese patients(22).

Weight SDS of the hypothalamic obese children was significantly lower than the simple obese patients but when corrected for height SDS (i.e. expressed as BMI SDS) no differences were observed between simple and hypothalamic obese groups which suggest that both obese groups were proportionate to each other. Most of the hypothalamic obese children are usually under the GH therapy which has been shown to reduce fat mass, increase skeletal muscle mass, and exercise and motor performance after 12 and 24 months of start of GH therapy (308). Studies have shown long term GH replacement to induce lipolysis, cause reduction in fat mass, and increase lean mass (35, 309). This might be a factor accounting for the significant difference in the weight SDS between simple and hypothalamic obese children in our study. It has been observed that this effect is reversed after cessation of GH therapy as found by Oto *et al.* (2014) (310). This study on young PWS patients found a significant increase in BMI SDS over a period of 24 months after GH cessation and a trend towards increase in subcutaneous and visceral body fat distribution (310).

BMI (in kg/m^2 or SDS) is a good measure of assessing the cardiovascular disease risk in general population. Studies have shown a strong association of increased BMI SDS scores in childhood as predictors of adulthood obesity and its metabolic complications (311). A non-significant but relatively lower BMI SDS in hypothalamic obese children than simple obese children may be due to the fact that simple obese children in our study may not be a representative sample of the general population, as cases of obesity attending the tertiary care centre are severely obese [median BMI SDS 3.91 (0.91)] or resistant to the community weight management programs. Additionally, the nature of obesity might possibly be different from those of simple obesity because children with Prader Willi Syndrome and craniopharyngioma are generally short for their age with more fat and less muscle per unit of body weight and so they are likely to become obese even in the presence of a normal caloric intake. Weight SDS may therefore be a more sensitive predictor of obesity than BMI SDS in these patients. A relatively lower BMI in hypothalamic obese group than simple obese group could also be an indication of the effect of treatment in addition to the dietetic intervention in these children. Furthermore, waist circumference has been suggested as a strong predictor of adiposity in children and adolescents than BMI (312) but BMI SDS is more commonly used in population based studies for practical reasons.

Although not all children with Prader Willi syndrome in our study were obese, the median BMI SDS of PWS children in lean category was more than 1 SDS as compared to

healthy lean controls (median BMI SDS <0 SDS).This may represent the transitional phase between the initial changes in metabolism (i.e. reduced energy expenditure and increased fat mass) and hyperphagia towards an obese status as suggested by Miller *et al.* (2011) in their study. They followed 79 children with PWS and 84 siblings over a period of 10 years (307). This study found a gradual progression of PWS patients in 7 distinct nutritional phases as opposed to the previously described traditional 2 stage nutritional phases which suggested a switch towards hyperphagia and obesity between 18-36 months of life (313). Gathering physical activity data in our group of patients would help determine how much of this difference could be explained by physical activity. Published data suggests that three month, easy to follow, well accomplished, exercise significantly increases calf muscle mass, reduces calf skin-fold, and exercise endurance in PWS children (37). A lower bone mineral density suggest lower limb-muscle activity and hence a lower muscle mass and muscle power (39). The limb and body movements of hypothalamic obese children are therefore slow and less intense even in the presence of a comparable time of activity to lean healthy children (40). Adiposity in hypothalamic obese children (PWS) has been shown to increase with age, such as in patients age 12 years and above in a study by Brambilla *et al.* (1997) (39). However, we did not observe a significant positive correlation of BMI SDS with age in our hypothalamic obese participants.

3.6.2 Body composition

In our study, lean index (expressed as kg/m^2) was significantly higher in simple obese than healthy lean group. Given that our simple obese participants were taller, heavier, and had a high lean mass might suggest that in fact height and lean mass contribute to a higher BMI SDS scores in simple obese, followed by the hypothalamic obese participants. This is in line with findings from Metcalf *et al.*(314), who reported three major findings from the Earlybird diabetes cohort of children age 7-12 years, in Plymouth, UK. Firstly; BMI and fat mass correlated with height at each annual visit similar to that of height with other measures of adiposity (fat%, leptin, and insulin). Secondly; body mass and fat mass were more closely correlated with fat%, leptin and insulin rather than their height independent formulations and thirdly; children who grew faster gained the most weight(314). Further analysis by Wells and Cole suggested that independent of increase in adiposity, height was significantly associated with insulin resistance, while lean mass was associated with both leptin and insulin resistance in children especially girls measured annually (315). The associations of height and lean mass with insulin resistance in their study were never above 60% which suggested that un-explained variations related to diet, social class, physical activity, and other unknown confounding factors might also be playing their role as

environmental predictors of cardiovascular risk. Moreover, from our study, we found that fat mass, fat%, and fat index (fat mass dependent on height) were significantly higher in both of our obese than lean groups. Furthermore, hypothalamic obese children with PWS have a reduced visceral to subcutaneous fat ratio on dual energy X-ray absorptiometry and visceral adipose tissue is correlated with subcutaneous fat and BMI (316). This suggests that subcutaneous fat measurement can possibly be a surrogate measure of the elevated risks associated with high BMI in adults.

The method for the measurements of body composition in our study was based on foot-to-foot bioelectrical impedance analysis. Although this has been validated for use in children (317) and adults (317, 318), studies have shown that it may overestimate fat-free mass and underestimate fat mass (319, 320) and the measurements can vary largely between DEXA and foot-to-foot impedance analysis and according to the gender (321). Body composition of all participants could not be measured primarily due to age of the study participants. Furthermore, the initial plan was to measure only basic anthropometric data such as height and weight; however, the ease of use and the nature of body composition in hypothalamic obesity encouraged us to also measure the body composition data using non-invasive, portable, and validated device. Therefore, body composition measures of some of the participants recruited before the start of use of TANITA® were not available and the results of body composition should therefore be interpreted with great caution due to lower number of readings available for analysis.

3.6.3 Relationship of obesity with socioeconomic status

Our data showed significantly lower SIMD scores in the simple obese than healthy lean while a trend was observed in the hypothalamic group (lean and obese). SIMD rank and quintiles significantly negatively correlated with BMI SDS in healthy group (healthy lean and simple obese). Although our participants sample is not representative of general population, this finding is in line with the Scottish government reports attributing a high prevalence of obesity to areas with low SIMD scores and quintiles (7, 11). This is primarily explained by the association between obesity and socioeconomic class or possibly by the recruitment of more affluent lean control group. Other studies also found a significant negative association of socioeconomic status with obesity (322). Although some countries are in a plateau phase of obesity prevalence but this has been at the expense of increasing socioeconomic disparity (323, 324). Parents and families with low socioeconomic status are likely to follow unhealthy food choices, have less awareness of health benefits of good food choices, live in more deprived areas with limited access to

physical exercise and leisure facilities, and have limited access to fresh fruits and vegetables. However, some studies have found an upward trend of obesity in highly educated (325) and high socioeconomic group with long working h.

3.6.4 Dietary energy and macronutrients intake

Obesity is a state of positive energy balance occurring as a result of increased energy intake against a reduced energy expenditure (326). Population based studies and large meta-analyses have mentioned a reduced intake of dietary fibre, increased intake of readily available carbohydrates and fats by obese people including children and this increased intake is associated with increased weight (327). Here we show that the proportional intake of the dietary fat (fat %) and proteins (protein %) were higher while that of carbohydrate was lower in the obese group, particularly simple obese group. Dietary fibre is important for colonic health and a lower intake has been reported in obese population (328, 329). Our data suggest a clear downward trend in the percentage recommended intake of dietary fibre from lean towards obese.

Simple obese children reported 32.6% and 34.6% lower intake of energy than their predicted basal metabolic rate at recruitment and after 2-3 months respectively. Similar results have been reported in the literature for obese children with central adiposity in the US (329). The lower percentage of average recommended intake of energy in the hypothalamic lean, hypothalamic obese and simple obese groups can either be attributed to reduced energy requirements, reduced energy expenditure, or due to the actual effect of dietetic management, or it may represent under-reporting of dietary habits. We found that the healthy group (healthy lean and simple obese) but not the pathological group (hypothalamic lean and obese) showed a significant negative correlation of BMI SDS with percentage of recommended estimated intake of energy (%EAR), intake of dietary carbohydrates, and dietary fibre. This finding suggested that the observed lower dietary energy and macronutrient intakes in patients with Prader-Willi syndrome and craniopharyngioma irrespective of the lean or obese status were possibly related to the reduced energy requirements and reduced energy expenditure rather than under-reporting. This is in line with the studies reporting lower energy intake in the hypothalamic obese group compared to the reference population (330-334). Moreover, using Schofield equation or Goldberg cut offs may not be relevant while considering pathological conditions such as PWS or craniopharyngioma as these equations assume stable health status. Despite this evidence, we still are cautious to rule out under-reporting in the hypothalamic disease group due to large variation and hence less power in our data.

Furthermore, our assumption needs confirmation via physical activity and body energy metabolism studies in our cohort, although these have been shown to affect obesity in PWS as mentioned in chapter 1 section 1.3.5.1(22, 39, 335). On the other hand, disparity of BMI SDS scores with energy intake (lower BMI SDS-higher energy intake) in the healthy group suggested under-reporting in our simple obese group. Recording a reliable dietary history is a challenge as many studies have pointed towards the tendency of the obese population to under report(336). This reporting error has been shown to be consistently associated with increasing BMI SDS of the participants (337, 338) and increasing age (329). The former evidence is supported by our findings.

None of the dietary macronutrients or energy intake as %EAR was significantly associated with BMI SDS in adjusted and un-adjusted multivariate regression analysis except an association of proportion of fat intake ($p=0.054$) at recruitment. Moreover, change in BMI SDS (ΔBMI SDS) did not correlate with changes in energy intake over the period of study. This further indicated that apart from the above mentioned possible factors, limitations related to the dietary assessment method and bias on behalf of the patients related to interpretation of portion sizes might also play their role in determining this association. The food diaries were not weighed which might have resulted bias in portion size interpretation and hence false differences in macronutrients and energy intake. The diaries used were for a single day and may not reflect the frequency of foods taken on daily or weekly basis. Furthermore, foods taken by the child at school might have not have been reported by the parents. We tried to address these potential limitations by explaining the food diary in detail to the participant and/or the parent and asking them to preferentially choose a weekday to fill the diary. Use of multiple pass food diaries might help address issues related to memory bias and under-reporting by participants (339), and is therefore suggested for future studies involving these groups.

Despite possible under-reporting, we observed that the proportion of recommended dietary fibre and carbohydrate intake was lower in the simple obese compared to the healthy lean participants and a lower intake of dietary fibre and carbohydrate intake was negatively correlated with BMI SDS when both healthy lean and simple obese groups were studied together. Population based studies have suggested lower intake of dietary fibre in the simple obese population (340). Moreover, the use of more readily available sources of energy such as glucose has further contributed to this low fibre intake. Diets with readily available sources of energy(such as glucose) have a high glycaemic index which induces hormonal changes (such as hyperinsulinemia and hypoglucogonemia) that has been shown to promote voluntary food intake by up to 81% compared to 51% in low glycaemic index foods (341). The typical Western diet is composed of a higher proportion of fats and it has

been reported in longitudinal studies in adolescents that diets high in fat are devoid of fruits and vegetables which are important sources of dietary fibre (342). Additionally, the dietary choices of obese children may be influenced by the family meal plans and it has been observed that obese children usually have obese parents whose diet is rich in fats and low in fibre intake (15). Proportional intake of fats was marginally associated with BMI SDS in our cohort, and it has been suggested that the consumption of high fat diet is a consistent factor for obesity in paediatric population (343). Other factors such as unhealthy food choices with low dietary fibre (340), education, socioeconomic status, and awareness regarding high fibre diets could also be amongst other contributory factors to low fibre intake in simple obese group (17, 344).

Although the time period between the two assessments was relatively short (median 2.92 months) it allowed us to investigate changes in anthropometry and dietary intake with weight management in short term. Weight management in dietetic clinics for overweight and obese patients in Yorkhill hospital has two main goals a) to stop the progression of weight gain followed by b) reduction in the current over-weight by no more than 0.5kg/week. We observed no major significant differences in the anthropometric measurements and dietary intake between and within the groups. However, there was strikingly lesser variation in the change in BMI SDS/month in the simple obese participants compared to the lean groups. This finding can have three probable interpretations; firstly, it suggests the resistance of obese people to changes in BMI SDS and secondly, it might suggest that the weight management plan are at least successful to keep the change in BMI SDS/month close to zero which is also supported by the finding that more simple obese patients had ΔBMI SDS/month <0 SDS compared to healthy lean participants. Thirdly, the simple obese participants had probably reached their "limit of adiposity" to accommodate any further change in BMI SDS. However, long term follow-up data of these patients would help in confirming any of the three possible interpretations.

3.7 Conclusions

In conclusion, simple and hypothalamic obese patients differ from each other in the nature of their obesity; hypothalamic obese patients being shorter and with lower fat mass compared to simple obese. Correlation of change in BMI SDS with change in energy intake as %EAR, dietary fibre and carbohydrates only in the non-pathological group suggest that the actual dietary consumption of the hypothalamic obese and lean patients might be less and that their energy expenditure and satiety (controlled by hypothalamus) might play a more important role in determining their energy intake. Additionally, dietary

intervention in the hypothalamic lean or obese groups should take into account this reduced energy intake to avoid under or over nutrition. On the other hand, simple obese participants seem to under-report which limits the real association of dietary intake with body composition. Differences in the "nature" of anthropometry and body composition between the simple and hypothalamic obese groups therefore make them better suited for comparison to test our main hypothesis.

In subsequent chapters, this anthropometric and dietary information will be used as the basis to investigate the association of the gut microbiota metabolic activity and diversity with anthropometric data and dietary intakes to support or refute our hypothesis of gut microbiota in relation to obesity.

Chapter 4: Differences in Bacterial Metabolites of Gut Microbiota in Simple and Hypothalamic Obesity

4.1 Chapter Outlines

This chapter explores differences in the faecal bacterial metabolites between simple and hypothalamic obese groups and their relationship with diet and weight change.

4.2 Introduction

4.2.1 Short chain fatty acids and human health

Gut microbiota may in part affect obesity by degrading complex dietary polysaccharides (and some proteins) to 1-6 carbon organic compounds, called short chain fatty acids (SCFA). The most important of these SCFA are acetate (C2), propionate (C3), and butyrate (C4), the molar ratios of which varies between 48:29:23 and 70:15:15 respectively with a mean ratio of 60:20:20 (75). The exact amount of SCFA produced by the gut microbiota in humans is difficult to determine due to difficulties in access to the proximal colon (i.e. *in-vivo*) and measurement of SCFA both in the lumen and the portal circulation to give an estimate of the total amount produced. However, the rate of production of SCFA considerably varies with the amount and type of substrate available for bacterial fermentation, gastrointestinal tract transit time, and composition of the gut microbiota (345, 346).

There are estimates that SCFA, primarily acetate, propionate, and butyrate may contribute to approximately 10% of the daily energy requirements (347). Acetate is involved in *de novo* hepatic lipogenesis (162). Propionate is related to beneficial effects such as inhibition of lipogenesis (via 3-hydoxy, 3-methylglutaryl co-enzyme A reductase), hypocholesterolemia (via redistribution of cholesterol from plasma to the liver), hepatic and intestinal gluconeogenesis (348), intestinal GPR41- induced induction of satiety via hormone Peptide YY (PYY), and eating behaviour through leptin (97). Butyrate is one of the main SCFA required by the colonocytes as an energy source for cellular metabolism. It exerts anti-proliferative effects on colon cancer cells, stimulates apoptosis and positively affects cellular differentiation, and proliferation of normal colonic epithelium (111, 349). Studies have shown the potential anti-obesity and anti-inflammatory effect of butyrate by alleviating metabolic stress, maintaining β-cell function and protecting inflammatory response (113).

4.2.2 Gut bacterial metabolites in relation to obesity

Pioneering studies in gnotobiotic mouse models (GF mice) suggested a potential causative role of the gut microbiota in the development of obesity. GF mice were shown to be resistant to obesity despite higher caloric intake compared with their lean wild-type counterparts (162). This was attributed to reduced energy harvest in the colon from the diet and increased expression of energy sensors such as AMP kinase that stimulate energy generation and inhibit anabolic pathways. Transplantation of gut microbiota from conventionally raised (CONV) mice resulted in the development of an obese phenotype which meant that this "amicrobiotic" environment may have acted as a caloric restrictor for protection against obesity in GF mice (66). One study found reduced diversity of gut microbiota, reduction in Bacteroidetes, and increase in Firmicutes with development of obesity on high fat feeding (258). These differences in gut microbiota may be driving differences in the faecal SCFA between lean and obese individuals. However, results from human studies have been controversial due to small study samples, inter-individual variations, differences in diet, and other confounding factors. A recent study by Rahat *et al.* (2014) has suggested that a higher faecal SCFA in overweight and obese adults than in lean adults might be due to an increased production of SCFA and more dietary energy indirectly available to the host, by a different pattern of gut microbiota (175). A crossectional study by Payne *et al.* (2011) found significantly higher levels of faecal butyrate, propionate, and iso-butyrate in obese than lean children (95). Schwiertz *et al.* (2010) found 20% higher total SCFA and a significantly higher concentration and proportion of propionate in the faecal samples of obese than the lean volunteers (350).

In this context, several controversial differences in gut microbiota between lean and obese individuals have been suggested. In children, an increase in *Staphylococcus aureus* and reduction in *Bifidobacteria* has been associated with development of obesity (258). Some studies revealed a reduced diversity of gut microbiota, reduction in *Bacteroidetes* and increased *Firmicutes* in obese vs. lean individuals (159). *Faecalibacterium prausnitzii* has been associated with reduced inflammation and increased numbers of this bacteria are thought to play a protective role in IBD and diabetes (351) while *Akkermansia muciniphila* has been associated with weight reduction (352).

4.2.3 Gut bacterial metabolic activity in relation to diet

Changes in the diversity of gut microbiota may occur with change in energy intake, nutrient load, or nutrient composition which highlights diet as an important factor that interacts with gut microbiota (265). Gut microbiota and their capability to produce SCFA

are thought to co-evolve as an adaptive response to the change in dietary intake which varies with geography (155). Differences or change in dietary patterns might cause a dramatic transient change in the gut microbiota population at species and group level (217). However, these changes are not consistent between different studies possibly due to differences in experimental settings, dietary substrates used, and biological variation between individuals. A study by Kim *et al.* showed a reduction in faecal SCFA, reduction in *Firmicutes*, increase in *Bacteroidetes*, and reduction in *Firmicutes: Bacteroidetes* ratio in six overweight and obese adults who were kept on a strict vegetarian diet for 28 days (170). Lastly, Belobrajdic *et al.* (2012) showed a significant increase in the faecal output and total SCFA pool in the caecum of Sprague-Dawley rats independent of obesity phenotype with the introduction of at least 8% dietary resistant starch (174).

4.2.4 Factors contributing to the variation in the gut bacterial metabolites and diversity

Several factors have been proposed to help explain the higher faecal short chain fatty acid levels in obese populations. These include; increased production by "obesogenic" gut microbiota, altered symbiotic relationships for the utilization of nutrients in the gut lumen between different groups of gut microbiota, decreased mucosal absorption, and increased transit time (260). However, generally, it is recognised that changes in gut microbiota are associated with changes in the SCFA profile of the faecal samples (166). Anatomical changes in the gut may also influence the variation in the gut microbial diversity and metabolic activity. This is exemplified by gastric bypass surgery in individuals who show a sustained post-surgical weight loss (353). The change in gut physiology might be due to the reduction in the amount of dietary substrate intake, changes in bile circulation, anatomical changes to the normal passage of the food and acids, and changes in parasympathetic innervation. However, some authors suggest that gut microbiota play their role in causing weight loss by ways independent of these effects (354).

4.2.5 Controversies in the relationship of gut microbiota metabolites with obesity

Whereas the studies discussed above point towards the gut microbiota as a factor in obesity pathogenesis, several studies have contradicted this notion by showing no compositional differences or changes in gut microbiota with dietary intervention between lean and obese individuals (95) and that such changes in the gut microbiota are not always associated with obesity (260). Some studies have found significantly higher proportion of

Firmicutes and higher *Firmicutes* to *Bacteroidetes* ratio in lean rather than obese individuals (350). Additionally, a compositional shift in gut microbiota induced by intervention with supposedly beneficial gut microbiota such as *Lactobacillus salivarius*-ls33 (171) or with high fat diet (222) may not be associated with the change in functionality or markers of increased energy harvest by the resident gut microbiota. As opposed to findings of the pioneering study by Backhed and colleagues (162), some studies found significantly higher weight gain in germ-free mice than conventionally raised mice on high fat diet which suggested that other mechanisms might be involved in weight gain induced by high fat diet independent of gut microbiota (165). Some authors have suggested a reciprocal relationship between caloric density and gut microbiota because on one hand gut microbiota are modulated by increased caloric density while on the other hand they extract energy from the diet and increase energy salvage in the form of SCFA. The balance of this relationship is affected by a variety of genetic factors influencing the expression of certain genes that may be peculiar to obese and lean individuals.

This discussion leads us to the debate as to whether changes in gut bacteria are a cause or an effect in obesity genesis and the role of diet in this interplay. Studies done on experimental animals are not always reciprocated in humans due to anatomical and physiological differences and differences in diet and lifestyle of animals and human subjects. Several controversial data on the gut microbiota diversity in lean vs. obese population suggest that the functionality, and not the actual structural organisation, of the gut microbiota might be more important (199). Although the core gut microbiome remains relatively stable throughout the life of an individual, shifts in the gut luminal environment due to changes in the availability of substrate for fermentation may cause shifts in the relative abundance and proportions of gut microbiota (263).

No study has thus far been able to prove a causal relationship between the gut microbiota and obesity. We have therefore used a unique model to look at this relationship by comparing a group of obese children and young adults who are prone to develop obesity either because of a genetic disorder (e.g. Prader-Willi Syndrome) or a tumour that erodes the hypothalamic satiety centre (e.g. craniopharyngioma) and compare their gut bacterial diversity and metabolic activity with a group of children who have obesity of undetermined cause and healthy lean controls. We have attempted to account for the dietary intake by recording the 24 hour food diary and we also explored alterations with weight change. A causative role of gut microbiota would be expected to reveal significant differences in microbial metabolic activity between the "bacterial induced" simple obesity and hypothalamic or "pathological" obesity caused by hyperphagia (66).

4.3 Purpose of this chapter

- To compare gut bacterial metabolic activity by measuring the concentration of faecal SCFA, hydrogen sulphide, lactate, and ammonia profile between simple and hypothalamic obese groups at the time of recruitment and at follow-up after 2-3 months.
- To assess changes in gut bacterial metabolites between and within the groups with weight change over a period of 2-3 months and explore relationships with weight change.
- To assess the association of demographic, anthropometric, and dietary predictor variables with gut bacterial metabolites using univariate and multivariate analysis.

4.4 Patients and Methods

A detailed description of participants and the methods used is given in chapter 2. Briefly, simple obese (n=16), hypothalamic obese (n=10), and hypothalamic lean (n=12) were recruited from the endocrine and dietetic clinics at Royal Hospital for Sick Children Glasgow. Healthy lean (n=27) participants were recruited from the community. Two faecal samples along with body composition data and 24 h food diary were collected from each participant at an interval of 2-3 months as described in section 2.2.3.

Laboratory methods

All faecal samples were processed for the measurement of SCFA using gas chromatography (section 2.3.6). Hydrogen sulphide (free, total, bound) was measured with a colorimetric assay according to the methylene blue reaction (section 2.3.7), ammonia using automated ammonia analyser (HANNAH Electrical HI93715) (section 2.3.4), pH using a benchtop pH meter (section 2.3.3), and lactate (D, L, and total isomers) with an enzymatic assay (section 2.3.8). Values for SCFAs and BCFA in freeze dried faecal material were expressed as µg/g dry or wet faeces. Free, bound, and total sulphide were expressed as µmol/g dry or wet faeces. Faecal NH_3 and lactate were expressed as mg/g dry or wet faeces.

Dietary assessment

All participants or their parents on behalf of the participant were handed over a form to complete a 24 h food diary. They were encouraged to complete the diary prospectively; however, some participants filled it in retrospect. The diaries were analysed using Windiet® 2005 software (Robert Gordon University Aberdeen UK). All foods entered into the software were analysed for total caloric intake and major macronutrient intake per day. The amounts of macronutrients (fats, carbohydrates, and proteins) were then expressed as

percentage proportion of total caloric intake. The proportion of recommended estimated average energy requirements (%EAR) and dietary fibre intake as percent recommendations (%DF) by the UK Scientific Advisory Committee on Nutrition (SACN) 2011 report (303) while the recommended nutritional intake of proteins (%RNI) was calculated from the Department of Health 1991 recommendation (COMA 1991). Portion sizes for all unspecified foods were estimated from the information based on published data from National Diet and Nutrition Survey (NDNS) (355). Foods not mentioned in the software were added as per 100 grams dietary values to the software from the information given by the major supermarket chains such as Tesco and ASDA stores in the UK. A total of 99 new foods were added to the database.

4.5 Statistics

Anderson Darling test of normality showed non-normal distribution of the data, therefore non-parametric analysis was applied. All values are expressed as median and inter-quartile range unless otherwise stated. Due to the difference in the nature of the groups, differences between two primary outcome variables were determined by Mann-Whitney U test. Correlation between variables was measured with Spearman Rank correlation. No adjustment for multiple testing or false discovery rates was done for these tests. All significant p-values in the results section should therefore be considered as nominally significant.

Univariate regression analysis for all faecal SCFA, BCFA, hydrogen sulphide (free, total, & bound), lactate (D & L), and ammonia at the time of recruitment and at after 2-3 months were individually assessed for demographic, anthropometric, and dietary predictors (Table 4.1). All associations with $p \leq 0.1$ were then analysed in a stepwise regression analysis with and without adjustment for pathology (i.e. presence or absence of Prader-Willi Syndrome and craniopharyngioma).

Associations of change (Δ = after 2-3 months – at recruitment) in SCFA, BCFA, hydrogen sulphide, lactate, and ammonia with the time elapsed between assessments and changes in the above mentioned anthropometric, body composition, and dietary predictors (in table 4.1) between the two time points. In a similar way; the association of change in these parameters on the response variables at 2-3 months were also assessed. All associations with $p \leq 0.1$ were then analysed in a stepwise regression analysis with and without adjustment for pathology.

All data was first compiled on Excel spreadsheets, and then analysed using statistical software Minitab 16® (Minitab Corporation, Coventry, UK)

Table 4.1: Demographic, anthropometric, and dietary predictors used in regression analysis

Age (years)
Gender
SIMD rank and quintiles
Time elapsed between assessments
BMI SDS
ΔBMI SDS/month
% Body fat
Body fat mass (kg)
Fat free mass (kg)
Carbohydrates (g)
Fat intake (g)
Protein (%)
Carbohydrates (%)
Fat intake (%)
% Recommended dietary fibre intake (%)
% Recommended intake of proteins (%RNI)
% Estimated average requirements (%EAR)
Faecal pH
Percentage faecal water (%H_2O)

4.6 Results

4.6.1 Differences in faecal SCFA between groups

4.6.1.1 Differences in faecal SCFA between obese subjects of different aetiology

No significant differences in the absolute concentration as well as the proportion of individual SCFA and BCFA were observed between simple and hypothalamic obese participants at recruitment and after 2-3 months except for the proportion of acetate (hypoth. vs. simple Obese, median (IQR) C2%; (65.57(10.83) vs. 60.57(6.25), p=0.022) and iso-butyrate (simple vs. hypoth. obese median (IQR) iC4%= 3.45(1.88) vs. 1.86(1.7), p=0.010) only at recruitment (table 4.2). A trend towards higher proportion of iC5 (p=0.051) and C5 (p=0.083) in simple obese patients was observed only at recruitment (Table 4.2, Table 4.3).

Molar ratios of different SCFA (particularly acetate/butyrate and lactate/butyrate) may indicate the predominance of peculiar pathways of substrate utilisation and transfer between different groups of gut microbes in order to maintain symbiotic relationship. At recruitment, there was a trend towards a higher molar ratio of acetate to butyrate (C2/C4) in hypothalamic obese than simple obese group [median (IQR) C2/C4= 5.18(3.02) vs. 4.14(3.03), p=0.06] (Table 4.4). However, this trend was reversed at follow-up as the ratio of acetate to butyrate was significantly lower for hypothalamic obese than simple obese group (median (IQR) C2/C4 ratio; 4.74(1.74) vs. 6.35(1.83), p=0.050) in the dry faeces (Table 4.4).

4.6.1.2 Differences in faecal SCFA between lean subjects

The absolute concentration of total and individual SCFA did not differ between healthy lean and hypothalamic lean participants at both presentation and at follow up (Table 4.2, Table 4.3) except for the proportion of propionate (C3%) at presentation which was significantly higher in healthy lean than the hypothalamic lean group [healthy lean vs. hypoth. lean, median (IQR) C3%=13.6(2.92) vs. 10.8(2.17), p=0.04] (Table 4.2).

Molar ratios of acetate/propionate were significantly higher in hypothalamic lean than healthy lean group in both dry (p=0.03, Table 4.2) and wet faeces (p=0.02, Table 4.3) at presentation while only trend was observed after 2-3 months (p=0.09, Table 4.3). Hypothalamic lean group also had a significantly higher lactate/butyrate ratio in dry faeces (p=0.05, Table 4.4) and a trend in acetate/butyrate ratio (p=0.06) only after 2-3 months.

4.6.1.3 Differences in faecal SCFA between non-pathological groups (healthy lean and simple obese)

Significant differences in the proportion of propionate were found between the two groups only for the second sample. The simple obese group had a significantly higher proportion of propionate than the healthy lean in dry (median (IQR) %C3; Simple obese 17.1(5.89), Healthy lean 13.3(3.32), p=0.01) as well as wet faecal sample (median (IQR) %C3; Simple obese 16.6(6.03), healthy lean 13.3(3.32), p=0.03) (Table 4.3). Acetate/propionate ratio was significantly higher (p=0.04) while the propionate/butyrate ratio was significantly lower (p=0.04) in healthy lean than simple obese group only for dry faeces (Table 4.4).

4.6.1.4 Differences in faecal SCFA between pathological groups (Hypothalamic lean vs. hypothalamic obese)

At the time of recruitment, the total SCFA concentration in dried faecal sample was significantly higher in the hypothalamic obese group in both dry (median (IQR); 681.6(395) vs. 406.6(280.1) µmol/g dry faeces, p=0.037) and wet faeces (median (IQR); 543.7(298.5) vs. 277.4(257.8) µmol/g wet faeces, p=0.01).

For the individual SCFA, the hypothalamic obese group showed a consistent and significantly higher concentration of propionate than the hypothalamic lean group at recruitment in both dry faeces [median (IQR); hypoth. obese 84.2(62.7) vs. hypoth. Lean 45.2(37.69) µmol/g dry faeces, p=0.015] and wet faeces [median (IQR); hypoth. obese 68.28(53), hypoth. Lean 29.68(25.26) µmol/g wet faeces, p=0.01]. Similar results were observed in faecal samples after 2-3 months in dry [median (IQR) C3; hypoth. Obese 86.03(72.4), hypoth. Lean 47.56(29.78) µmol/g dry faeces, p=0.018) and wet faeces (median (IQR) C3; hypoth. obese 58.6(52.8), hypoth. Lean 33.90(20.48) µmol/g wet faeces p=0.04) (Table 4.2, Table 4.3). The proportion of propionate was also significantly

higher in hypothalamic obese than lean group but only after 2-3 months in both dry (p=0.004, table 4.2) and wet faeces (p=0.004, Table 4.3).

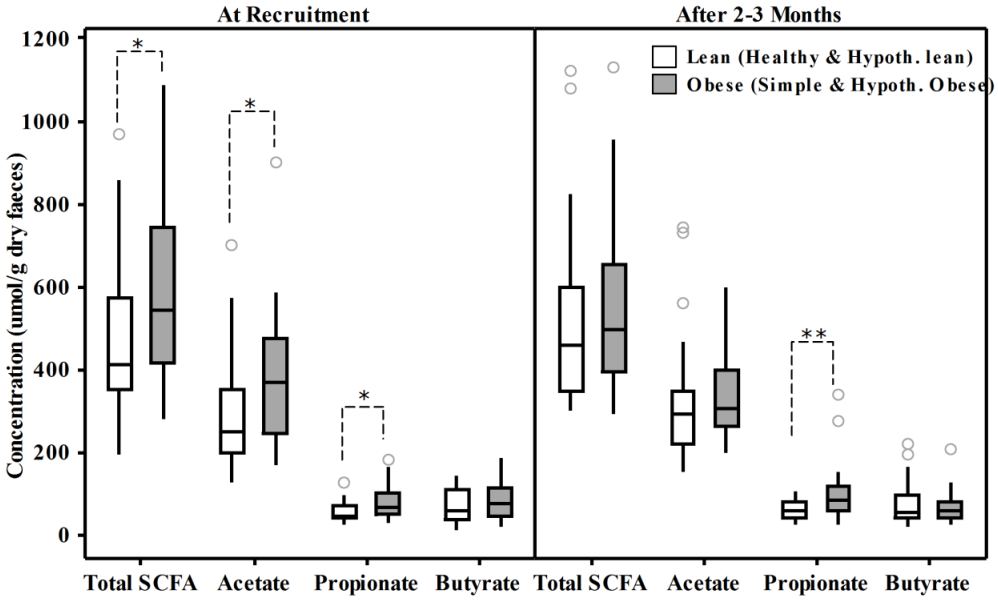
Concentration of butyrate was significantly higher in hypothalamic obese than lean group only in wet faecal sample [median (IQR); hypoth. obese 74.09(58.30) vs. hypoth. lean 29.5(38.5) µmol/g, p<0.01] (Table 4.3). Amongst medium chain fatty acids, only the proportion of octanoic acid (C8%) was significantly higher in hypothalamic lean than obese group at recruitment both in dry [median (IQR) hypoth. Lean 1.26(1.33) vs. hypoth. obese 0.57(0.80), p=0.01] and wet faecal samples [median (IQR) 1.22(1.33) vs. 0.58(0.80), p=0.01] (table 4.3).The concentration of BCFA, isobutyric acid was significantly higher in hypothalamic obese group (p=0.04, Table 4.3) only in wet faecal sample.

Consistent with a higher concentration of propionate in hypothalamic obese group, the ratio of acetate/propionate was significantly lower (p=0.04, Table 4.4) while that of propionate/butyrate was significantly higher (p=0.002, Table 4.4) in hypothalamic obese than lean group (both in dry and wet faecal samples) at the time of recruitment (Table 4.4).

4.6.2 Total and major individual SCFA concentrations according to lean (healthy lean and hypothalamic lean) and obese (simple and hypothalamic obese) phenotype

A tendency in the concentration of total and major individual SCFA was seen in the two obese groups compared to the two lean groups. Therefore, participants were grouped according to their phenotype into lean (healthy lean and hypothalamic lean) and obese (simple and hypothalamic obese) groups. Obese phenotype showed significantly higher concentration of total SCFA (p=0.028), acetate (p=0.028), and propionate (p=0.011) at recruitment (Figure 4.1). Concentration of propionate remained significantly higher in obese phenotype than lean phenotype even after 2-3 months (p=0.010) (Figure 4.1).

Figure 4.1: Boxplot showing concentration of total SCFA, acetate (C2), propionate (C3), and butyrate (C4) (expressed as µmol/g dry faeces) according to lean (healthy lean and hypothalamic lean) and obese (simple and hypothalamic obese) phenotype, at the time of recruitment and after 2-3 months.



Blank circles indicate outliers, * indicate $p<0.05$, ** indicate $p=0.01$

Table 4.2: Concentration (µmol/g) and proportion (%) of total and individual short chain fatty acids and branched-chain fatty acids (freeze dried) at the time of recruitment (A) and after 2-3 months (B).

Parameter	**Hypoth Lean (n=12)**		**Hypoth Obese(n=10)**		**Healthy Lean(n=27)**		**Simple Obese(n=16)**	
Sample No.	A	B	A	B	A	B	A	B
Concentration (µmol/g dry faeces) median (IQR)								
Acetate (C2)	256.3(241.2)	292.5(108.9)	427.3(241.9)	380.8(125.3)	246.6(146.4)	290.1(190.7)	327.1(198.9)	290.4(149.6)
Propionate (C3)	**45.21(37.69)†**	**47.56(29.8)†**	**84.2(62.7)†**	**86.0(72.4)†**	49.88(28.50)	65.60(40.68)	63.0(52.6)	82.9(48.3)
Iso-butyrate (iC4)	12.29(5.45)	13.98(4.95)	11.52(8.88)	11.29(7.39)	13.47(6.12)	14.55(6.46)	14.34(4.40)	11.71(7.94)
Butyrate (C4)	44.1(41.4)	55.68(35.02)	95.3(73.3)	59.16(31.90)	59.91(71.46)	61.1(79.7)	75.1(71.4)	58.0(46.6)
Iso-valerate (iC5)	13.31(7.06)	15.69(6.56)	12.52(9.52)	11.38(7.05)	14.50(6.53)	15.96(8.52)	16.82(7.42)	11.53(9.64)
Valerate (C5)	10.66(11.51)	14.59(8.74)	10.24(10.2)	14.85(11.08)	12.85(6.50)	15.03(8.55)	15.04(6.53)	12.61(11.71)
Iso-caproic acid (iC6)	0.00 (0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)
Caproic acid (C6)	0.00(8.62)	0.00(8.16)	1.40(4.68)	0.87(4.98)	3.40(7.51)	3.13(9.22)	3.21(9.13)	2.48(7.82)
Heptanoic acid (C7)	0.000(1.85)	0.00(3.85)	0.00(1.568)	0.00(0.000)	0.00(2.88)	0.00(0.60)	0.00(0.87)	0.00(0.63)
Octanoic acid (C8)	5.660(3.140)	4.66(4.63)	3.88(5.47)	3.30(1.98)	4.23(4.72)	4.56(4.63)	3.08(5.05)	3.32(4.03)
Total SCFA	**406.6(280.1)†**	448.0(152.9)	**681.6(395.0)†**	590.6(244.9)	413.9(224.5)	479.7(333.7)	494.0(309.7)	487.9(247)
Proportion (%)median(IQR)								
Acetate (C2%)	64.93(7.41)	64.44(6.29)	**65.6 (10.83)‡**	64.48(6.86)	60.93(9.09)	60.18(8.65)	**60.57(6.25)‡**	61.45(6.57)
Propionate (C3%)	**10.8(2.17)¥**	**11.88(3.48)†**	14.57(7.17)	**14.81(5.69)†**	**13.64(3.12)¥**	13.31(3.32)	12.59(2.40)	17.09(5.89)
Iso-butyrate (iC4%)	2.83(2.74)	3.26(1.85)	1.86(1.74)*	2.54(1.645)	2.72(2.27)	3.23(2.42)	3.45(1.88)*	2.03(2.370)
Butyrate (C4%)	10.28(5.96)	13.0(3.97)	12.62(5.66)	10.57(2.59)	13.82(4.19)	14.1(6.77)	14.77(7.75)	11.65(3.99)
Iso-valerate (iC5%)	3.21(3.31)	3.435(2.35)	**1.85(1.44)‡**	2.54(1.865)	3.26(2.87)	3.71(2.94)	**2.99(3.31)‡**	2.41(3.29)
Valerate (C5%)	2.98(3.49)	3.10(2.23)	1.73(2.41)	2.53(1.16)	2.86(1.98)	2.69(2.32)	2.98(1.70)	2.80(3.00)
Iso-caproic (iC6%)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)
Caproic acid (C6%)	0.00(2.39)	0.00(1.80)	0.21(0.62)	0.22(0.81)	0.97(1.70)	0.66(1.38)	0.72(1.69)	0.42(1.07)
Heptanoic acid (C7%)	0.00(0.54)	0.00(0.66)	0.00(0.248)	0.00(0.00)	0.00(0.53)	0.00(0.15)	0.00(0.12)	0.00(0.13)
Octanoic acid (C8%)	**1.26(1.33)†**	0.93(0.94)	**0.57(0.80)†**	0.47(0.66)	0.81(1.02)	0.67(0.82)	0.74(1.30)	0.54(0.87)

The values are expressed as median and interquartile range. A; at recruitment, B; after 2-3 months, n; number of participants in each group.
† indicate significant differences between hypoth. lean and hypoth. obese, * indicate significant differences between healthy lean and simple obese
‡ indicate significant differences between hypoth obese and simple obese, ¥ indicate significant differences between hypoth lean and healthy lean

Table 4.3: Concentration (µmol/g) and proportion (%) of total and individual short chain fatty acids and branched-chain fatty acids (wet faeces) at the time of recruitment (A) and after 2-3 months (B).

Parameter	**Hypoth Lean (n=12)**		**Hypoth Obese(n=10)**		**Healthy Lean(n=27)**		**Simple Obese(n=16)**	
Sample No.	A	B	A	B	A	B	A	B
Concentration (µmol/g wet faeces)median(IQR)								
Acetate (C2)	191.3(228.5)	200.1(132.6)	340.8(198.0)	243.8(119.1)	162.8(131.0)	197.4(147.9)	210.9(178.5)	216.4(174.4)
Propionate (C3)	**29.68(25.26)†**	33.92(20.48)	**68.3(53.0)†**	58.7(52.8)	32.88(24.45)	47.91(31.99)	44.84(39.13)	61.6(51.3)
Iso-butyrate (iC4)	9.08(2.56)	9.73(3.56)	9.06(7.21)	7.26(4.81)	9.05(4.48)	10.07(4.30)	9.91(3.96)	8.57(5.38)
Butyrate (C4)	**29.5(38.5)†**	34.64(35.59)	**74.09(58.30)†**	38.79(22.77)	37.17(56.60)	43.70(60.27)	56.0(61.7)	38.5(53.1)
Iso-valerate (iC5)	9.91(3.04)	10.09(3.04)	9.61(7.21)	7.26(4.46)	9.83(4.22)	10.87(4.14)	10.95(3.80)	8.48(5.50)
Valerate (C5)	7.63(7.43)	9.74(5.56)	8.66(7.90)	9.38(9.32)	8.75(4.69)	10.12(5.91)	10.40(5.64)	8.25(8.23)
Iso-caproic acid (iC6)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)
Caproic acid (C6)	0.00(5.54)	0.00(4.52)	1.08(3.60)	0.67(3.39)	2.99(5.48)	2.06(6.63)	2.09(6.78)	1.02(5.50)
Heptanoic acid (C7)	0.00(1.19)	0.00(2.38)	0.00(1.22)	0.00(0.00)	0.00(1.98)	0.00(0.48)	0.00(0.59)	0.00(0.35)
Octanoic acid (C8)	4.05(2.37)	2.89(2.08)	3.05(4.15)	2.57(1.37)	2.86(3.20)	3.30(3.14)	2.04(2.87)	1.98(3.33)
Total SCFA	**277.4(257.8)†**	309.6(186.9)	**543.7(298.5)†**	379.4(214.8)	271.2(205.4)	312.4(251.8)	334.5(271.4)	334.1(264.9)
Proportion (%)median(IQR)								
Acetate (C2%)	64.94(7.48)	64.48(6.28)	65.60(10.83)	64.49(6.89)	60.63(9.12)	60.17(8.41)	60.44(4.02)	61.96(5.07)
Propionate (C3%)	10.80(2.17)	11.88(3.49)	14.57(7.17)	14.79(5.69)	13.64(2.92)	13.31(3.32)	12.59(1.88)*	16.66(6.03)*
Iso-butyrate (iC4%)	2.82(2.74)	3.26(1.86)	1.855(1.748)	2.54(1.65)	3.16(2.20)	3.23(2.45)	3.51(2.14)	2.04(2.58)
Butyrate (C4%)	9.97(5.96)	12.99(3.97)	12.60(5.66)	10.57(2.59)	14.21(5.11)	14.08(6.77)	15.07(7.03)	12.04(4.53)
Iso-valerate (iC5%)	3.21(3.31)	3.43(2.35)	1.84(1.44)	2.53(1.86)	3.69(3.07)	3.69(2.98)	3.25(3.54)	2.70(3.56)
Valerate (C5%)	2.69(3.49)	3.09(2.23)	1.73(2.42)	2.52(1.18)	2.86(1.88)	2.69(2.32)	3.10(1.41)	2.75(2.49)
Iso-caproic (iC6%)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)	0.00(0.00)
Caproic acid (C6%)	0.00(2.39)	0.00(1.79)	0.20(0.63)	0.22(0.80)	1.02(1.70)	0.69(1.37)	0.87(1.72)	0.19(1.03)
Heptanoic acid (C7%)	0.00(0.54)	0.00(0.66)	0.00(0.24)	0.00(0.00)	0.00(0.54)	0.00(0.13)	0.00(0.15)	0.00(0.07)
Octanoic acid (C8%)	**1.22(1.33)†**	0.93(0.94)	**0.57(0.80)†**	0.47(0.62)	0.87(1.15)	0.66(0.81)	0.79(1.30)	0.51(1.01)

The values are expressed as median and interquartile range. A; at recruitment, B; after 2-3 months, n; number of participants in each group. † indicate significant differences between hypoth. lean and hypoth. obese

Table 4.4: Molar ratios of different SCFA in freeze dried faeces (dry faeces) and faecal samples (wet faeces) of all participants at recruitment and after 2-3 months.

Parameter	**Hypoth lean (n=12)**		**Hypoth obese(n=10)**		**Healthy lean(n=27)**		**Simple obese(n=16)**	
Sample No.	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Dry faeces								
Lactate:C4	0.08(0.09)	**0.08(0.09)***	0.05(0.04)	0.06(0.04)	0.07(0.05)	**0.05(0.05)***	0.06(0.06)	0.06(0.05)
C2:C3	**6.01(2.74)¥**	5.425(2.15)	4.32(3.55)	4.43(1.70)	**4.42(1.26)¥**	**4.74(1.74)***	4.840(1.03)	**3.89(1.12)***
C2:C4	6.17(3.37)	4.86(1.58)	5.185(3.02)	**6.35(1.82)‡**	4.38(1.92)	4.11(2.34)	4.14(3.03)	**4.74(1.61)‡**
C3:C4	1.03(0.62)	0.90(0.48)	1.205(1.07)	1.47(0.22)	0.94(0.362)	0.82(0.56)	0.90(0.70)	1.35(1.13)
Wet faeces								
Lactate:C4	0.03(0.05)	0.05(0.052)	0.01(0.011)	0.02(0.03)	0.03(0.04)	0.03(0.03)	0.03(0.05)	0.03(0.04)
C2:C3	**6.01(2.74)¥**	5.42(2.15)	4.32(3.55)	4.43(1.71)	**4.34(1.19)¥**	4.74(1.74)	4.85(1.23)	4.06(0.97)
C2:C4	6.17(3.37)	4.86(1.58)	5.18(3.02)	6.35(1.83)	4.21(1.89)	4.11(2.34)	4.05(1.97)	4.67(1.75)
C3:C4	1.03(0.62)	0.90(0.48)	1.20(1.07)	1.47(0.23)	0.94(0.39)	0.82(0.56)	0.87(0.48)	1.17(0.87)

The values are expressed as median and interquartile range. A; at recruitment, B; after 2-3 month, n; number of participants in each group.
* indicate significant differences between healthy lean and simple obese
‡ indicate significant differences between hypoth. obese and simple obese
¥ indicate significant differences between hypoth. lean and healthy lean

4.6.3 Percentage faecal water

At the time of recruitment, hypothalamic obese group had a significantly higher percentage water content than simple obese [median (IQR): %H2O= hypoth. Obese 78.6 (7.23), simple obese 70.3 (12.73), p=0.04] and hypothalamic lean groups [median (IQR): %H2O= hypoth. obese 78.6(7.23) vs. hypoth. Lean 73.12(12.24), p=0.02] (Figure 4.1, table 4.5). However, no significant differences were found between any of the groups after 2-3 months (Table 4.5, Figure 4.2).

4.6.4 Faecal Ammonia in dry and wet faeces

At recruitment, healthy lean participants showed a significantly higher faecal ammonia levels than simple obese [median (IQR): healthy lean 3.6(1.79) simple obese 2.1(1.91), p=0.02] and hypothalamic lean group [median (IQR): healthy lean 3.6(1.79), hypoth. Lean 2.37(1.60), p=0.04] (Table 4.5, Figure 4.3).

After 2-3 months, faecal ammonia was only marginally-significantly lower in hypothalamic obese group than the hypothalamic lean group in wet faeces (Hypoth. obese vs. lean; median (IQR): 0.48(0.29) vs. 0.82(0.54) µg/ml, p=0.05) (Figure 4.3, Table 4.5).

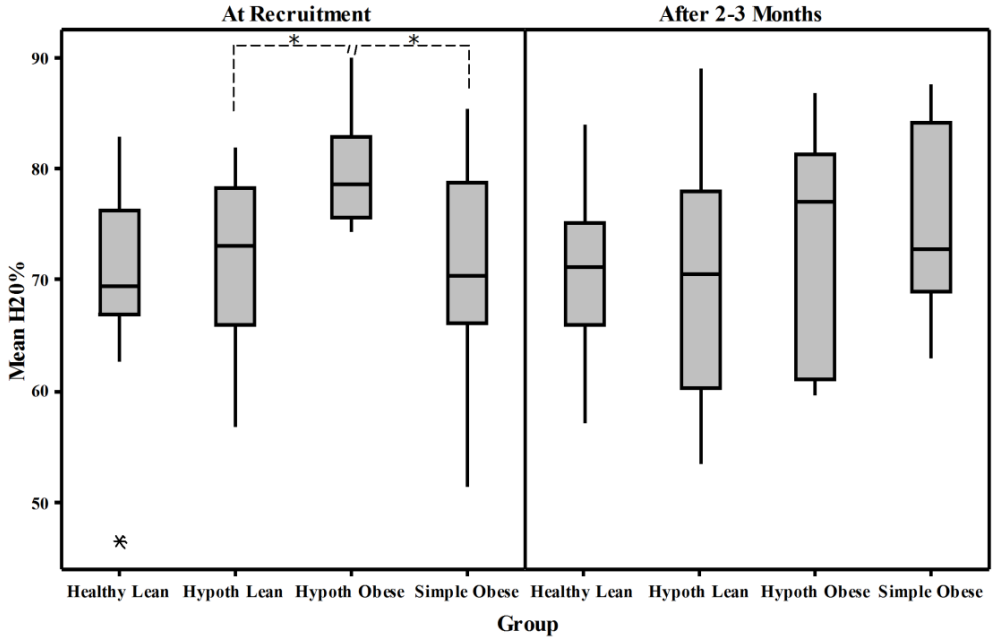
4.6.5 Faecal D, L, and total lactate

No significant differences were observed for D, L, and total lactate at the time of recruitment and after 2-3 months between any of the groups apart from hypothalamic lean group which had a significantly higher D-lactate than hypothalamic obese group (dry faeces; p=0.03 and wet faeces; p=0.01, Table 4.5) and total lactate (wet faeces; p=0.01, Table 4.5) at the time of recruitment.

4.6.6 Faecal pH and faecal hydrogen sulphide

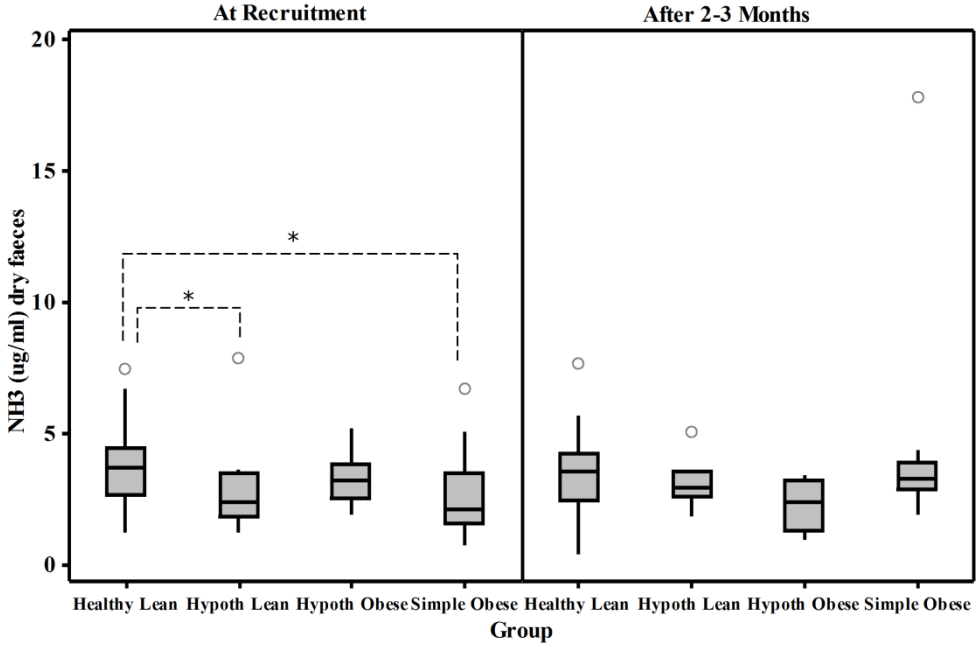
There was no significant difference in the faecal pH (Figure 4.4, Table 4.5) and hydrogen sulphide (free, bound, and total) between any of the groups at the time of recruitment and after 2-3 months (Table 4.5).

Figure 4.2: Boxplot of faecal water (%) content in faecal samples of all 4 groups at recruitment and after 2-3 months.



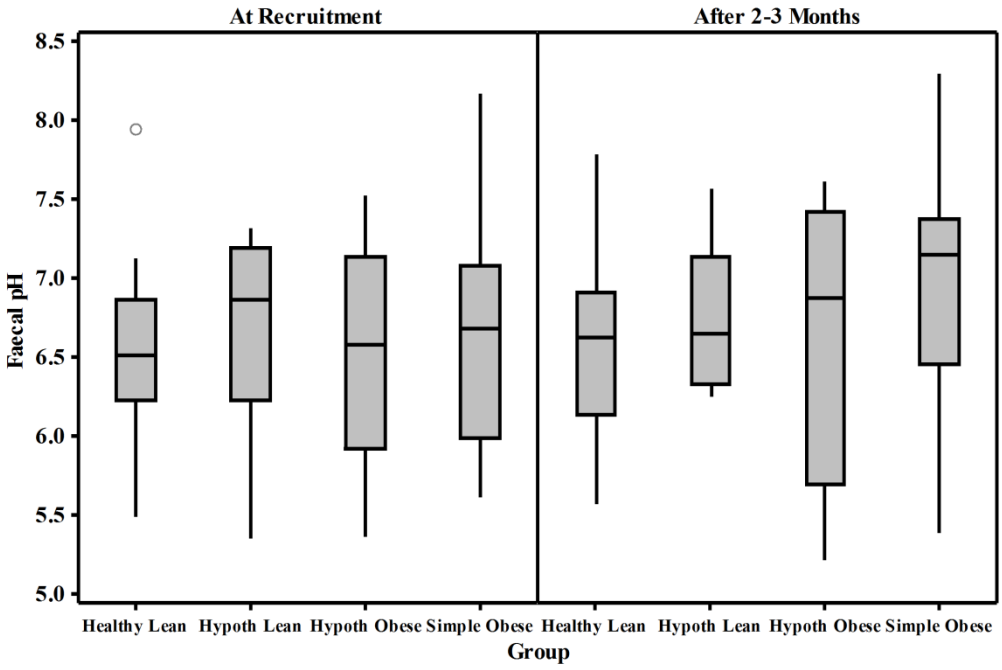
* indicate p<0.05, Hypoth.; hypothalamic (lean or obese)

Figure 4.3: Boxplot of faecal ammonia concentration (per freeze dried faecal material) at recruitment and after 2-3 months for all 4 groups.



Blank circles represent outliers, * indicate p<0.05. The top most outlier in "after2-3 months" panel belongs to an obese subject whose faecal sample had urine contamination. Hypoth.; hypothalamic (lean or obese)

Figure 4.4: Boxplot of faecal pH at recruitment (sample A) and after 2-3 months (sample B) of all 4 groups.



Blank circle represent outliers. Hypoth.; hypothalamic (lean or obese)

Table 4.5: Concentration of hydrogen sulphide (free, total, bound), lactate (D, L, and total), ammonia, faecal pH, and % water content in the dry and wet faecal samples of all participants at recruitment and at after 2-3 months.

Parameter	**HYPOTH LEAN**		**HYPOTH OBESE**		**HEALTHY LEAN**		**SIMPLE OBESE**	
Sample No.	A	B	A	B	A	B	A	B
Faecal pH	6.86(0.96)	6.65(0.81)	6.58(1.22)	6.87(1.73)	6.51(0.65)	6.62(0.78)	6.69(1.09)	7.15(0.93)
Median H_20%	73.12(12.24)	70.46(17.77)	**78.65(7.23)‡**	77.01(20.19)	69.38(9.39)	71.18(9.25)	**70.31(12.73)‡**	72.75(15.25)
Dry Faeces								
NH_3 (µg/ml)	**2.37(1.60)¥**	2.91(0.93)	3.22(1.33)	2.41(1.94)	**3.66(1.79)*¥**	3.54(1.73)	**2.12(1.91)***	3.25(1.01)
Free H_2S (µmol/g)	0.01(0.03)	0.02(0.06)	0.02(0.04)	0.01(0.019)	0.02(0.054)	0.04(0.03)	0.03(0.02)	0.03(0.04)
Total H_2S (µmol/g)	0.13(0.24)	0.19(0.10)	0.09(0.23)	0.12(0.24)	0.16(0.17)	0.15(0.24)	0.25(0.32)	0.20(0.22)
Bound H_2S (µmol/g)	0.18(0.18)	0.15(0.09)	0.08(0.18)	0.11(0.25)	0.13(0.18)	0.11(0.22)	0.20(0.29)	0.17(0.20)
D-lactate (mg/g)	**2.59(1.01)†**	2.64(1.36)	**1.98(0.86)†**	1.86(0.24)	2.16(0.63)	1.91(0.39)	1.9(1.09)	1.84(1.08)
L-lactate (mg/g)	2.24(1.49)	2.05(1.23)	1.83(1.22)	1.78(0.57)	2.01(0.61)	1.82(0.33)	1.7(0.85)	1.79(1.03)
Total Lactate (mg/g)	4.78(2.07)	4.79(2.54)	3.80(1.80)	3.66(0.79)	4.15(0.94)	3.77(0.63)	3.81(1.71)	3.67(2.09)
D/L ratio	1.05(0.24)	1.07(0.14)	1.03(0.11)	0.98(0.11)	1.04(0.06)	1.04(0.06)	1.07(0.14)	1.04(0.09)
Wet Faeces								
NH_3 (µg/ml)	**0.78(0.46)†**	0.82(0.54)	**0.56(0.38)†**	0.48(0.288)	1.02(0.56)	0.91(0.54)	0.71(1.06)	0.93(0.66)
Free H_2S (µmol/g)	0.05(0.07)	0.11(0.18)	0.077(0.15)	0.05(0.13)	0.07(0.10)	0.12(0.12)	0.07(0.08)	0.07(0.12)
Total H_2S (µmol/g)	0.54(0.76)	0.73(0.75)	0.41(0.92)	0.67(0.65)	0.54(0.52)	0.63(0.59)	0.79(1.00)	0.72(0.86)
Bound H_2S (µmol/g)	0.66(0.57)	0.61(0.79)	0.38(0.79)	0.56(0.61)	0.47(0.52)	0.42(0.54)	0.70(0.99)	0.66(0.72)
D-lactate (mg/g)	**0.68(0.41)†**	0.79(0.64)	**0.47(0.14)†**	0.64(0.27)	0.69(0.33)	0.56(0.18)	0.58(0.48)	0.41(0.44)
L-lactate (mg/g)	0.50(0.49)	0.61(0.55)	0.40(0.22)	0.62(0.23)	0.58(0.28)	0.48(0.23)	0.47(0.49)	0.41(0.43)
Total Lactate (mg/g)	**1.15(0.86)†**	1.42(1.17)	**0.84(0.29)†**	1.28(0.50)	1.24(0.53)	1.06(0.36)	1.11(0.94)	0.82(0.88)
D/L ratio	1.05(0.24)	1.07(0.14)	1.03(0.11)	0.98(0.11)	1.04(0.06)	1.04(0.06)	1.07(0.14)	1.04(0.09)

The values are expressed as median and interquartile range. A; sample at recruitment, B; sample after 2-3 months.H_2O; water, NH_3; ammonia, H_2S; Hydrogen sulphide, D/L; ratio of D and L lactate. † indicate significant differences between hypoth. lean and hypoth. obese, * indicate significant differences between healthy lean and simple obese, ‡ indicate significant differences between hypoth obese and simple obese, ¥ indicate significant differences between hypoth lean and healthy lean

4.6.7 Changes in gut microbiota metabolic parameters within individual groups over the period of follow-up

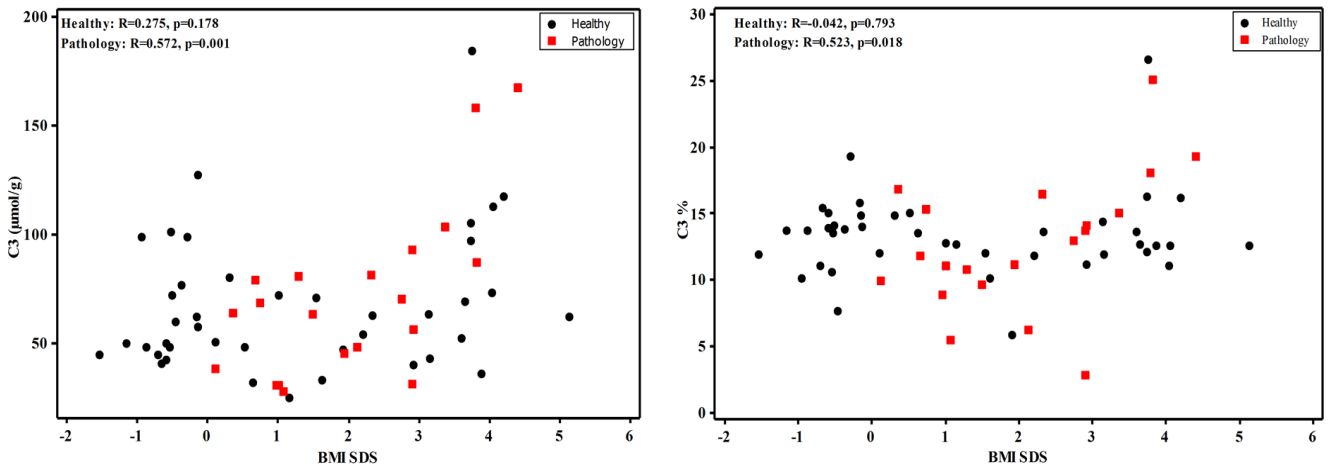
No significant differences in the median concentration and proportion of the total and individual SCFA (Table 4.2, Table 4.3), hydrogen sulphide (free, bound, and total), lactate (D, L, and D/L ratio), ammonia, faecal pH, and % faecal H_2O content were observed over the period of follow-up within each group (Table 4.5).

4.6.8 Correlation of SCFA and BCFA with BMI SDS according to their pathology (healthy vs. pathological)

Whether the various faecal metabolites correlated with BMI SDS was tested in the pathological (hypothalamic lean and obese) and healthy group (healthy lean and simple obese) of participants.

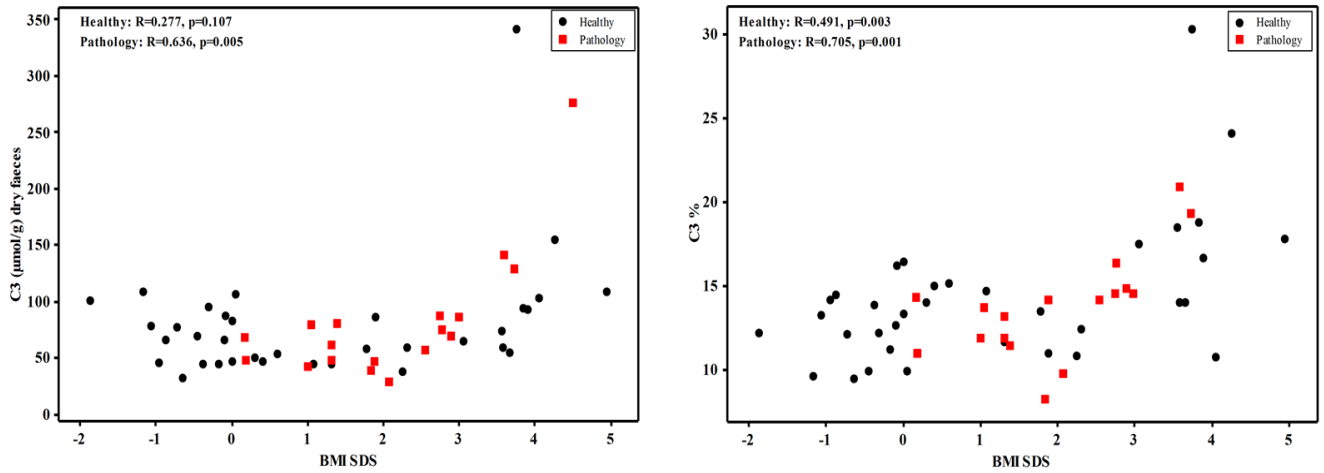
Participants grouped as a pathological group showed consistently significant positive correlation BMI SDS with concentration and proportion of propionate both at recruitment and after 2-3 months (figure 4.5 and figure 4.6), while healthy group showed a significant positive correlation with the proportion of propionate only after 2-3 months (Figure 4.6 and Table 4.6). Additionally, the pathological group also showed a strong negative correlation of BMI SDS with proportion of octanoic acid at recruitment and branched chain fatty acids (iso-butyric and iso-valeric acid) after 2-3 months (Table 4.6).

Figure 4.5: Correlation of BMI SDS with concentration and proportion of propionate (µmol/g) between Healthy (healthy lean + simple obese) and pathological groups (hypothalamic lean + hypothalamic obese) at recruitment.



C3; propionate concentration or proportion in freeze dried faeces

Figure 4.6: Correlation of BMI SDS with concentration of propionate (µmol/g) between Healthy (healthy lean + simple obese) and pathological groups (hypothalamic lean + hypothalamic obese) after 2-3 months.



C3; propionate concentration or proportion in freeze dried faeces

Table 4.6: Spearman-Rank Correlations of all SCFA and BCFA with BMI SDS according to pathology (healthy with no pathology and patients with pathology).

	Healthy (Healthy Lean + Simple Obese)		**Pathological** (Hypoth. Lean + Hypoth. Obese)	
SCFA (µmol/ml and %)	**R**	**p-value**	**R**	**p-value**
Correlations of BMI SDS with SCFA and BCFA at recruitment				
Acetate (C2)	0.239	0.132	0.364	0.115
Propionate (C3)	0.215	0.178	**0.572**	**0.008***
Iso-butyrate (iC4)	0.160	0.317	0.289	0.217
Butyrate (C4)	0.106	0.510	0.052	0.827
Iso-valerate (iC5)	0.103	0.524	0.085	0.722
Valerate (C5)	0.191	0.232	0.128	0.591
Iso-caproic acid (iC6)	-0.060	0.709	-0.021	0.930
Caproic acid (C6)	-0.185	0.247	0.155	0.515
Heptanoic acid (C7)	-0.069	0.669	-0.328	0.158
Acetate (C2%)	-0.001	0.995	-0.167	0.482
Propionate (C3%)	-0.042	0.793	**0.523**	**0.018***
Iso-butyrate (iC4%)	0.052	0.747	0.042	0.862
Butyrate (C4%)	-0.062	0.702	-0.363	0.116
Iso-valerate (iC5%)	-0.117	0.465	-0.343	0.139
Valerate (C5%)	0.037	0.820	-0.028	0.908
Caproic acid (C6%)	-0.120	0.454	-0.181	0.445
Heptanoic acid (C7%)	-0.193	0.227	0.153	0.519
Octanoic acid (C8%)	-0.051	0.750	**-0.536**	**0.015***
Total SCFA	0.222	0.164	**0.446**	**0.049***
Correlations of BMI SDS with SCFA and BCFA after 2-3 months				
Acetate (C2)	0.055	0.755	0.454	0.059
Propionate (C3)	0.277	0.107	**0.636**	**0.005***
Iso-butyrate (iC4)	-0.052	0.765	0.224	0.371
Butyrate (C4)	-0.285	0.097	-0.427	0.077
Iso-valerate (iC5)	-0.204	0.240	-0.436	0.071
Valerate (C5)	-0.174	0.319	0.275	0.270
Iso-caproic acid (iC6)	-0.092	0.601	0.099	0.696
Caproic acid (C6)	-0.075	0.667	-0.125	0.622
Heptanoic acid (C7)	-0.126	0.471	-0.207	0.411
Acetate (C2%)	0.061	0.726	-0.307	0.215
Propionate (C3%)	**0.491**	**0.003***	**0.705**	**0.001***
Iso-butyrate (iC4%)	-0.319	0.062	-0.264	0.291
Butyrate (C4%)	-0.222	0.200	**-0.585**	**0.011***
Iso-valerate (iC5%)	-0.173	0.319	**-0.548**	**0.019***
Valerate (C5%)	-0.138	0.431	-0.105	0.677
Caproic acid (C6%)	-0.116	0.507	0.103	0.685
Heptanoic acid (C7%)	-0.067	0.704	-0.125	0.622
Octanoic acid (C8%)	-0.101	0.565	-0.363	0.139
Total SCFA	0.058	0.739	0.448	0.062

* Indicate significant correlations, R; Spearman Rank correlation.

4.6.9 Univariate and multivariate analysis of dietary, demographic and anthropometric factors as predictors of gut bacterial metabolites

4.6.9.1 Univariate analysis of dietary, demographic and anthropometric factors as predictors of gut bacterial metabolites

Determinants of faecal bacterial metabolites (SCFA, BCFA, sulphide (free, bound, & total), D- & L-lactate, and ammonia) and pH were explored. These included demographic characteristics, dietary, and anthropometric variables tested in a univariate linear regression analysis (Table 4.1). Various significant associations were seen at recruitment as well as after 2-3 months (Table 4.7, Table 4.8, Table 4.9, Table 4.10).

Of particular note, concentration of total SCFA, acetate, propionate, and butyrate were significantly negatively associated with faecal pH and % faecal water both at the time of recruitment (Table 4.7) and after 2-3 months (Table 4.9). Moreover, concentration of propionate was significantly positively associated with fat mass (kg) and fat free mass (kg) at the time of recruitment (Table 4.7).

Table 4.7: Univariate analysis of demographic, dietary, and anthropometric variables at the time of recruitment as predictors of gut bacterial metabolites.

Predictors		Total SCFA (dry)	C2 (dry)	C3 (dry)	iC4 (dry)	C4 (dry)	iC5 (dry)	C5 (dry)	C6 (dry)	C7 (dry)	C8 (dry)
Age (years)	β-coef.		-0.008					0.492	0.197		
	p-value		0.08					0.002	0.047		
	R^2-adj		3.40%					13.30%	4.90%		
SIMD Quintiles	β-coef.									0.411	
	p-value									0.02	
	R^2-adj									7.10%	
Time elapsed (h)	β-coef.										0.316
	p-value										0.092
	R^2-adj										3.10%
BMI SDS	β-coef.		0.003	0.018							
	p-value		0.054	0.005							
	R^2-adj		4.60%	11.20%							
% body fat	β-coef.							0.092			
	p-value							0.088			
	R^2-adj							5.24%			
Body fat mass (kg)	β-coef.							0.09			
	p-value							0.068			
	R^2-adj							6.20%			
% actual energy consumed	β-coef.							-0.01			
	p-value							<0.001			
	R^2-adj							17.00%			
Energy intake (kcal)	β-coef.				21.45		0.004				
	p-value				0.09		0.015				
	R^2-adj				3.80%		9.80%				

Table 4.7 continued

Predictors		Total SCFA	C2 (dry)	C3 (dry)	iC4 (dry)	C4 (dry)	iC5 (dry)	C5 (dry)	C6 (dry)	C7 (dry)	C8 (dry)
Fat intake (g)	β-coef.				1.4		0.112	0.095	0.047		
	p-value				0.034		0.002	0.032	0.069		
	R^2-adj				7.10%		16.9%	7.3%	4.80%		
Protein intake (g)	β-coef.				1.355		0.126				
	p-value				0.027		0.002				
	R^2-adj				7.90%		16.7%				
Fat intake (%)	β-coef.				0.358		0.286	0.202			
	p-value				0.105		0.01	0.106			
	R^2-adj				3.40%		11.40%	3.40%			
Carbohydrate intake (%)	β-coef.				-0.49		-0.288				
	p-value				0.058		0.002				
	R^2-adj				5.40%		17.20%				
DF % recommended intake	β-coef.									0.016	
	p-value									0.033	
	R^2-adj									7.20%	
% estimated average req (% EAR)	β-coef.							-0.06			
	p-value							0.1			
	R^2-adj							3.50%			
Faecal pH	β-coef.	-145.22	-0.002	-0.007	0.025	-40.3	4.49			-0.986	
	p-value	<0.001	0.002	0.004	0.095	<0.001	0.001			0.014	
	R^2-adj	20.30%	14.60%	11.90%	3.10%	35.74%	17.50%			8.50%	
Faecal % H2O	β-coef.	10.03	0.022		-0.34		-0.368				
	p-value	0.001	0.002		0.098		<0.001				
	R^2-adj	16.20%	14.30%		3.10%		21.80%				

Predictors which had a significant association or a tendency for association ($p<0.1$) are shown only.β-coef.; beta coefficient, SIMD; Scottish Index of multiple deprivation, time elapsed; time elapsed between sample production and processing in the lab, Protein% RNI; percentage recommended nutritional intake of proteins (%RNI), %EAR; percentage of estimated average requirements of calories for UK population, C2; acetate, C3; propionate, iC4; iso-butyrate, C4; butyrate, iC5; iso-valerate, C5; valerate, iC6; iso-caproic acid, C6; Caproic acid, C7; heptanoic acid, C8; Octanoic acid

Table 4.8: Univariate analysis of demographic, dietary, and anthropometric variables as predictors of other gut bacterial metabolites at the time of recruitment.

Predicators		Lactate:C4	C2: C3	C2: C4	C3: C4	NH_3	Free-H_2S	Bound-H_2S	Total-H_2S	D-Lactate	L-lactate	Total Lactate
Age (Years)	β-coef.		-0.1706	0.199		0.099	-0.0022	-0.012	-0.013			
	p-value		0.034	0.05		0.007	0.061	0.073	0.077			
	R^2-adj		5.70%	4.70%		11%	4.70%	4.30%	4.10%			
Time elapsed (h)	β-coef.	0.006			0.04	-1.932				0.064		0.115
	p-value	0.025			0.097	0.052				0.047		0.066
	R^2-adj	7.30%			2.90%	5.10%				5.40%		4.40%
BMI SDS	β-coef.					-0.218				-0.066		
	p-value					0.07				0.087		
	R^2-adj					4.20%				3.6		
% body fat	β-coef.									-0.01		
	p-value									0.065		
	R^2-adj									6.80%		
Body fat mass (kg)	β-coef.									-0.011	-0.009	-0.021
	p-value									0.018	0.092	0.036
	R^2-adj									12.50%	5.30%	9.50%
Fat free mass (kg)	β-coef.						-0.0005					
	p-value						0.01					
	R^2-adj						15.70%					
% actual energy intake	β-coef.		0.004	0.004	0.0006			0.0006	0.0007			
	p-value		0.09	<0.001	0.052			0.001	0.001			
	R^2-adj		3.90%	26.40%	5.70%			24.10%	24%			

Table 4.8 continued

Predicators		**Lactate:C4**	**C2: C3**	**C2: C4**	**C3: C4**	**NH_3**	**Free-H_2S**	**Bound-H_2S**	**Total-H_2S**	**D-Lactate**	**L-lactate**	**Total Lactate**
Energy intake (Kcal)	β-coef.		-0.002			0.001						
	p-value		0.089			0.014						
	R^2-adj		3.90%			11.3%						
Fat intake (g)	β-coef.		-0.044			0.017		0.002	0.002			
	p-value		0.044			0.08		0.084	0.08			
	R^2-adj		6.20%			4.70%		4.90%	5%			
Protein intake (g)	β-coef.	0.0004				0.019				0.006	0.006	
	p-value	0.098				0.059				0.07	0.09	
	R^2-adj	4.20%				5.90%				5.40%	4.40%	
Carbohydrate intake (g)	β-coef.					0.008						0.012
	p-value					0.014						0.056
	R^2-adj					11.20%						6.20%
Dietary fibre intake (g)	β-coef.					0.09						
	p-value					0.012						
	R^2-adj					11.90%						
Fat intake (%)	β-coef.		-0.131									
	p-value		0.05									
	R^2-adj		5.90%									
Protein intake (%)	β-coef.	0.004		0.125								
	p-value	0.017		0.039								
	R^2-adj	10.70%		6.70%								

Table 4.8 continued

Predicators		**Lactate:C4**	**C2: C3**	**C2: C4**	**C3: C4**	**NH_3**	**Free-H_2S**	**Bound-H_2S**	**Total-H_2S**	**D-Lactate**	**L-lactate**	**Total Lactate**
Carbohydrate intake (%)	β-coef.		0.098		-0.013							
	p-value		0.08		0.088							
	R^2-adj		4.20%		4%							
Dietary fibre recommended intake (%)	β-coef.					0.015						
	p-value					0.018						
	R^2-adj					10.30%						
Protein % RNI	β-coef.			0.006			0.0001	0.001	0.001			
	p-value			0.012			0.031	<0.001	<0.001			
	R^2-adj			10.50%			8.50%	25.00%	24.90%			
% Estimated Average Requirement	β-coef.						0.0003	0.005	0.005			
	p-value						0.021	<0.001	<0.001			
	R^2-adj						10.00%	28.70%	28.80%			
Faecal pH	β-coef.	0.041		1.389	0.187							
	p-value	<0.001		<0.001	0.087							
	R^2-adj	30.30%		19.80%	3.40%							
Faecal %H2O	β-coef.	-0.002					-0.001	-0.006	-0.008			
	p-value	0.015					<0.001	0.017	0.003			
	R^2-adj	9.20%					27.10%	9.50%	14.70%			

Predictors which had a significant association or a tendency for association ($p<0.1$) are shown only. β-coef.; beta coefficient, SIMD; Scottish Index of multiple deprivation, time elapsed; time elapsed between sample production and processing in the lab, Protein% RNI; percentage recommended nutritional intake of proteins (%RNI), %EAR; percentage of estimated average requirements of calories for UK population, NH_3; ammonia, lactate:C4; lactate to butyrate ratio, C2:C3; acetate to propionate ratio, C2:C4; acetate to butyrate ratio, C3:C4; propionate to butyrate ratio

Table 4.9: Univariate analysis of demographic, dietary, and anthropometric variables as predictors of gut bacterial metabolites after 2-3 months.

Predicators		Total SCFA (dry)	C2 (dry)	C3 (dry)	iC4 (dry)	C4 (dry)	iC5 (dry)	C5 (dry)	C6 (dry)	C7 (dry)	C8 (dry)
Age (Years)	β-coef.							NS	0.297		
	p-value								0.004		
	R^2-adj								13.20%		
BMI SDS	β-coef.				-0.77		-0.783				
	p-value				0.018		0.052				
	R^2-adj				8.70%		5.40%				
% body fat	β-coef.				-0.094		-0.123				
	p-value				0.037		0.03				
	R^2-adj				9.40%		10.00%				
Body fat mass (kg)	β-coef.	3.679		1.933	-0.079		-0.11				
	p-value	0.053		<0.001	0.019		0.014				
	R^2-adj	7.70%		34.20%	12.40%		13.50%				
Fat free mass (kg)	β-coef.			2.185							
	p-value			0.001							
	R^2-adj			24.60%							
% actual energy consumed	β-coef.										-0.003
	p-value										0.055
	R^2-adj										5.60%
Energy intake (kcal)	β-coef.				0.002		0.004				
	p-value				0.089		0.011				
	R^2-adj				4%		10.80%				
Fat intake (g)	β-coef.			0.442		0.371	0.057		0.038		0.022
	p-value			0.1		0.101	0.022		0.087		0.047
	R^2-adj			3.40%		3.50%	8.60%		4%		6%
Carbohydrate intake (g)	β-coef.				0.015		0.025				
	p-value				0.078		0.021				
	R^2-adj				4.40%		8.80%				
Dietary fibre (g)											
Dietary Fat (%)	β-coef.	8	4.284	1.925		1.285					0.084
	p-value	0.019	0.046	0.028		0.08					0.017
	R^2-adj	9.10%	6.10%	7.80%		4.3					9.40%

Table 4.9 continued

Predicators		**Total SCFA (dry)**	**C2 (dry)**	**C3 (dry)**	**iC4 (dry)**	**C4 (dry)**	**iC5 (dry)**	**C5 (dry)**	**C6 (dry)**	**C7 (dry)**	**C8 (dry)**
Protein intake %	β-coef.				-0.314		-0.331				
	p-value				0.007		0.023				
	R^2-adj				12.40%		8.40%				
Carbohydrate intake (%)	β-coef.	-6.75		-1.957							-0.060
	p-value	0.046		0.024							0.086
	R^2-adj	6.10%		8.40%							4.10%
% Estimated Average Requirements	β-coef.						0.055				
	p-value						0.024				
	R^2-adj						8.30%				
Faecal pH	β-coef.	-222.8	-128.3	-41.88		-48.79	1.775		-1.74	-0.56	
	p-value	<0.001	<0.001	<0.001		<0.001	0.098		0.072	0.051	
	R^2-adj	44.70%	35.80%	24.50%		45.30%	3.90%		5%	6.20%	
Faecal %H2O	β-coef.	12.98	8.791	2.562	-0.172	2.178	-0.266				-0.067
	p-value	<0.001	<0.001	0.001	0.008	0.002	0.001				0.064
	R^2-adj	27.20%	30.30%	16.70%	11.10%	15.40%	18.80%				4.60%

Predictors which had a significant association or a tendency for association ($p<0.1$) are shown only. β-coef.; beta coefficient, SIMD; Scottish Index of multiple deprivation, time elapsed; time elapsed between sample production and processing in the lab, Protein% RNI; percentage recommended nutritional intake of proteins (%RNI), %EAR; percentage of estimated average requirements of calories for UK population, C2; acetate, C3; propionate, iC4; iso-butyrate, C4; butyrate, iC5; iso-valerate, C5; valerate, iC6; iso-caproic acid, C6; Caproic acid, C7; heptanoic acid, C8; Octanoic acid

Table 4.10: Univariate analysis of demographic, dietary, and anthropometric factors as predictors of other gut bacterial metabolites after 2-3 months.

Predicators		Lactate:C4	C2: C3	C2: C4	C3: C4	NH_3	Free-H_2S	Bound-H_2S	Total-H_2S	D-Lactate	L-lactate	Total Lactate
Time elapsed (h)	β-coef.				0.094							
	p-value				0.035							
	R^2-adj				6.40%							
BMI SDS	β-coef.		-0.306	0.286	0.195		-0.004					
	p-value		0.003	0.015	<0.001		0.08					
	R^2-adj		14.80%	9.20%	25.20%		4.90%					
% body fat	β-coef.		-0.037		0.023							
	p-value		0.015		0.006							
	R^2-adj		13.30%		17.10%							
Body fat mass (kg)	β-coef.		-0.036		0.025		-.0004					
	p-value		0.002		<0.001		0.08					
	R^2-adj		22.70%		39.70%		6.90%					
Fat free mass (kg)	β-coef.		-0.041		0.029							
	p-value		0.008		<0.001							
	R^2-adj		16.10%		30.70%							
Dietary fibre intake (g)	β-coef.			-0.065	-0.031							
	p-value			0.098	0.084							
	R^2-adj			3.60%	4.10%							
Fat intake (%)	β-coef.		-0.04									
	p-value		0.075									
	R^2-adj		4.50%									
CHO%	β-coef.		0.037									
	p-value		0.095									
	R^2-adj		3.70%									
Dietary Fibre intake (%)	β-coef.				-0.006							
	p-value				0.07							
	R^2-adj				4.70%							
Faecal pH	β-coef.	0.034		1.195		1.34						
	p-value	<0.001		<0.001		0.038						
	R^2-adj	25.90%		23.60%		9.17%						
Faecal %H2O	β-coef.	-0.002					-0.001	-0.008	-0.009			
	p-value	0.001					0.026	<0.001	<0.001			
	R^2-adj	21.80%					9.10%	23.90%	24.20%			

Predictors which had a significant association or a tendency for association ($p<0.1$) are shown only. β-coef.; beta coefficient, SIMD; Scottish Index of multiple deprivation, time elapsed; time elapsed between sample production and processing in the lab, Protein% RNI; percentage recommended nutritional intake of proteins (%RNI), %EAR; percentage of estimated average requirements of calories for UK population, H_2S; hydrogen sulphide, NH_3; ammonia, lactate:C4; lactate to butyrate ratio, C2:C3; acetate to propionate ratio, C2:C4; acetate to butyrate ratio, C3:C4; propionate to butyrate ratio.

4.6.9.2 Independent predictors of gut bacterial metabolites at the time of recruitment (multivariate analysis)

All associations at the time of recruitment that were either significant ($p<0.05$) or showed a tendency ($p \leq 0.1$) on univariate analysis were entered together in a step-wise multiple linear regression analysis with and without adjustment for pathology.

4.6.9.2.1 Association of faecal SCFA at the time of recruitment

Total SCFA at the time of recruitment were significantly negatively associated with pH and positively with % faecal H_2O on unadjusted (for pathology) multivariate step-wise regression analysis (pH; p=0.005, β= -111 and $\%H_2O$ p=0.04, β= 5.9, R^2=29.6%) (Table 4.11). Acetate was significantly negatively associated with pH; p=0.02, β=-60, BMI SDS; p=0.05, β=18, and % faecal water; p=0.04, β=5.9, R^2-Adj=33%). Propionate was negatively associated with pH and positively with BMI SDS (pH; p=0.007, β=-17.9, $\%H_2O$; p=0.004, β= 6.6, R^2-Adj=22.53%) while butyrate was only significantly associated with faecal pH (p<0.0001, β=-40.3, R^2-Adj=35.37%) (Table 4.11).

When adjusted for pathology, the association of % faecal water with total SCFA and that of BMI SDS with acetate was no longer significant (Table 4.11).

4.6.9.2.2 Association of faecal ammonia, sulphide and lactate

Faecal NH_3 was significantly associated with age and dietary fibre intake in grams in unadjusted multiple step-wise regression analysis (age; p=0.004, β=0.125, DF in g; p=0.03, β=0.072, R^2-adj=29.18%)(Table 4.12).Faecal free, bound, and total sulphide were significantly negatively associated with $\%H_2O$ content [p=0.02, β=-0.001 (R^2-adj=25.40%), p=0.005, β=-0.001 (R^2-adj=16.07%), and p=0.001, β=-0.007 (R^2-adj=21.40%) respectively] (Table 4.12). Similarly, faecal D and total lactate were significantly negatively associated with body fat mass in kg and positively associated with dietary intake of protein in grams (R^2-adj=35%.25 for D lactate, R^2-adj=35%.04 for total lactate) (Table 4.12).

However, on adjustment for pathology in multiple regression analysis, the significant association of D lactate with fat mass and that of total lactate with protein intake in grams was no more observed (p=0.218 for D lactate with fat mass & p=0.060 for protein intake in grams). In contrast, the association of acetate: butyrate ratio with proportion (%) of protein intake significantly increased when adjusted for pathology compared to unadjusted association (p=0.02, R^2- adj=38.1%, β=0.146 for % protein intake).

Table 4.11: Multivariate regression analysis for the association of dietary, anthropometric and demographic factors with gut bacterial metabolites at the time of recruitment. Analysis is presented both with and without adjustment for pathology (hypothalamic disorder). Predictors which had a significant association are shown only.

Predicators	R^2 Unadj/adj for pathology	Age (Years)		SIMD Quintiles		BMI SDS		Protein intake (g)		Dietary fat intake (%)		DF intake (%)		Faecal pH		Faecal %H_2O	
		B	p	β	p	β	p	β	p	B	p	β	p	β	p	β	p
Total SCFA	26.96 26.36													-111 -115.8	0.005 0.004	5.9 5.161	0.039 0.09
C2	27.94 26.33					18 15.83	0.049 0.086							-60 -68.9	0.028 0.014		
C3	22.53 21.26					6.6 6.41	0.004 0.007							-17.9 -17.9	0.007 <0.001		
iC4	9.78 9.38													2.4 2.222	0.03 0.219		
C4	35.7 34.8													-40.3 -40.25	<0.001 <0.001		
iC5	38.69 43.71							0.118 0.146	0.001 0.29							-0.37 -0.34	<0.001 0.009
C5	16.17 12.99									0.34 0.86	0.02 0.016						
C6	8.41 8.42	0.25 0.19	0.023 0.106														
C7	26.1 26.47			0.46 0.49	0.006 0.004							0.013 0.015	0.038 0.023	-0.85 -0.92	0.023 0.016		
C8	NS NS																

β ; beta coefficient, p; p-value, R^2 Unadj/adj for pathology; R^2 value (%) unadjusted and adjusted for pathology (hypothalamic lean and hypothalamic obese vs. healthy lean and simple obese), SIMD; Scottish Index of multiple deprivation, time elapsed; time elapsed between sample production and processing in the lab, DF; dietary fibre, C2; acetate, C3; propionate, iC4; iso-butyrate, C4; butyrate, iC5; iso-valerate, C5; valerate, iC6; iso-caproic acid, C6; Caproic acid, C7; heptanoic acid, C8; Octanoic acid NS; non-significant

Table 4.12: Multivariate step-wise regression analyses of anthropometric, dietary, and demographic factors with molar SCFA ratios, Ammonia, sulphide, lactate at the time of recruitment.

Predicators	R^2 Unadj/adj for pathology	Age (years)		Time elapsed (h)		Body fat (kg)		% Actual energy intake		Fat intake (g)		Protein intake (g)		Dietary fibre intake (g)		Protein intake %		Faecal pH		Faecal %H2O	
		β	p	B	p	β	P	β	p	β	p	B	p	β	p	β	p	β	p	β	p
Lactate:C4	41.51 36.51			0.005 0.006	0.049 0.029											0.004 4E-04	0.024 0.092			-0.001 -0.001	0.08 0.153
C2: C3	11.02 12.91							0.004 0.003	0.065 0.32	-0.046 0.211	0.033 0.171										
C2: C4*	40.4 38.1							0.003 0.004	0.002 0.002							0.1 0.127	0.56 0.02	0.88 0.97	0.029 0.014		
C3: C4	20.12 18.61			0.06 0.482	0.067 0.086			0.001 2.714	0.001 0.01												
NH_3	29.18 23.25	0.125 0.023	0.004 0.816											0.072 NS	0.028						
Free-H_2S	25.4 25.99																			-.001 -9E-04	0.023 0.086
Bound H_2S	16.07 11.65																			-0.007 -0.009	0.005 0.007
Total- H_2S	21.42 11.65																			-0.01 -0.009	0.001 0.007
D-Lactate	35.25 41.74					-0.169 -0.013	0.001 0.218					0.008 0.008	0.03 0.026								
L-lactate	9.18 12.59					-0.011 -0.01	0.07 0.102														
Total Lactate	35.04 29.35			0.153 0.094	0.024 0.019	-0.305 -0.012	0.003 0.035					0.015 0.008	0.03 0.059								

Analysis is presented both with and without adjustment for pathology (hypothalamic disorder) Predictors which had a significant association are shown only.β; beta coefficient, p; p-value, R^2 Unadj/adj for pathology; R^2 value (%) unadjusted and adjusted for pathology, time elapsed; time elapsed in h between sample production and processing in the lab, NH_3; ammonia, lactate:C4; lactate to butyrate ratio, C2:C3; acetate to propionate ratio, C2:C4; acetate to butyrate ratio, C3:C4; propionate to butyrate ratio, NS; non-significant.* $p<0.05$ after adjustment for pathology

4.6.9.3 Independent predictors of faecal bacterial metabolites after 2-3 months

Each selected demographic, anthropometric, and dietary predictor were individually analysed by univariate regression analysis (Table 4.9, Table 4.10). All significant variables with $p<0.05$ and those showing tendency ($p<0.1$) were included in multiple stepwise regression analysis with and without adjustment for pathology, to explore their independent association.

4.6.9.3.1 Association of SCFA after 2-3 months

Unadjusted step-wise regression analysis showed a significant negative association of total SCFAs with pH and $\%H_2O$ (pH; $p<0.0001$, β= -191, $\%H_2O$; $p=0.007$, β=9.2 R^2-Adj=67.07%). Acetate was significantly negatively associated with faecal pH and positively with $\%H_2O$ (pH; $p<0.0001$, β=-103, $\%H_2O$; $p=0.002$, β=5.8, R^2-adj. = 61.4%) (Table 4.13). Propionate was significantly associated with body fat mass and negatively with faecal pH (fat mass in kg; $p<0.0001$, β=1.81, pH; $p<0.0001$, β= -47, R^2-Adj=59.58%) while butyrate was only significantly negatively associated with pH ($p<0.0001$, β= -39.6% R^2-Adj=48.62%) (Table 4.13). Multiple regression analysis adjusted for pathology didn't affect these associations except in branched chain fatty acids (isobutyric and isovaleric acids) where the unadjusted association of energy intake was no more significant after adjustment for pathology (Table 4.13).

4.6.9.3.2 Association of faecal ammonia, sulphide, and lactate after 2-3 months

Faecal (dry) bound and total sulphide were significantly negatively associated with $\%H_2O$ ($p<0.0001$, β= -0.008 R^2-adj=23.9% for bound sulphide and $p<0.0001$, β= -0.009 R^2-adj=24.2% for total sulphide) in unadjusted multiple step-wise regression analysis. Faecal ammonia was associated with pH ($p=0.04$, β=1.34, R^2-Adj=9.2%) while faecal lactate (D, L and total lactate) after 2-3 months were not significantly associated with any predictor on multivariate analysis (Table 4.14).

Adjustment for pathology in multiple regression analysis only affected the association of free sulphide with body fat in kg ($p=0.27$ after adjustment compared to $p=0.002$ before adjustment).

Table 4.13: Multivariate step-wise regression analyses of anthropometric, dietary, and demographic factors with molar SCFA ratios, Ammonia, sulphide, lactate after 2-3 months. Analysis is presented both with and without adjustment for pathology (hypothalamic disorder) Predictors which had a significant association are shown only.

Response variable	R^2 Unadj/adj for pathology	Body fat mass (kg)		Actual energy intake (%)		Energy intake (kcal)		Body Fat (%)		Faecal pH		Faecal H_2O (%)	
		β	p	β	p	β	p	B	p	β	p	β	p
Total SCFA	67.07							5.6	0.06	-191	<0.001	9.2	0.007
	63.73							6.827	0.171	-185.01	<0.001	9.49	0.02
C2	56.13							0.053	0.053	-103	<0.001	5.8	0.002
	54.96							3.387	0.062	-103.2	<0.001	5.817	0.002
C3	59.89	1.81	<0.001							-47	<0.001		
	55.5	1.212	0.183							-39.02	0.008		
iC4	21.09					0.003	0.005					-0.27	0.001
	39.79					0.002	0.342					-0.244	0.008
C4*	48.62									-39.6	<0.001	1.23	0.06
	54.58									-45.75	<0.001	0.908	0.153
iC5	52.63					0.006	<0.001					-0.291	0.002
	46.29					0.004	0.115					-0.303	0.008
C5	NS												
	NS												
C6	14.75									-2.1	0.037		
	12.38									-2.07	0.052		
C7	6.2									-0.56	0.051		
	5.52									-0.56	0.051		
C8	24.31			-0.003	0.022							-0.09	0.01
	19.35			-0.004	0.024							-0.087	0.015

β ; beta coefficient, p; p-value, R^2 Unadj/adj for pathology; R^2 value (%) unadjusted and adjusted for pathology, C2; acetate, C3; propionate, iC4; iso-butyrate, C4; butyrate, iC5; iso-valerate, C5; valerate, iC6; iso-Caproic acid, C6; Caproic acid, C7; heptanoic acid, C8; Octanoic acid, * indicate p=0.04 after adjustment for pathology

Table 4.14: Multivariate step-wise regression analyses of anthropometric, dietary, and demographic factors with molar SCFA ratios, Ammonia, sulphide, lactate after 2-3 months. Analysis is presented both with and without adjustment for pathology (hypothalamic disorder) Predictors which had a significant association are shown only.

Predicators	R^2 Unadj/adj for pathology	Time elapsed (h)		BMI SDS		%body fat		Body fat (kg)		Faecal pH		% faecal H_2O	
		β	p	B	p	β	p	B	p	β	p	β	p
Lactate:C4	33.42									0.024	0.014	-0.002	0.026
	33.42									0.024	0.014	-0.002	0.026
C2: C3	NS												
	NS												
C2: C4*	27.02			0.27	0.028					1.00	0.003		
	34.59			0.165	0.2					0.978	0.003		
C3: C4**	49.6	0.1	0.031			-0.024	0.063	0.038	<0.001				
	52.73	0.082	0.074			-0.037	0.068	0.038	0.028				
NH_3	9.25									1.34	0.038		
	9.25									1.3	0.045		
Free-H_2S	14.26							-0.001	0.027				
	0.53							-0.0002	0.624				
Bound H_2S	23.9											-0.008	<0.001
	22.79											-0.008	0.001
Total- H_2S	24											-0.009	<0.001
	22.79											-0.009	0.001
D-Lactate	NS												
	NS												
L-lactate	NS												
	NS												
Total Lactate	NS												
	NS												

β; beta coefficient, p; p-value, R^2 Unadj/adj for pathology; R^2 value (%) unadjusted and adjusted for pathology, time elapsed; time elapsed between sample production and processing in the lab, NH_3; ammonia, lactate:C4; lactate to butyrate ratio, C2:C3; acetate to propionate ratio, C2:C4; acetate to butyrate ratio, C3:C4; propionate to butyrate ratio, NS; non-significant. * indicate p=0.010 and ** indicate p=0.044 after adjustment for pathology

4.6.9.4 Association of gut bacterial metabolites after 2-3 months with change in demographic, anthropometric, and dietary predictors (i.e. response at 2-3 months vs. change in predictors between recruitment and after 2-3 months)

Whether change in demographic, anthropometric, and dietary predictors determines the concentration of gut bacterial metabolites in the faecal samples at the time of follow-up, was studied by univariate analysis. All significant associations in univariate analysis were then analysed by multiple regression analysis.

Univariate analysis

Higher SCFA at follow-up were associated negatively with change in pH while branched chain fatty acids at follow-up were associated with changes in the intake of dietary components particularly carbohydrates and proteins (Table 4.15).

Multivariate analysis

Change in pH (Δ pH) predicted the concentration of follow-up total SCFA (p=0.004, β= -153, R^2-Adj=27.28%), acetate (p=0.01, β= -76, R^2-Adj=23.2%), propionate (p=0.01, β= -35.06, R^2-Adj=11.35), and butyrate (p=0.004,β= -33.3 R^2-Adj=18.06%) in adjusted & unadjusted multivariate analysis (table 21) while faecal sulphide and lactates at follow-up were not significantly associated with change in any of the predictor (Table 4.16).

Table 4.15: Univariate analysis of demographic, dietary, and anthropometric variables as predictors of other gut bacterial metabolites after 2-3 months with change in anthropometric and dietary predictors (Δ). Predictors which had a significant association or a tendency for association (p<0.1) are shown only.

Predictors	R^2-adjusted	C2	C3	iC4	C4	iC5	C5	C6	C7	Lact:C4	C2: C4
Δ BMI SDS	β-coef.								4.09		
	p-value								0.088		
	R^2-adj								3.80%		
Δ BMI SDS/month	β-coef.								4.05		
	p-value								0.09		
	R^2-adj								1.97%		
Δ Energy intake (Kcal)	β-coef.			0.002		0.003					
	p-value			0.064		0.026					
	R^2-adj			6%		9.50%					
Δ Fat intake (g)	β-coef.			0.036		0.044					
	p-value			0.09		0.098					
	R^2-adj			4.80%		4.40%					
Δ Carbohydrates intake (g)	β-coef.			0.017		0.026					
	p-value			0.041		0.012					
	R^2-adj			7.80%		12.50%					
Δ Dietary fibre intake (g)	β-coef.							-0.241			
	p-value							0.027			
	R^2-adj							9.40%			
Δ Protein intake (%)	β-coef.			-0.196		-0.214					
	p-value			0.013		0.036					
	R^2-adj			12.30%		8.30%					
Δ Carbohydrates intake (%)	β-coef.								-0.055		
	p-value								0.02		
	R^2-adj								10.60%		
DF% recommended intake	β-coef.	-1.02						-0.046			
	p-value	0.06						0.026			
	R^2-adj	6.30%						9.50%			
Faecal pH	β-coef.	-73.5	-35.6		-33.29		-5.409	-2.96		0.023	0.96
	p-value	0.029	0.014		0.004		0.001	0.006		0.047	0.021
	R^2-adj	8.60%	11.3		15.70%		21.90%	14.30%		7.80%	9.70%

Table 4.16; Univariate analysis of demographic, dietary, and anthropometric variables as predictors of other gut bacterial metabolites after 2-3 months with change in anthropometric and dietary predictors (Δ). Predictors which had a significant association or a tendency for association (p<0.1) are shown only.

Response variables at 2-3 months	R^2 Unadj/Adj for pathology (%)	Δ BMI SDS		Δ Carbohydrate (g)		Δ Dietary fibre intake (g)		Δ Protein intake (%)		Δ Carbohydrate (%)		Δ Dietary Fibre (%)		Δ faecal pH	
		β	p	B	p	β	p	B	p	β	p	β	p	β	P
Total SCFA	27.28													-153	0.004
	15.16													-156.1	0.004
C2	23.19											-1.03	0.05	-76	0.01
	22.64											-1.134	0.041	-78.16	0.012
C3	11.3													-35.6	0.01
	9.24													-35.63	0.015
iC4	12.27							-0.2	0.01						
	5.8							-0.107	0.614						
C4**	15.7													-33.3	0.004
	24.17													-35.32	0.002
iC5	12.5			0.03	0.01										
	7.65			0.003	0.969										
C5	21.9													-5.41	0.001
	21.31													-5.308	0.001
C6**	25.33					-0.19	0.04							-2.77	0.007
	37.07					-0.069	0.261							-2.707	0.006
C7	20.58	5.1	0.03							-0.06	0.01				
	18.43	5.05	0.033							-0.055	0.012				
Lactate:C4*	7.8													0.023	0.047
	18.99													0.021	0.047
C2: C4**	9.7													0.964	0.021
	25.92													1.06	0.006

β; beta coefficient, p; p-value, R^2 Unadj/adj for pathology; R^2 value (%) unadjusted and adjusted for pathology, Δ; measurement at follow-up – measurement at presentation. CHO; carbohydrates, DF; dietary fibre. Delta BMISDS/month was not significantly associated with any metabolite in multivariate regression analysis and is therefore not presented in the table, * indicate p<0.05 and ** indicate p<0.01 after adjustment for pathology

4.6.10 Differences in gut bacterial metabolites between the groups with weight loss or weight gain

Weight loss over the period of follow-up was calculated as change in BMI SDS per month (Δ BMI SDS/month) for all the groups

Differences in gut bacterial metabolites between the groups with weight loss or weight gain

Changes in the concentration of total SCFA and major individual SCFA (acetate, propionate, and butyrate) were not significantly different between the groups with weight loss or weight gain.

Association of gut bacterial metabolites with weight loss or weight gain

To determine whether change in weight expressed as Δ BMI SDS/month is associated with change in gut bacterial metabolites (Δ) between two time points over the period of 2-3 months; regression analysis was done using Δ SCFA, Δ H_2S, Δ lactate, and Δ NH_3 as response or dependent variables and Δ BMI SDS/month as predictor or outcome variable . Change in none of the gut bacterial metabolite was significantly associated with change in weight (weight loss or weight gain) when adjusted for pathology. The only exception was the significant negative association of lactate: butyrate ratio with pathology-adjusted Δ BMI SDS/month in healthy group (simple obese and healthy lean) (R^2-adj=7.84%, β= -0.016, p=0.028).

To assess whether gut bacterial metabolites at follow-up (i.e. time point B) are determined by Δ BMI SDS/month; regression analysis was done for total and individual SCFA, H_2S, lactate, and NH_3 at follow-up. None of the metabolite, except heptanoic acid, had a tendency of positive association with change in BMI SDS/month when adjusted for pathology (R^2-adj=1.95%, β=4.05, p=0.09).

4.7 Discussion

Several animal and human studies (164, 177, 217) have looked at the relationship of gut microbial metabolites in relation to obesity. However, the results are controversial and there is no definite conclusion regarding the cause or effect relationship in the role of gut microbiota in obesity onset and pathogenesis. To test whether gut microbiota play a causative role in the aetiology of obesity by producing excess SCFAs, the current study compared the gut bacterial metabolic activity between children /young adults with known and unknown cause of obesity.

Our results suggest that apart from differences in faecal water, proportion of acetate and iso-butyric acid at recruitment, there was no significant difference in the gut bacterial metabolites (total SCFA, H_2S, ammonia, and D &L lactate) between the simple and hypothalamic obesity. No difference in metabolic activity between simple and hypothalamic obese groups suggests that metabolic activity of the gut microbiota is not different between obesity of different aetiology which contradicts the causative role of gut microbiota in the aetiology of obesity. Moreover, higher SCFA concentration in obese (simple & hypothalamic obese) than lean (healthy & hypothalamic lean) phenotype in our study indicate that previously observed differences in the concentration of SCFA are the result of differences in dietary intake. In our study the absence of difference between obesity of different aetiology indicates that simple and hypothalamic obese people might share the same dietary pattern distinct from lean people resulting in the increased metabolic potential of gut microbiota in obesity.

Published data suggest that availability and/or changes in the type and amount of substrate in the gut lumen determine the concentration and pattern of SCFA in obese individuals (166, 217). Furthermore, high fat diet has been shown to have a pronounced but similar effect on the gut microbiota in obese and non-obese groups of Sprague Dawley rats, supporting the role of dietary substrate on the gut microbiota and their metabolic potential (199). However, changes in gut microbiota due to different pattern of diets in obese individuals, irrespective of the cause of obesity, might then acquire the capacity to harvest energy from the available substrates in the gut lumen as suggested by Turnbaugh *et al.*(2006) from mouse models(159) and Ley *et al.*(2006) in human studies (164). However, this will not be implicated in the primary onset but possibly in the propagation of adiposity and increase in cardiovascular risk.

It can be argued that SCFA in faecal samples are the result of a net difference in production in the gut lumen and absorption in the colon and therefore might not be good indicators of energy harvesting capability of the gut microbiota. This question will be

addressed by *in-vitro* batch culture fermentation in the next chapter which suggested no difference in the energy harvesting capability of gut microbiota of the two obese groups (chapter 5). Whether differences in dietary patterns between obese and lean phenotype determine the SCFA production capability of the gut microbiota in our study, was explored but the interpretation of the results are limited by dietary underreporting on behalf of our participants and the validity of method of dietary assessment used (chapter 3).

In contrast to our expectation, median percentage faecal water was significantly higher in the hypothalamic obese than simple obese group at recruitment. Reduced gastric emptying and prolonged gastrointestinal transit time due to hormonal disturbances (particularly reduced PYY and persistently increased ghrelin) in Prader Willi syndrome has been linked to constipation (356, 357). Additionally, studies have reported gastric wall necrosis and rupture due to severely reduced gastric emptying in PWS patients (358). Although, a questionnaire-based study have reported soft (but not watery) stools in 49.2% patients compared to 7.9% hard stools in Prader-Willi syndrome (359),this evidence is subjective, as it was based on mailed questionnaires filled by the parents compared to an objective measurement of gastrointestinal transit time by Kuhlmann *et al.*(2014) (356). Despite this evidence, increased water content in hypothalamic obese patients in our study could be attributed to a shorter transit time (as opposed to the reported prolonged transit time) leading to a state of malabsorption in children with hypothalamic obesity, the mechanism for which is however unknown to us, but it may be associated with the muscular hypotonia observed in this population. Moreover, hypothalamic obese group might have higher food and fibre intake which might be holding more water in the gut lumen compared to other groups. This is however not suggested by the dietary data but it is impossible to rule out this possibility as under-reporting was large in our cohort. Lastly, this effect could be due to high inter-individual variations (as suggested by a wide IQR) within hypothalamic obese group and lack of statistical power and hence suggest more study participants to account for this variation.

In addition to the percentage water content, hypothalamic obese group also had a significantly higher proportion of acetate compared to simple obese group at recruitment. Furthermore, proportion of acetate had a tendency ($p=0.062$) towards positive association with percentage faecal water in hypothalamic obese group in general regression analysis suggesting that higher proportion of acetate was predicted by higher percentage of faecal water. With decrease in faecal water by 4% after 2-3 months, the proportion of acetate was no more significant. Moreover, increased proportion of acetate at recruitment could also be due to the preferential utilisation of lactate to generate butyrate by lactate-utilising

butyrate-producing bacteria such as *E. halii* and *Anaerostipes caccae*, thus sparing the utilization of acetate (292).

The proportion of BCFA, iso-butyric acid, was significantly higher (p=0.010) in the simple obese group compared to hypothalamic obese group at the time of recruitment. These results are in line with a crossectional study by Payne *et al.* (2012), who found a higher concentration of iso-butyric acid in simple obese Swiss children as compared to lean children (95). Although, these results may not be comparable with the two obese groups of our study, the results of this study at least in part indicate differences between obese and lean groups. However, their study did not give an indication of the relationship of BCFA in obese children with diet as dietary data was not recorded. The concentration and proportion of BCFA are generally increased when fermentable carbohydrate is limited in the colon and also from endogenous sources of proteins in the form of sloughed cells. In accordance with this, we found a significant negative association of proportional intake of carbohydrates with BCFA (isobutyric and iso-valeric acid) at the time of recruitment. This was further supported by the finding that the proportion of both iso-butyric and iso-valeric acids were significantly negatively correlated with BMI SDS in healthy as well as pathological group.

BCFAs, iso-butyric acid and iso-valeric acid, are produced as a result of protein fermentation particularly branched amino acids such as valine and leucine from exogenous protein sources of proteins (114) or from endogenous sloughed colonic epithelium (360). A study on faecal samples of 4 different species (humans, horses, rats, and pegs) has shown a consistently strong correlation between percentage of iso-butyric and iso-valeric acid in these species fed on different diets, irrespective of the amount of SCFA, age, diet, and living conditions (114). Although the proportional intake of proteins was significantly higher in simple obese vs. healthy lean group after 2-3 months, none of the BCFAs was significantly associated with the amount or proportion of protein intake in multivariate regression analysis. We therefore suggest the use of stronger and more reliable methods of dietary assessment such as multiple days weighed food diaries with food frequency questionnaires to elucidate difference in dietary intake of carbohydrates and proteins as our dietary assessment method (24 h food diaries) might have failed to show these differences.

Octanoic acid or caprylic acid, an eight carbon medium chain saturated fatty acid, is naturally found in the milk of mammals (such as goats) and in minor concentrations in coconut and palm oil. Caprylic acid has been shown to inhibit the activity of several pathogenic bacteria such as *E. coli* (O157: H7 strain) (361), *Salmonella enterica* (362), *Clostridium difficile*(363), *Listeria monocytogenes* (364), Furthermore, the active acylated form of ghrelin requires n-octanoic acid at serine residue on its chain and studies in fish

(barfin flounder) (365)and mouse models (366)have shown that ingested fatty acids (such as octanoic acid)increased the acylated form of ghrelin and hence are substrates for ghrelin acylation(365). Very little is known about the source and microbial producers of caprylic acid in the gut. Previous studies in this department reported lower faecal levels of caprylic acid in Crohn's disease patients compared to healthy children(126). Recent unpublished data from this department have shown that caprylic acid is produced by the degradation of medium chain triglycerides (MCTs) in a formula feed (Modulen®). No study to date has reported faecal caprylic acid in obese children and young adults. Negative correlation of the proportion of caprylic acid with BMI SDS in PWS patients might suggest a lower intake of MCTs and hence lower production of caprylic acid in this group. However, we have no dietary record of the intake of MCTs from our food diaries. MCTs are absorbed via passive diffusion from the gut into portal circulation and do not require bile salts for absorption unlike long-chain fatty acids. They are therefore used in malabsorption states as a source of energy. Whether negative correlation of caprylic acid with BMI SDS in PWS patients represents increasing malabsorption and faster transit time with increasing BMI, need to be investigated in future studies.

Propionate was consistently and significantly higher and significantly correlated with BMI SDS in the two obese groups

Our results showed consistently higher faecal propionate in simple and hypothalamic obese groups. Moreover, both the concentration and proportion of faecal propionate was significantly positively correlated with BMI SDS in the pathological group (hypothalamic lean and obese) but not in the healthy (healthy lean and simple obese) groups.

Obesity is generally associated with metabolic dysfunction. Furthermore, higher levels of propionate have been associated with hypocholesterolemia (96), reverse cholesterol transport, anti-lipogenesis, improved satiety through hormone PYY, decreased meal size by increased leptin (which induces suppression of food intake through receptors expressed in central nervous system) (97), and antiproliferative effect on colonic cancer cells (98). However, this paradoxical increase in "beneficial propionate" in our obese groups has also been reported by other studies (95, 172, 176, 350). Propionate is primarily produced by Bacteroidetes. Members of Bacteroidetes such as *B. thetaiotaomicron* have the ability to respond to the diverse environmental fluctuation by producing a range of glycosyl hydrolases that can redirect its energy harvesting capability to degrade mucin in the presence of a low dietary fibre intake (367). Upon exposure to diet containing simple sugars, caeca of gnotobiotic mice with *B. thetaiotaomicron* express genes that encode enzymes for degrading only the host glycans (367). This makes them better suited to the

situation of "increased energy but less fibre intake". It may therefore be more important to look into the substrate source of increased propionate in these groups rather than just the increased propionate itself. Population based studies in humans have reported a reduced intake of dietary fibre and higher amounts of the readily available sources of dietary carbohydrates in the diets of obese people (343). This might explain the abundance of Bacteroidetes and the consequently higher concentration of propionate reported in obese people. An apparent increase in the "beneficial" propionate might be an indication of a higher carbohydrate but less dietary fibre-rich food (172).

Moreover increased propionate in our obese groups might suggest increased propionate-induced hepatic gluconeogenesis which plays its part in causing insulin resistance (368). Insulin resistance is one of the hallmarks of diabetes, to which obese people are generally prone. However, a recent study have suggested that propionate exert beneficial metabolic actions by stimulating intestinal gluconeogenesis to which propionate is a substrate (348). Glucose from intestinal gluconeogenesis is sensed by the portal vein glucose sensors which transmits signals to the brain to promote beneficial effects on the food intake and energy metabolism (348).

SCFA especially propionate also act as a ligand for G protein coupled receptor GPR41 (also called FFAR3). These receptors are expressed in adipose tissue, pancreas, spleen, liver, enteroendocrine L cells, and mononuclear cells. These receptors have been shown to improve insulin sensitivity and secretion via stimulation of GLP-1 from L-cells (99). The results are however controversial as other studies have found a reduction in insulin sensitivity with activation of GPR41 which indicate other mechanisms involved which are poorly known (99). Propionate and acetate added to a culture medium containing adipocytes and pre-adipocytes reduced lipolysis by 50% and the same effect was seen in C57BL/6 mice fed with propionate and acetate preparations (100).This anti-lipolytic effect was abolished when GPR41deficient knock-out mice were used, implicating that propionate and acetate exert this effect through GPR41 (100).

Faecal ammonia is associated with dietary fibre intake, pH and age

We found significantly higher faecal ammonia in dry faecal sample of healthy lean group than the simple obese and hypothalamic lean groups at the time of recruitment. To our knowledge, we are reporting differences in faecal ammonia between lean and obese children for the first time as no study has thus far studied faecal ammonia in relation to obesity.

In the colon, faecal pH and carbohydrate availability are the major negative determinants of peptides and amino acid fermentation in the large intestine; carbohydrate availability being stronger factor than faecal pH (135). Our dietary data showed a lower

amount and percentage intake of dietary carbohydrates in healthy lean group as compared to the hypothalamic lean group which might indicate that host mucins and dead bacteria are used for the production of ammonia. However, this was not the case for simple obese group which may be due to a significant effect of under-reporting. The levels of faecal ammonia increased with a significant change in the percentage intake of proteins in simple obese group as reported in the data after 2-3 months; which conforms with another study in which increase in the intake of meat protein was associated with a significant increase in the production of SCFA and ammonia fed to human volunteers (73). Birkett *et al.*(1996) showed a reduction in the faecal ammonia and phenols with increase in the intake of resistant starch although there was no change in the urinary ammonia, phenol, urea, and total nitrogen (136). Russell *et al.*(2011) showed similar faecal ammonia concentration in obese human volunteers on high protein medium carbohydrate, high protein low carbohydrate, and weight maintenance diets for 8 weeks (137).However, faecal ammonia in our study was significantly positively associated with intake of dietary fibre and age at presentation and with pH after 2-3 months and not with protein intake.

Faecal sulphide were not significantly different between the two obese and lean groups

We found no significant difference in the faecal concentration of free, bound, and total sulphide between any of the four groups. To our knowledge this is the first study to report faecal hydrogen sulphide in children with simple and hypothalamic obesity. Although extensively studied in relation to inflammatory bowel disease (126); faecal hydrogen sulphide in obese human beings including children has not been studied. Similar levels of hydrogen sulphide in all our groups might suggest similar relative abundance of SRBs, similar gut luminal conditions in terms of transit time and pH, and similar capability of maintaining the redox potential in the gut lumen.

Whereas physiological concentrations of hydrogen sulphide has beneficial effects on gut health (124), a number of potentially adverse effects of excess hydrogen sulphide have been suggested (119) as discussed in chapter 1 section 1.2.2.6.

We found significant negative association of faecal sulphide with % faecal water content at recruitment and after 2-3 months irrespective of the study group in a multivariate regression analysis. Chassard *et al.*(2012) reported a significantly lower faecal sulphide, molecular hydrogen, and significantly higher methane in healthy lean women as compared to women with constipated irritable bowel syndrome (IBS) (128). This was coupled with significantly higher lactate utilising bacteria and methanogenic archaea, and significantly lower sulphate reducing bacteria in healthy lean women as compared to constipated IBS women. Although total SCFA, acetate and propionate were similar between the two

groups; levels of butyrate were significantly lower in the constipated IBS group signifying the fact that hydrogen sulphide inhibited oxidation of butyrate (128).

Interestingly, the change in faecal sulphide (Δ) in our study was determined by the proportion of fat and carbohydrate intake in grams (for bound sulphide) in addition to % water content (for total sulphide).This result should be considered with caution as our obese group might have underreported their diet but other studies have also found changes in faecal sulphide with changes in faecal consistency and dietary intervention (121, 129, 369). A recent study on gnotobiotic mouse model showed an increase in caecal sulphides, increase in a prominent SRB; *Desulfovibrio piger,* and a decrease in acetate and propionate on high fat and low complex polysaccharide diet. This was in addition to increased gene expression of sulfatase; a mucosal peptidoglycan degrading enzyme sulfatase produced by Bacteroides (121). De Preter *et al.*(2010) showed a significant reduction of sulphide in faecal samples incubated with oligofructose-inulin by *in vitro* fermentation (129). In another study, volunteers who had SRBs in their faeces, showed a reduction in faecal sulphide with oligofructose along with increase in the total SCFA although the population of SRBs did not change with intervention(130). However, this is controversial as some studies have shown higher sulphate reducing bacteria in African population consuming high fibre diet than in African-Americans consuming low fibre diet (131).

Systemic hydrogen sulphide is a gasotransmitter vasodilator and has several beneficial cardiovascular and anti-obesity effects (120, 124). Shen *et al.*(2013) have shown that gut microbiota play a key role in regulating the bioavailability of hydrogen sulphide in the systemic circulation (132). They found significantly lower tissue and intestinal free H_2S in germ free vs. conventionally housed animals. In contrast, a study by Flannigan *et al.* (2011) on Swiss Webster mice showed no difference in faecal H_2S between germ free and conventional mice (120). The difference may be attributed to different mouse models, experimental conditions and sample size between the two studies. Our study did not look into the systemic levels of hydrogen sulphide to compare the faecal sulphide with that in systemic circulation. It will be interesting to correlate gut luminal H_2S which is being considered as harmful metabolite with H_2S in systemic circulation with its aforementioned beneficial actions and whether gut microbiota or obesity plays any role in determining this balance. Furthermore, based on the available data, the source and mechanisms of faecal sulphide production is not known in our group of patients. We also cannot comment on the proportion of faecal sulphide contributed by the microbial production from the available luminal substrates (prokaryotic contribution) and that contributed by the host mucin peptidoglycans and peptides (eukaryotic contribution) (120).

Faecal Lactate

Overall; there was no significant difference in the concentration of faecal D, L and total lactate and D/L ratio between simple and hypothalamic obese groups. Generally, healthy lean and hypothalamic lean groups had a tendency towards higher faecal lactate as compared to the two obese groups. However, this was only significantly higher in hypothalamic lean than hypothalamic obese group in wet faecal samples at presentation but not in the freeze dried samples and at follow-up. Since lactate is a metabolic intermediate during the production of butyrate, propionate, and acetate; its presence in lower quantities in obese might indicate active utilisation of lactate in obese group. This in turn might have resulted in the relatively higher total and major individual SCFA in the two obese groups as compared to the lean groups. This finding also conforms with findings from other studies in children where higher faecal lactate was found in lean children as compared to obese children (95).Higher levels of lactate in our lean groups might indicate a higher lactate producing *Bifidobacterium* spp. and studies have shown a positive association of lactate producing *Bifidobacteria* with leanness and that a higher *Bifidobacteria* in early infancy may predict normal weight in later childhood (258). In contrast, lower levels of lactate in plasma has been associated with presence of increased *Bifidobacteria* in the gut (370).

Concentration of faecal bacterial metabolites may be explained by increased production *in vivo* and is not affected by the presence or absence of pathology.

Extensive multivariate regression analysis of faecal bacterial metabolites with dietary, anthropometric, and demographic predictors suggested that faecal SCFA and the changes in faecal SCFA over the period of study were strongly negatively associated with the change in pH of the faeces both at the time of recruitment and after 2-3 months. This is expected, as the production of SCFA in the gut lumen reduces the pH in the gut lumen and the pH rises as the rate of SCFA production decrease along the distal end of the gut. Colonic pH decreases to ~5.5 at the caecum and proximal part of the ascending colon due to SCFA production (70-140 mol/kg) and rises to ~6.5-6.8 in the descending colon and rectum where the production of SCFA is lower (~20-70 mmol/kg) compared to the proximal part. Interestingly this association remained unchanged even after adjustment for the presence of pathology which suggested that any possible differences in gut physiology due to Prader- Willi syndrome or craniopharyngioma may not be affecting the metabolic potential of the gut microbiota and that the availability of substrate might be a more important determinant of this association. However, none of the gut bacterial metabolites was significantly associated with the dietary macronutrients or energy intake. Branched

chain fatty acids were significantly associated with energy intake at follow-up only, but were no more significant when the analysis was adjusted for pathology.

Nevertheless, the significant association of propionate both with and without pathology was striking. This was discussed above. Additionally, presence of a disease seems to alter the molar ratios of butyrate with acetate, propionate, and lactate which might be driven by the utilization of more lactate than acetate to generate butyrate in our hypothalamic obese group.

Weight loss or weight gain (expressed as Δ BMI SDS/month) was not associated with changes in bacterial metabolites.

Differences in weight loss and weight gain between the groups were not significantly different and were discussed in chapter 3. We explored the association of weight change (Δ BMI SDS/month) with changes in gut bacterial metabolites and we found no significant differences in the concentration of faecal gut bacterial metabolites between obesity of different aetiology (simple vs. hypothalamic obese), between the two lean groups (healthy lean vs. hypothalamic lean), and between obese (simple and hypothalamic obese) and lean (healthy lean and hypothalamic lean) phenotypes. None of the predictors was associated with any of the metabolites at follow-up or with the change in metabolites between the two time points even after adjustment for pathology. Studies have reported changes in the gut microbial diversity and metabolic activity with changes in weight; weight loss being associated with an increase in Bacteroidetes, total SCFA, and major individual SCFA such as acetate (163, 164). However, weight change is also associated with change in dietary pattern which is also reported to alter the gut microbial communities, at least transiently, if not permanently (216). Furthermore, weight loss or weight gain is associated with changes in the endocrine and neuronal pathways of food intake (371, 372), which may also contribute to the change in metabolism of gut microbial metabolites. No significant differences in gut bacterial metabolites in our study might therefore suggest functional resilience of the gut microbiota diversity and metabolic activity in our participants which does not vary significantly with obesity or pathology, at least within a period of 2-3 months. It is however difficult to implicate whether differences in weight gain/loss over an extended period of follow-up would bring up differences in metabolism and diversity of gut microbiota in our cohort. This will need a long term close follow-up of these participants.

4.8 Conclusion

Data presented in this chapter indicate no major differences in the gut bacterial metabolic activity between simple and hypothalamic obesity suggesting that obesity and not the cause

of obesity determine differences in the metabolic potential and that other factors such as diet and lifestyle might be more important in the cause of obesity than gut microbiota.

In subsequent chapters this potential is further explored by in-vitro batch culture fermentation of gut microbiota to see whether fermentation capacity or energy harvesting capability of gut microbiota is different between simple and hypothalamic obese groups under similarly mimicked gastrointestinal conditions.

Chapter 5: *In Vitro* Fermentation Capacity of Gut Microbiota from Children with Simple and Hypothalamic Obesity

5.1 Chapter Outlines

This chapter investigated the energy harvesting capacity of gut microbiota between children with obesity of different aetiology through *in vitro* batch culture fermentation studies using a range of fermentable carbohydrates (apple pectin, raw-potato starch, wheat bran, raftilose, and maize starch). Furthermore, changes in fermentation capacity with dietary management in simple and hypothalamic obese groups were assessed.

5.2 Introduction

The on-going pandemic of obesity in adults and children is a major public health issue and an economic burden in both the developed and developing world due to its association with cardiovascular diseases, diabetes and hypertension (chapter 1, section 1.3)(19). Obesity is generally regarded as a state of positive energy balance resulting from the availability of excess energy in the body related to lower energy expenditure. This has been linked to low levels of physical activity(373), increased energy intake(374, 375), genetic predisposition(376), inflammation(377), and hormonal disturbances(378).

As discussed in the previous chapter, the gut microbiota may play a key role in deriving energy from the diet as they degrade fermentable carbohydrates into short chain fatty acids, the amount of which varies with the amount of dietary fibre intake. Chronic excess production of 200 extra kcal/day energy by this route would result in increased energy availability and hence increase in weight by 1kg in a year and 10 kg over 10 years (169). Compositional differences and changes in the gut microbiota with dietary intervention in obese animal and human studies suggested increased efficiency in energy harvesting capability of the "obese" gut microbiome. The obese microbiome is associated with lower bacterial richness and diversity, increased relative abundance of Firmicutes, and reduced Bacteroidetes (162). In study of 12 lean and 9 obese adults on diet containing either 2400 kcal/day or 3400 kcal/day, Jumpertz *et al.* (2011) reported that on the same diet, a 20% increase in Firmicutes was associated with faecal energy loss of approximately 150 Kcal collected over 3 days in lean individuals but not in obese (265). However, this evidence was limited by the fact that the calculations of the difference in faecal energy were based on the assumption that all the subjects had equal nutrient digestion and

absorption statuses. Furthermore, despite similar reported gut transit time, no significant differences were seen in the energy loss in faeces in obese subjects on weight maintenance, 2400 kcal/day, or 3400 kcal/day diets. The differences seen in the gut microbial population between obese and lean subjects may not be related to the results for faecal energy loss as the samples for estimating gut microbiota composition were spot faecal samples taken at two different times than those for the faecal energy studies which were 3 day stool collections. In contrast, studies in genetically obese ob/ob mice on low fat diet and wild-type mice on high fat Western diet showed a progressive increase in Firmicutes over 15 weeks in the obese but not in the lean mice. Additionally, changes in energy harvesting capability were not associated with changes in the gut microbiota (such as increase in relative abundance of Firmicutes) (220). Another study found similar gut microbiota in RELMβ knock-out mice (resistant to obesity) and wild-type mice on high fat diet independent of the genetic or lean/obese phenotype (224). Differences observed between these studies may have been due to differences in the source of samples (faecal vs. caecal sample), in experimental settings, methods of DNA extraction, use of different animal models, and different macronutrients and fibre proportion of the diets. A convincing conclusion is therefore still awaited to explain the association of gut microbiota with energy harvest.

In the previous chapter, we reported no significant differences in the faecal SCFA and other bacterial metabolic products between simple and hypothalamic obese groups and that the two obese groups had a tendency towards a higher total faecal SCFA than the two lean groups. In particular, the concentration and proportion of propionate was consistently significantly higher in hypothalamic obese than lean groups both at recruitment (concentration; $p=0.015$ & proportion; $p=0.004$) and after 2-3 months (concentration; $p=0.018$ & proportion; $p=0.004$) (chapter 4, section 4.6.1.4) and was significantly positively correlated with BMI SDS. Similarly, the proportion of propionate was significantly higher in simple obese vs. healthy group ($p=0.01$). However, the amount of faecal SCFA is the result of a difference in the production and absorption of SCFA as the material moves from proximal colon to the rectum. Whether increased SCFA in the faecal samples are due to increased production or is an effect of malabsorption is not known. Thus it is important to measure production rates to understand these results.

Several factors have been identified that affect the production of SCFA in the gut such as; the availability of substrate in the form of fermentable carbohydrates and gut transit time (379, 380). Sampling from the caecum of experimental animals gives a more accurate estimation of SCFA concentration in the lumen of the gut after dietary fibre intervention than that measured in faeces (381). However, this approach is not practical in

human studies, although measurement of intestinal SCFA have been reported in studies on sudden death victims (382, 383). Cummings and colleagues in their study analysed SCFA in jejunal, ileal, and colonic content and portal, hepatic, and peripheral femoral blood in victims of sudden death in 1987 (n=6, age range: 16-89 years, mean age: 57 years) (382). The authors observed a progressive rise in pH from 5.6 in the caecum to 6.7 in the descending colon with an inverse trend in the SCFA concentration falling from 131 ± 0.9 mmol/kg content in the caecum to 80 ± 17 mmol/kg colonic content in the descending colon. SCFA concentration rose by 10 fold between ileum and caecum (382). Molar ratios of acetate: propionate: butyrate was 57:22:21 in the whole colonic content, not varying between the segments (382). The results were important given that it was the first study to give an indication of the colonic concentration of SCFA in humans, however there were several confounders that could potentially limit the interpretation of these results. There was no history of diet or the gastrointestinal health or other conditions that could potentially affect fermentation capability. Additionally, the age range was very wide given the differences in the fermentation capability varying with age and the inter-individual variation in the given data was very high due to small numbers. Furthermore, the cause of their death might also have affected the levels of the SCFA in the gut as well as portal, hepatic, and peripheral blood due to sudden metabolic stress and shock (gunshot, road traffic accident) or due to chronic compromised blood supply (coronary heart disease). McFarlane and Gibson in their study on two sudden death victims reported results similar to Cummings *et al.*(383). In addition, these authors reported that the *in vitro* batch culture fermentation of the colonic content without any added substrate showed higher concentration of SCFA in the proximal colon than distal colon. In contrast, the production of branched chain fatty acids iso-butyrate and iso-valerate were significantly higher in distal than proximal colon. Furthermore, the authors found higher methane and methanogenic archaea in distal colonic content of one person while higher sulphide and sulphate reducing bacteria in the colonic content of the other, suggesting differences in the gut microbial composition and physiology with substrate availability and anatomy of the large intestine (383). This study was also limited by number of participants making the results less representative.

Further to this evidence, variation in fermentability of the dietary fibre (such as non-starch polysaccharides) may differentially affect faecal and caecal SCFA, in which case caecal SCFA might underestimate the true picture of colonic fermentation for some fibres. In a study by Edwards and Eastwood (1995), male Wister rats (n=60) were housed for four weeks on basal diet containing 45 g/kg non-starch polysaccharide followed by supplementation with 50g/kg either of guar gum, xanthan, tragacanth, karaya, gellen, or

ispaghula for further four weeks (384). Guar gum was shown to increase caecal SCFA due to rapid fermentation, while another group of NSP (tragacanth, xanthan, and karaya) increased faecal SCFA and faecal water but not caecal SCFA suggesting that the fermentability of dietary fibre is related to the site of SCFA accumulation (384). Campbell and colleagues conducted a study in 50 male Sprague-Dawley rats fed with control diet with or without cellulose, fructo-oligosaccharide, xylo-oligosaccharide, or raftilose for 13 days. After 13 days intervention, the authors did not find significant correlation of faecal SCFA with caecal SCFA concentration despite strong correlation of the faecal microbiota with caecal microbiota composition (346). This suggests rapid absorption of SCFA in the large bowel shortly after being produced and hence demonstrates the fact that fibres degraded in the proximal large bowel may not be comparable to those fermented partially while the faecal microbiota composition is still reproducibly comparable between caecum and faeces.

In-vivo fermentation studies, where the dietary fibre fermentation actually takes place, is the ideal approach to assess the energy harvesting capability of gut microbiota from dietary fibre. However, this is very difficult to achieve in humans. Bellier*et al.*(1995) measured *in-vivo* caecal fermentation capacity in 12 adult and post-weaning rabbits by caecal cannulation. They found significantly lower total SCFA, lower molar proportion of butyrate, and higher ammonia in the caecum of post-weaned rabbits but not in adult rabbits. Changes in the pattern of fermentation products were observed according to the circadian rhythm only in post weaned rabbits (385). James (1972) measured the digestion and absorption of orally ingested lactose and sucrose in 8 malnourished children by jejunal perfusion with a multi-lumen tube and found that apparently well absorbed sugars may not always be coupled with good tolerance of lactose (386). Florent *et al.*(1985) attempted to assess *in vivo* caecal and ileal lactic and volatile fatty acids (VFA) concentration by triple lumen tube intubation upon administration of 20 g twice daily lactulose for eight days in eight volunteers (387). They observed a reduction in caecal pH and increase in lactate, acetate, and total VFA after eight days intervention. Interestingly, no changes in faecal pH, lactate and VFA were observed with intervention suggesting a reliable and early detection of changes in metabolic activity of the caecal gut microbiota (387). Moreover, *in-vivo* ileal (388) and colonic perfusion studies (389) have been conducted to study availability of Bifidobacteria in the ileum or the absorption of salt and water with colonic SCFA perfusion respectively. Translation of these studies to routine and clinical studies involving humans is still a major issue to address when considering *in vivo* studies. Furthermore, the inaccessibility of the colon, limitations in reproducibility, inability to account for the complex interaction of food with enzymes and hormones in the gut, and the ethical issues

involved in *in vivo* studies make them difficult to achieve in humans. This makes the less powerful *in vitro* batch or continuous culture fermentation studies, conducted in controlled environment mimicking gastrointestinal tract, a more likely and convenient choice to study the fermentation capacity of gut microbiota. These methods have been shown to be reproducible, repeatable, and well controlled (273, 274).

With regard to obesity, there is very limited evidence from animal models studying the fermentation capacity of gut microbiota in relation to obesity. Recently, Condezo-Hoyos *et al.* (2014) conducted *in-vitro* batch culture fermentation of faecal samples of obese (n=3) and lean (n=3) mice incubated with apple pectin derived from a special variety of apple, Granny Smith. Obese mice were fed with high fat diet while lean mice were fed standard normal diet. After 12 h of incubation in batch cultures, relative abundance of *Bacteroidetes* significantly decreased in lean control cultures compared to obese control cultures. However, in obese cultures incubated with apple pectin as a substrate, relative abundance of *Bacteroidetes* also decreased significantly. SCFA analysis revealed that only acetate was significantly reduced in the cultures of obese mice incubated with apple pectin compared to obese control cultures (390). The conclusions from this study should however be interpreted with caution as the authors used a different diet regimen for each group for un-explained reasons (high fat diet for obese and standard diet for lean), used a composite of faecal samples from all members of the same group (which might have masked the pattern of SCFA and bacterial diversity from individual mice), and was also limited by few numbers (n=3).

In humans, several studies have investigated the effect of dietary fibre interventions on gut bacterial metabolic parameters in obese (391, 392) and lean humans (393, 394). However, only three human studies have investigated the differences in metabolic potential of gut microbiota by *in-vitro* fermentation in simple obese and healthy lean people (95, 172, 395).

Payne *et al.* (2012) in their *in-vitro* continuous culture fermentation study involving one lean and one obese child showed a similar "butyrogenic effect" of high and normal energy nutrient load with no effect on propionate. This was coupled with increased butyrogenic bacteria from phylum Firmicutes (*Roseburia/E.rectale* in the obese child and *E.halii* in the lean child under high energy Western diet and the reverse was found on normal energy load) (95). The results of this study are interesting but less convincing as there was only one lean and one obese child in the study and the differences observed may be a subject of inter-individual variation and not attributable to the obesity phenotype. Yang *et al.* (2013b) showed an increase in the molar propionate to total SCFA ratio and a lower proportional acetate in faeces from obese than lean adults through *in-vitro* batch

culture fermentation, and a significant positive correlation of propionate with BMI (kg/m^2) of participants (172). This study was limited because the authors used faecal samples that were stored for more than 24 h and then processed which may have affected the balance of aerobic and anaerobic gut bacterial population. Sarbini *et al.*(2013) investigated the effect of α-gluco-oligosaccharides (GOS) using *in-vitro* batch cultures and reported no significant difference in the production of SCFA between lean and obese adults (395). Although the production rates of acetate and propionate were observed to be similar in both lean and obese adults, the ratio of acetate to propionate was significantly lower in the obese than the lean subjects. Changes in gut microbiota with GOS and inulin were similar in lean and obese. Levels of *Bifidobacteria* increased with GOS compared to control (with no GOS), *Bacteroides/Prevotella* increased with both GOS and inulin, while *Faecalibacterium prausnitzii* decreased with both GOS and inulin (395). Although this study had pH controlled *in-vitro* conditions, the numbers were again very small (n=4) and are limited to provide conclusive evidence for differences in fermentation capacity between lean and obese phenotypes.

In the presence of limited and inconclusive evidence, it remains to be determined whether the obese phenotype differs from the lean phenotype in terms of fermentation capacity. Furthermore, is any difference in fermentation capacity between lean and obese phenotype determined by the gut microbiota populations. In an attempt to rule out this reverse causality, we conducted *in-vitro* batch culture fermentation studies on fresh faecal samples from a larger group of children and young adults with obesity of different aetiology and healthy lean children to give a better indication of the fermentation capacity of gut microbiota (i.e. production and rate of production of SCFA). Our main aim was to investigate the cause or effect relationship of gut microbiota with obesity, for which reason we conducted this study to assess whether the fermentation capacity of gut microbiota was any different between obesity of different aetiology.

We used a range of different commonly consumed dietary fibres as substrates to assess if the response of gut microbiota to individual fibres was "general" (i.e. fermenting all fibres uniformly; as a proxy for a general increase in the capacity) or whether any differences in microbiota were "substrate-selective" (i.e. more specific based on the predominant components of the diet of the participants). Published studies have shown a selective pattern of both gut microbiota and SCFA production by different dietary substrates used *in-vitro* (Table 5.1).

Table 5.1: Predominant SCFA produced by fermentation of selected fibres for this study and the predominant gut microbiota involved in their production.

Fibre	Predominant SCFA produced	Predominant Bacteria affected	Reference
Apple Pectin	Butyrate	*Bifidobacteria, Lactobacilli, Bacteroides fragilis, Enterococci*	(396, 397)
Raw Potato Starch	Propionate, acetate	*Bifidobacteria, Lactobacilli, Enterobacteria, Streptococci*	(398)
Raftilose	Acetate	*Bifidobacteria, Lactobacilli*	(392, 399)
Wheat bran	Butyrate	*Bifidobacteria, lactobacilli, Eubacteria*	(394)
Maize starch	Butyrate, propionate	*Ruminococcus bromii* and *Eubacterium rectale*	(400, 401)

In our study; raftilose & raw potato starch produced the most acetate, wheat bran & raw potato starch produced the most propionate, and raw potato starch & maize starch produced the most butyrate

The fermentability of dietary fibre by the gut microbiota depends on its water-solubility, chemical structure, particle size, lignification, and other ingested food components (402). The pattern of short chain fatty acids production depends on the type of fibre and gut microbiota residing in the colon (403). Excess SCFA particularly acetate is regarded as more obesogenic, while butyrate and propionate are regarded beneficial for colonic and general health (98, 404). Choosing a range of dietary fibres with a predominant pattern of SCFA production is therefore advisable to elucidate the differences in pattern of SCFA between lean and obese people.

To the best of our knowledge, this is the first study looking at the differences in the fermentation capacity of gut microbiota between obese children/young adults of different aetiology (hypothalamic obesity vs. simple obesity) and between the obese and lean phenotypes through *in vitro* batch culture fermentation studies.

5.3 Subjects and Methods

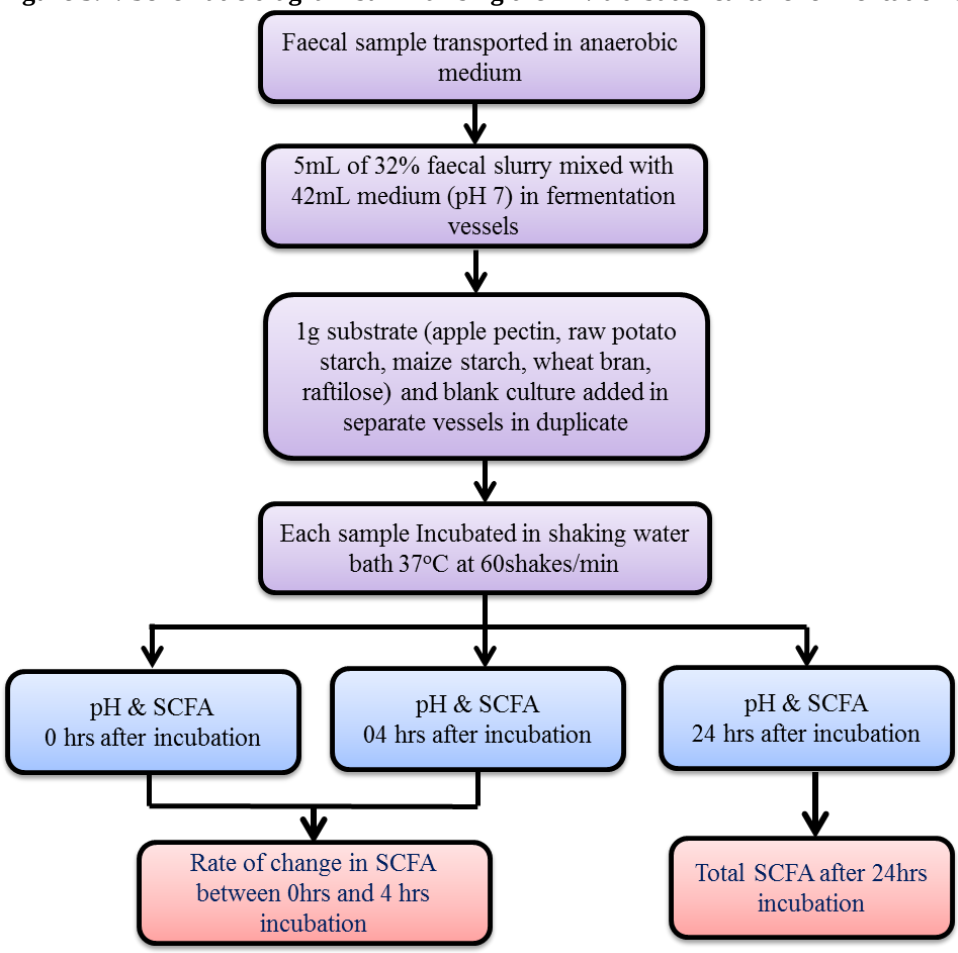
5.3.1 Patients and methods

Detailed information about the subjects and their recruitment is given in chapter 2. Briefly, each participant giving written informed consent was asked to give two faecal samples at an interval of 2-3 months with dietary and body composition data. Faecal samples were collected and transported in an anaerobic environment to the laboratory within 4 h after being produced. Each sample was processed for *in vitro* batch culture fermentation (Figure 5.1). Detailed explanation of the *in-vitro* batch culture fermentation is given in chapter 2, section 2.3.5. Fermentation supernatant obtained from the incubated sample at 0 h, 4 h, and 24 h of incubation was used to measure pH (using a benchtop pH meter) and short chain

fatty acids (using gas chromatography (chapter 2, section 2.3.6). Approximately, 4,500 samples were analysed for SCFAs over a period of 8 months.

Note: Obtaining two faecal samples, body composition measurements, and the comparisons done between the two time points were primarily aimed at the assessment of changes in fermentation capacity with weight management. Changes in anthropometric and body composition measurement are explored in detail in chapter 3.

Figure 5.1: Schematic diagram summarising the *in-vitro* batch culture fermentation studies.



5.3.2 Substrates

Pectin derived from apple source (cat. No.76282) and raw potato starch (containing 10% resistant starch) (Cat no. S2004) were both obtained from Sigma Aldrich. Raftilose (Orafti P95®), a short chain inulin fructo-oligosaccharide [degree of polymerization (DP) = 3-5] extracted from chicory root (*cichoriumintybus*) was kindly provided by BeneoOrafti (Tienen Belgium). This oligofructose has 95% raftilose and 5% other sugars such as glucose, lactose, and sucrose. Un-treated non-pre-digested wheat bran was obtained from

Infinity foods co. UK. Maize starch Hi-Maize® TM 260 (Cat. no. KK10283) was kindly provided by National Food Innovation Manchester. HI MAIZE® has high amylose content from corn and is a type 2 resistant starch containing 60% resistant starch and 40% slowly digestible starch.

5.4 Data sorting

Sorting and analysing of the data obtained from 24 h *in-vitro* batch culture fermentation was a complex task. Participants were in four groups; each participant had two faecal samples incubated for 24 h, using 5 substrates and a blank (with faecal inoculum but no substrate), and each substrate incubated in duplicate. Furthermore, samples in duplicate were taken from the incubated fermentation vessels for SCFA analysis at three time points (0h, 4h, and 24 h). Total of 151 samples were collected from all the recruited participants and their parents whenever they agreed to take part (n=30). This generated about 4500 faecal samples for SCFA extraction and analysis with GC-FID.

All data in the form of area under the curve obtained from GC-FID software was first converted into molar concentrations using a formula (explained in chapter 2, section 2.3.6). Each sample analysis was transferred to a single sheet and then screened for mismatch (high %CV) between the duplicates. Any erroneous and abnormal samples were scrutinised and the samples re-extracted. The duplicates were averaged, data was first organised in a single sheet containing all participants, with all dietary substrates and the blank, all time points taken at 0h, 4h and 24h incubation, and both samples taken at interval of 2-3 months. This sheet was used to generate descriptive statistics in Minitab® 16. All data could not be analysed in a single worksheet therefore this main sheet was further split into sub-sheets of time points (0 h, 4 h, and 24 h) for each sample (at recruitment and after 2-3 months). Sheet with 24 h data was used for statistics related to fermentation end point (24 h) while sheets for 0h and 4 h were combined to calculate the rate of SCFA production between 0-4 h incubation. Anthropometric data was incorporated into each sheet to correlate SCFA production or the rate of SCFA production with BMI SDS. Each sheet was also sub-divided based on the dietary substrate to find differences between the groups based on dietary substrates. Within each sheet and sub-sheet, data had to be unstacked several times for the purpose of analysis.

5.5 Statistics

Non-parametric statistics were used for analysis due to the non-normal distribution of the data based on Anderson-Darling test of normality. Median and interquartile range were

used for calculations and statistics. Mann-Whitney U test was used to compare two groups for different variables. P-values of less than 0.05 were considered significant. Due to the difference in the nature of the groups, comparisons were done using Mann-Whitney U test between hypothalamic obese vs. simple obese, hypothalamic obese vs. hypothalamic lean, healthy lean vs. simple obese, and healthy lean vs. hypothalamic lean. No adjustment for multiple testing or false discovery rates was done for these tests. All significant p-values should therefore be considered as nominally significant.

Change in total and individual SCFA (Δ) between samples at recruitment and samples after 2-3 months was measured as a difference of measurement at follow-up minus measurement at presentation. Spearman rank correlations were used to find correlations between BMI SDS and SCFA production on ranked data. General regression analysis was used to find associations between total SCFA and time elapsed in processing the samples for *in-vitro* fermentation.

5.6 Results

5.6.1 Differences in pH of incubated faecal samples after 24 h at recruitment and after 2-3 months

At the time of recruitment, there was no significant difference in culture pH after 24 h incubation between those seeded with faeces of children with obesity of different aetiology, between the two lean groups, and between lean and obese groups for any of the substrate and the blank (Table 5.2, Figure 5.2).

However, after 2-3 months follow up, the cultures from the simple obese group tended to have a lower pH than all other groups but this was significantly lower only compared with the hypothalamic obese (p=0.04) and healthy lean groups (p=0.02) in cultures containing raw potato starch (Table 5.2, Figure 5.3).

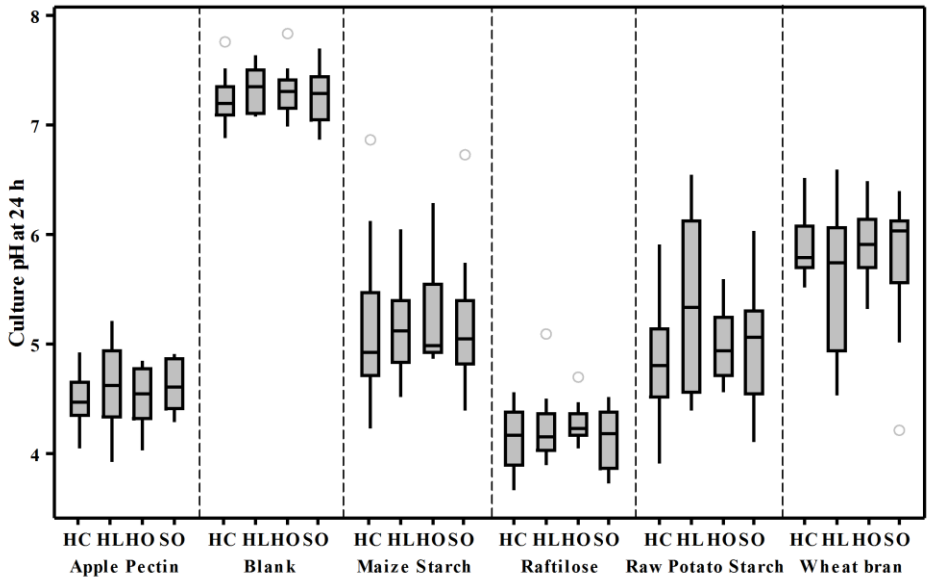
Table 5.2: pH of the faecal samples after 24 h incubation period at the time of recruitment and after 2-3 months.

Substrate	**Healthy Lean**		**Hypoth. lean**		**Hypoth. Obese**		**Simple Obese**	
	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Sample A	n=27		n=12		n=10		n=15	
Apple Pectin	4.47	0.32	4.68	0.56	4.54	0.49	4.61	0.45
Blank	7.20	0.25	7.36	0.34	7.26	0.29	7.30	0.39
Maize Starch	4.93	0.75	5.17	0.55	4.98	0.72	5.06	0.59
Raftilose	4.17	0.48	4.18	0.31	4.28	0.24	4.18	0.51
Raw Potato Starch	4.81	0.61	5.23	1.47	4.93	0.63	5.06	0.75
Wheat bran	5.79	0.38	5.81	0.94	5.91	0.52	6.04	0.56

Sample B		n=24		n=10		n=9		n=13
Apple Pectin	4.50	0.47	4.43	0.40	4.56	0.93	4.35	0.72
Blank	7.11	0.35	7.12	0.41	7.37	0.26	7.15	0.49
Maize Starch	4.98	0.31	4.93	0.50	4.97	0.73	4.83	0.47
Raftilose	**4.02¥**	0.23	**4.21¥**	0.22	4.27	0.61	4.02	0.78
Raw Potato Starch	**4.76***	0.51	5.10	0.53	**4.91‡**	1.28	**4.54*‡**	0.30
Wheat bran	5.65	0.34	5.71	0.70	5.95	0.58	5.59	0.56

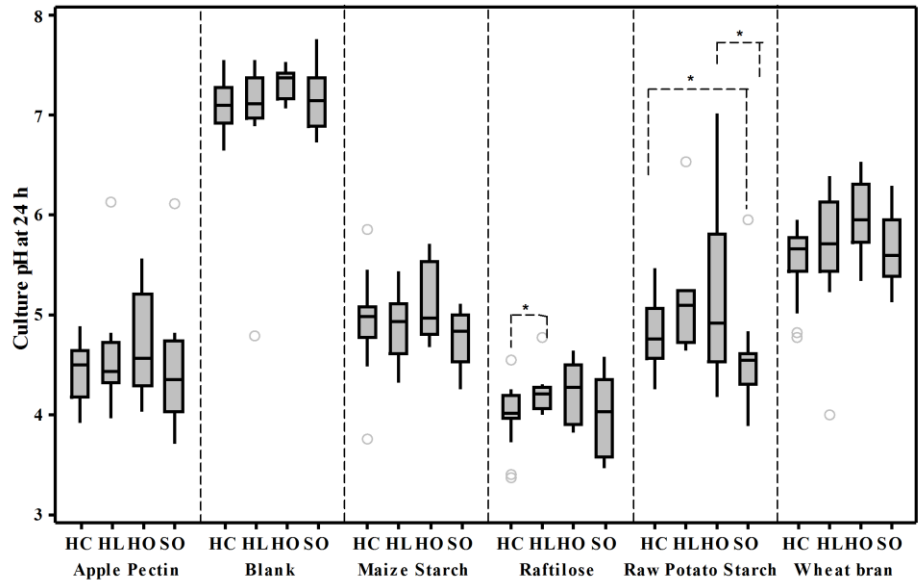
¥ indicate significant differences between hypothalamic lean and healthy lean, ‡ indicate significant differences between hypothalamic obese and simple obese, * indicate significant differences between healthy lean and simple obese

Figure 5.2: Culture pH of the 24 h incubated sample at the time of recruitment



Blank circles represent outliers. HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; simple obese.

Figure 5.3: Culture pH of the 24 h incubated sample after 2-3 months of recruitment



Blank circles represent outliers. HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; simple obese. * indicate $p<0.05$ on Mann Whitney U test

5.6.2 Differences in the production of total and major SCFA after 24 h incubation

Differences in total SCFA at recruitment and after 2-3 months

There was no significant difference in the concentration of total SCFA between any of the groups at the time of recruitment (Table 5.3, Figure 5.4&Figure 5.6) and after 2-3 months (Table 5.4, Figure 5.5&Figure 5.7), although the two obese groups (hypothalamic and simple obese) had a tendency to produce higher SCFA than the two lean groups.

Differences in individual SCFA at recruitment

With regard to individual SCFA, no significant differences were observed between simple and hypothalamic obese groups except the concentration of acetate which was significantly higher in simple obese than hypothalamic obese participants in cultures containing maize starch [median (IQR) µmol/ml: simple obese; 51.09(20.40) vs. Hypoth. obese; 40.06(21.60), p=0.04].

Although, tendencies towards higher individual SCFA concentration and proportions (acetate, propionate, and butyrate) were observed in the two obese groups than the two lean groups, only few reached statistical significance due to large variation in the data particularly in the obese. Only, the proportion of acetate was significantly higher in hypothalamic obese than hypothalamic lean group in cultures containing wheat bran [median (IQR) %; hypoth. obese; 65.14 (16.10) vs. hypoth. Lean; 57.89 (8.41), $p<0.05$] (Table 5.3). Additionally, proportion of butyrate was marginally significantly higher in hypothalamic obese than hypothalamic lean group only for cultures containing wheat bran ($p=0.052$, Table 5.3).

Table 5.3: Concentration and proportion of total and major individual SCFA after 24 h incubation of faecal samples with substrates and the blank culture at the time of recruitment.

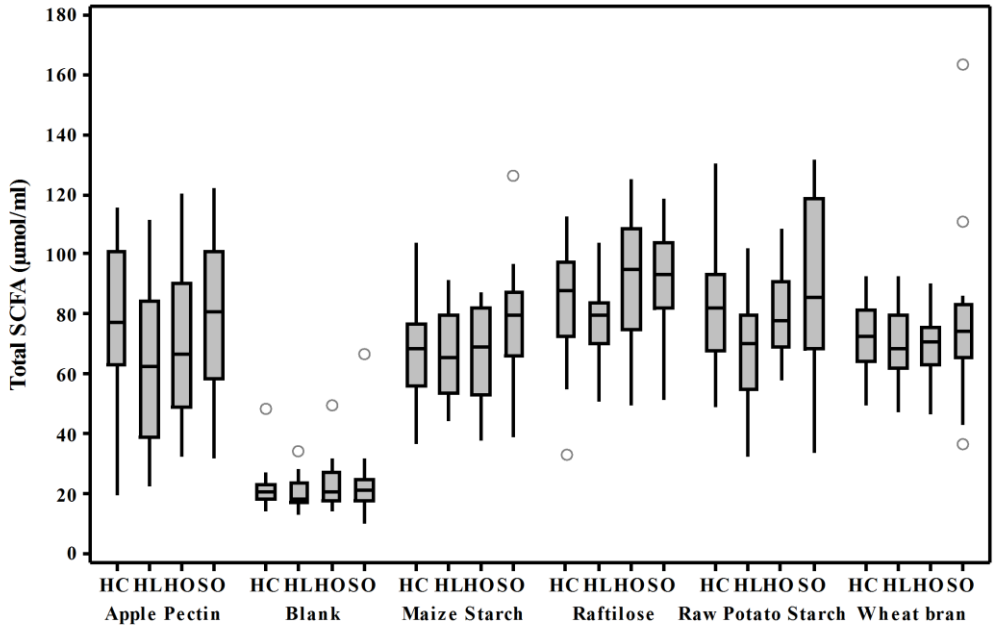
	Healthy Lean (n=27)		**Hypoth. Lean (n=12)**		**Hypoth. Obese (n=10)**		**Simple Obese (n=15)**	
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Blank								
Total SCFA	20.57	4.49	18.03	6.66	20.45	9.39	21.21	7.04
C2	11.83	2.66	11.39	3.39	11.46	2.53	11.81	4.68
C3	3.11	1.03	2.32	1.08	3.39	2.66	3.56	1.86
C4	2.24	1.08	2.07	0.73	2.77	3.95	1.84	0.83
C2%	56.12	9.21	61.27	7.20	52.72	16.57	58.01	11.32
C3%	14.97	6.12	13.11	2.37	15.30	7.44	15.39	4.92
C4%	12.00	3.85	9.73	2.94	11.81	12.69	9.21	4.62
Apple Pectin								
Total SCFA	77.55	38.05	62.76	45.62	66.89	41.41	80.83	42.88
C2	54.68	24.99	45.54	28.14	49.11	20.22	50.90	14.21
C3	7.71	4.62	6.62	8.44	7.66	9.72	8.44	7.13
C4	14.30	14.47	10.36	10.07	9.03	11.30	8.95	17.61
C2%	71.91	7.59	74.76	12.77	73.42	14.04	75.63	14.89
C3%	9.31	5.61	8.70	9.32	12.08	10.99	11.77	8.63
C4%	14.66	12.03	15.66	11.07	12.24	11.93	10.32	14.16
Maize Starch								
Total SCFA	68.38	20.59	65.63	26.03	69.22	29.00	79.55	21.12
C2	42.32	17.84	43.29	18.08	**40.06‡**	21.60	**51.09‡**	20.40
C3	6.61	5.91	6.17	6.31	7.51	13.88	6.70	6.18
C4	8.29	17.48	8.53	11.02	9.66	17.33	6.39	17.75
C2%	68.40	13.93	68.69	20.78	57.98	27.77	68.00	20.26
C3%	9.92	7.50	11.24	10.48	10.06	13.81	9.99	9.35
C4%	11.91	14.04	13.49	23.55	13.96	21.42	14.33	17.22
Raftilose								
Total SCFA	87.8	25.3	79.6	14.0	95.3	33.4	93.4	21.5
C2	69.9	25.5	69.3	24.1	74.6	21.2	82.4	25.4
C3	5.9	7.2	2.1	2.3	10.0	12.8	5.9	9.2
C4	4.2	7.1	3.9	10.9	8.6	14.5	2.0	3.5
C2%	87.3	18.9	89.5	13.4	74.7	26.4	88.8	22.1
C3%	6.4	7.2	2.7	2.6	10.0	14.6	6.1	10.8
C4%	4.7	7.6	5.8	13.5	8.7	11.4	2.1	5.0

Table 5.3 continued

	Healthy Lean (n=27)		**Hypoth. Lean (n=12)**		**Hypoth. Obese (n=10)**		**Simple Obese (n=15)**	
	Median	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Raw Potato Starch								
Total SCFA	82.22	25.73	70.45	24.92	77.71	21.75	85.44	50.39
C2	51.59	20.92	46.00	24.16	52.41	18.75	61.85	36.94
C3	7.89	4.85	6.18	4.27	9.20	4.89	8.72	6.30
C4	10.01	15.79	8.12	13.11	16.28	8.93	14.09	14.66
C2%	67.85	16.14	66.83	18.75	61.30	10.71	68.87	14.85
C3%	9.82	5.49	10.10	6.74	11.06	8.43	10.38	6.35
C4%	13.62	13.92	12.45	16.33	20.21	11.57	16.83	16.64
Wheat bran								
Total SCFA	72.62	17.53	68.42	18.02	70.53	12.66	74.12	17.74
C2	45.12	10.51	46.82	16.28	39.14	11.21	47.31	13.62
C3	9.87	4.01	9.24	5.62	10.78	7.99	10.86	6.45
C4	11.74	8.08	9.61	6.08	15.15	11.02	9.41	6.38
C2%	65.00	5.79	**65.14†**	16.10	**57.89†**	7.41	67.11	8.57
C3%	13.03	5.13	12.35	8.82	15.20	7.93	14.66	6.38
C4%	14.99	11.15	**12.68†**	8.84	**18.56†**	13.55	13.14	4.61

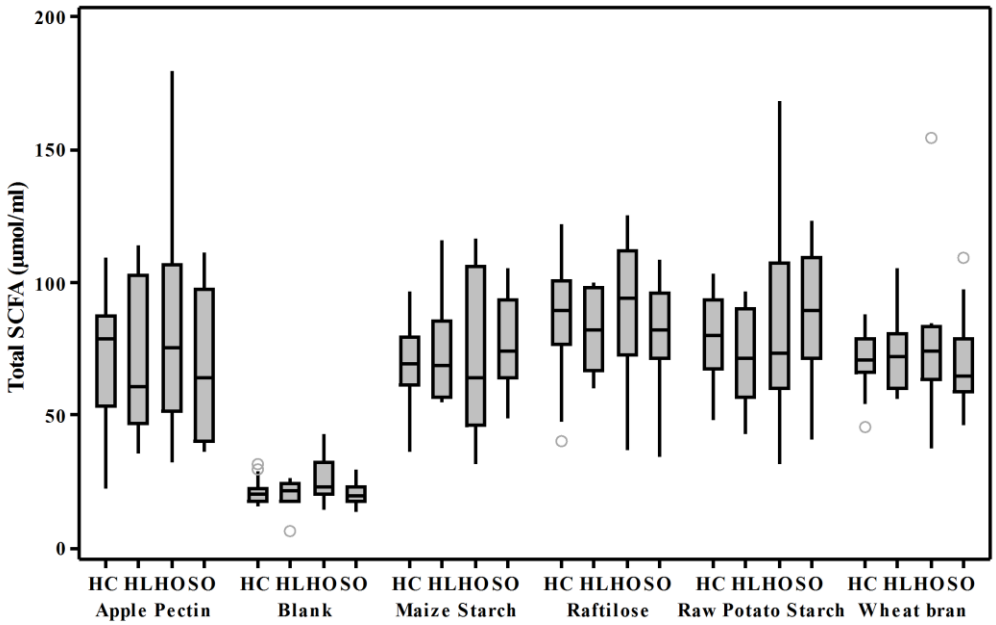
C2; acetate, C3; propionate, C4; butyrate, concentration are expressed as µmol/ml while proportions are expressed as %, † indicate significant differences between hypoth. lean and hypoth. obese, ‡ indicate significant differences between hypothalamic obese and simple obese

Figure 5.4: Concentration of total SCFA at the time of recruitment in all the groups after 24 h incubations.



Blank circles represent outliers. HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; simple obese.

Figure 5.5: Concentration of total SCFA after 2-3 months in all the groups after 24 h incubations.



Blank circles represent outliers. HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; simple obese

Figure 5.6: Pattern of change in SCFA concentration between 0 and 24 h incubations in all dietary substrates at the time of recruitment

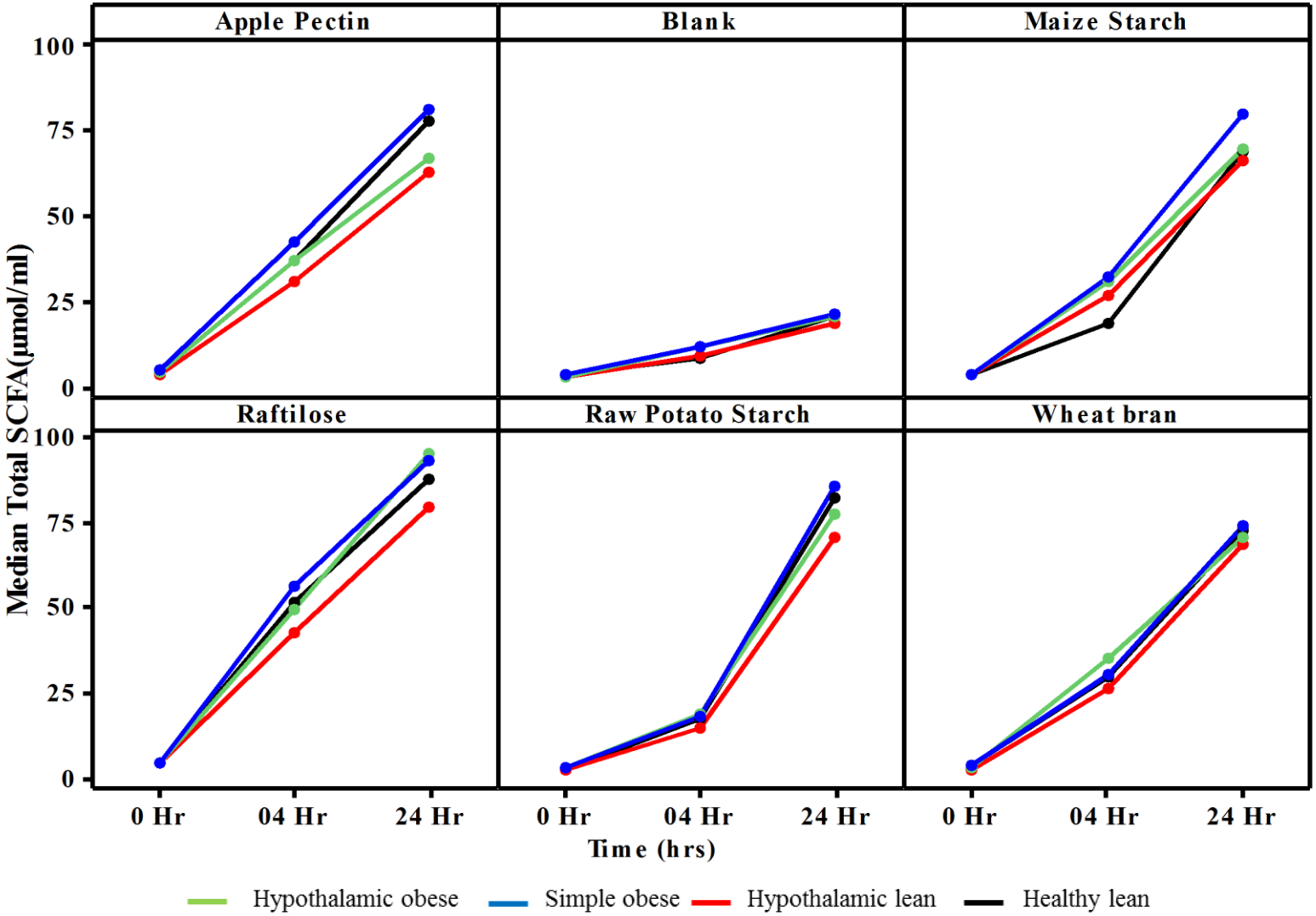
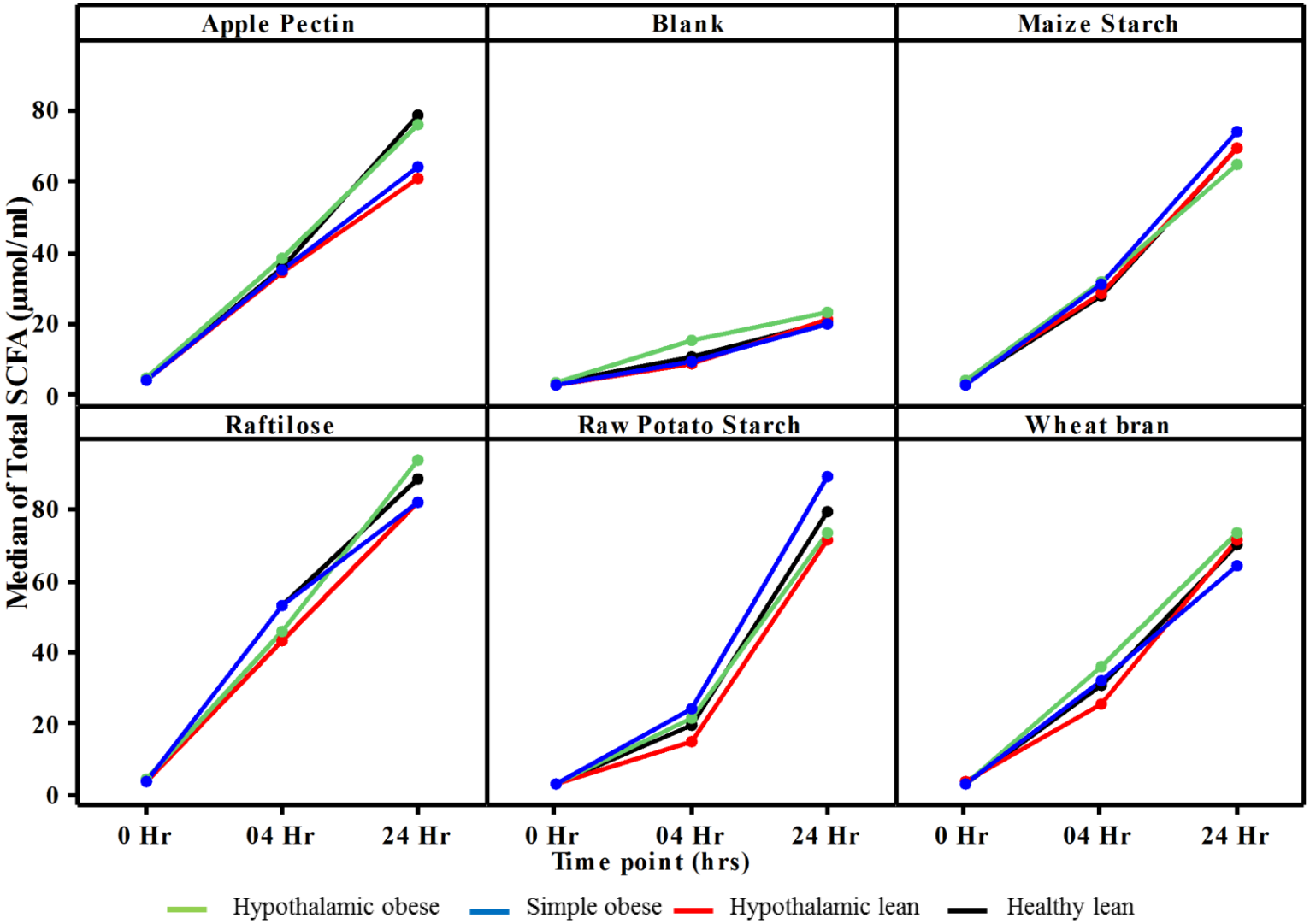


Figure 5.7: Pattern of change in SCFA concentration between 0 and 24 h incubations in all dietary substrates after 2-3 months



Differences in the concentration and proportion of individual SCFA after 2-3 months

No peculiar pattern of higher or lower concentration and proportion of acetate and butyrate were seen in the any of the groups and in any substrate. However, consistent with samples at recruitment, tendencies towards higher concentration and proportion of propionate was observed in obese (simple and hypothalamic) than lean groups (hypoth. lean and healthy lean). Only the proportion of propionate was significantly higher in simple obese vs. healthy lean in apple pectin (median (IQR) C3%: simple obese; 12.02(12.96) vs. healthy lean: 8.56(3.06), p=0.02) (Table 5.4, Figure 5.8). Overall, there was a large variation in the simple and hypothalamic obese groups than hypothalamic lean and healthy lean groups in all substrates.

Table 5.4: Concentration and proportion of total and major individual SCFA after 24 h incubation of faecal samples with different fibres and the blank after 2-3 months.

	Healthy Lean (n=24)		**Hypoth. Lean (n=10)**		**Hypoth. Obese (n=9)**		**Simple Obese (n=13)**	
Variable	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Blank								
Total SCFA	20.31	4.62	21.24	6.93	22.90	11.47	19.35	5.45
C2	10.59	2.59	11.78	3.09	13.05	3.53	11.09	4.32
C3	3.12	0.96	2.56	1.49	3.51	3.70	3.02	1.37
C4	2.30	0.88	2.19	0.88	2.42	5.09	1.97	0.63
C2%	54.09	6.43	61.96	12.20	56.40	21.79	57.31	10.00
C3%	15.11	3.18	12.52	4.90	15.40	7.36	15.87	6.10
C4%	11.94	3.29	10.51	1.71	10.83	7.57	10.18	3.00
Apple Pectin								
Total SCFA	78.39	33.93	60.64	55.91	75.40	55.20	63.73	57.10
C2	52.75	21.42	42.79	42.64	49.55	42.67	42.07	28.43
C3	6.45	4.37	8.06	9.16	8.19	12.98	7.51	13.47
C4	13.83	14.15	12.33	9.31	12.60	9.37	9.46	13.04
C2%	71.35	8.41	70.57	9.16	67.64	16.90	70.92	23.87
C3%	**8.56***	3.06	7.26	11.90	13.93	14.17	**12.02***	12.96
C4%	18.04	9.84	18.16	12.07	17.20	5.04	11.23	22.24

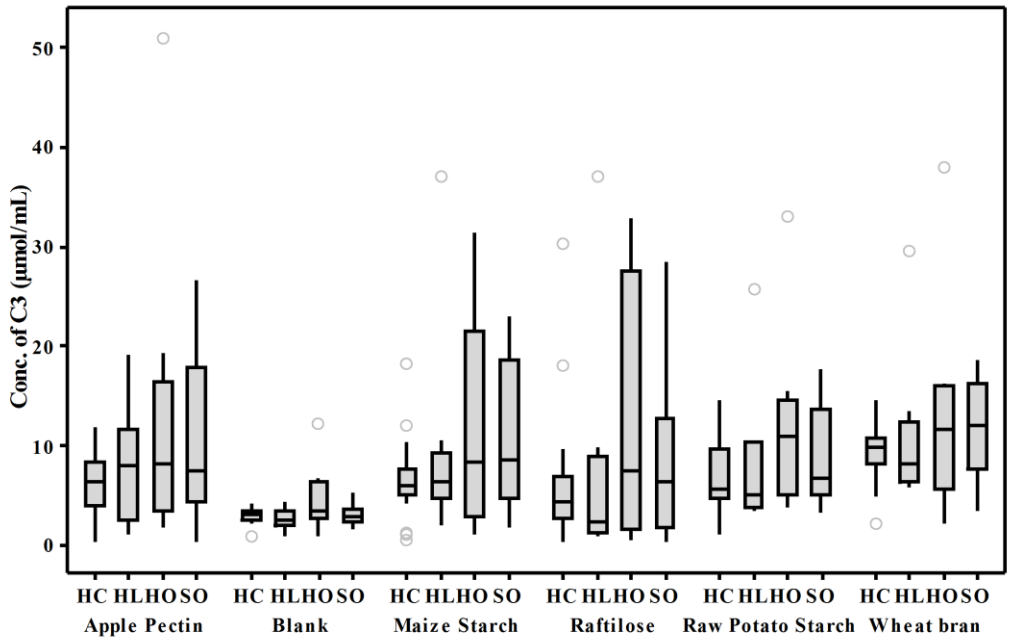
Table 5.4 continued

	Healthy Lean (n=24)		Hypoth. Lean (n=10)		Hypoth. Obese (n=9)		Simple Obese (n=13)	
Variable	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
Maize Starch								
Total SCFA	69.35	18.03	68.88	28.74	64.20	59.30	73.76	28.82
C2	41.60	15.27	39.26	24.34	36.11	34.24	47.67	28.94
C3	6.09	2.54	6.36	4.65	8.50	18.62	8.58	14.00
C4	15.62	7.81	14.02	11.26	14.43	14.52	13.10	10.80
C2%	61.38	9.92	62.71	19.69	65.99	31.78	64.08	18.46
C3%	9.13	3.23	9.38	6.76	9.38	25.44	12.43	11.75
C4%	21.89	10.94	24.29	21.12	15.68	10.49	19.48	17.18
Raftilose								
Total SCFA	89.09	23.75	81.97	31.67	94.19	39.31	82.06	24.99
C2	76.73	21.93	53.11	35.97	73.50	47.08	70.65	35.50
C3	4.41	4.13	2.48	7.60	7.60	25.91	6.39	11.01
C4	4.25	5.02	8.64	19.45	6.61	9.91	2.57	4.52
C2%	88.29	8.43	82.69	35.36	84.66	27.22	87.26	14.32
C3%	6.06	4.75	3.86	7.49	6.54	24.64	9.14	11.75
C4%	4.99	6.42	13.45	24.74	9.09	9.65	2.91	5.28
Raw Potato Starch								
Total SCFA	79.98	25.89	71.48	32.83	73.40	47.10	89.36	37.72
C2	47.22	18.69	43.78	19.85	49.02	41.62	58.18	24.35
C3	5.75	5.00	5.09	6.54	11.05	9.55	6.88	8.45
C4	17.06	9.35	10.57	10.72	15.34	12.26	11.99	14.17
C2%	65.67	11.36	64.32	13.61	64.92	16.18	72.22	15.85
C3%	9.07	4.97	8.59	7.08	14.92	10.71	10.93	8.70
C4%	22.29	10.30	17.76	16.94	17.97	7.77	17.64	12.96
Wheat bran								
Total SCFA	70.49	12.72	71.87	20.54	73.70	19.90	64.61	19.79
C2	43.57	9.31	40.34	14.68	44.42	19.29	41.90	13.02
C3	9.97	2.57	8.21	6.00	11.65	10.37	12.14	8.61
C4	13.81	5.67	11.22	4.97	13.04	7.16	9.80	3.60
C2%	61.22	7.32	61.79	10.42	60.24	15.55	59.90	14.32
C3%	12.93	3.13	11.21	5.56	14.15	15.07	17.06	7.87
C4%	18.24	6.31	17.80	10.50	17.18	7.42	15.64	9.16

C2; acetate, C3; propionate, C4; butyrate, concentrations are expressed as μmol/ml. Proportions are expressed as %, * indicate significant differences between healthy lean and simple obese

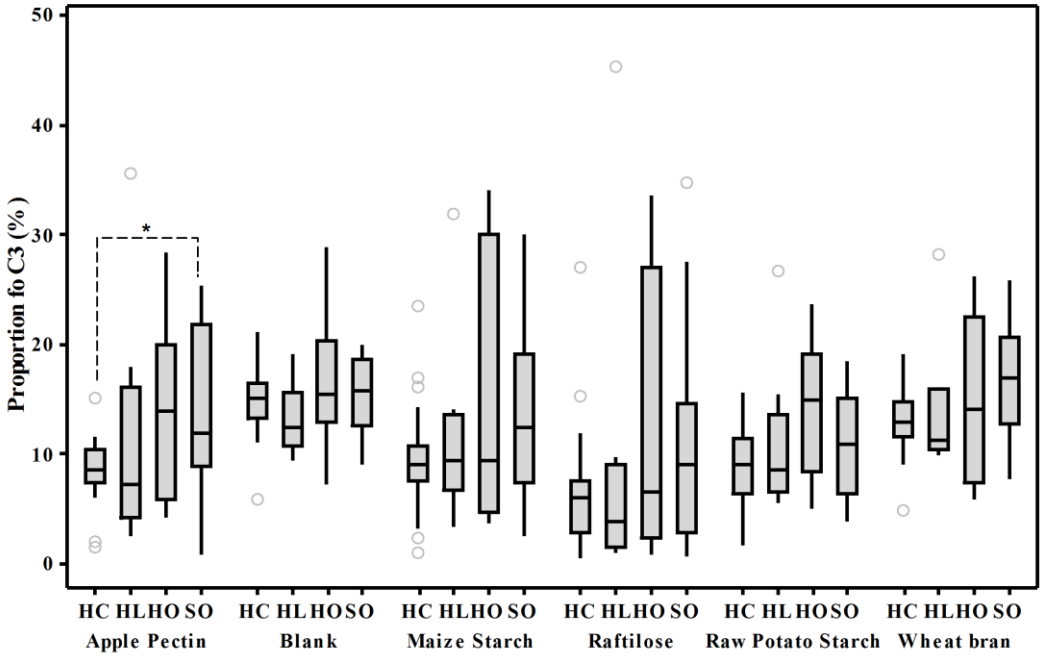
Figure 5.8: Concentration of Propionate in 24 h incubated culture in all groups after 2-3 months.

a)



Blank circles represent outliers, * indicate significant differences. HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; simple obese. Note the huge variation in concentration and proportion of propionate in hypothalamic obese group.

Figure 5.8b:Proportion of Propionate in 24 h incubated cultures in all groups for different fibres after 2-3 months.



Blank circles represent outliers, * indicate significant differences. HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; simple obese. Note the huge variation in concentration and proportion of propionate in hypothalamic obese group.

5.6.3 Change in pH and the production of SCFA between 0 h and 4 h of incubation

It is likely that the rate of fermentation tends to slow down or plateau towards maximum at 24 h. SCFA at the end point of fermentation (i.e. at 24 h) indicate only the total fermentation capability of the gut microbiota. However, it poorly indicates the rate or speed of fermentation and the rate at which this plateau or maximum levels are reached. Therefore, we measured the change in pH and change in total and individual SCFA between 0h and 4 h of incubation to estimate the rate of SCFA production. This was assessed by calculating the difference between measurements at 4 h and 0h of incubation.

Change in pH between 0-4 h at recruitment and after 2-3 months

No significant difference was seen in the change in pH between any of the groups in all substrates and the blank cultures both at recruitment and after 2-3 months (Table 5.5).

Table 5.5: change in pH (ΔpH= pH at 4 h – pH at 0 h) for all substrates between 0 h and 4 h of incubation at recruitment and after 2-3 months.

	Healthy Lean		**Hypoth. Lean**		**Hypoth. Obese**		**Simple Obese**	
Variable	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Δ pH between 0 & 4 h at recruitment								
Apple Pectin	-1.81	0.51	-1.77	0.35	-1.81	0.47	-1.93	0.39
Blank	0.04	0.14	0.11	0.11	0.02	0.11	0.03	0.11
Maize Starch	-0.62	1.08	-0.7	1.42	-0.59	0.49	-0.58	0.68
Raftilose	-2.56	0.65	-2.32	0.61	-2.59	0.78	-2.44	0.73
Raw Potato Starch	-0.44	0.52	-0.23	0.53	-0.28	0.51	-0.34	0.95
Wheat Bran	-0.70	0.38	-0.58	0.24	-0.53	0.39	-0.65	0.45
Δ pH between 0 & 4 h after 2-3 months								
Apple Pectin	-1.75	0.74	-1.87	0.67	-1.98	0.61	-2.16	0.69
Blank	0.04	0.10	0.06	0.44	0.04	0.31	0.04	0.22
Maize Starch	-0.69	0.62	-0.59	1.10	-0.99	1.69	-0.98	1.64
Raftilose	-2.46	0.44	-2.34	0.95	-2.57	0.60	-2.82	0.88
Raw Potato Starch	-0.43	0.50	-0.25	0.63	-0.66	1.04	-0.42	0.72
Wheat Bran	-0.62	0.26	-0.47	0.37	-0.69	0.47	-0.74	0.32

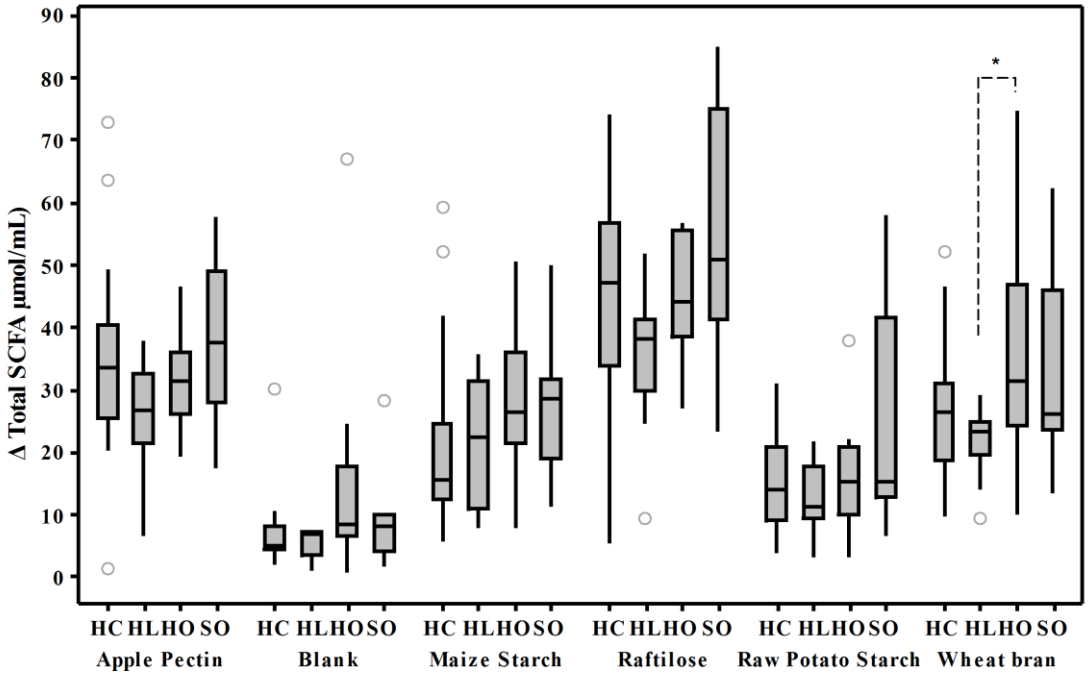
Change in total and individual SCFA between 0-4 h at recruitment

Overall, no significant difference was observed in the change in total SCFA concentration between simple and hypothalamic obese groups. However, the two obese groups (simple obese in particular) showed an increased change in the concentration of total SCFA in all substrates than the two lean groups which was only significant in cultures containing wheat bran between hypothalamic obese and hypothalamic lean participants (p=0.02, Figure 5.9,

Table 5.6). As a general trend, hypothalamic obese group showed a tendency towards higher change in concentration and proportion of propionate and butyrate.

Change in the concentration of propionate between zero and 4 h incubation was significantly higher in hypothalamic obese than hypothalamic lean group in the blank cultures (p=0.02), cultures containing maize starch (p=0.008), raftilose (p=0.02), and wheat bran (p=0.006) (Table 5.6, Figure 5.10). Similarly, hypothalamic obese group also showed a significantly higher change in the concentration of butyrate than simple obese (p=0.04, Table 5.6) and hypothalamic lean group (p=0.04, Table 5.6) only in the blank culture.

Figure 5.9: Δ total SCFA expressed as µmol/ml between 0 h and 4 h incubation at recruitment.



HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; simple obese.*indicate significant difference on Mann Whitney U test (p=0.02)

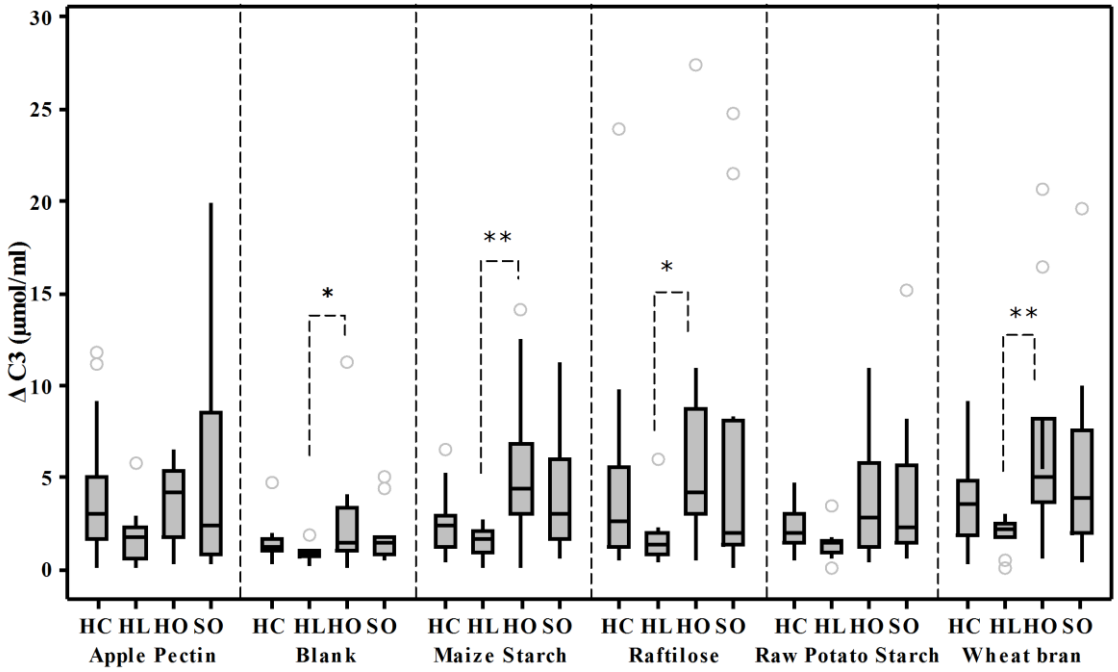
Table 5.6: change in total and major individual SCFA between 0 h and 4 h of incubation at recruitment.

	Healthy Lean		**Hypoth. Lean**		**Hypoth. Obese**		**Simple Obese**	
Variable	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Apple Pectin								
Δ Total SCFA	33.69	14.76	26.83	11.06	31.47	9.84	37.77	21.26
Δ C2	28.96	10.89	23.31	9.62	25.28	8.22	30.53	7.67
Δ C3	3.05	3.38	1.74	1.65	4.14	3.54	2.33	7.72
Δ C4	1.08	1.43	1.95	2.29	1.85	3.77	0.98	2.66
Δ C2%	0.01	5.95	-2.70	13.56	-1.30	12.06	3.90	19.87
Δ C3%	2.53	5.59	0.00	5.40	0.98	5.24	-1.22	13.30
Δ C4%	-2.41	8.70	1.00	7.47	1.67	7.63	-4.18	5.90
Blank								
Δ Total SCFA	5.16	3.89	6.87	3.75	8.53	11.20	8.19	5.97
Δ C2	3.62	1.77	4.54	1.99	5.35	6.95	5.02	3.31
Δ C3	1.23	0.63	**0.85**†	0.38	**1.45**†	2.35	1.40	0.94
Δ C4	0.51	0.83	**0.58**†	0.88	**1.11**†‡	0.82	**0.74**‡	0.74
Δ C2%	-9.60	11.04	-8.02	6.64	-11.48	11.40	-6.72	12.56
Δ C3%	8.35	9.25	3.18	4.81	2.57	6.46	3.41	7.26
Δ C4%	-1.50	5.67	0.09	7.71	3.03	5.82	-1.84	6.55
Maize Starch								
Δ Total SCFA	15.70	12.04	22.47	20.33	26.71	14.80	28.67	12.89
Δ C2	11.41	10.28	17.39	16.92	17.56	9.07	18.39	13.53
Δ C3	2.40	1.71	**1.67**†	1.16	**4.4**†	3.82	**2.96**	4.24
Δ C4	1.29	1.19	1.46	2.31	2.85	2.53	1.38	2.01
Δ C2%	-4.34	14.26	-0.10	17.57	-4.61	11.48	0.93	16.58
Δ C3%	3.91	6.28	-1.32	6.83	3.41	9.04	0.94	9.88
Δ C4%	-0.11	8.17	0.43	8.52	2.57	6.40	-2.89	5.37
Raftilose								
Δ Total SCFA	47.29	22.94	38.23	11.27	44.31	17.22	51.18	33.64
Δ C2	39.21	15.51	35.87	12.23	32.22	17.39	41.44	18.31
Δ C3	2.57	4.40	**1.31**†	1.19	**4.19**†	5.69	1.96	6.75
Δ C4	1.55	2.11	2.26	3.04	3.00	5.42	1.29	2.54
Δ C2%	2.08	10.38	**-1.24**†	11.09	**-9.95**‡†	14.09	**4.38**‡	14.62
Δ C3%	1.16	4.51	-0.45	5.80	2.01	5.07	-1.50	12.14
Δ C4%	-2.96	7.64	0.88	6.88	0.39	11.26	-3.47	5.73
Raw Potato Starch								
Δ Total SCFA	14.31	11.89	11.47	8.47	15.31	10.61	15.32	28.78
Δ C2	8.57	8.96	8.64	5.63	9.25	8.82	11.77	23.06
Δ C3	1.96	1.57	1.38	0.61	2.80	4.49	2.27	4.24
Δ C4	0.91	1.18	1.09	1.43	2.05	2.22	1.13	1.85
Δ C2%	-4.26	8.16	-1.45	10.04	**-12.57**‡	11.80	**1.74**‡	16.59
Δ C3%	4.48	6.60	1.91	6.20	6.26	7.48	0.36	11.43
Δ C4%	-1.38	7.09	-0.09	7.46	2.05	6.27	-4.47	5.85
Wheat bran								
Δ Total SCFA	26.47	12.54	**23.52**†	5.15	**31.65**†	22.68	26.31	22.19
Δ C2	17.70	9.72	18.14	4.60	21.65	12.80	19.86	14.69
Δ C3	3.49	3.00	**2.19**†	0.76	**5.04**†	4.55	3.82	5.53

Δ C4	1.76	2.19	2.18	2.84	3.44	4.01	1.53	2.51
Δ C2%	-6.68	11.09	-4.39	12.57	-9.26	8.33	2.64	15.80
Δ C3%	4.77	6.09	-0.07	3.67	4.89	5.92	1.44	10.45
Δ C4%	-0.55	9.92	2.14	10.02	3.17	6.97	-2.32	6.85

C2; acetate, C3; propionate, C4; butyrate, Δ indicate change between 0 and 4 h (4 h- 0h), † indicate significant differences between hypothalamic lean and hypothalamic obese, ‡ indicate significant differences between hypothalamic obese and simple obese, (Mann Whitney U test). Concentrations are expressed as µmol/ml and proportion as %.

Figure 5.10: Change in propionate (Δ) expressed as µmol/ml between 0 h and 4 h incubation at recruitment.



HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; simple obese. Blank circles represent outliers. * indicate p<0.05, ** indicate p<0.01 on Mann Whitney U test

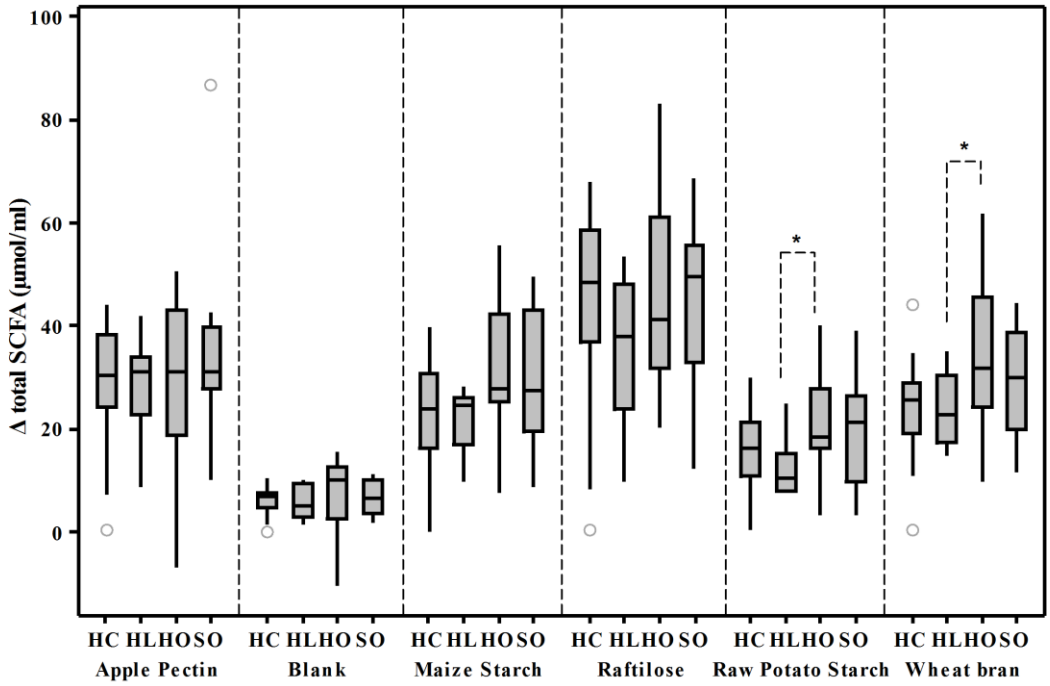
Change in total and individual SCFA production between 0 & 4 h incubation after 2-3 months

Consistent with the results at recruitment, no significant differences were observed between simple and hypothalamic obese groups in the change in production of total SCFA between 0 h and 4 h incubation after 2-3 months. As a general trend, simple and hypothalamic obese groups, particularly the latter, had a higher change in the SCFA production than the two lean groups. Hypothalamic obese group showed a significantly higher change in total SCFA than hypothalamic lean group in cultures containing raw potato starch (p=0.04) and wheat bran (p=0.04) (Figure 5.11, Table 5.7)

In terms of the concentration and proportion of individual SCFA, no significant difference was observed between simple and hypothalamic obese groups and between the two obese and the two lean groups in any of the substrates and the blank except that the

proportion of propionate was significantly lower in hypothalamic obese groups than hypothalamic lean group in the blank culture ($p<0.05$) (Table 5.7).

Figure 5.11: Δ total SCFA expressed as µmol/ml between 0 h and 4 h incubation after 2-3 months.



HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; simple obese.
* indicate $p<0.05$ on Mann Whitney U test

Table 5.7: change in total and major individual SCFA between 0 h and 4 h of incubation after 2-3 months.

Delta B	**Healthy Lean**		**Hypoth. Lean**		**Hypoth. Obese**		**Simple Obese**	
Variable	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Apple Pectin								
Δ Total SCFA	30.62	13.78	31.20	11.08	31.27	24.36	31.35	11.81
Δ C2	25.61	10.36	23.86	11.16	20.85	12.27	27.12	8.02
Δ C3	2.54	1.87	1.15	3.31	2.69	7.94	2.86	5.10
Δ C4	2.30	2.80	1.63	2.63	2.69	3.01	2.23	4.86
Δ C2%	3.67	7.98	-1.03	12.46	-1.21	8.16	-2.00	8.49
Δ C3%	0.22	3.61	0.18	3.15	0.43	9.68	0.20	4.21
Δ C4%	-2.64	6.76	-1.87	7.21	1.78	7.43	0.87	5.05
Blank								
Δ Total SCFA	7.22	2.98	5.35	6.61	10.37	10.22	6.84	6.55
Δ C2	4.17	2.11	4.11	3.77	6.94	6.38	4.31	3.89
Δ C3	1.40	0.59	0.65	1.13	1.23	2.67	1.30	0.95
Δ C4	0.86	0.72	0.72	0.98	1.17	1.11	0.90	1.15
Δ C2%	-9.43	10.10	-7.56	13.78	-4.89	23.14	-5.56	9.96
Δ C3%	5.03	5.82	**2.59**†	5.11	**0.44**†	5.69	2.77	7.73
Δ C4%	-1.28	5.84	1.41	4.96	1.47	4.80	0.34	6.52

Table 5.7 continued

Delta B	**Healthy Lean**		**Hypoth. Lean**		**Hypoth. Obese**		**Simple Obese**	
Variable	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Maize Starch								
Δ Total SCFA	24.04	14.74	24.59	9.06	28.05	17.26	27.56	23.34
Δ C2	16.45	10.16	17.58	9.73	19.72	11.43	16.50	17.87
Δ C3	2.40	1.43	1.49	1.65	3.29	7.34	3.19	4.35
Δ C4	2.61	2.05	2.10	3.21	3.45	1.65	2.73	4.45
Δ C2%	-1.58	13.77	-4.13	19.87	-5.26	15.15	-3.87	21.02
Δ C3%	1.77	4.89	1.37	7.46	-1.25	10.89	0.20	6.38
Δ C4%	-2.26	6.60	0.57	8.63	2.74	6.77	1.54	6.96
Raftilose								
Δ Total SCFA	48.48	21.89	37.94	24.28	41.22	29.19	49.49	22.44
Δ C2	41.89	14.56	25.44	18.40	27.73	27.15	36.40	14.50
Δ C3	2.36	3.93	1.00	2.34	2.78	13.28	3.23	9.74
Δ C4	2.58	2.73	2.07	4.85	3.82	1.23	2.39	2.83
Δ C2%	1.98	12.15	0.92	17.35	-2.41	19.56	1.34	15.46
Δ C3%	0.20	8.17	-1.06	7.89	1.79	23.74	-0.30	9.09
Δ C4%	-2.14	7.10	-0.14	9.77	2.77	9.86	-1.24	7.84
Raw Potato Starch								
Δ Total SCFA	16.33	10.24	**10.68**†	7.23	**18.57**†	11.64	21.61	16.48
Δ C2	10.79	8.23	7.50	3.30	13.76	13.63	13.92	10.00
Δ C3	2.14	0.94	1.36	2.04	2.19	4.07	3.05	3.01
Δ C4	1.92	1.54	1.31	2.26	2.33	0.92	2.01	3.20
Δ C2%	-3.40	8.57	-11.14	22.34	-8.59	11.72	-2.55	14.87
Δ C3%	2.21	6.28	3.59	7.66	2.22	13.03	0.42	8.55
Δ C4%	-2.26	6.30	3.07	10.02	3.58	4.46	0.00	7.73
Wheat bran								
Δ Total SCFA	25.87	9.86	22.96	13.25	31.99	21.46	30.21	18.54
Δ C2	18.55	6.41	16.48	6.97	19.68	12.44	19.40	7.01
Δ C3	3.25	2.31	1.70	2.42	4.51	7.44	4.44	5.18
Δ C4	3.08	2.54	2.52	3.28	3.76	3.92	3.14	4.85
Δ C2%	-4.51	12.89	-11.10	14.01	-7.17	7.58	-6.49	13.97
Δ C3%	2.65	7.62	4.46	6.25	2.22	10.11	2.46	9.06
Δ C4%	-0.22	7.01	1.14	6.91	4.34	10.44	2.72	6.94

C2; acetate, C3; propionate, C4; butyrate, Δ indicate change between 0 and 4 h (4 h- 0h), concentrations are expressed as µmol/ml and proportion as %, † indicate $p<0.05$ between hypoth. lean and hypoth. obese(Mann Whitney U test).

5.6.4 Changes in pH and production of total and major individual SCFA at 24 h incubation over the period of follow-up

Change (increase or decrease) in pH and total and major individual SCFA after 24 h incubation over the period of follow up was calculated by the difference of measurements after 2-3 months than at recruitment.

Changes in pH after 24 h incubation over the period of follow-up

No significant difference in the change in pH was observed between any of the groups except in wheat bran where the change was significantly higher in hypothalamic lean as compared to healthy lean participants (p=0.006, Table 5.8).

Table 5.8: change in pH for all substrates at 24 h incubation between samples at recruitment and after 2-3 months (change in pH= pH after 2-3 months– pH at recruitment).

	Healthy Lean		**Hypoth lean**		**Hypoth Obese**		**Simple Obese**	
Substrate	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Apple Pectin	-0.01	0.63	-0.12	0.78	0.03	1.26	-0.39	0.88
Blank	-0.15	0.41	-0.25	0.49	0.06	0.52	-0.14	0.50
Maize Starch	-0.12	0.65	-0.22	0.80	-0.09	0.93	-0.36	0.29
Raftilose	-0.05	0.59	0.08	0.30	-0.05	0.50	-0.19	0.69
Raw Potato Starch	-0.02	0.42	-0.05	0.91	-0.23	1.60	-0.49	0.91
Wheat Bran	**-0.26¥**	0.64	**0.35¥**	0.66	0.02	0.92	-0.47	0.57

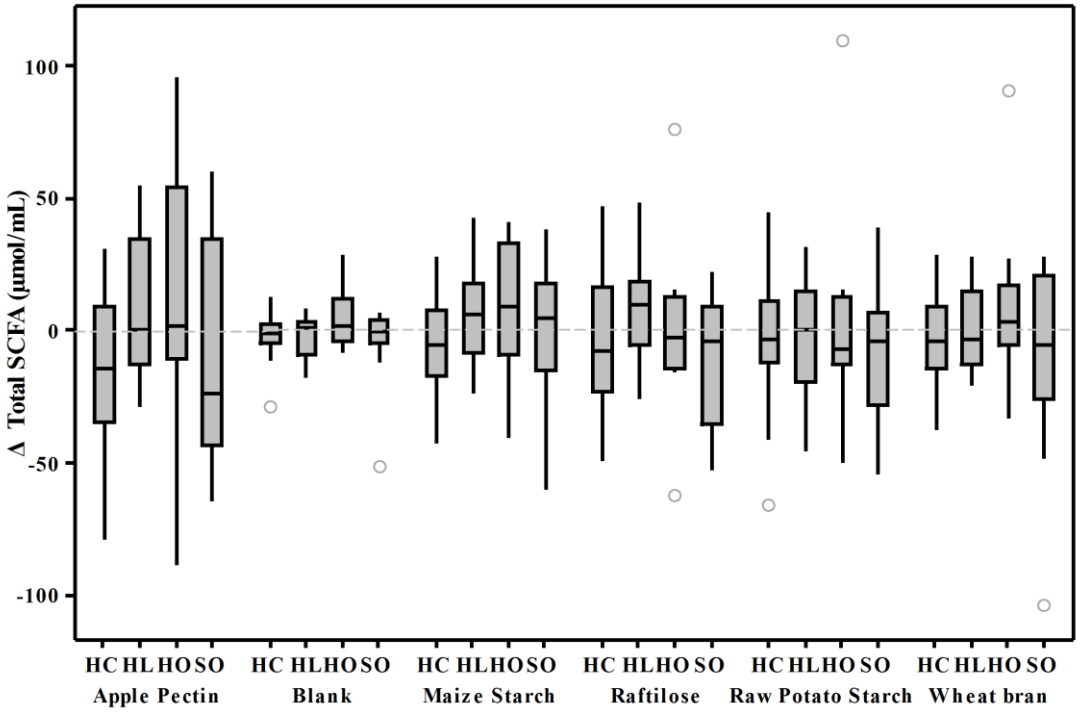
¥ indicate p=0.006 between healthy lean and hypothalamic lean (Mann Whitney U test).IQR; Inter-quartile range

Changes in total and major individual SCFA after 24 h incubation over the period of follow-up

No significant difference in the change in concentration of 24 h total and major individual SCFA (acetate, propionate, and butyrate) were observed between samples incubated at recruitment versus those incubated after 2-3 months between any of the group and in any substrate (Table 5.9, Figure 5.12).

The proportion of major individual SCFA (acetate, propionate, and butyrate) were not significantly different between any of the groups for most of the substrates. However, significantly higher change in the proportion of acetate was observed in healthy lean vs. hypothalamic lean in cultures containing apple pectin (p=0.032) (Table 5.9). Change in the proportion of propionate was significantly higher in hypothalamic lean vs. hypothalamic obese in cultures containing wheat bran [median (IQR) µmol/ml: hypoth. lean; 5.32(10.01) vs. hypoth. obese; -2.02(10.84), p=0.038] (Table 5.9) while change in the proportion of butyrate was significantly higher in cultures containing raw potato starch [median (IQR) µmol/ml: hypoth. lean; 6.45(11.51) vs. hypoth. obese; -3.88(17.15), p=0.042] (Table 5.9).

Figure 5.12: change in total SCFA after 24 h of incubation between the time of recruitment and after 2-3 months (change in total SCFA= total SCFA after 2-3 months – total SCFA at recruitment).



HC; healthy lean control, HL; hypothalamic lean, HO; hypothalamic obese, SO; simple obese. Dotted line indicates no change. Blank circles represent outliers

Table 5.9: change in total and major individual SCFA after 24 h of incubation between recruitment and after 2-3 months (change in total SCFA= total SCFA after 2-3 months – total SCFA at recruitment).

	Healthy Lean		**Hypoth lean**		**Hypoth Obese**		**Simple Obese**	
Variable	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Apple Pectin								
Change in Total SCFA	-14.28	43.81	0.59	47.14	2.10	64.60	-23.70	77.80
Change in C2	-6.17	29.10	-1.43	33.91	2.41	39.23	-18.76	41.28
Change in C3	-1.15	6.24	0.11	6.68	2.37	11.73	-0.08	12.74
Change in C4	-1.54	9.64	2.37	10.98	3.37	17.35	-1.30	12.79
Change in C2%	**-1.40¥**	8.35	**-5.31¥**	12.42	-9.62	18.58	-4.53	25.57
Change in C3%	-0.09	5.70	1.38	11.39	2.00	10.08	-1.52	15.77
Change in C4%	-0.11	9.10	5.40	7.32	1.29	13.08	0.76	10.22
Blank								
Change in Total SCFA	-1.21	7.53	0.75	12.94	1.78	15.97	-0.39	8.42
Change in C2	-0.23	2.55	0.13	8.56	1.96	7.10	1.00	5.21
Change in C3	0.10	1.49	0.71	1.97	-0.12	4.04	-0.38	1.71
Change in C4	-0.02	1.02	-0.26	1.27	-0.18	5.72	-0.03	1.12
Change in C2%	-0.08	8.97	0.39	7.57	1.03	25.17	-0.07	10.80
Change in C3%	1.10	4.90	1.55	7.90	-1.14	3.95	-0.59	5.51
Change in C4%	0.88	3.03	0.64	3.67	-1.03	13.41	1.05	4.31

Table 5.9 continued

	Healthy Lean		Hypoth lean		Hypoth Obese		Simple Obese	
Variable	Median	IQR	Median	IQR	Median	IQR	Median	IQR
Maize Starch								
Change in Total SCFA	-5.45	24.88	5.92	26.22	8.97	42.65	4.75	32.49
Change in C2	-4.43	12.58	-3.94	27.63	4.36	37.01	-8.71	22.88
Change in C3	-0.59	6.07	0.67	6.86	0.63	17.50	0.84	10.72
Change in C4	5.04	12.63	5.61	9.51	0.16	23.70	1.83	8.11
Change in C2%	-6.49	16.92	-10.38	25.36	-0.20	55.30	-0.47	14.66
Change in C3%	-1.25	8.40	1.09	12.21	-0.23	20.54	0.31	10.23
Change in C4%	9.91	15.82	16.22	18.53	1.61	33.94	4.20	7.40
Raftilose								
Change in Total SCFA	-7.85	39.23	9.92	24.33	-3.00	27.10	-4.20	44.87
Change in C2	-4.54	29.89	-2.99	24.25	1.77	35.30	-5.49	35.04
Change in C3	-0.74	3.63	1.60	5.69	0.25	25.02	-0.53	8.41
Change in C4	0.65	8.36	0.73	18.58	0.06	14.22	-0.27	2.53
Change in C2%	-0.73	19.15	-6.12	21.45	3.04	32.76	-0.98	16.14
Change in C3%	-0.24	7.10	2.99	6.12	-0.67	17.96	-1.35	8.12
Change in C4%	0.77	8.68	0.09	20.57	1.89	9.66	0.21	1.79
Raw Potato Starch								
Change in Total SCFA	-3.42	23.32	0.08	34.20	-6.70	25.70	-4.13	34.77
Change in C2	-7.40	28.91	-3.66	26.37	-3.20	42.50	-4.02	23.83
Change in C3	-0.85	6.37	0.25	8.86	0.28	11.27	0.98	6.21
Change in C4	4.25	10.06	4.32	5.19	2.06	22.37	1.76	13.56
Change in C2%	-9.65	16.89	-9.30	16.29	1.97	27.22	-4.54	20.57
Change in C3%	-1.40	5.96	0.34	7.80	3.68	6.61	0.30	4.26
Change in C4%	7.27	12.22	**6.45**†	11.51	**-3.88**†	17.15	2.02	12.31
Wheat bran								
Change in Total SCFA	-3.73	23.73	-3.60	27.19	3.20	22.30	-5.60	46.80
Change in C2	-3.30	8.42	-6.24	27.01	1.90	21.08	-6.74	17.25
Change in C3	-0.76	5.07	2.58	7.85	-0.87	12.21	1.53	7.91
Change in C4	1.27	11.33	2.32	6.17	-1.39	10.00	0.44	8.16
Change in C2%	-3.94	10.14	-7.32	18.05	1.31	18.64	-2.59	14.00
Change in C3%	0.14	6.27	**5.32**†	10.06	**-2.02**†	10.84	1.24	6.71
Change in C4%	3.87	13.43	6.68	11.86	-1.30	12.77	1.91	5.80

C2; acetate, C3; propionate, C4; butyrate, † indicate significant differences between hypoth. lean and hypoth. obese, ¥ indicate significant differences between hypothalamic lean and healthy lean (Mann Whitney U test). Concentrations are expressed as µmol/ml and proportion as %.

5.6.5 Changes in the rate of production (between 0-4 h incubation) of total and individual SCFA over the period of follow-up

To assess the changes in the rate of production of total and major individual SCFA between incubated samples over the period of follow-up, the rate of production after 2-3

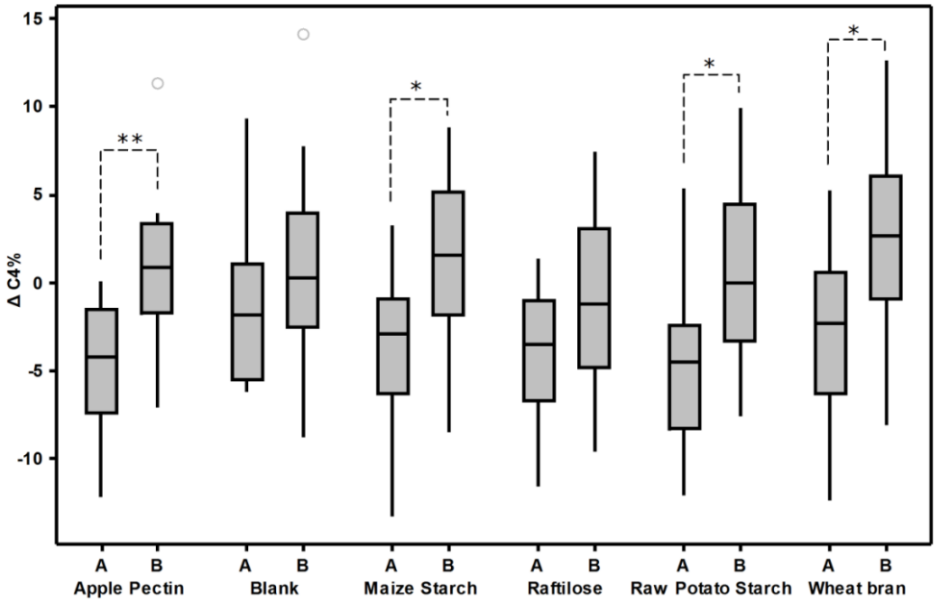
months was subtracted from the rate of production at recruitment for participants in all the groups.

Change in the rate of production of SCFA= rate of production (between 0-4 h) after 2-3 months – rate of production (between 0-4 h) at recruitment

The change in the rate within each individual group (intra-group differences) and between different groups (inter-group differences) was then compared.

Within the groups, only simple obese group showed a significant increase in the change in proportion of butyrate (Δ C4%) between samples at recruitment versus samples after 2-3 months in cultures containing apple pectin [median(IQR)%: -4.18(5.89) vs. 0.87(5.05), p=0.004], maize starch [median(IQR)%: -2.88(5.37) vs. 1.54(6.96), p=0.02], raw potato starch [median(IQR)%: -4.46(5.86) vs. 0.00(7.71), p=0.036], and wheat bran [median(IQR)%: -2.32(6.86) vs. 2.72(6.94), p=0.02] (Figure 5.13).

Figure 5.13: Change in the proportion of butyrate (ΔC4%) in simple obese group between 0 h and 4 h incubation between samples incubated at recruitment versus those incubated after 2-3 months.



A; samples at recruitment, B; samples after 2-3 months. Blank circles represent outliers. * indicate p<0.05 and ** indicate p<0.01 (Mann Whitney U test).

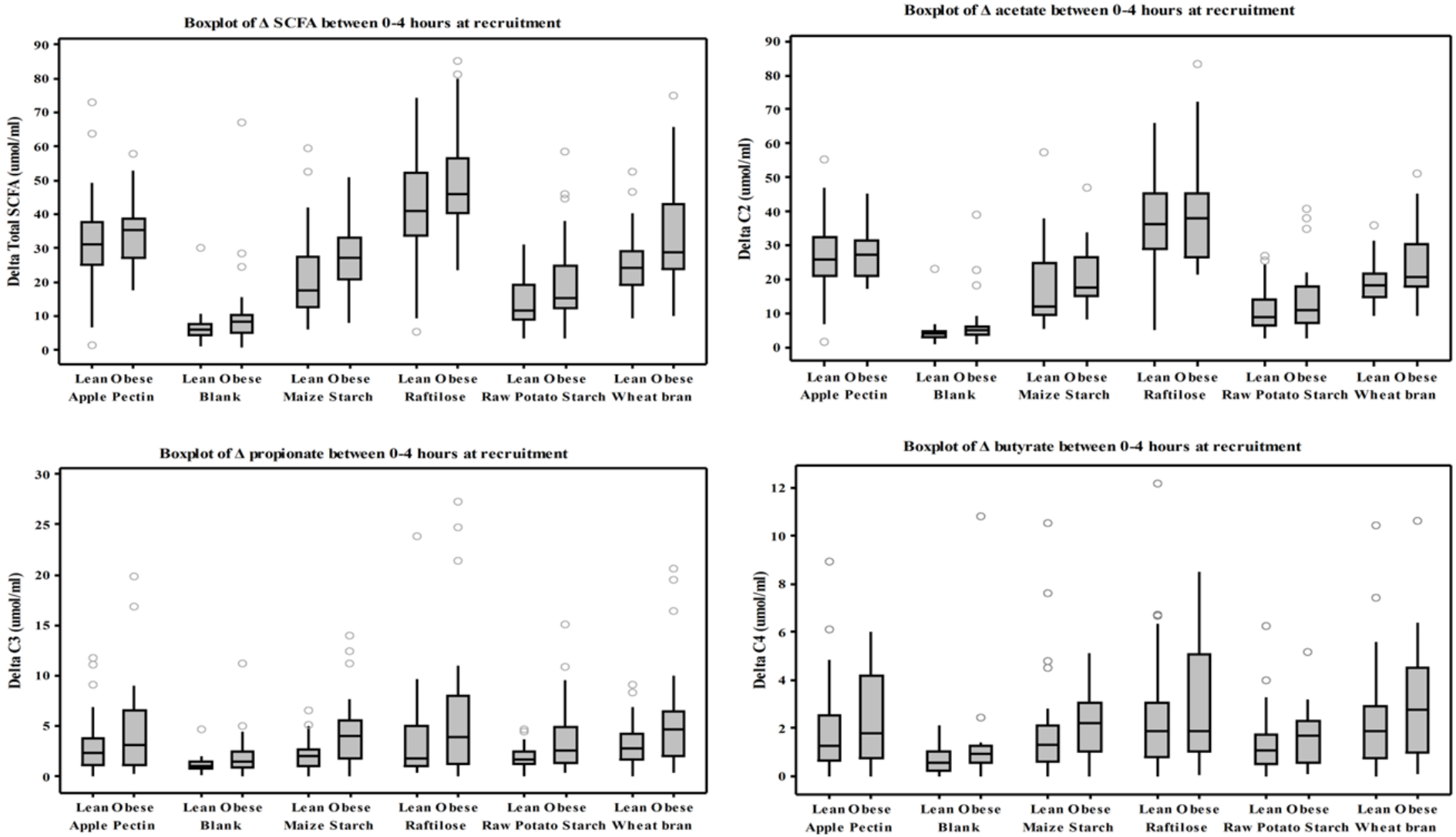
5.6.6 Differences in the rate of total and individual SCFA production between 0-4 h according to obese or lean phenotype and according to the presence or absence of pathology

When participants were grouped together based on their lean (healthy lean and hypothalamic lean) or obese (simple and hypothalamic obese) phenotype, no significant differences were found in the 0-4 h rate of production of total and major individual SCFA both at the time of recruitment (Figure 5.13) and after 2-3 months (Figure 5.14). However, obese phenotype had a characteristically uniform and consistent tendency towards higher rate of total SCFA, acetate, propionate, and butyrate production than the lean phenotype both at the time of recruitment (Figure 5.13) and after 2-3 months (Figure 5.14).

With regard to the proportion of individual SCFA, no significant difference was observed in the rate of change in the proportion of 0-4 h acetate, propionate, and butyrate both at recruitment and after 2-3 months. There was no peculiar pattern for the distribution of proportion of acetate and propionate both at recruitment and after 2-3 months. However, the proportion of butyrate at 2-3 months showed a consistent tendency to be non-significantly higher in the obese phenotype than lean phenotype (Figure 5.16).

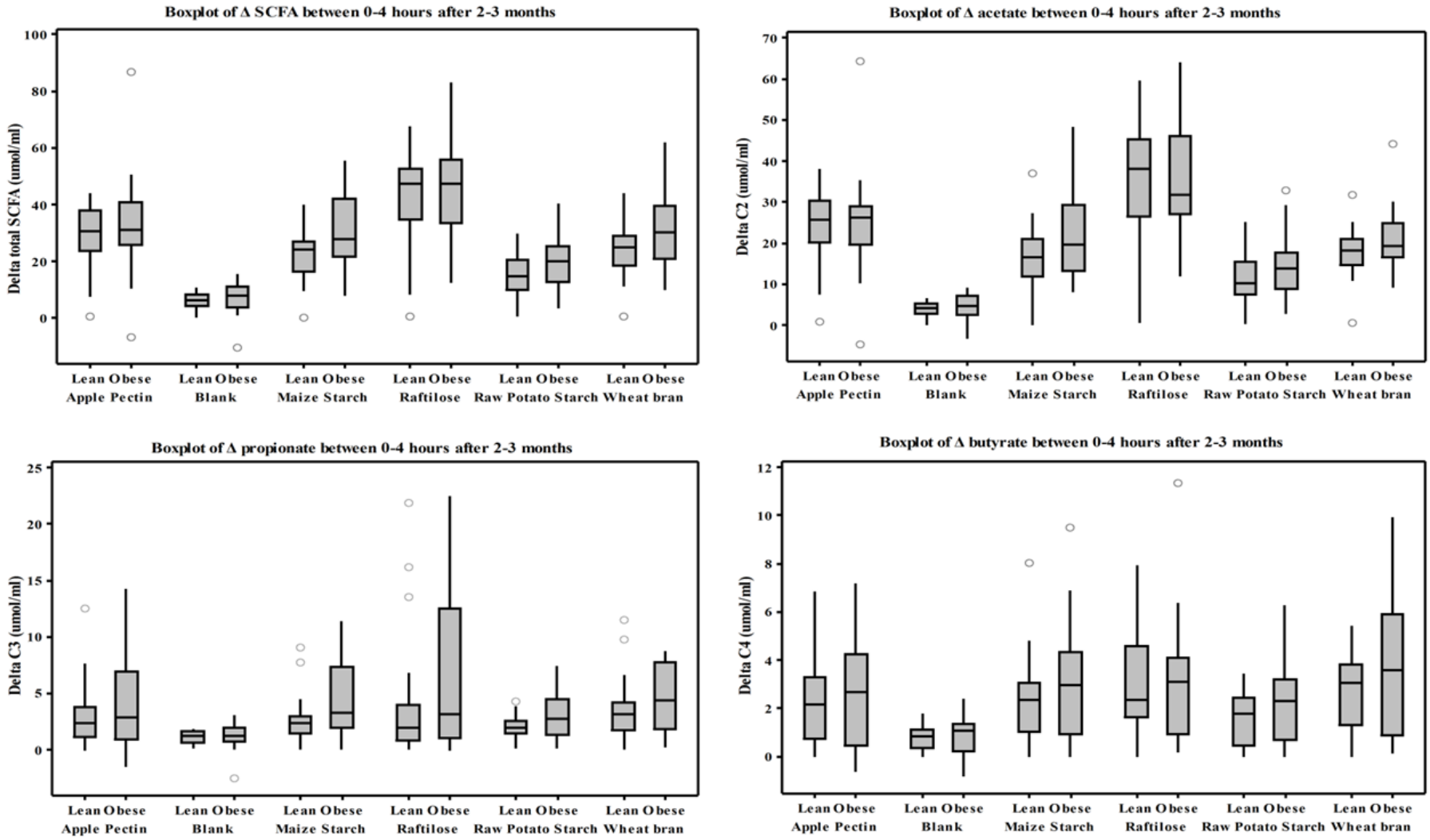
When participants were grouped into pathological group (hypothalamic lean and obese) and healthy group (simple obese and healthy lean) based on the presence or absence of pathology, no significant difference or peculiar pattern of SCFA production was seen between the two groups.

Figure 5.14: : Boxplots showing the rate of change in the concentration of total and individual SCFA between 0-4 h according to obese and lean phenotype at recruitment



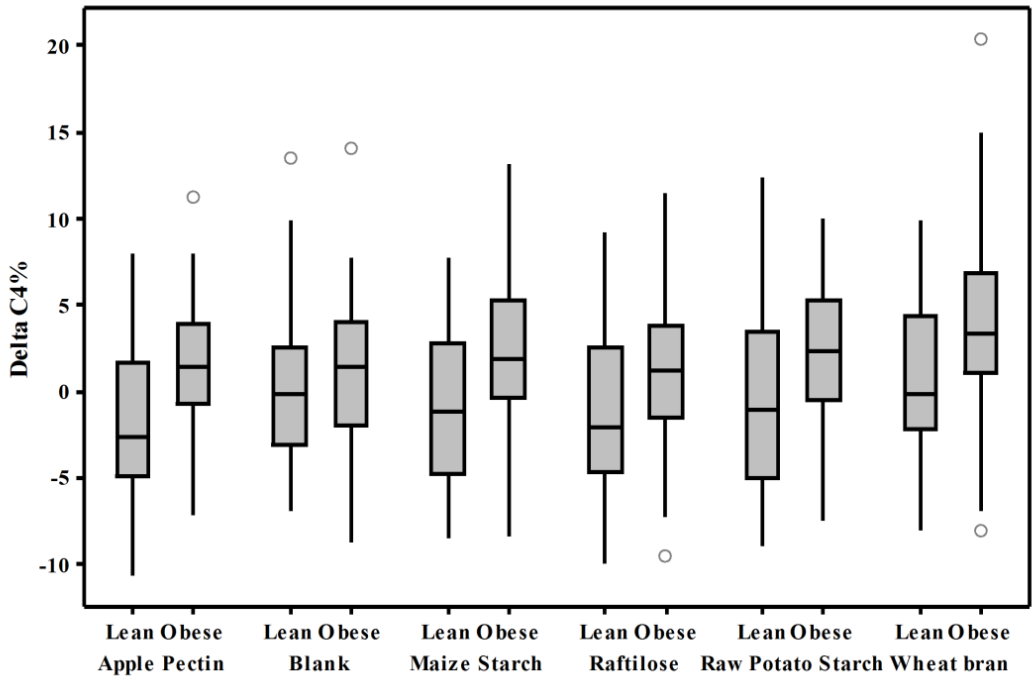
Blank circles represent outliers. Δ indicate change in concentration between 0-4 h. Lean; hypothalamic lean and healthy lean, Obese; simple and hypothalamic obese

Figure 5.15: Boxplots showing the rate of change in the concentration of total and individual SCFA between 0-4 h according to obese and lean phenotype after 2-3 months



Blank circles represent outliers. Δ indicate change in concentration between 0-4 h. Lean; hypothalamic lean and healthy lean, Obese; simple and hypothalamic obese

Figure 5.16: Boxplots showing the rate of change in proportion of butyrate between 0-4 h according to obese and lean phenotype after 2-3 months.



Blank circles represent outliers, C4; butyrate, Lean; healthy lean and hypothalamic lean, obese; hypothalamic obese and simple obese

5.6.7 Correlation of BMI SDS with total SCFA production and the rate of SCFA production between 0-4 hour incubation at recruitment and after 2-3 months

Spearman rank correlations were done to assess the correlation of BMI SDS with total SCFA production and the rate of SCFA production between 0-4 hour incubation at the time of recruitment and after 2-3 months.

Correlation of BMI SDS with the total SCFA after 24 h incubation

No significant correlations were found between BMI SDS and the total SCFA production after 24 h incubation at the time of recruitment in any of the groups for all the substrates. However, after 2-3 months, the healthy lean group showed significant negative correlations in cultures containing maize starch (R=-0.510, p=0.015), Raftilose (R=-0.468, p=0.028), and the blank cultures (R=-0.418, p=0.047) (Table 5.10). Similarly, hypothalamic obese showed significant negative correlations in cultures containing raftilose (R=-0.717, p=0.045) while hypothalamic lean group showed significant positive correlation in blank cultures (R=0.678, p=0.045) and (Table 5.10).

Correlation of BMI SDS with the rate of SCFA production between 0-4 h

Consistent with total SCFA production after 24 h incubation at recruitment, no significant differences were seen in the rate of total SCFA production between 0-4 h incubation at the time of recruitment (Table 5.10). After 2-3 months, healthy lean group showed a significant negative correlation of BMI SDS with the rate of total SCFA production in cultures containing apple pectin (R=-0.452, p=0.039) and raftilose (R=-0.455, p=0.038). In contrast, BMI SDS in Simple obese group had a tendency towards positive correlation with the rate of SCFA production which was only significant in raw potato starch (R=0.612, p=0.026) (Table 5.10).

Correlation of BMI SDS with the production of 24 h SCFA and the rate of SCFA production according to phenotype (lean or obese) and pathology (healthy or pathological)

To assess the correlation of BMI SDS with SCFA production and rate of SCFA production based on their phenotype or the presence or absence of pathology, participants were grouped into lean and obese phenotype (based on phenotype) or healthy and pathological groups (based on pathology) (Table 5.11).

No significant correlations were found in the 24 h total SCFA production at the time of recruitment and after 2-3 months in any of the groups (lean/obese or healthy/pathological) except for a positive correlation in cultures containing apple pectin in obese phenotype only at recruitment (R=0.469, p=0.021) (Table 5.11).

Rate of SCFA production was significantly negatively correlated with BMI SDS with lean phenotype at recruitment only in cultures containing raftilose (R=-0.421, p=0.026) (Table 5.11).

Table 5.10: Correlation of BMI SDS with total SCFA production after 24 h incubation and the rate of SCFA production between 0-4 h incubation at the time of recruitment and after 2-3 months in all the study groups.

	Healthy Lean Control				**Hypothalamic Lean**				**Hypothalamic Obese**				**Simple Obese**			
	At recruitment		**At 2-3 months**		**At recruitment**		**At 2-3 months**		**At recruitment**		**At 2-3 months**		**At recruitment**		**At 2-3 months**	
Variable	R	p	R	p	R	p	R	p	R	p	R	p	R	p	R	p
Total SCFA after 24 h incubation																
Apple Pectin	-0.052	0.795	-0.355	0.105	-0.226	0.503	0.262	0.495	-0.578	0.062	-0.390	0.340	0.412	0.143	0.126	0.767
Blank	0.062	0.765	**-0.428**	**0.047***	-0.280	0.404	**0.678**	**0.045***	0.029	0.931	-0.380	0.353	0.528	0.052	0.204	0.661
Maize Starch	-0.263	0.185	**-0.510**	**0.015***	-0.281	0.402	-0.068	0.862	-0.279	0.406	-0.156	0.712	0.490	0.151	0.222	0.597
Raftilose	0.141	0.483	**-0.468**	**0.028***	-0.047	0.890	0.234	0.545	-0.364	0.271	**-0.717**	**0.045***	0.534	0.112	-0.032	0.940
Raw Potato Starch	0.066	0.745	-0.247	0.267	-0.466	0.149	-0.167	0.651	0.315	0.346	-0.254	0.544	0.003	0.994	0.196	0.641
Wheat Bran	0.014	0.945	-0.396	0.068	-0.405	0.217	0.265	0.776	-0.360	0.277	0.165	0.697	0.281	0.432	0.243	0.562
Rate of SCFA production between 0-4 h																
Apple Pectin	-0.058	0.788	**-0.452**	**0.039***	-0.203	0.550	**-0.752**	**0.051**†	-0.279	0.435	-0.259	0.501	0.513	0.088	0.266	0.379
Blank	-0.247	0.233	-0.232	0.326	-0.108	0.753	-0.510	0.242	0.353	0.317	0.085	0.841	0.514	0.087	0.377	0.204
Maize Starch	0.169	0.408	0.006	0.981	0.425	0.255	0.675	0.096	-0.217	0.548	0.215	0.578	0.258	0.419	0.304	0.313
Raftilose	0.087	0.673	**-0.455**	**0.038***	0.060	0.861	-0.679	0.094	-0.639	0.047	-0.319	0.403	0.319	0.313	0.391	0.187
Raw Potato Starch	0.016	0.939	-0.056	0.808	0.219	0.517	-0.076	0.871	0.151	0.676	0.147	0.707	0.214	0.504	**0.612**	**0.026***
Wheat Bran	0.112	0.585	-0.257	0.260	-0.086	0.801	0.052	0.923	-0.038	0.916	0.265	0.492	0.610	0.412	0.473	0.102

R; Spearman Rank correlations, p; p-value, *indicate significant correlations ($p<0.05$), † indicate $p=0.052$ (Spearman Rank)

Table 5.11: Correlation of BMI SDS with total SCFA production after 24 h incubation and the rate of SCFA production between 0-4 h incubation at the time of recruitment and after 2-3 months according to their phenotype (lean or obese) and pathology (pathology and healthy).

	Lean (Healthy & Hypoth. lean)				Obese (Simple & Hypoth. obese)				Healthy (Healthy lean & Simple obese)				Pathology (Hypoth. Lean & Hypoth. obese)			
	At recruitment		At 2-3 months		At recruitment		At 2-3 months		At recruitment		At 2-3 months		At recruitment		At 2-3 months	
Variable	R	p	R	p	R	P	R	p	R	P	R	p	R	p	R	p
Total SCFA after 24 h incubation																
Apple Pectin	-0.249	0.131	-0.346	0.061	**0.469**	**0.021***	-0.180	0.422	0.014	0.928	-0.187	0.281	0.285	0.251	0.113	0.665
Blank	-0.064	0.709	-0.335	0.071	0.210	0.324	-0.138	0.550	0.202	0.194	-0.113	0.516	0.068	0.790	0.352	0.181
Maize Starch	0.164	0.282	-0.281	0.132	0.038	0.881	-0.104	0.644	-0.263	0.111	0.091	0.605	0.246	0.236	-0.058	0.605
Raftilose	-0.077	0.648	-0.513	0.004	-0.024	0.910	-0.356	0.104	0.193	0.208	-0.294	0.087	0.225	0.369	-0.090	0.731
Raw Potato Starch	-0.086	0.609	-0.339	0.067	0.148	0.489	-0.175	0.437	0.147	0.339	0.067	0.701	0.319	0.197	0.015	0.955
Wheat Bran	-0.137	0.414	-0.224	0.235	0.325	0.121	-0.249	0.263	0.129	0.406	-0.177	0.309	0.113	0.657	0.055	0.833
Rate of SCFA production between 0-4 h																
Apple Pectin	-0.206	0.235	-0.326	0.091	0.237	0.288	0.063	0.779	0.146	0.375	0.048	0.788	0.220	0.381	-0.285	0.285
Blank	-0.272	0.108	-0.186	0.353	0.325	0.140	0.128	0.580	0.134	0.411	0.124	0.490	**0.473**	**0.047***	0.236	0.398
Maize Starch	0.180	0.286	0.090	0.655	0.210	0.925	0.204	0.363	0.318	0.043	0.281	0.113	0.205	0.414	0.455	0.077
Raftilose	0.017	0.923	**-0.421**	**0.026***	-0.012	0.958	0.131	0.560	0.220	0.167	-0.046	0.794	0.186	0.459	-0.141	0.602
Raw Potato Starch	-0.046	0.787	-0.154	0.435	0.232	0.299	0.371	0.089	0.256	0.107	0.271	0.121	0.234	0.349	0.389	0.137
Wheat Bran	-0.041	0.808	-0.155	0.441	0.093	0.680	0.287	0.195	0.241	0.129	0.217	0.218	0.363	0.139	0.389	0.152

R; Spearman Rank correlations, p; p-value, *indicate significant correlations ($p<0.05$), † indicate $p=0.052$ (Spearman Rank)

5.6.8 Correlation of BMI SDS with production of acetate, propionate, and butyrate after 24 h incubation at recruitment and after 2-3 months

Spearman rank correlations were done to assess the correlation of BMI SDS with acetate, propionate, and butyrate production after 24 hour incubation at the time of recruitment and after 2-3 months in individual groups.

Correlation of BMI SDS with acetate in individual groups

Significant negative correlations were observed between BMI SDS and acetate in cultures containing apple pectin in hypothalamic lean (R=-0.616, p=0.044) and raftilose in hypothalamic obese group (R=-0.680, p=0.031) only at the time of recruitment (Table 5.12).

Correlation of BMI SDS with propionate in individual groups

A general tendency towards positive correlation of BMI SDS with propionate was observed in the two obese groups which was statistically significant only in the blank cultures with no substrates (R=0.583, p=0.029) and cultures containing apple pectin (R=0.676, p=0.008) only at the time of recruitment (Table 5.12). In contrast, healthy lean participants showed significant negative correlation of BMI SDS with propionate in cultures containing maize starch (R=-0.563, p=0.006) and a tendency in apple pectin (p=0.052), raftilose (p=0.072), and wheat bran (p=0.072) (Table 5.12).

Correlation of BMI SDS with butyrate in individual groups

Simple obese participants showed a tendency towards a positive correlation of BMI SDS with butyrate which was significant for raw potato starch (R=0.718, p=0.004) and wheat bran at recruitment and maize starch (R=0.627, p=0.022) after 2-3 months (Table 5.12). In contrast, healthy lean participants showed a tendency towards negative correlation of BMI SDS with butyrate which was statistically significant for apple pectin (R=-0.444, p=0.038), maize starch (R=-0.678, p=0.001), and raw potato starch (R=-0.538, p=0.010) after 2-3 months (Table 5.12).

Table 5.12: Correlation of BMI SDS with acetate, propionate, and butyrate after 24 h incubation at recruitment and after 2-3 months.

	Healthy Lean Control				**Hypothalamic Lean**				**Hypothalamic Obese**				**Simple Obese**			
	At recruitment		**At 2-3 months**		**At recruitment**		**At 2-3 months**		**At recruitment**		**At 2-3 months**		**At recruitment**		**At 2-3 months**	
Variable	**R**	**p**	**R**	**p**	**R**	**p**	**R**	**p**	**R**	**p**	**R**	**p**	**R**	**p**	**R**	**p**
Acetate production after 24 h incubation																
Apple Pectin	0.009	0.964	-0.415	0.055	**-0.616**	**0.044***	-0.290	0.485	0.372	0.290	-0.041	0.916	0.148	0.613	-0.404	0.171
Blank culture	0.123	0.550	-0.256	0.251	0.226	0.503	-0.351	0.393	-0.500	0.141	0.593	0.121	0.104	0.723	-0.358	0.230
Maize Starch	-0.044	0.829	-0.020	0.928	-0.422	0.196	-0.022	0.959	-0.094	0.795	-0.269	0.484	0.147	0.601	-0.504	0.079
Raftilose	0.028	0.890	-0.342	0.119	-0.156	0.648	-0.609	0.109	**-0.680**	**0.031***	-0.553	0.123	-0.226	0.437	-0.276	0.361
Raw Potato Starch	0.058	0.772	0.088	0.698	0.041	0.904	-0.047	0.912	-0.102	0.780	-0.384	0.307	-0.008	0.977	-0.469	-0.106
Wheat Bran	0.135	0.503	-0.173	0.441	-0.241	0.475	0.279	0.504	0.002	0.995	-0.363	0.337	0.008	0.977	-0.500	0.082
Propionate production after 24 h incubation																
Apple Pectin	-0.076	0.708	**-0.419**	**0.052†**	-0.296	0.376	-0.039	0.928	0.509	0.133	0.038	0.923	**0.676**	**0.008***	0.004	0.989
Blank culture	-0.003	0.989	-0.336	0.126	-0.033	0.922	-0.390	0.339	-0.171	0.637	0.326	0.430	**0.583**	**0.029***	0.357	0.231
Maize Starch	-0.227	0.255	**-0.563**	**0.006***	-0.213	0.529	0.465	0.245	0.199	0.581	-0.058	0.882	0.384	0.158	0.005	0.987
Raftilose	-0.212	0.287	-0.388	0.075	-0.330	0.322	-0.053	0.900	0.346	0.328	0.109	0.780	0.617	0.019	0.271	0.371
Raw Potato Starch	-0.038	0.849	-0.298	0.178	0.247	0.463	0.050	0.907	0.153	0.673	0.109	0.780	0.309	0.282	0.049	0.875
Wheat Bran	0.095	0.637	-0.391	0.072	-0.096	0.778	-0.109	0.798	0.229	0.524	-0.253	0.511	0.437	0.118	0.196	0.521
Butyrate production after 24 h incubation																
Apple Pectin	-0.186	0.354	**-0.444**	**0.038***	-0.194	0.567	-0.354	0.390	0.365	0.300	-0.149	0.701	0.455	0.111	0.520	0.069
Blank culture	-0.010	0.959	-0.285	0.199	0.237	0.483	-0.505	0.202	-0.323	0.362	-0.060	0.888	0.418	0.137	0.433	0.139
Maize Starch	-0.406	0.036	**-0.678**	**0.001***	0.403	0.219	-0.162	0.701	-0.428	0.217	-0.107	0.784	0.422	0.117	**0.627**	**0.022***
Raftilose	0.063	0.753	-0.324	0.141	-0.048	0.899	-0.013	0.976	-0.113	0.755	0.054	0.899	0.422	0.133	**-0.556**	**0.049***
Raw Potato Starch	-0.101	0.617	**-0.538**	**0.010***	0.673	0.023	-0.453	0.260	-0.385	0.272	-0.143	0.713	**0.526**	**0.053†**	0.320	0.287
Wheat Bran	0.029	0.885	-0.205	0.360	0.025	0.942	0.260	0.534	-0.487	0.153	-0.137	0.725	**0.718**	**0.004***	**0.551**	**0.051†**

* indicate p<0.05, † indicate p=0.05, R; Spearman Rank correlations, p; p-value,

Correlation of BMI SDS with the production of acetate, propionate, and butyrate 24 hour incubation according phenotype (lean or obese) and pathology (healthy or pathological)

To assess the correlation of BMI SDS with acetate, propionate, and butyrate according to lean/obese phenotype or presence/absence of pathology, participants were grouped into lean (healthy and hypothalamic lean) and obese (simple and hypothalamic obese) phenotype or into pathological (hypothalamic lean and obese) and healthy (healthy lean and simple obese) groups.

Correlations of BMI SDS with acetate, propionate, and butyrate according to phenotype

Lean phenotype showed significant negative correlations of BMI SDS with acetate, propionate, and butyrate in most dietary substrates and the blank cultures particularly in samples collected after 2-3 months (Table 5.13). In contrast, obese phenotype showed a consistent tendency towards positive correlations of BMI SDS particularly with propionate and butyrate (Table 5.13).

Acetate was significantly negatively correlated with BMI SDS in lean phenotype after 2-3 months in cultures containing apple pectin (R=-0.384, p=0.036) and raftilose (R=-0.450, p=0.013) (Table 5.13). Obese phenotype only showed a significant negative correlation of BMI SDS with acetate only in cultures containing wheat bran in samples collected after 2-3 months (R=-0.464, p=0.030).

Propionate in obese phenotype was significantly positively correlated with BMI SDS in cultures containing apple pectin (R=0.592, p=0.002) at recruitment while lean phenotype showed significant negative correlations with raftilose (R=-0.350, p=0.031) at recruitment and in blank cultures (R=-0.374, p=0.042) after 2-3 months (Table 5.13).

Butyrate showed strong negative correlations with BMI SDS in the lean phenotype for cultures containing apple pectin (R= -0.399, p=0.029), maize starch (R=-0.448, p=0.013), raw potato starch (R=-0.555, p=0.002), and the blank cultures (R=-0.466, p=0.013) after 2-3 months (Table 5.13). In contrast, no significant correlations between BMI SDS and butyrate were observed in the obese phenotype at recruitment as well as after 2-3 months (Table 5.13).

Correlations of BMI SDS with acetate, propionate, and butyrate according to pathology

When participants were grouped according to the presence or absence of pathology, no significant correlations were found between BMI SDS and acetate, propionate, and butyrate at the time of recruitment or after 2-3 months, except a significant negative correlation of butyrate in healthy group in blank cultures after 2-3 months (R= -0.407, p=0.015). This was suggestive of negligible effect of pathology on the correlations of BMI SDS with individual SCFAs.

Table 5.13: Correlation of BMI SDS with acetate, propionate, and butyrate production after 24 h incubation according to phenotype (lean or obese) and presence or absence of pathology (pathological or healthy), at the time of recruitment and after 2-3 months.

	Lean (Healthy & Hypoth. lean)				Obese (Simple & Hypoth. obese)				Healthy (Healthy lean & Simple obese)				Pathology (Hypoth. Lean & Hypoth. Obese)			
	At recruitment		**At 2-3 months**		**At recruitment**		**At 2-3 months**		**At recruitment**		**At 2-3 months**		**At recruitment**		**At 2-3 months**	
	R	**p**	**R**	**p**	**R**	**p**	**R**	**p**	**R**	**p**	**R**	**p**	**R**	**p**	**R**	**p**
Acetate production after 24 h incubation																
Apple Pectin	-0.233	0.179	**-0.384**	**0.036***	0.292	0.166	-0.288	0.193	-0.003	0.987	-0.284	0.098	0.227	0.364	0.075	0.775
Blank culture	0.104	0.538	-0.099	0.604	-0.240	0.259	-0.155	0.503	0.021	0.893	0.012	0.945	-0.147	0.561	0.343	0.194
Maize Starch	-0.096	0.566	0.004	0.984	0.173	0.408	-0.312	0.157	0.188	0.217	0.177	0.309	-0.243	0.331	-0.050	0.850
Raftilose	-0.050	0.764	**-0.450**	**0.013***	-0.382	0.066	-0.348	0.113	0.119	0.441	-0.265	0.124	-0.137	0.588	-0.261	0.312
Raw Potato Starch	-0.083	0.619	-0.108	0.571	0.034	0.876	-0.282	0.204	0.161	0.296	0.218	0.208	0.243	0.331	0.027	0.917
Wheat Bran	0.124	0.458	-0.012	0.950	0.096	0.657	**-0.464**	**0.030***	-0.316	0.201	-0.193	0.268	0.091	0.558	0.006	0.982
Propionate production after 24 h incubation																
Apple Pectin	-0.207	0.213	-0.209	0.268	**0.592**	**0.002***	-0.001	0.998	0.101	0.512	0.083	0.637	0.322	0.192	0.129	0.621
Blank culture	-0.171	0.311	**-0.374**	**0.042***	0.320	0.127	0.156	0.499	0.175	0.262	0.012	0.944	0.285	0.251	0.412	0.113
Maize Starch	-0.213	0.199	-0.297	0.110	0.282	0.173	0.016	0.943	0.058	0.704	0.102	0.561	0.098	0.699	0.178	0.495
Raftilose	**-0.350**	**0.031***	-0.332	0.073	0.406	0.049	0.179	0.425	0.027	0.860	0.124	0.479	**0.461**	**0.054**†	0.279	0.278
Raw Potato Starch	-0.095	0.569	-0.204	0.280	0.212	0.321	0.023	0.919	0.084	0.586	0.102	0.561	0.321	0.194	0.320	0.210
Wheat Bran	-0.144	0.388	-0.326	0.078	0.347	0.097	0.030	0.894	0.179	0.244	0.110	0.529	0.380	0.120	-0.022	0.933
Butyrate production after 24 h incubation																
Apple Pectin	-0.228	0.169	**-0.399**	**0.029***	0.395	0.056	0.142	0.528	-0.061	0.694	-0.269	0.119	0.181	0.471	0.082	0.752
Blank culture	-0.140	0.409	**-0.460**	**0.011***	-0.163	0.448	-0.065	0.779	-0.141	0.367	**-0.407**	**0.015***	0.254	0.310	0.281	0.291
Maize Starch	-0.221	0.181	**-0.448**	**0.013***	0.054	0.796	0.337	0.125	-0.100	0.513	-0.233	0.179	-0.060	0.814	-0.334	0.191
Raftilose	-0.023	0.893	-0.119	0.532	0.040	0.852	0.136	0.548	-0.100	0.517	-0.268	0.120	0.141	0.576	-0.019	0.942
Raw Potato Starch	-0.084	0.617	**-0.550**	**0.002***	0.133	0.534	0.109	0.628	0.047	0.761	**-0.334**	**0.050**†	0.410	0.091	-0.113	0.666
Wheat Bran	-0.147	0.379	-0.160	0.399	0.147	0.492	0.160	0.477	-0.034	0.828	-0.215	0.215	0.242	0.333	0.083	0.752

* indicate $p<0.05$, † indicate $p=0.05$, R; Spearman Rank correlations, p; p-value,

5.6.9 Differences in sample collection and processing time (in h) between the groups and its effect on SCFA production capability

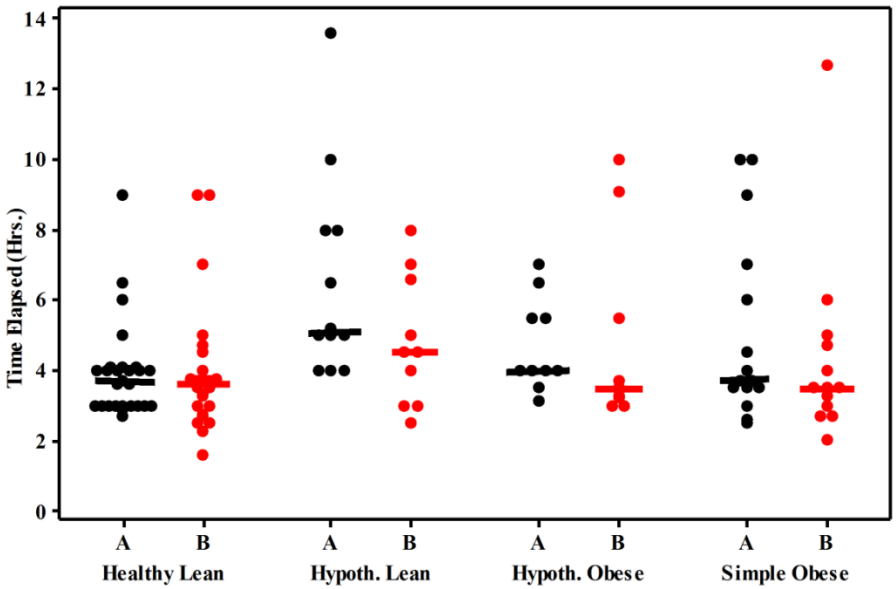
At the start of the study, the aim was to process all faecal samples in the laboratory within 4 h after being produced. However, for reasons explained earlier, 40/151 samples (n=23 at recruitment and n=17 after 2-3 months) were processed more than 4h after they were produced. The median processing time was significantly higher in hypothalamic lean group than healthy lean group [median (IQR) h: hypoth. lean; 5.1(3.7) vs. healthy lean; 3.7(1.1)] (table 5.14, figure 5.17).

Table 5.14: Comparison of sample processing times (time elapsed) between different groups at recruitment after 2-3 months

Group	Processing time at recruitment (h)				Processing time at 2-3 months (h)			
	Median	IQR	Min	Max	Median	IQR	Min	Max
Healthy Lean	3.7†	1.1	2.7	9.0	3.6	1.6	1.6	9.0
Hypoth. Lean	5.1†	3.7	4.0	13.6	4.5	3.6	2.5	8.0
Hypoth. obese	4.0	1.5	3.1	7.0	3.5	2.3	3.0	10.0
Simple Obese	3.7	3.5	2.5	10.0	3.5	1.7	2.0	12.7

† indicate significant difference between healthy lean and hypothalamic lean at recruitment (Mann-Whitney U test). Min; minimum, max; maximum time elapsed in h, IQR; interquartile range.

Figure 5.17: Individual value plot showing distribution of sample processing times in all the groups at recruitment (A, in black colour) and after 2-3 months (B, in red colour).



A; sample processing time at recruitment, B; sample processing time after 2-3 months. Each dot represent a participant at that time point while the horizontal bars in each column represent median.

Whether SCFA production after 24 h incubations were affected by the difference in sample processing time was assessed by Spearman rank correlations. Hypothalamic lean group showed a consistent and strong negative correlation of SCFA production in apple pectin with sample processing time at recruitment (R= -82.8, p=0.001) and after 2-3 months (R= -85.0, p=0.004) (table 5.15, figure 5.18, figure 5.19). Healthy lean group also showed a negative correlation of SCFA with sample processing time in cultures containing apple pectin at recruitment (R= -51.9, p=0.006) (Table 5.15, Figure 5.17) and in cultures containing maize starch (R= -0.432, p=0.040), raftilose (R= -0.440, p=0.036), and blank cultures (R= -49.4, p=0.017) after 2-3 months (Table 5.15, Figure 5.18).

Table 5.15: Correlation of SCFA production at 24 h incubation with sample processing times (Spearman rank correlations)

Substrate	**Healthy Lean Control**		**Hypothalamic Lean**		**Hypothalamic Obese**		**Simple Obese**	
	R	P	R	P	R	p	R	p
Correlation of time elapsed with24 h total SCFA at recruitment								
Apple Pectin	**-0.519**	**0.006†**	**-0.828**	**0.001†**	-0.603	0.065	-0.208	0.476
Blank	-0.318	0.113	0.138	0.670	0.306	0.389	-0.360	0.206
Maize Starch	-0.270	0.173	0.132	0.684	-0.155	0.668	-0.261	0.347
Raftilose	-0.363	0.063	-0.278	0.382	-0.057	0.876	-0.107	0.715
Raw Potato Starch	-0.122	0.546	0.190	0.554	-0.257	0.474	-0.036	0.902
Wheat Bran	0.027	0.895	-0.448	0.144	-0.226	0.530	-0.440	0.116
Correlation of time elapsed with24 h total SCFA after 2-3 months								
Apple Pectin	-0.290	0.179	**-0.851**	**0.004†**	-0.125	0.748	-0.147	0.632
Blank	**-0.494**	**0.017***	-0.637	0.065	-0.057	0.893	0.120	0.696
Maize Starch	**-0.432**	**0.040***	-0.369	0.329	-0.016	0.967	-0.100	0.746
Raftilose	**-0.440**	**0.036***	**-0.717**	**0.030***	-0.309	0.418	0.337	0.260
Raw Potato Starch	-0.302	0.161	-0.462	0.211	-0.189	0.626	-0.045	0.883
Wheat Bran	-0.276	0.203	-0.201	0.604	0.051	0.896	0.142	0.642

*indicate $p<0.05$, † indicate $p<0.01$, R; Spearman rank correlation, p; p-value

Figure 5.18: Scatter plot showing correlation/association of 24 h SCFA with sample processing time (in h) for different dietary substrates at recruitment

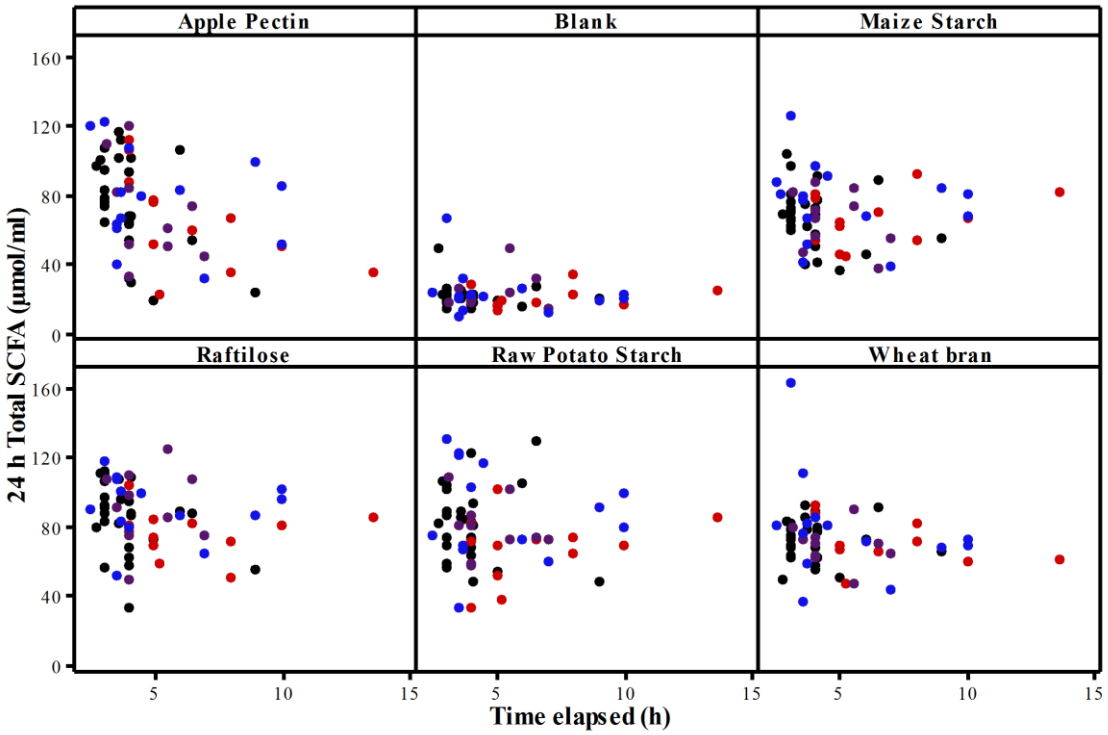
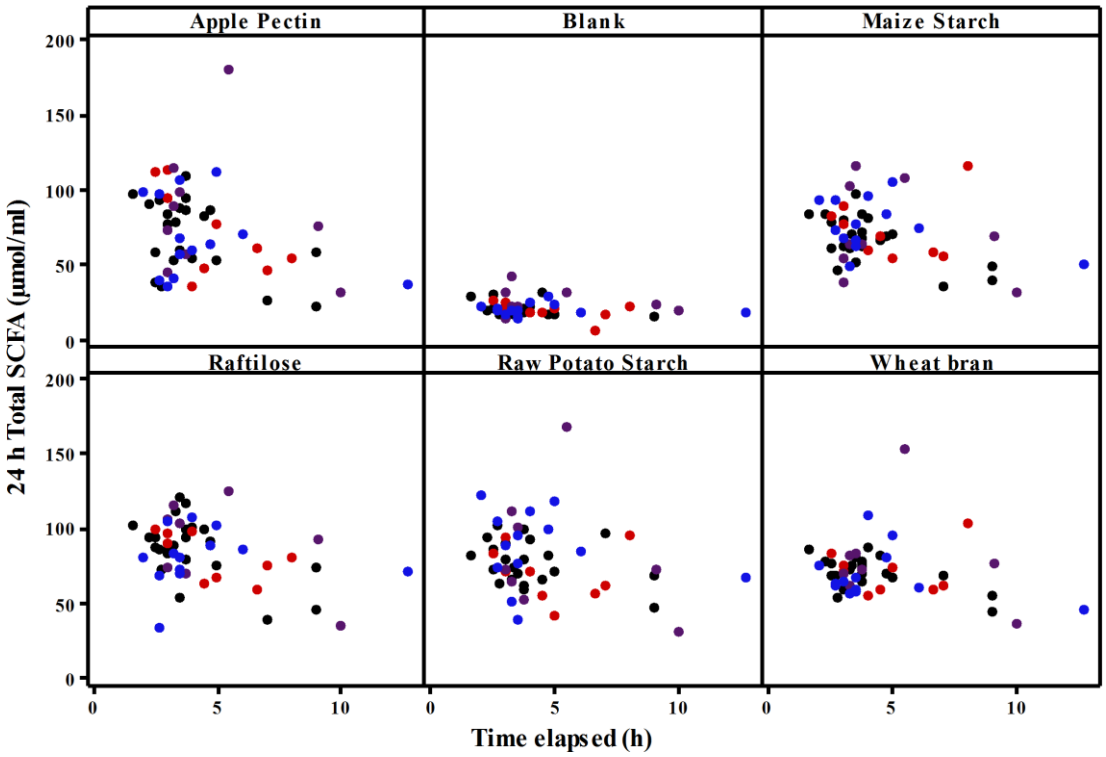


Figure 5.19: Scatter plot showing correlation/association of 24 h SCFA with sample processing time (in h) for different dietary substrates after 2-3 months



Black dots; healthy lean, Red dots; hypothalamic obese, Purple dots; hypothalamic lean, Blue dots; simple obese

Correlations analysis showed strong negative correlations of hypothalamic lean and healthy lean group with the sample processing times across different substrates. However,

general regression analysis adjusted for participants' group (used as an interaction term) showed significant negative associations of 24 h total SCFA production with sample processing time independent of the participants group both at the time of recruitment and after 2-3 months. General regression analysis (adjusted for participant groups) showed significant negative associations between sample processing time (in h) and the production of total SCFA after 24 h incubation in cultures containing apple pectin, both at recruitment (β= -6.274, R^2-adj=16.46%, p=0.001) and after 2-3 months (β= -4.597, R^2-adj=10.01%, p=0.010). Furthermore, significant negative associations between sample processing time and total SCFA production in samples after 2-3 months were also seen in cultures containing maize starch(β= -2.954, R^2-adj= 10.37%, p=0.016), raftilose (β= -3.425, R^2-adj=7.59%, p=0.007), and blank cultures (β= -0.6900, R^2-adj=12.02%, p=0.041). Marginally significant effect (p=0.048) of hypothalamic obese group was seen only in cultures containing maize starch after 2-3 months.

5.7 Discussion

Despite an exceptional diversity of complex dietary carbohydrates in human diets, our metagenome encodes only few enzymes for the digestion of oligo- and polysaccharides. The majority of these complex non-digestible, fermentable carbohydrates are degraded by a wide array of enzymes secreted by up to 1000 known species of gut microbiota into short chain fatty acids which contribute up to 10% towards our daily energy needs (75). Whether this energy generating capability depends on the resident gut microbiota in obese people or whether the nature of diet modulates the efficiency of gut microbiota in these people is controversial.

To the best of our knowledge this is the first study to address the cause or effect relationship between gut microbiota and obesity by comparing obese groups of known and unknown aetiology. Our results suggest no major difference in the fermentation capacity of gut microbiota between simple and hypothalamic obese patients which indicates that the increased energy harvesting capability of gut microbiota reported in other studies may not be causally attributed to obesity. Differences in diet and energy intake, physical activity, genetic propensity towards obesity, or other environmental factors between lean and obese populations may cause changes in the gut microbiota that in turn determine the changes in energy extraction capability from the diet. Moreover, non-significant differences in the production of total SCFA after 24 h incubation between the lean and obese groups also suggest a similar but slightly increased energy harvesting capability in obese than lean group which may indicate that increased harvesting capacity in obese individuals follows

obesity onset. With lower than anticipated study participants and several comparisons necessary to explore differences between the groups using a Mann-Whitney U test might have resulted in false discovery and over-interpretation of some significant results. Results from differences in p-value greater than 0.01 but less than 0.05 should therefore be interpreted with caution. Furthermore, very high interquartile range in our study participants particularly in our obese group reduced the chances of observing significant results.

These results are in accordance with findings from Sarbini *et al*.(2011) (405) who found no significant difference in the fermentation capacity of gut microbiota between lean and obese adults incubated with galacto-oligosaccharides or inulin. Similarly, in the study by Yang *et al.* (2013) (172), no significant difference was observed in total SCFA production after 24 h incubation. Additionally, Payne *et al.* (2012) (95) observed a similar response of normal and high energy nutrient load on the production of SCFA in continuous cultures of faecal bacteria between lean and obese children. Whereas these results agree with our findings, these studies are limited by fewer study numbers, methodological issues, and fewer dietary substrates tested. Our study had a larger cohort and we included a control group. Most of the samples (111/151, ~73% samples) were analysed within 4 h after being produced and were maintained with an anaerobic and cold environment. Furthermore, we analysed the fermentation capacity with a range of dietary fibres producing different pattern of SCFA to test our hypothesis compared to less substrates in previous studies.

Reduction in pH in the gut lumen implicates changes in the gut luminal milieu and increased SCFA production particularly due to the formation of SCFA particularly acetate, which *in vivo,* enhances the absorption of sodium and water from the lumen (406). Lower pH in the incubated samples of our simple obese patients compared to lean group after 2-3 months was suggestive of increased SCFA production particularly acetate. However, in contrast to our expectations, there was a net decrease in the proportion of acetate after 2-3 months which might indicate active utilization of acetate into butyrate or less likely, propionate. As shown in figure 5.12, the rate of change in the proportion of butyrate in simple obese group increased by approximately 5% in the second sample after 2-3 months compared to samples at recruitment which might indicate utilisation of acetate to butyrate by acetate-utilising butyrate-producing bacteria. However, butyrate can also be produced in the gut by conversion of lactate to butyrate by lactate-utilizing butyrate-producing bacteria such as *E. halii* and *Anaerostipes caccae* strains which have been shown to convert lactate to butyrate when pH of the gut lumen decreases (86). However, we did not measure lactate levels in our incubated samples that could give an indication of any activity of this cross-feeding pathway. Moreover, *in-vitro* conditions with only one dietary fibre substrate per

culture unlike diverse food components in the gut *in vivo* may not be the ideal environment to allow and establish these cross feeding pathways and matrix interactions in the fermentation vessels.

Measuring total and individual SCFAs give an idea about the total production but the rate at which these SCFA are produced tend to reduce over the period of incubation due to substrate exhaustion and end product inhibition in the *in-vitro* batch cultures. So we estimated the rate of production by calculating the difference of total and individual SCFA between 0 and 4 h incubation. Consistent with the total amounts after 24 h, the rate of production of total SCFA and acetate production was similar between the two obese groups. Simple and hypothalamic obese groups had a tendency towards a higher rate of change in SCFA production than the two lean groups particularly propionate and butyrate which suggested that gut microbiota of obese participants are efficient in energy extraction compared to the lean. In support of this, the rate of SCFA production between 0-4 h incubation was positively correlated with BMI SDS in obese phenotype but not in lean phenotype.

A significantly higher rate of propionate production between 0-4 h incubation and tendency towards higher concentration and proportion of propionate after 24 h in obese (simple and hypothalamic obese) compared to lean groups (healthy lean and hypothalamic lean) suggested increased propionate producing capability of the gut microbiota in obese phenotype irrespective of the aetiology of obesity. Furthermore, production of propionate was positively correlated with BMI SDS within the obese phenotype. These findings further re-confirmed our results from the faecal samples where we observed increased concentration and proportion of propionate in obese (simple and hypothalamic obese) than lean groups (healthy lean and hypothalamic lean). Similar results were also shown by other studies in the form of lower acetate to propionate ratio (405) and increased concentration and proportion of propionate (172) in obese adults compared to lean.

As discussed in chapter 4 section 4.7, higher levels of propionate have been associated with hypocholesterolemia, reverse cholesterol transport (96), improved satiety through hormone PYY, and decreased meal size via increased leptin (97). Furthermore, it has been shown to exert antiproliferative action on colon cancer cells in experimental cell models (98). The capability of gut microbiota of obese participants to produce this beneficial propionate consistently across different dietary substrates suggests higher propionate-producing bacteria residing in the colon of obese patients. Several studies have found higher faecal propionate in obese versus lean children and adults along with higher relative abundance of propionate producing Bacteroidetes (95, 172, 176, 350). Bacteroidetes express a diverse range of glycosyl-hydrolases capable of degrading a

variety of exogenous dietary and endogenous host glycans allowing this group of bacteria to survive in diverse ecological systems, not only in the gut. As reported in our study in chapter 3, section 3.6.7, and also in a population based study (340), obese people consume significantly lower amounts and proportions of dietary fibre and higher amount of readily available carbohydrates such as simple sugars (which are absorbed from the small intestine) compared to lean population. This suggests that endogenous source of host glycans might be an important and predominant source due to low fibre intake and hence might indicate a "stress situation" of low fibre intake. The capability of obese groups to ferment dietary substrate to produce propionate at a much higher rate than lean groups might therefore be an indication of the ability of obese gut ecosystem to be well-equipped for the low-fibre stress. This might also indicate that the gut microbiota re-shape themselves according to the type of substrate available for utilisation in the gut.

Tendency to produce higher butyrate across most of the dietary fibres was observed in the hypothalamic obese group at recruitment. Furthermore, BMI SDS was significantly positively correlated with butyrate production in the obese phenotype but not in lean phenotype. This could either be due to increased production of butyrate by butyrate producing bacteria or increased production of butyrate by conversion of acetate or lactate to butyrate by acetate- and lactate-utilising bacteria such as *Roseburia* spp. */E. rectale* group (166). As discussed in previous chapter, section 4.7 and also given that the amount of dietary substrate and faecal slurry added to the batch cultures were the same, this trend of increased butyrate was also seen in the spot faecal samples of the same group which strengthens our assumption that higher butyrate in this group might be due to a higher rate of conversion of acetate or lactate to butyrate. This trend towards higher butyrate and lower acetate reversed at follow up in hypothalamic obese group accompanied by an increased pH, indicating that the increased butyrate in the samples at presentation might have been mainly due to the utilization of acetate or lactate into butyrate (166).

Children and young adults attending Yorkhill endocrine and dietetic clinic are monitored for changes in weight and general growth while they are following a prescribed healthy diet and physical activity. However, during the course of the recruitment, a very high rate of non-attendance to the clinic appointments was observed by the researcher suggesting low compliance. No significant changes in dietary macronutrients intake were observed within the groups except for a significant increase in percentage intake of daily protein in simple obese group and in percentage intake of recommended dietary fibre in healthy lean group (chapter 3, section 3.5.9.1). Despite this, a non-significant decrease in median BMI SDS (↓ΔBMI SDS) was observed in all the groups over the period of 2-3 months (chapter 3, Table 3.5). Accordingly, the fermentation capacity of gut microbiota

decreased in the two obese groups for most of the fibres after 2-3 months. Although changes in the concentration of total and major individual SCFA were not significantly different between and within the groups, significant changes in the proportion of SCFA within the groups (such as increase in the rate of change in butyrate in simple obese) suggested that dietetic weight management might beneficially affect the pattern of SCFA production although the total SCFA concentration remains unchanged. Simple obese groups showed significantly higher proportion of butyrate at follow up than at presentation in all fibres except raftilose. This indicates that shifts in the metabolic priorities of gut microbiota towards beneficial SCFA with weight management might be more important than the actual change in total energy production. Whether this response is an effect of altered dietary behaviour or it was an effect of a changed pattern of gut microbiota with dietetic weight management was further assessed by the estimation of dietary intake by 24 h food diary and next generation sequencing of the faecal gut microbiota. Analysis of 24 h food diary showed a significant increase only in the percentage intake of proteins in simple obese group. This might be expected to increase the fermentation of proteins to branched chain fatty acids rather than butyrate depending on the amount of the available fibre and composition of gut microbiota. Alternatively, pathways of butyrate production might have been activated in simple obese group by butyrate producing bacteria such as *Roseburia/E.rectale* group. Although faecal gut microbiota profile showed increased butyrogenic *Roseburia* species belonging to the phylum Firmicutes in obese groups, this is yet to be confirmed in the samples obtained from *in-vitro* fermentation studies as we did not study the gut microbiota in faecal samples obtained via *in-vitro* fermentation. Lastly, this discrepancy between the changes in dietary intake in obese participants and changes in rate of SCFA production in simple obese group may be due to lack of compliance and significant under-reporting of the diet in this group.

The human gut ecosystem is a symbiotic association of aerobic and anaerobic (obligate and facultative) microbiota, the association being outweighed by the anaerobes. The overall fermentation capability and the pattern of SCFA production might be affected by the relative abundance of gut microbiota *in vivo*. In our study, there was a significant impact of the sample processing time on the production of SCFA in cultures containing apple pectin, maize starch, raftilose and the blank cultures both at the time of recruitment and after 2-3 months independent of the study group.

Evidence regarding the effect of sample storage at room temperature, cold (0 ^{o}C or 4 ^{o}C) or freezing conditions (-20 ^{o}C or -80 ^{o}C) on the relative abundance of the gut microbial communities is controversial. Lauber *et al.*(2010) studied the effect of temperature (20 ^{o}C, 4 ^{o}C, -20 ^{o}C, and -80 ^{o}C) and various storage conditions for 3 or 14 days on the soil (n=2),

skin, and faecal gut microbial communities analysed by high throughput 16S rRNA sequencing (298). Clustering of the gut microbiota communities was observed based on the individual sample community and not based on the temperature and storage conditions. Furthermore, the community composition remained stable even after 14 days of storage at 20 ^{o}C, 4 ^{o}C, -20 ^{o}C, and -80 ^{o}C (298). Similar results were also found in study by Roesch *et al.*(2009) in faecal samples from 4 study subjects after 72 h storage of faecal sample at room temperature (407). Carroll *et al.* (2012) in their study on two healthy and two IBS patients found no significant difference in the relative abundance of Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria after storage for 24 h at room temperature and another subset of samples for 6 months at -80 ^{o}C (297). Although overall phylogenetic diversity of gut microbiota remained stable, these studies did observe changes at lower (family) level taxa [such as significant increase in Lachnospiraceae at -20^{o}C storage, reduction in Bacteroideaceae at -80 ^{o}C in study by Lauber *et al.* (2010) and non-significant increase in Actinobacteria over 24 h in study by Carrol *et al.* (2012)]. Lower taxonomic level changes in gut microbiota might influence the fermentation capacity of the specific dietary substrates in in-vitro batch cultures such as that of apple pectin by members of Bacteroideaceae. Furthermore, the sample size of these studies was too small to account for inter-individual variations. Study by Hervais *et al.* (2005) found no effect of storage of sample at 0 ^{o}C for 3 or 6 h or freezing at -18 ^{o}C for 24 h on the cellulose and starch degradation and gas production by ruminal microbiota (408). McBurney and Thompson observed similar substrate fermentibility rankings with oat bran, wheat bran, kidney beans, and guar gum for all 6 faecal sample donors despite the difference in the 24 h SCFA and gas production (381). SCFA and gas production were affected by the source of inoculum, the substrate, and the interaction of the inoculum with the substrate (381). This suggests that the differences seen in our study may not be solely attributable to the processing time of the sample analysis but other factors related to the study participants and the nature of the dietary substrate might also be playing a role in determining differences in fermentation capacity of gut microbiota. In this context, differences in the functionality of gut microbiota (SCFA production) in our study with time might represent natural variation in the study subjects and not the effect of difference in sample processing time. However, the effect on the composition of microbiota on this difference in functionality is not known to us in this study and will be an interesting future aspect to investigate.

Total SCFA production in apple pectin showed a consistent negative correlation with the processing time of the sample in our study. Apple pectin is fermented by *Bifidobacteria*, *Lactobacilli*, *Bacteroides fragilis*, and *Enterococci* (397). Although

significant reduction in the ratio of Firmicutes to Bacteroides have been reported due to long term storage (>50 days) in -80 ^{o}C, a non-significant reduction in Bacteroideaceae was also observed by Lauber *et al.* (2010) in short term (up to 24 h) storage as mentioned above (298). Therefore a reduction in the population of Bacteroidetes might have reduced the extent of SCFA production from fermentation of apple pectin in our study. However, study conducted by Hoyos *et al.* (2014) on faecal sample incubated with apple pectin in obese and lean rats found that the obese rats faecal cultures incubated with apple pectin as a substrate resulted in significant reduction in the relative abundance of *Bacteroidetes* compared to obese cultures without apple pectin (390). The relationship of apple pectin with Bacteroidetes is therefore controversial and needs further investigation.

5.8 Conclusion

In conclusion, our results from *in-vitro* batch culture fermentation studies suggest that fermentation capacity of the gut microbiota do not differ between obesity of different aetiology implicating that factors other than gut microbiota energy harvesting capability may be causally related to the aetiology of obesity. Differences between lean and obese phenotypes are blunted by inter-individual variations particularly in the obese groups. Significantly higher rate of propionate production in obese vs. lean groups is in confirmation with our findings from faecal samples. Furthermore, processing time of the samples negatively affects the production of SCFAs independent of phenotype and pathology.

Whether no difference in functionality extends to the composition of gut microbiota has been explored in subsequent chapter.

Chapter 6: Preliminary Analysis of the Gut Microbiota Composition in Simple and Hypothalamic Obesity

6.1 Outline

We explored gut bacterial metabolic activity in previous sections to see any difference between simple and hypothalamic obese groups. No differences were observed in the faecal metabolites, so the energy harvesting capability of the gut microbiota was further explored by *in-vitro* fermentation studies which confirmed findings from spot faecal samples. Whether changes exist in gut microbiota composition between the different aetiology of obesity and against lean controls was further studied by applying next generation sequencing of the 16S rRNA gene. This chapter outlines preliminary results of this analysis.

Note: Although in the original plan of this PhD we wanted to study selected gut bacteria through quantitative polymerase chain reaction (qPCR), the continuous decreasing cost of high throughput sequencing and emerging collaborations with world leading bioinformaticians within the team allowed us to explore the global faecal microenvironment with these state-of-the-art techniques in all samples from our participants.

6.2 Introduction

The continuous increase in prevalence of obesity worldwide is attributed to several factors such as consumption of energy dense foods, sedentary lifestyle, and familial predisposition but none of these fully explain the aetiology of obesity (15). Obesity is a polygenic disorder but only a small proportion (up to 10%) of obesity is attributed solely to the mutations in the expression of obesogenic traits (such as leptin gene) in obese population (14).

Gut microbiota residing primarily in the proximal colon; carry a whole metagenome which out-numbers human metagenome by 100 times. Since this "microbial organ" has close ties with the physiology of the host and outer environment at the same time, several biochemical functions such as energy harvest from complex polysaccharides to produce SCFA, production of vitamins such as B6 and B12 and other hormonal effects are established in the human host through this relationship with gut microbiota.

6.2.1 Gut microbiota as a cause of obesity

Recently, gut microbiota have been suggested as one of the environmental factors that might affect host energy storage and homeostasis (162). Gut microbiota transplanted into the germ-free mice resulted in ~60% increase in body fat and plasma insulin despite reduced chow intake. Gut microbiota were shown to stimulate hepatic *de novo* lipogenesis by the expression of carbohydrate response element binding proteins and sterol response element binding protein 1, stimulation of hepatic triglyceride storage by supressing fasting induced adipocyte factor, and decrease hepatic and muscle fatty acid oxidation (89). Further to this, evidence also suggested increased energy harvesting capability in the form of SCFA in obese than lean animals (159) and humans (164). Gut microbiota were also shown to play a key role in chronic low-grade inflammation commonly found in obesity (168). These aspects were discussed in detail in preceding chapters of this thesis.

Phylum-wide differences in gut microbiota composition with higher relative abundance of Firmicutes and lower Bacteroidetes were shown in obese than lean phenotype(162). This finding was supported by several other studies from genetic animal models of obesity (203) and diet-induced animal models of obesity (211). However, data from human subjects suggested species and genus level differences between lean and obese such as *Lactobacillus reuteri* (237), *E. coli* (170), and *Staphylococcus aureus*(239) (chapter 1, table 1.5 & 1.6). The mere presence of the gut microbiota in conventionally raised mice compared to amicrobiotic environment in germ-free mice was enough to show higher levels of energy metabolites such as pyruvic acid, citric, fumaric, and malic acid, and higher rate of clearance of cholesterol and triglycerides (213).

The association of changes in markers of aberrant metabolism with gut microbiota supported the causative role of gut microbiota composition in metabolic syndrome. Reversal of metabolic abnormalities with weight loss were shown to be associated with reversal of Firmicutes to Bacteroidetes ratio suggesting that changes in glucose and lipid metabolism in an individual might be influenced by changes in gut microbial composition. Furthermore, the manipulation of the gut microbiota such as introduction of Bifidobacteria have been shown to improve diabetes and endotoxemia (184). Colonic fermentation pattern determine the energy harvesting capability from the diet and a higher Firmicutes to Bacteroidetes ratio in obese than lean people have been shown to be positively correlated with the production of SCFA in the colon (176). Additionally, the anti-obesity effect of conjugated linoleic and linolenic acid derived from polyunsaturated fatty acid in obese women were shown to be produced by the action of *Bifidobacteria, F. prausnitzii,* and *Lactobacilli*. Both linoleic and linolenic acid stimulate PPARα and PPARγ receptor to

exert anti-inflammatory effects and also improve lipid and glucose metabolism (increase HDL, reduce LDL cholesterol, increased insulin sensitivity). The production of these compounds from dietary PUFA is unaffected by intervention with prebiotic which suggest that indigenous gut microbiota might be determining this potential (242). Gut microbiota relative abundance at various taxonomic levels in obese versus lean people and their relationship with pattern of faecal or caecal SCFA has been shown to differ between different studies as discussed in chapter 1 (Table 1.6, Table 1.10).

Evidence regarding this causative role was further supported by human mono- and dizygotic twin studies which showed that obese related twins and their mothers had had lower microbial diversity, shared community structure (higher Firmicutes and lower Bacteroidetes in obese than lean) and metabolic pathways (Firmicutes enriched with transport system genes and Bacteroidetes enriched with carbohydrate metabolism genes) for the degradation of dietary substrate compared to lean (409). Developments in the field of metagenomics revealed differences in the functionality of gut microbiota i.e. the expression of genes related to distinct metabolic pathways in lean versus obese. Microbiota of obese animals and humans were shown to have low gene counts related to metabolic functions (410) and expressed genes involved in detoxification, stress response, and biosynthesis of cobalamin, essential and non-essential amino acids, and gluconeogenic pathways (212, 214, 409, 410). On the other hand, lean animals and humans exhibited genes capable of synthesizing vitamin B6, fermenting plant polysaccharides, butyrate, and propionate (212, 214, 409, 410). Furthermore, the transmissibility of compositional and functional characteristics from obese to lean animals (159) and vice versa (212) in experimental studies strengthened the causative role of gut microbiota in determining host energy homeostasis.

The above mentioned evidence suggested a causative role of gut microbiota in the aetiology of obesity. However, data from both animal and human studies hint towards certain limitations in these studies that need to be taken into account when interpreting these results. For example, germ-free animals with "amicrobiotic environment" were suggested to be resistant to obesity by Backhed *et al.*(162); however, study by Fleissner *et al.* (2010) contradicted this finding as GF mice in their study developed 2-3 fold more weight than CONV mice on a high fat diet and the phylum wide differences in gut microbiota were driven by a single class of Erysipelotrichaceae in Firmicutes (165). Moreover, evidence also suggested that structural differences between lean and obese may not be consistent and may not be important from functional prospects as some studies found high Firmicutes to Bacteroidetes ratio in lean than obese subjects but these differences were unrelated to the metabolic differences in the two groups (220, 224). Yet

others found no differences in the gut microbiota community and metabolic activity between lean and obese adults (260). Some gut microbes such as *L. reuteri* associated with obesity are used as probiotics supplements which have been shown to reduce the absorption of non-cholesterol sterols from the gut through deconjugation of intraluminal bile acids, reduce low-density lipoproteins and apolipoprotein B compared to placebo (411).

Furthermore, differences in the anatomy and physiology of animal models from that of humans is documented and extrapolation of findings in experimental studies may therefore need careful revision. Additionally, differences in the metabolome and microbiome has been observed even between different strains of the same animal models (such as C57BL/6J vs. C57BL/6N) (228). Some differences might also be attributed to the gender of the experimental animals as suggested by Won *et al.* (2013) (214).This, in addition to other methodological, host, and environmental differences in experimental conditions add to the complexity of the relationship. The exact mechanism of how these changes might affect obesity phenotype is still not known.

6.2.2 Gut microbiota as a consequence of obesity

Although differences in gut microbiota between lean and obese phenotype were observed in studies but the effect of diet in determining this capacity was outlined as a primary driver than the gut microbiota themselves (217). Differences in dietary patterns between lean and obese create a peculiar environment that suits both gut microbiota and host to maintain this symbiotic relationship albeit at a new threshold that may result in increased energy harvest and obesity (166). Changes in gut microbiota have been observed with changes in dietary habits such as anorexia nervosa (236). Patients with this dietary behaviour have been shown to have higher methanogens than lean and obese (236). Some interventional studies reported changes in gut microbiota with change in proportion of macronutrients in isocaloric diets (223), while others reported changes in energy harvest with changes in the nutrient load (95, 265) or caloric restriction coupled with exercise (263). Change in the nature of the diet such as a switch over to a strictly vegetarian diet was shown to improve markers of the metabolic syndrome (170). Using diet, dietary supplementation with prebiotics, or diet with live cultures of gut microbiota (probiotics) as therapeutic alternative for the treatment of obesity has therefore been attempted to treat diet-induced obesity-related increase in metabolic alteration (193, 412). However, the gut microbiota restructure or develop “resistance” to the intervention over the period of weeks to months to blunt the effect of pre-, pro- or antibiotics (413). Faecal transplantation

studies attempted in human studies reported changes in the gut microbial community and improvement in the insulin sensitivity and markers of inflammation at least until the period of follow-up (414). However, the authors observed a gradual decrease in response and restructuring of gut microbiota over the period of 4 weeks. The fact that germ free animals were able to develop the obese phenotype upon transplantation of gut microbiota from lean or obese donors might have been due to the lack of immunogenic response that develops after birth and after exposure to environmental antigens (415). Additionally, these animals develop alternative preferential metabolic pathways to meet the body energy requirements such as reduced energy expenditure compared to CONV mice and the ability to absorb more glucose compared to conventionalised animals (165). This highlights the importance of innate immune and genetic factors of the host that could potentially affect the colonisation of certain preferred set of gut microbiota in early infancy and hence the metabolic phenotype.

Pattern of colonisation of gut microbiota in early infancy and childhood may influence the immune status and community structure of the gut microbiota in later life. Children born through normal vaginal delivery harbour gut microbiota communities broadly similar to their parents (138, 147, 150) while those born via caesarean section harbour gut microbiota unrelated to maternal gut microbial community. Additionally, the presence of certain species and genera (such as *Staphylococcus aureus*) in early childhood are correlated with obesity while others with leanness (such as *Bifidobacteria*) in later life (229). Population based studies suggest that children exposed to antibiotics within 6 months of their life are prone to develop obesity in later childhood (251) which indicate the role of antibiotics in modulation of gut microbiota and hence energy balance. Furthermore, children exposed to the commonly used antibiotic amoxicillin had lower *Bifidobacteria* and *Bacteroides* compared to children not exposed to antibiotics (150). Although obesity in childhood potentially contributed by gut microbiota is associated with obesity in adulthood in 50-70% of cases, only 20% of obese adults are reported to be obese during their childhood (20). The link between obesity in childhood and obesity in adulthood is therefore missing and the role of gut microbiota in this missing link is still poorly understood.

6.2.3 Conclusion and aim of this chapter

On the balance of evidence, the debate remains to determine whether gut microbiota cause obesity or they are a consequence of a changed dietary pattern in obese population to harvest increased energy from the diet. We attempted to prove this “reverse causality” by a human observational study; comparing two groups of obese children/young adults, one

with a known of cause of obesity (such as Prader Willi Syndrome or craniopharyngioma) and another with no known cause of obesity (commonly referred to as simple obesity or classical obesity). Our hypothesis was based on the assumption that if gut microbiota were to be implicated as a cause of obesity, we would expect to see differences in the gut microbiota between obesity of different aetiology and vice-versa if they were an effect of diet. Bacterial metagenome was sequenced by high throughput next generation sequencing to address this hypothesis.

This is an on-going research project. 16S rRNA gene amplification using barcoded fusion primers was done initially to sequence bacterial metagenome of all our participants which is still under statistical bio-informatics analysis. Furthermore, metagenomic libraries were created for 96 samples from participants in all our groups to look into the differences in functional representation of genes in obesity of different aetiology. However, due to time restraints for the timely completion of this PhD, we have only been able to present the preliminary analysis of the data from 16S rRNA gene sequencing while data for microbial metagenomics is not included in this thesis and will be published as soon as it is analysed.

6.3 Patients and methods

6.3.1 Patients

Detailed description of participants and the methods used is given in chapter 2. Briefly, simple obese (n=16), hypothalamic obese (n=10), and hypothalamic lean (n=12) participants were recruited from the endocrine and dietetic clinics at Royal Hospital for Sick Children Glasgow. Healthy lean (n=27) participants were recruited from the community. Two faecal samples along with body composition data and 24 h food diary were collected from each participant at an interval of 2-3 months as described in chapter 2, section 2.2.4.

6.3.2 Laboratory methods (details in chapter 2, section 2.3.10)

Preparation of the amplicon pool

Genomic bacterial DNA was extracted from all faecal samples using chaotropic method discussed in detail in chapter 2, section 2.3.9). 16S rRNA gene of the double stranded DNA was amplified by polymerase chain reaction targeting V4 region of the 16S rRNA gene using barcoded fusion reverse primers (Golay barcoded primers) (chapter 2, section

2.3.10.1). Each amplified sample was subjected to agarose gel electrophoresis (1% agarose gel in 1X TAE buffer) followed by extraction of DNA bands under UV light (chapter 2, section 2.3.10.2). Each DNA band was purified using QIAquick gel extraction kit (Cat. no. 28705 QIAGEN® Germany) (chapter 2, section 2.3.10.3). The concentration of resultant purified DNA was measured by Qubit® 2.0 flourometer (life® technologies, USA) (chapter 2, section 2.3.10.4).

Emulsion PCR and sequencing

Emulsion PCR followed by sequencing was done on illumina® Miseq platform (illumina®, USA) with 2 x 250 bp reads. This was done in Bioscience laboratory, University of Birmingham as we did not have these facilities within the University of Glasgow at the time of this study. The principle of this procedure is given in chapter 2, section 2.3.10.

6.4 Bioinformatics and Statistics

For the gut microbiota analysis, sequence reads from illumina Miseq® were filtered for quality to reduce ubiquitous artefacts. They were further followed by trimming to a fixed length, overlapped and consensus determined, chimeras were removed and operational taxonomic units (OTUs) constructed with UPARSE at 3% sequence difference (416). OTUs are groups of sequences that are intended to correspond to taxonomic clades or monophyletic groups. UPARSE is a recently devised pipeline (software) for the construction of OTUs by Edger (416). The reads were also taxonomically classified using the RDP stand-alone classifier. After filtering, only samples with more than 10,000 reads were used in the following analysis. To determine the impact of obesity, pathology and the result of dietary intervention on community structure, two-way nested permutational multivariate analyses of variances were performed using genera frequencies or UParse 3% OTU frequencies. Bray-Curtis distances were used following normalisation of relative frequencies.

Values for the relative abundance of gut microbiota were expressed as median percentage relative abundance and the frequency was expressed as log10 logarithmic scale. Kruskal-Wallis non-parametric ANOVA was used to calculate differences (significant at p-value <0.05) between the groups. To account for false positive or false negative significant differences due to multiple comparisons, Benjamini-Hochberg false discovery rate test (expressed as *pa*) was used and *pa* value <0.1 was considered significant. Non-metric

multidimensional scale (NMDS) plots were used to show the position of the bacterial community structure in relation to obese or lean phenotype. The NMDS scale is expressed as rank order of the bacterial community (and not the absolute or relative abundance). To group the similar communities, Bray-Curtis distances using matrix of dissimilarities were used.

All bioinformatics analysis and statistics discussed below was performed by Dr Christopher Quince and Dr Umar Ijaz from the School of Engineering, University of Glasgow.

6.5 Results

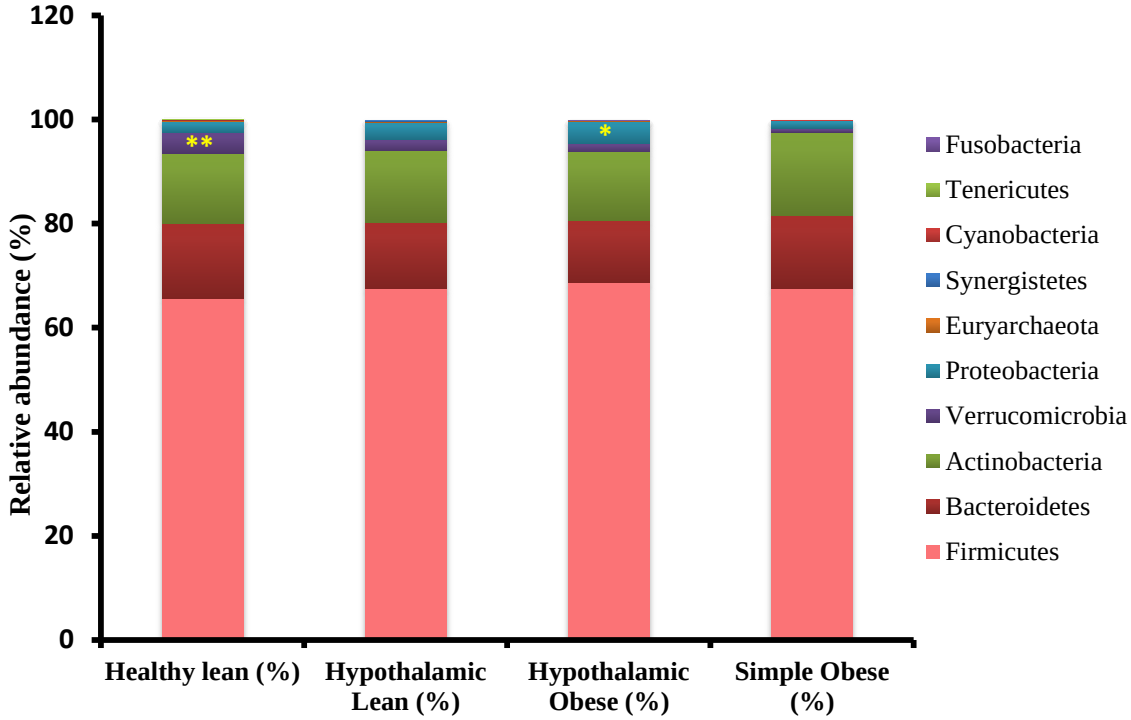
Two way nested permutational multivariate analysis of variance showed no significant differences in community genera composition between the sample at recruitment and after 2-3 months) (R^2=0.00364, *Pr* (>F) =0.9298) and OTU composition (R^2=0.00333, *Pr* (>F) =0.9974). Hence, all subsequent analysis was done combining all the samples at both time points (samples at recruitment + samples after 2-3 months) considering them as biological replicates of each other.

6.5.1 Relative abundance of gut microbiota composition

6.5.1.1 Phylum level differences in relative abundance between the groups

Relative abundance of majority of the phyla detected in the participants were contributed by Firmicutes (66.8%) and Bacteroidetes (16%), followed by Actinobacteria (11.9%) (Table 6.1, Figure 6.1). No significant differences in the relative abundance of major phyla were observed between simple and hypothalamic obese groups except Proteobacteria which was significantly higher in hypothalamic obese group compared to simple obese group (hypoth. obese; 4.3% vs. simple obese; 1.5%, $p<0.05$). Additionally, healthy lean group had a significantly higher relative abundance of Verrucomicrobia compared to simple obese group (healthy lean; 4.1% vs. simple obese; 0.70%, $p<0.01$) (Table 6.1, Figure 6.1). Phylum Archaea, consisting of methanogens, were not detected in simple obese group in contrast to the healthy lean participants.

Figure 6.1: Percentage relative abundance of different phyla in all the groups (samples at the time of recruitment are combined with samples after 2-3 months)



Different colours in the bars represent specific phyla given on the right.* indicate significant difference of Proteobacteria in hypothalamic obese group from simple obese. ** indicate significant difference of Verrucomicrobia in healthy lean from the simple obese group

Table 6.1: Relative abundance of phylum level gut microbial composition in all the groups

Phylum level taxonomy	Total (%)	Healthy lean (%)	Hypothalamic Lean (%)	Hypothalamic Obese (%)	Simple Obese (%)
Firmicutes	66.80	65.70	67.50	68.60	67.50
Bacteroidetes	16.00	14.40	12.70	12.00	14.10
Actinobacteria	11.90	13.30	13.80	13.20	16.00
Verrucomicrobia	2.80	**4.10**	2.20	**1.60**	**0.70**
Proteobacteria	2.30	2.10	3.30	**4.30**	**1.50**
Euryarchaeota	0.10	0.30	0.20	0.10	0.00
Synergistetes	0.00	0.00	0.20	0.00	0.00
Cyanobacteria	0.00	0.10	0.00	0.00	0.10
Tenericutes	0.00	0.10	0.00	0.00	0.00
Fusobacteria	0.00	0.00	0.00	0.10	0.00
Acidobacteria	0.00	0.00	0.00	0.00	0.00
Gemmatimonadetes	0.00	0.00	0.00	0.00	0.00
Lentisphaerae	0.00	0.00	0.00	0.00	0.00
Spirochaetes	0.00	0.00	0.00	0.00	0.00
TM7	0.00	0.00	0.00	0.00	0.00

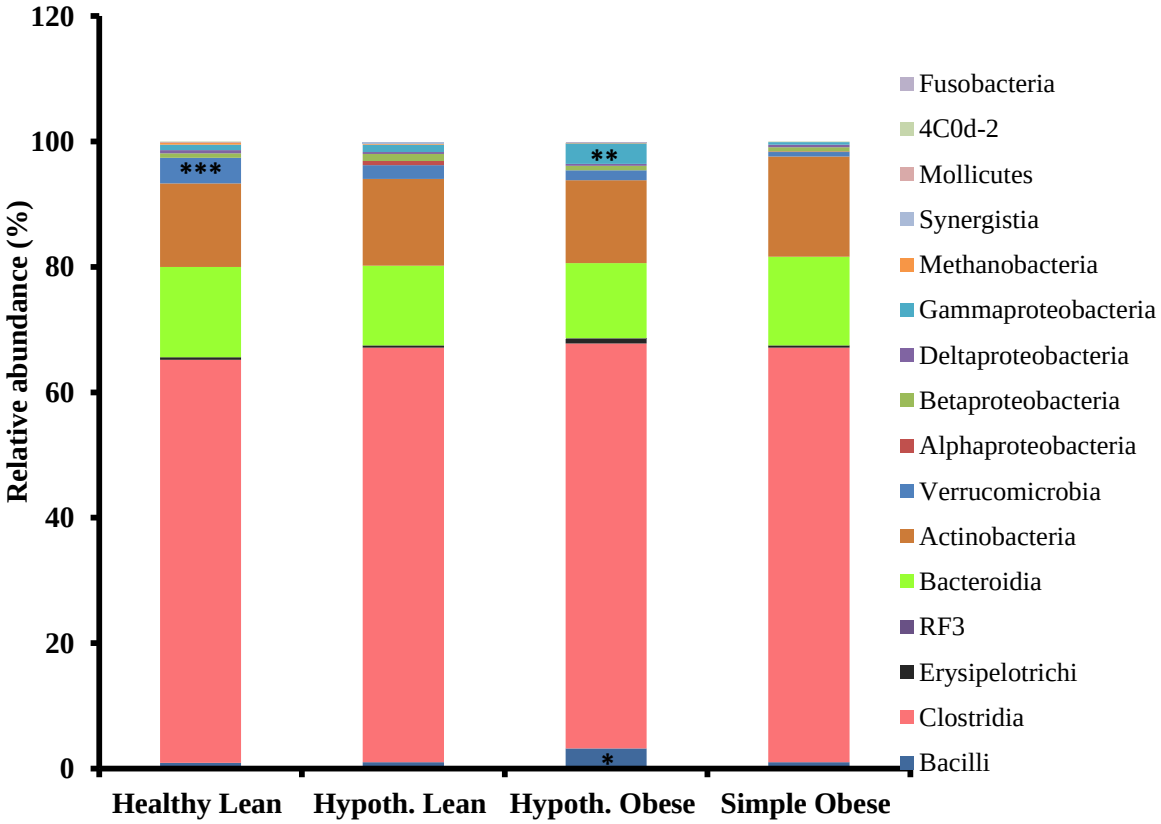
Figures in bold indicate significant differences between the groups

6.5.1.2 Class level differences in relative abundance between the groups

Differences in relative abundance at phylum level extended to the class level taxonomy. The difference between hypothalamic obese and simple obese in phylum Proteobacteria was contributed mainly by the class Gammaproteobacteria (hypoth. obese; 3.30% vs.

simple obese; 0.40%, p<0.01) (Table 6.2, Figure 6.2). However, despite no differences in Firmicutes at phylum level, the relative abundance of Bacilli, belonging to Firmicutes, was significantly different between the hypothalamic obese group and simple obese group (hypoth. obese; 3.20% vs. simple obese;1.00%, p<0.01) (Table 6.2, Figure 6.2). This indicates that differences at higher taxonomic level may not be sufficient to reveal differences at lower taxonomic levels which can impact the metabolism of the gut microbiota within and between the groups.

Figure 6.2: Percentage relative abundance of different classes in all the groups (samples at the time of recruitment are combined with samples after 2-3 months)



Different colours in the bars represent specific classes given on the right.* indicate significant difference of Bacilli in hypothalamic obese group from all other groups. ** indicate significant difference of Gammaproteobacteria in hypothalamic obese from the simple obese group, *** indicate significant difference of Verrucomicrobia in healthy lean from simple obese.

Table 6.2: Relative abundance of Class level gut microbial composition in all the groups

Phylum	Class	Total (%)	Healthy Lean (%)	Hypoth. Lean (%)	Hypoth. Obese (%)	Simple Obese (%)
Firmicutes	**Bacilli**	1.30	0.90	1.00	**3.20***	**1.00***
	Clostridia	64.80	64.30	66.10	64.60	66.10
	Erysipelotrichia	0.70	0.40	0.40	0.70	0.40
	CK-1C4-19	0.00	0.00	0.00	0.00	0.00
	RF3	0.00	0.00	0.00	0.10	0.00
Bacteroidetes	**Bacteroidia**	16.00	14.40	12.70	12.00	14.10
	Flavobacteria	0.00	0.00	0.00	0.00	0.00
	Sphingobacteria	0.00	0.00	0.00	0.00	0.00
Actinobacteria	**Actinobacteria**	11.90	13.30	13.80	13.20	16.00
Verrucomicrobia	**Verrucomicrobia**	2.80	**4.10***	2.20	1.60	**0.70***
Proteobacteria	**Alphaproteobacteria**	0.10	0.00	0.70	0.00	0.10
	Betaproteobacteria	0.70	0.70	1.10	0.70	0.70
	Deltaproteobacteria	0.40	0.50	0.30	0.30	0.40
	Epsilonproteobacteria	0.00	0.00	0.00	0.00	0.00
	Gammaproteobacteria	1.10	0.90	1.20	**3.30***	**0.40***
Euryarchaeota	**Methanobacteria**	0.10	0.30	0.20	0.10	0.00
	Thermoplasmata	0.00	0.00	0.00	0.00	0.00
Synergistetes	**Synergistia**	0.00	0.00	0.20	0.00	0.00
Tenericutes	**Mollicutes**	0.00	0.10	0.00	0.00	0.00
Cyanobacteria	**4C0d-2**	0.00	0.10	0.00	0.00	0.10
	Chloroplast	0.00	0.00	0.00	0.00	0.00
Fusobacteria	**Fusobacteria**	0.00	0.00	0.00	0.10	0.00
Gemmatimonadetes	**Gemm-5**	0.00	0.00	0.00	0.00	0.00
Lentisphaerae	**Lentisphaeria**	0.00	0.00	0.00	0.00	0.00
Spirochaetes	**Spirochaetes**	0.00	0.00	0.00	0.00	0.00
	Brachyspirae	0.00	0.00	0.00	0.00	0.00
TM7	**TM7-3**	0.00	0.00	0.00	0.00	0.00
Acidobacteria	**Acidobacteria-2**	0.00	0.00	0.00	0.00	0.00

*indicate significant differences between the two highlighted groups in a given row

6.5.1.3 Order level differences in relative abundance between the groups

At order level taxonomy, hypothalamic obese group had significantly higher relative abundance of Aeromonodales and Enterobacteriales in class Gammaproteobacteria than simple obese group (Aeromonodales: hypoth. obese; 0.80% vs. simple obese; 0.00%, $p<0.01$ and Enterobacteriales: hypothalamic obese; 1.60% vs. simple obese; 0.30%, $p<0.01$) (Table 6.3).

6.5.1.4 Family level differences

Differences in Aeromonodaceae and Enterobacteriaceae belonging the phylum Firmicutes were also observed at family level (Aeromonodaceae: hypoth. obese; 0.80% vs. simple obese; 0.00%, $p<0.01$ and Enterobacteriaceae: hypothalamic obese; 1.60% vs. simple obese; 0.30%, $p<0.01$) (Table 6.4). In addition, differences observed in Bacilli at class level between hypothalamic obese and simple obese were more pronounced in family Lactobacillaceae (Lactobacillaceae: hypoth. obese; 1.20% vs. simple obese; 0.50%, $p<0.05$) and Streptococcaceae (hypoth. obese; 1.90% vs. simple obese; 0.40%, $p<0.01$) (Table 6.4).

Table 6.3: Relative abundance of Order level gut microbial composition in all the groups

Phylum	Order	Total	Healthy Lean	Hypoth. Lean	Hypoth. Obese	Simple Obese
Euryarchaeota	Methanobacteriales	0.10	**0.30**	**0.20**	**0.10**	**0.00**
	E2	0.00	0.00	0.00	0.00	0.00
Firmicutes	Bacillales	0.00	0.00	0.00	0.00	0.00
	Gemellales	0.00	0.00	0.00	0.00	0.00
	Lactobacillales	1.20	0.80	1.00	**3.10***	**0.90***
	Turicibacterales	0.10	0.10	0.00	0.10	0.10
		3.00	3.00	2.80	3.20	2.80
	Clostridiales	51.60	51.90	57.50	49.40	52.50
	Coriobacteriales	10.20	9.30	5.90	12.00	10.90
	Desulfitobacterales	0.00	0.00	0.00	0.00	0.00
	SHA-98	0.00	0.00	0.00	0.00	0.00
	Erysipelotrichales	0.70	0.40	0.40	0.70	0.40
Fusobacteria	Fusobacteriales	0.00	0.00	0.00	0.10	0.00
Bacteroidetes	Bacteroidales	16.00	14.40	12.70	12.00	14.10
	Flavobacteriales	0.00	0.00	0.00	0.00	0.00
	Sphingobacteriales	0.00	0.00	0.00	0.00	0.00
Actinobacteria	Actinomycetales	0.10	0.10	0.20	0.20	0.10
	Bifidobacteriales	11.80	13.10	13.60	13.00	15.90
Verrucomicrobia	Verrucomicrobiales	2.80	**4.10***	2.20	1.60	**0.70***
Cyanobacteria	YS2	0.00	0.10	0.00	0.00	0.10
	Streptophyta	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Caulobacterales	0.00	0.00	0.00	0.00	0.00
	RF32	0.10	0.00	0.70	0.00	0.10
	Rhizobiales	0.00	0.00	0.00	0.00	0.00
	Rickettsiales	0.00	0.00	0.00	0.00	0.00
	Sphingomonadales	0.00	0.00	0.00	0.00	0.00
	Burkholderiales	0.70	0.60	1.10	0.70	0.70
	Neisseriales	0.00	0.00	0.00	0.00	0.00
	Desulfovibrionales	0.40	0.50	0.30	0.30	0.40
	Campylobacterales	0.00	0.00	0.00	0.00	0.00
	Aeromonadales	0.20	0.20	0.00	**1.50***	**0.00***
	Enterobacteriales	0.70	0.60	1.00	**1.60***	**0.30***
	Oceanospirillales	0.00	0.00	0.00	0.00	0.00
	Pasteurellales	0.10	0.10	0.10	0.10	0.00
	Pseudomonadales	0.10	0.10	0.10	0.10	0.00
	Xanthomonadales	0.00	0.00	0.00	0.00	0.00
Synergistetes	Synergistales	0.00	0.00	0.20	0.00	0.00
TM7		0.00	0.00	0.00	0.00	0.00
Tenericutes	Anaeroplasmatales	0.00	0.00	0.00	0.00	0.00
	RF39	0.00	0.10	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00
Gemmatimonadetes	Victivallales	0.00	0.00	0.00	0.00	0.00
Lentisphaerae	Spirochaetales	0.00	0.00	0.00	0.00	0.00
Spirochaetes	Brachyspirales	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00
Acidobacteria		0.00	0.00	0.00	0.00	0.00

*indicate significant differences between the two highlighted groups in a given row.

Table 6.4: Relative abundance of family level gut microbial composition in all the groups

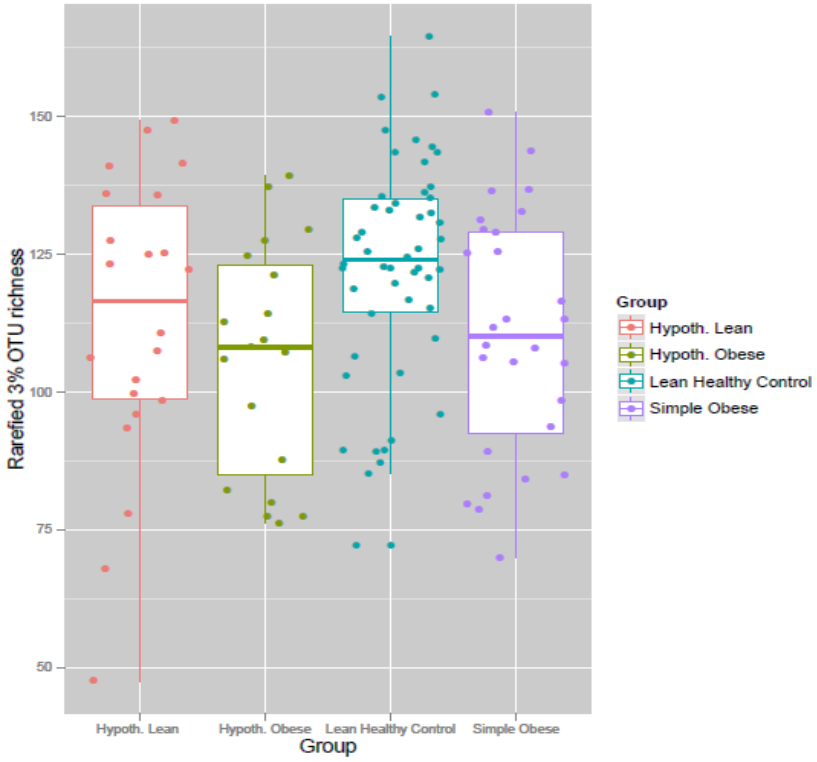
Phylum	Family	Total (%)	Healthy Lean (%)	Hypoth. Lean (%)	Hypoth. Obese (%)	Simple Obese (%)
Euryarchaeota	Methanobacteriaceae	0.10	**0.30**	0.20	0.10	**0.00**
Acidobacteria		0.00	0.00	0.00	0.00	0.00
Actinobacteria	Actinomycetaceae	0.10	0.10	0.20	0.20	0.10
	Un-named	1.10	1.50	0.70	1.10	1.80
	Bifidobacteriaceae	10.6	11.6	12.9	11.9	14.1
Bacteroidetes	Bacteroidaceae	4.70	4.80	7.90	4.40	4.20
	Porphyromonadaceae	0.40	0.60	0.50	0.80	0.40
	Prevotellaceae	9.10	5.10	2.30	6.10	8.30
	Rikenellaceae	0.70	1.50	1.10	0.30	0.40
	S24-7	0.30	0.70	0.00	0.10	0.10
	Barnesiellaceae	0.40	0.80	0.60	0.20	0.30
	Odoribacteraceae	0.10	0.20	0.20	0.10	0.10
	Paraprevotellaceae	0.30	0.70	0.20	0.10	0.40
Cyanobacteria		0.00	0.10	0.00	0.00	0.10
Firmicutes	Lactobacillaceae	0.40	0.00	0.10	**1.20***	**0.50***
	Streptococcaceae	0.80	0.80	0.90	**1.90***	**0.40***
	Turicibacteraceae	0.10	0.10	0.00	0.10	0.10
	Un-named	3.00	3.00	2.80	3.20	2.80
	Un-named	1.30	2.30	1.10	1.70	0.70
	Catabacteriaceae	0.10	0.20	0.10	0.00	0.10
	Christensenellaceae	0.00	0.10	0.00	0.00	0.10
	Clostridiaceae	0.40	0.40	0.50	0.40	0.30
	Lachnospiraceae	24.4	23.2	27.1	26.8	23.5
	Peptococcaceae	0.10	0.00	0.00	0.00	0.00
	Peptostreptococcaceae	1.00	1.10	1.00	1.80	1.20
	Ruminococcaceae	16.5	19.4	18.6	11.8	18.3
	Veillonellaceae	7.80	5.20	8.90	6.90	8.20
		2.80	3.10	2.50	2.30	3.00
	Coriobacteriaceae	7.40	6.30	3.40	9.70	7.90
	Desulfitobacteraceae	0.00	0.00	0.00	0.00	0.00
	Erysipelotrichaceae	0.30	0.10	0.10	0.40	0.10
	Coprobacillaceae	0.30	0.30	0.30	0.40	0.20
Fusobacteria	Fusobacteriaceae	0.00	0.00	0.00	0.10	0.00
Lentisphaerae	Victivallaceae	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Desulfovibrionaceae	0.40	0.50	0.30	0.30	0.40
	Un-named	0.10	0.00	0.70	0.00	0.10
	Alcaligenaceae	0.70	0.60	1.10	0.70	0.60
	Burkholderiaceae	0.00	0.00	0.00	0.00	0.00
	Oxalobacteraceae	0.00	0.00	0.00	0.00	0.00
	Neisseriaceae	0.00	0.00	0.00	0.00	0.00
	Campylobacteraceae	0.00	0.00	0.00	0.00	0.00
	Un-named	0.10	0.10	0.00	0.70	0.00
	Aeromonadaceae	0.10	0.10	0.00	**0.80***	**0.00***
	Enterobacteriaceae	0.70	0.60	1.00	**1.60***	**0.30***
	Pasteurellaceae	0.10	0.10	0.10	0.10	0.00
	Moraxellaceae	0.10	0.10	0.10	0.10	0.00
Spirochaetes	Spirochaetaceae	0.00	0.00	0.00	0.00	0.00
	Brachyspiraceae	0.00	0.00	0.00	0.00	0.00
Synergistetes	Dethiosulfovibrionaceae	0.00	0.00	0.00	0.00	0.00
	Synergistaceae	0.00	0.00	0.20	0.00	0.00
TM7	*Un-named*	0.00	0.00	0.00	0.00	0.00
Tenericutes	Anaeroplasmataceae	0.00	0.00	0.00	0.00	0.00
	Un-named	0.00	0.10	0.00	0.00	0.00
Verrucomicrobia	Verrucomicrobiaceae	2.80	**4.10***	2.20	1.60	**0.70***

*indicate significant differences between the two highlighted groups in a given row.

6.5.2 Richness of Operational taxonomic units (OTU) in all groups

There was a significant relationship (p-value = 0.0227) between group and rarefied OTU richness. However, both obese groups (simple and hypothalamic obese) had a lower diversity relative to Lean groups (healthy and hypothalamic lean) (Figure 6.3).

Figure 6.3: Boxplots of rarefied OTU richness in all groups.



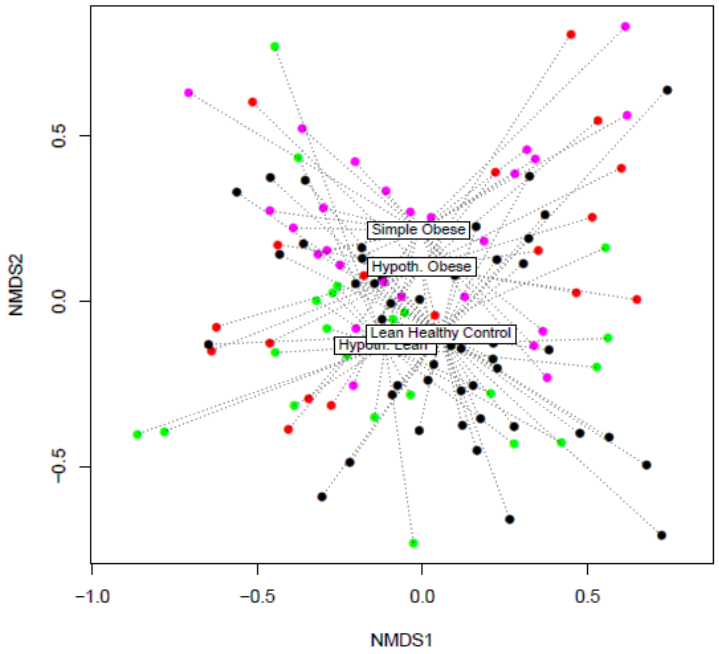
Colour coding for the groups is given on the right of the figure. Individual dot represent each participant. OTU; operational taxonomic unit based on 97% similarity

6.5.3 Impact of obesity and pathology on the community genera composition

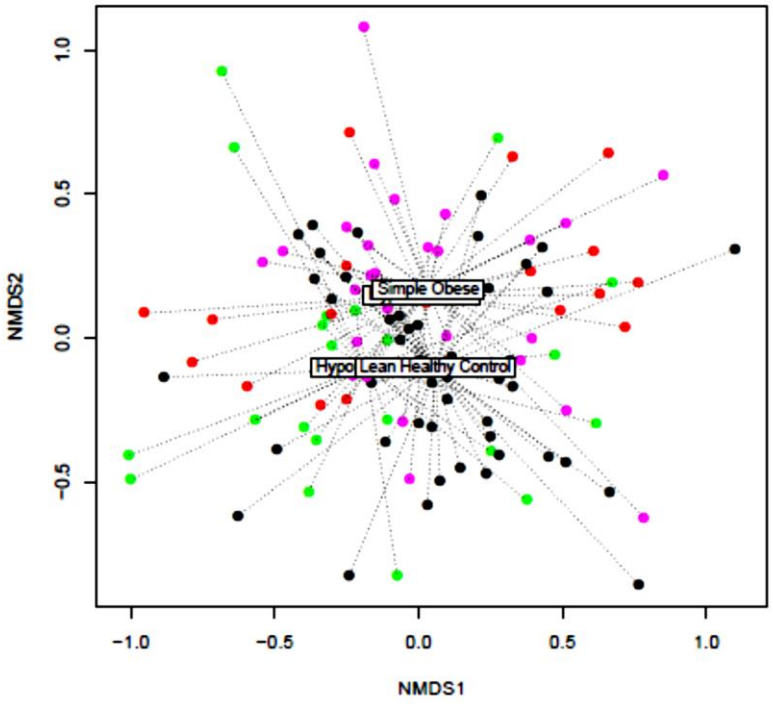
To determine the impact of obesity, pathology and the result of dietary intervention on genus level and OTU level community structure, two nested permutational multivariate analysis of variances were performed. The first used genera frequencies from Ribosomal Database Project (RDP) and the second the UPARSE 3% OTU frequencies. In both cases, Bray-Curtis distances were used following normalisation of relative frequencies. The results were similar, indicating a highly significant effect of obesity on community structure both at genera (R^2=0.0326, *Pr* (>F) =0.0005) and OTUs level (R^2=0.0287, *Pr* (>F) =9.99E-05) and a mildly significant impact of pathology at genus level (R^2=0.0166, *Pr* (>F) =0.0422) and OTU level (R^2=0.0157, *Pr* (>F) =0.0125) (Figure 6.4a, b). However, only 5% in the variation of communities was explained by pathology and adiposity together.

Figure 6.4: Non-metric multidimensional scale (NMDS) plot of genus level (a) and OTU level (b) community compositions using Bray-Curtis distances.

a. NMDs plot of genus level community composition



b. NMDS plot of OTU level community composition



NMDS1 and NMDS2 Scale is the rank-order of the bacterial community in a two dimensional space. Colour of the dot represent group of the participant; magenta dots-simple obese, red dots-hypothalamic obese, black dots-healthy lean, and green dots-hypothalamic lean. Each dot represents a participant. The line from each dot converges to a centre based on similarity of genera community to a central point. In Figure 6.4b, hypothalamic obese group is hidden behind simple obese group while hypothalamic lean group is hidden behind healthy lean group suggesting a high degree of similarity between the groups based on their phenotype.

6.5.4 Community composition of genera in relation to obesity and pathology

In the presence of no significant difference in the community genus and OTU level composition and subsequent clustering based on phenotype and not pathology, the analysis of OTU level and genus level composition was performed by grouping obese (simple + hypothalamic obese) and lean (healthy lean + hypothalamic lean) participants.

6.5.4.1 Community genera composition in relation to obesity

Of more than 200 genera identified, relative abundance of only 11 genera shown in table 6.5 were found to be significantly different between the lean and obese groups. All genera with a false discovery rate of less than 10% are shown. Relative abundance of *Dorea, Collinsella, Lactobacillus, Megamonas,* and *Gemmiger* were significantly higher in obese while *Veillonella, Pasteurellaceae, Alistipes, Oscillibacter, Clostridium cluster XVIII,* and *Rothia* were significantly higher in lean than obese group (Table 6.5). The most significant effects included a higher frequency of *Dorea* (Figure 6.5) and *Collinsella* in Obese participants and a lower frequency of *Veillonella* and *Alistipes* in lean participants (Table 6.5, Figure 6.5).

Table 6.5: Relative abundance (mean percentage abundance) of genera differing significantly between lean and obese groups.

Genera	Lean (%)	Obese (%)	*p*	*pa*
Dorea	1.26	2.57	3.66E-06	0.000235
Veillonella	1.95	0.266	6.09E-06	0.000235
Collinsella	3.04	6.27	1.66E-04	0.00427
Lactobacillus	0.0618	0.245	7.51E-04	0.0144
Unclassified Pasteurellaceae	0.0572	0.00696	1.56E-03	0.0213
Alistipes	0.522	0.182	1.66E-03	0.0213
Megamonas	0.253	2.17	2.10E-03	0.022
Oscillibacter	0.544	0.195	2.28E-03	0.022
Clostridium cluster XVIII	0.0251	0.00358	3.17E-03	0.0271
Rothia	0.00284	0.000559	6.80E-03	0.0524
Gemmiger	1.21	2.07	8.51E-03	0.0596

The mean %age abundances in the two groups are given along with the p-value from a Kruskal-Wallis non-parametric ANOVA (*p*) and the Benjamini-Hochberg false discovery rate (*pa*) to account for multiple comparisons. All genera with pa < 0.1 were judged significant.

6.5.4.2 Community genera composition in relation to pathology

In contrast to the much higher differences in the genera based on obese or lean phenotype, only four genera (*Coprococcus, Eggerthella, Collinsella, and Flavonifractor*) differed

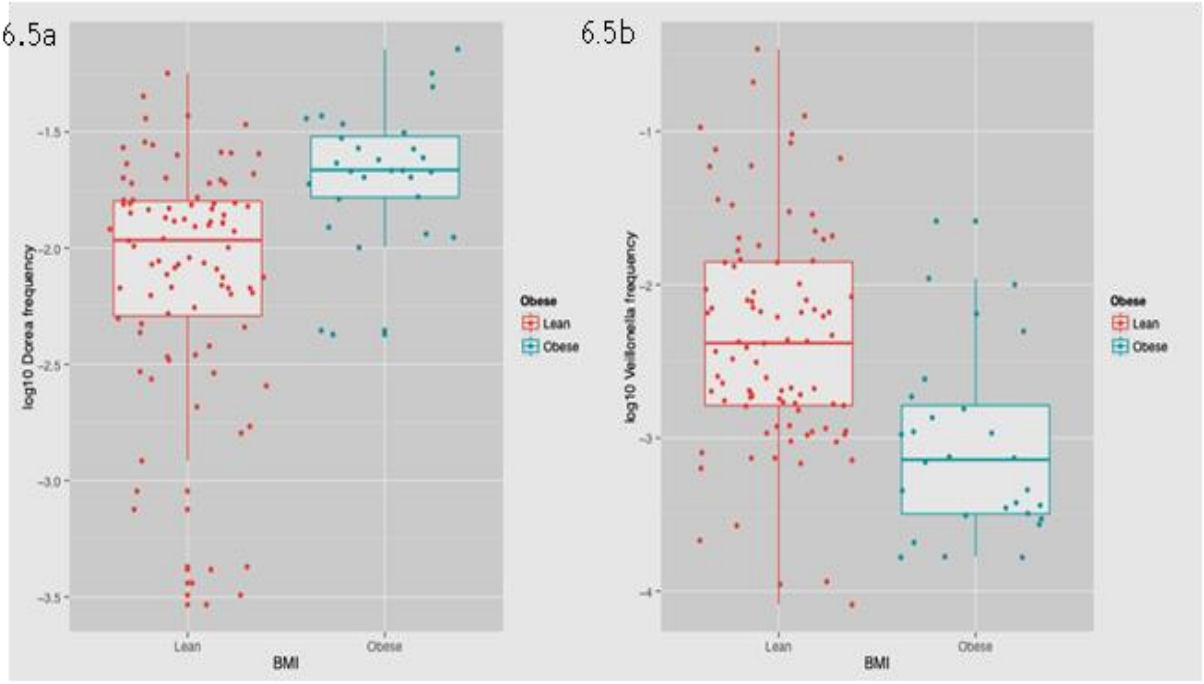
significantly in abundance between healthy and pathological children (Table 6.6). Coprococcus and Collinsella were significantly higher in healthy vs. pathological group (p=0.00031 & p=0.00164) while Eggerthella and Flavonifractor were significantly higher in pathological vs. healthy group (p=0.00039 & p=0.00248).

Table 6.6: Relative abundance (mean percentage abundance) of genera differing significantly between healthy (lean healthy + simple obese) and pathological (hypothalamic lean + obese) groups.

Genera	Healthy (%)	Pathology (%)	*P*	*pa*
Coprococcus	1.220	0.683	3.10E-04	0.0151
Eggerthella	0.156	0.196	3.92E-04	0.0151
Collinsella	4.150	3.140	1.64E-03	0.0422
Flavonifractor	0.077	0.267	2.48E-03	0.0478

Healthy; healthy lean + simple obese, pathology; hypothalamic lean + hypothalamic obese. The mean %age abundances in the two groups are given along with the p-value from a Kruskal-Wallis non-parametric ANOVA (*p*) and the Benjamini-Hochberg false discovery rate (*pa*) to account for multiple comparisons. Genera with pa < 0.1 were judged significant.

Figure 6.5:Boxplot of frequency (log10) of Dorea (a) and Veillonella (b) in lean and obese groups.



Dots represent individual participant

6.5.5 Community composition of OTU in relation to obesity and pathology

6.5.5.1 Community OTU composition in relation to obesity

Differences in OTU composition between lean and obese groups mirrored the genera level differences, with the two most significant OTUs corresponding to Dorea elevated in obese

(2.22% in obese vs. 0.958% in lean, p=2.79e-06) and Veillonella elevated in lean (1.99% in lean vs. 0.267% in obese, p=3.15e-06). In total, 17 OTUs differed significantly between lean and obese groups (Table 6.7).

Table 6.7: Relative abundance (mean percentage abundance) of OTUs differing significantly between lean and obese groups.

OTU	Assignment	Lean (%)	Obese (%)	*p*	*pa*
OTU17	Dorea sp.	0.9580	2.2200	2.79E-06	0.0005
OTU21	Veillonella sp.	1.9900	0.2670	3.15E-06	0.0005
OTU908	Bifidobacteria sp.	0.0128	0.0325	1.83E-05	0.0021
OTU58	_	0.1480	0.4380	1.08E-04	0.0091
OTU241	_	0.0916	0.0105	2.54E-04	0.0171
OTU798	_	0.0990	0.2220	5.19E-04	0.0262
OTU52	_	0.1980	0.0493	5.44E-04	0.0262
OTU657	_	0.5370	1.2600	8.41E-04	0.0344
OTU123	_	0.0589	0.0069	9.20E-04	0.0344
OTU71	_	0.3360	0.1020	1.70E-03	0.0572
OTU22	_	0.9380	1.6900	2.19E-03	0.0585
OTU883	_	6.5000	10.600	2.23E-03	0.0585
OTU247	_	0.0152	0.0006	2.28E-03	0.0585
OTU129	_	0.0942	0.0400	2.43E-03	0.0585
OTU240	_	0.0126	0.0009	3.24E-03	0.0724
OTU181	_	0.0125	0.0018	3.44E-03	0.0724
OTU26	_	0.3610	0.0484	4.45E-03	0.0883

Healthy; healthy lean and simple obese, Pathology; hypothalamic lean and hypothalamic obese. The mean %age abundances in the two groups are given along with the p-value from a Kruskal-Wallis non-parametric ANOVA (*p*) and the Benjamini-Hochberg false discovery rate (*pa*) to account for multiple comparisons. All OTUs with pa < 0.1 were judged significant.

6.5.5.2 Community OTU composition in relation to pathology

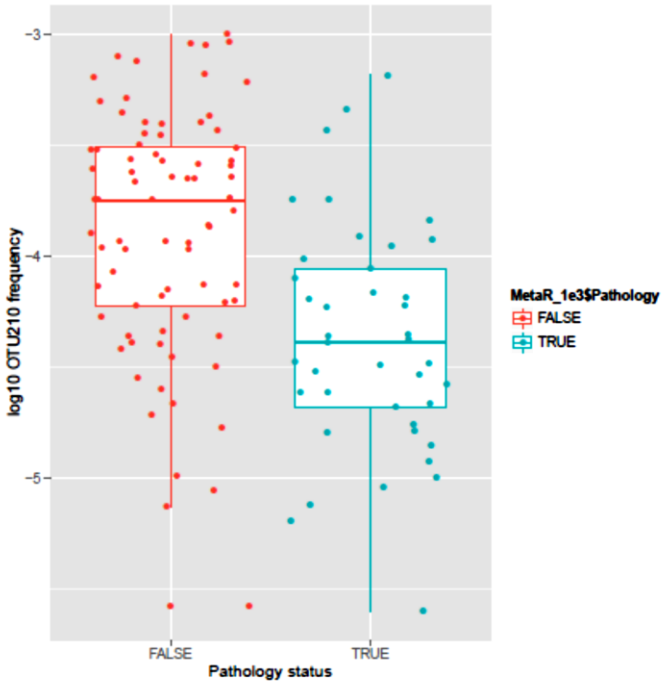
Remarkably, eighteen OTUs differed significantly in abundance between the healthy (healthy lean + simple obese) and pathological (hypothalamic lean + hypothalamic obese) groups (Table 6.8). The majority of these correspond to assorted Firmicutes that are more abundant in healthy group than pathological group. The most significantly different OTU between healthy and pathology group was OTU210 which is derived from the order Clostridiales (Figure 6.6). OTU908 assigned to *Bifidobacterium* spp. from Actinobacteria is also significantly higher in healthy group, and this is largely contributed by the simple obese group as the relative abundance of Actinobacteria is significantly higher in simple obese (16% in simple obese vs. 13.3% in healthy lean, $p<0.05$).

Table 6.8: Relative abundance (mean percentage abundance) of OTUs differing significantly between healthy and pathological groups.

OTU	Taxonomic assignment	Healthy (%)	Pathology (%)	*p*	*pa*
OTU210	Clostridiales order	0.0207	0.0051	3.96E-06	0.0008
OTU65	Lachnospiraceae family	0.2310	0.1850	4.65E-06	0.0008
OTU908	*Bifidobacteria sp.*	0.0233	0.0063	6.53E-05	0.0056
OTU26	*Coprococcus comes*	0.4550	0.2660	8.26E-05	0.0056
OTU61	*Eggerthella sp.*	0.1420	0.1890	8.26E-05	0.0056
OTU740	*Blautia sp.*	0.0211	0.0034	1.79E-04	0.0101
OTU34	Ruminococcaceae family	0.8600	0.2770	2.19E-04	0.0105
OTU66	Firmicutes phylum	0.1720	0.0909	1.30E-03	0.0507
OTU223	Clostridiales order	0.0052	0.0187	1.35E-03	0.0507
OTU246	Lachnospiraceae family	0.0176	0.0101	1.84E-03	0.0598
OTU86	-	0.1760	0.0511	2.18E-03	0.0598
OTU170	-	0.0245	0.0509	2.22E-03	0.0598
OTU622	-	0.0316	0.2030	2.31E-03	0.0598
OTU74	-	0.1090	0.0186	3.77E-03	0.0908
OTU841	-	0.0091	0.0349	4.27E-03	0.0936
OTU97	-	0.1840	0.1010	4.50E-03	0.0936
OTU382	-	0.0204	0.0164	4.83E-03	0.0936
OTU542	-	0.0504	0.0083	5.00E-03	0.0936

Healthy; healthy lean + simple obese, pathology; hypothalamic lean + hypothalamic obese. The mean %age abundances in the two groups are given along with the p-value from a Kruskal-Wallis non-parametric ANOVA (p) and the Benjamini-Hochberg false discovery rate (pa) to account for multiple comparisons. All OTUs with pa < 0.1 were judged significant.

Figure 6.6: Relative abundance of OTU210 in Control and Pathological children. Log relative frequency of OTU210 assigned to order Clostridiales in healthy and Pathological individuals.



Pathology status true: pathology group, pathology status false: healthy group

6.6 Discussion

In previous chapters we observed no significant differences in the gut microbial metabolic activity and fermentation capacity between obesity of different aetiology which hinted towards structural and/or functional similarity between the two obese groups. Preliminary analysis of the whole bacterial metagenome by high throughput next-generation sequencing complemented this work and offered insight into differences in gut bacterial compositional differences between hypothalamic obese and simple obese groups.

Taken as a whole, no significant differences were observed in gut microbial diversity between obesity of different aetiology at phylum, class, order, and family level except for phylum Proteobacteria which extended to the lower order taxonomy (class Gammaproteobacteria, order Enterobacteriales and Aeromonodales, family Enterobacteriaceae and Aeromonodaceae). Furthermore, hypothalamic obese group had a higher level of class Bacilli (including *Bacillus sp.*, *Lactobacilli* and *Mollicutes*).

No significant differences in the major phyla i.e. Firmicutes and Bacteroidetes between the simple and hypothalamic obese groups contradict the causal relationship of gut microbiota with obesity. Our study is the first line of evidence to explore this relationship in a human study. Furthermore, no significant difference in the relative abundance of these two major phyla between the lean (healthy lean and hypothalamic lean) and obese (simple obese and hypothalamic obese) groups contradicts previous major studies (162, 217). On the contrary, our study supports evidence which suggested no differences in Bacteroidetes and Firmicutes between lean and obese subjects (95, 260).

Hypothalamic obese group had significantly higher relative abundance of Proteobacteria than simple obese group and Bacilli (from phylum Firmicutes).

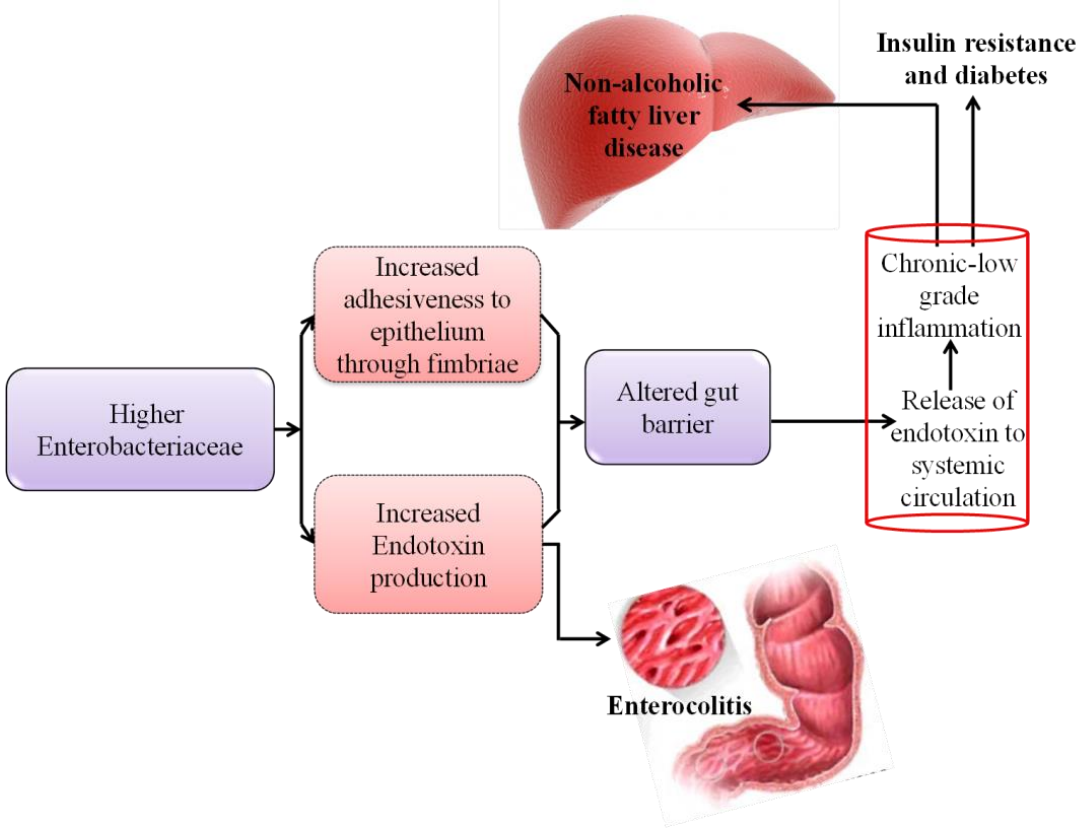
Enterobacteriaceae, commonly known as “Enterobacteria” is the most important family of the phylum Proteobacteria. They are Gram negative facultative anaerobes and include many important pathogenic microorganisms such as *E.coli, Salmonella, Yersinia pestis, Klebsiella, Shigella, Proteus, Citrobacter,* and *Enterobacter* apart from other harmless symbionts (417). Gut microbiota in this group perform mixed acid fermentation producing lactate, acetate, succinate, ethanol, 2, 3-butanediol and formate in varying amounts.

Additionally, members of Proteobacteria produce endotoxin (such as *Shigella* and *E. coli*) which is released when cellular apoptosis and cell wall disintegration occurs. This may cause systemic inflammatory response when released into the systemic circulation through a compromised gut barrier (168). Higher levels of Proteobacteria have been

correlated with chronic low-grade inflammation in obesity in Sprague Dawley rats (199) and human adults (167), type 2 diabetes (262), and late-onset necrotising enterocolitis in premature infants with compromised gut barrier function (418).

Studies have shown the presence of chronic low grade inflammation in children and adults with PWS compared to obese non-PWS controls such as raised post-prandial CRP, complement component 3, and pro-inflammatory IL-18 (419), and raised IL-6 and markers of neutrophil activation (CD66b & CD11b) (420). Interestingly, the inflammatory status in these studies was not related to BMI or BMI SDS and the relationship with insulin resistance was controversial suggesting other mechanisms for chronic low grade inflammation unrelated to adiposity and insulin sensitivity. Thus far, no study has investigated whether chronic low-grade inflammation in PWS is related to endotoxin-producing pro-inflammatory gut microbiota. Our study might be the first evidence linking chronic low-grade inflammation in PWS patients to the presence of increased endotoxin producing gut microbiota, independent of BMI. Increased capability of Enterobacteria to adhere to the colonic epithelium with the help of type 1 fimbriae might help in facilitating disruption of gut barrier and translocation of endotoxin into the systemic circulation. Patients with PWS have been reported to have higher incidence of non-alcoholic fatty liver disease related to chronic low grade inflammatory status (421). Furthermore, obese non-PWS adults with non-alcoholic fatty liver disease have been shown to have higher relative abundance of Gammaproteobacteria in addition to *Lactobacilli* and members of Lachnospiraceae (422). Therefore, a higher relative abundance of Gammaproteobacteria in hypothalamic obese group in our study might link with increased incidence of NAFLD in PWS through endotoxin-induced chronic low-grade inflammation. However, we did not have data regarding the systemic inflammatory markers and hepatic health status that could prove this explanation and is therefore a potential new area for us to investigate in near future (Figure 6.7).

Figure 6.7: Proposed mechanism for the relationship of increased Enterobacteria with chronic low grade inflammation in hypothalamic obese children/young adults in our study



Increased endotoxin production and the capability of these bacteria to adhere to the epithelium may result in altered gut barrier through altered expression of tight junction proteins allowing the release of endotoxin into the systemic portal circulation, chronic low grade inflammation, insulin resistance, and non-alcoholic fatty liver diseases. Additionally, increase endotoxin release into the gut epithelium may cause enterocolitis.

Hypothalamic obese group also had a higher relative abundance of Bacilli; which amongst other *Bacillus* species also includes *Lactobacilli. Lactobacilli* mainly produce lactate as a product of their fermentation which is either utilised in the synthesis of butyrate, propionate, or acetate. Heterofermentative *Lactobacilli* (such as *Leuonoctoc, Oenococcus, and Weissella)* also produce ethanol and carbon dioxide via pentose phosphate pathway. Butyrate, but not propionate is the primary route of utilisation of lactate. Although strains belonging to the genus Lactobacillus (such as *L. reuteri*) are correlated with obesity, other Lactobacilli (such as *L. plantarum, L. casei, L. paracasei*) are positively associated with leanness (236). Probiotics, which selectively stimulate groups of gut microbiota to beneficially affect the human gastrointestinal tract are mostly composed of different strains from the genus *Lactobacillus* and are shown to reduce obesity associated metabolic inflammatory markers (412, 423). Additionally, *Bifidobacteria* and *Lactobacilli* in mother's milk are crucial for the colonisation of non-pathogenic symbionts in the infant's gut (149). Therefore, genomic variability within Lactobacillus group demands a strain level analysis, rather than a more general approach, to assess any association with obesity.

Lower methanogenic bacteria and higher sulphate-reducing bacteria (Deufovibrio sp.) in obese than lean

Excess production of SCFA in the gut results in accumulation of hydrogen and reduced SCFA which can inhibit further fermentation due to the accumulation of reducing equivalents (NADH and NADPH) as a result of oxidation-reduction reaction. Removal of molecular hydrogen from these reducing equivalents is necessary for the acceptance of further electrons in glycolysis and Krebs's cycle. In the gut, homoacetogenic bacteria, sulphate reducing bacteria and methanogenic archaeacompete for the acceptance of hydrogen to generate acetate via Wood-Ljundahl pathway, hydrogen sulphide or methane respectively (121). Complete absence of methanogens in the simple obese or presence in reduced numbers in hypothalamic obese participants compared to lean participants suggest that sulphate reducing bacteria (SRBs) outcompete methanogens in accepting hydrogen to generate hydrogen sulphide. Published data suggest that higher SRBs level is associated with a negligible or extremely lower methanogens in the distal gut (122). Presence of methanogenic archaea has been associated with leanness (236, 237) and in anorexia nervosa (240). However, a study by Zhang *et al.* (2009) showed higher gene copies of methanogens in obese and association of hydrogen producing Prevotellaceae with hydrogen consuming *Methanobacteriales* in obese adults but not in lean and post-gastric bypass patients (167). In our study, although methanogens were absent or reduced in the two obese groups, there was no significant difference in the relative abundance of Desulfovibrionaceae and Prevotellaceae between lean and obese groups. This suggests that the association of methanogenic archaea with leanness or obesity is dependent on the interaction of gut microbial communities that varies between individuals and may be dependent on the dietary pattern of individual.

Verrucomicrobia (represented by Akkermansia muciniphila) were higher in lean than obese

We found significantly higher Verrucomicrobia at phylum, class, and family level in healthy lean group compared to simple obese group (4.1% in healthy lean vs. 0.70% in simple obese). Verrucomicrobia, primarily represented by *Akkermansia muciniphila* are mucin degrading bacteria residing in the mucus layer of the colonic epithelium (424). They represent up to 3-5% of the total bacterial population in healthy subjects, however the relative abundance may vary between individuals (424, 425). Higher levels of Verrucomicrobia have been observed in normal weight (254) and gastric bypass patients

(167) compared to obese subjects. Moreover, higher levels of *Akkermansia muciniphila* have been shown to be inversely associated with adiposity and measure of adiposity, metabolic endotoxemia, and markers of metabolic syndrome (247, 248). Our finding is therefore in accordance with these studies. Whether higher levels of *A. muciniphila* are a marker of good health or disease is controversial. A recent study by Everard *et al.*(2013) in animal models have shown reduced level of *Akkermansia muciniphila* in both genetic model of leptin deficient *ob/ob* mice (3,300 fold lower than lean) and high fat fed mice (100 fold lower than lean) (231). Supplementation with viable, but not heat-killed *Akkermansia muciniphila* was shown to improve metabolic disorder and diabetes by enhancing the markers of adipocyte differentiation, lipid oxidation (such as carnitine palmitoyltransferase 1), and glucose homeostasis (by improving fasting hyperglycaemia by 40% reduction in gluconeogenic enzyme glucose-6-phosphatase) (231). Interestingly, these effects were independent of changes in dietary intake by the experimental animals. In contrast, supplementation with prebiotic oligofructose was shown to recover the population of *Akkermansia muciniphila*. Whether this was a direct effect of the oligofructose on the selective growth of this species or it was due to the presence of other cross-feeding pathways that favoured increase in *A. muciniphila* was not shown. Earlier study by Collado *et al.* (2008) and Santacruz *et al.* (2010) showed higher levels of *Akkermansia muciniphila* in pregnant women gaining normal weight between 1st and 3rd trimester of pregnancy compared to those who gained excessive weight during that period (247, 248).

In contrast, the mucus-degrading capability of *Akkermansia* in human health is controversial as degradation of mucus is associated with reduction in thickness of mucus layer and exacerbation of inflammatory bowel disease. Furthermore, the effect of prebiotics fibre on the levels of *A. muciniphila* is controversial as Jakobsdottir *et al.* (2013) found positive association of fibre-free diet with *A. muciniphila* (226).

Obese phenotype is associated with lower OTU richness compared to lean phenotype

Regardless of the aetiology of obesity, we found a significantly lower OTU richness in obese than lean phenotype. A discussed in chapter 1, evidence regarding compositional differences in the relative proportion of gut microbiota at various taxonomic levels from phyla to species level is controversial both in experimental and human studies in obese vs. lean (159, 164, 222, 260, 350, 410). However, in these studies, there is a general agreement on a reduced diversity and richness of gut microbiome in obese vs. lean animals and humans. Our finding is novel, as to our knowledge, this is the first evidence from human studies to suggest that lower gut microbiota richness is independent of the type of obesity

(simple or hypothalamic obesity). This evidence further implicates that factors other than obesity such as dietary, environmental, and lifestyle factors may be much stronger determinants of the colonisation of gut microbial communities.

A recent study by Le Chatelier *et al.*(2013) showed a bimodal distribution of 292 individuals based on their low or high gene counts (more than 480,000 genes or less than 480,000 genes respectively). Obese adults (40% of study subjects) with lower gene count had higher counts of Proteobacteria and Bacteroidetes at phylum level, higher genomic potential to produce potentially harmful metabolites such as genes for β glucoronide and aromatic amino acids degradation, and had increased markers of metabolic syndrome and diabetes in contrast to high-gene count adults (410). Body mass index and body weight were the only significantly negatively associated parameters with gene counts amongst biochemical (insulin, HOMA IR, adiponectin, leptin, triglycerides, ALT, free fatty acids, hsCRP, fiaf,) and anthropometric (BMI, body weight, and body fat %) parameters.

In our study, differences in gut microbiota between lean and obese phenotypes were driven mostly by genera and OTUs belonging to the phylum Firmicutes, obese subjects showing higher relative abundance of *Dorea*, *Collinsella*, *Lactobacilli*, *Megamonas*, and *Gemmiger* while lean subjects had a higher relative abundance of genera *Veillonella, Pasteurellaceae, Alistipes, Oscillibacter, Clostridial cluster* XVIII, and *Rothia*. Taxonomic richness of Firmicutes in obese subjects have been shown to be linked to a lower functional diversity and expression of genes for metabolic pathways (409). On the contrary, some authors suggest that the functional representation of gut microbiota such as Bacteroidetes is equal to that of Firmicutes although the relative abundance is low and the disturbance of this equilibrium is a hallmark of obese phenotype (252). A recent study by Cottilard *et al.* (2013) has shown reduced bacterial richness and diversity, higher dys-metabolism and low-grade inflammation in obese vs. lean humans. Although dietary intervention improved gene richness and metabolism in obese, it is less efficient to improve the low grade inflammation (252).

Reduction in the OTU richness might suggest functional redundancy in the metabolic cross-feeding pathways due to a "monotonous diet" usually rich in fat and depleted in fermentable carbohydrates. Presence of fermentable carbohydrates allows the development of symbiotic associations between various taxonomic clades and enzyme system containing complex glycosylhydrolases for the generation of metabolic end products necessary for survival of gut microbiota (64, 426). These associations fail to develop when diet with abundant simple sugars is used(86). High fat diet containing higher amounts of saturated fat (such as palm oil) are also absorbed in the small intestine and are shown to reduce the diversity of gut microbiota and stimulate distal gut signalling

pathways to increase the expression of lipid metabolism-related genes in the distal colonic epithelium (223). This evidence is further supported by interventional studies highlighting the influence of introduction of probiotics on the gut microbiota richness and diversity at least transiently if not permanently (427). In their study, Wang *et al.* (2014) showed that introduction of three candidate probiotics each containing *Lactobacillus paracasei*, *L. rhamnosus*, and *Bifidobacterium animalis* to high-fat fed mice for 12 weeks significantly attenuated the gut microbiota OTU composition of obese metabolic phenotype. Of 83 OTUs altered by probiotics, 26 OTUs which were positively associated with metabolic syndrome were reduced and 13 OTUs that were negatively correlated with metabolic syndrome were promoted as a result of probiotics feeding. This was coupled with increase in caecal acetate and reduction in hepatic and adipose tissue TNFα expression (427). Furthermore, introduction of high fibre diet (such as inulin type fructans) in obese women has been shown to reverse the relative abundance of the predominant gut microbial communities (such as Firmicutes and Bacteroidetes), stimulation of previously suppressed microbes (such as *Bifidobacterium* and *F. prausnitzii*), and development of new cross-feeding pathways evidenced by the changes in the levels of metabolic end products (such as SCFA and lactate) (428). These changes in turn had a positive influence on improving anthropometric parameters, metabolic endotoxemia, and fasting glycaemia.

Bacterial genera and OTUs composition clustered based on their phenotype, and not pathology

Despite subtle differences in the gut microbiota composition between simple and hypothalamic obese groups, genus level and OTU level composition in obese phenotype clustered together and distinctly from lean phenotype (healthy lean and hypothalamic lean). Clustering of gut microbiota of obese phenotype distinct from the lean phenotype have been shown both in experimental animal (217) and human studies(410). However, our study indicates that adiposity and measures of adiposity are more closely related to the structure of gut microbiota than the cause of adiposity and, as mentioned above, it further implicates that gut microbiota are not causally related to the aetiology of obesity. However, microbial species which are different between the two types of obesity might be important for adiposity and cardiovascular risk.

Despite inter-individual differences, evidence suggests similarity of gut microbial community and their metabolic activity between members of the same community and between mother and off-springs distinct from unrelated individuals (248, 409). Obese related twins and their mothers had had lower microbial diversity, shared community

structure (higher Firmicutes and lower Bacteroidetes in obese than lean) and metabolic pathways (Firmicutes enriched with transport system genes and Bacteroidetes enriched with carbohydrate metabolism genes) for the degradation of dietary substrate compared to lean (409). Clustering of the gut microbiota of obese unrelated individuals with different aetiology therefore suggest common "exposure" factors such as diet, environment, and lifestyle that make them similar to each other in gut microbiota diversity at genera level, independent of obese phenotype. Although the core microbiome colonising the infant gut is determined by several factors discussed in detail in chapter 1, exposure to environmental factors play an important role in modifying gut microbiota. For example, study by De Fillipo *et al.* (2010) showed clustering of gut microbiota from African and European children in early days of life which then became grossly distinct over the period of months to years after exposure to different diets (African diet rich in plant fibre vs. European diet rich in fat and low in fibre) (155). Similarly, studies in centenarian population (≥100 years age) also indicate distinct clustering of the gut microbiota based on their community dwelling (hospitalization, day-care centres, and community dwelling)(429). Additionally, diet was a separate factor that produced sub-clusters within the same group. These findings further support the role of environmental factors in clustering individuals together distinctly from others (429).

Distinct clustering based on OTUs in our study might also suggest distinct grouping of gut microbiome in symbiotic relationship in obese vs. lean phenotypes. In the recent study by Ridaura *et al.*(2013), animals with obese transplanted microbiota exhibited higher expression of genes involved in detoxification and stress response, biosynthesis of cobalamin, essential and non-essential amino acids and gluconeogenic pathways. In contrast, animals with lean- transplanted microbiota exhibited genes capable of fermenting plant polysaccharides, butyrate, and propionate (212). Similarly, Won *et al.* (2013) found 48 distinct metabolic products in urine of obese subjects to be significantly up-regulated compared to the lean subjects (214). In this context, several discriminating gut microbial metabolic products have also been identified in the urine of obese vs. lean people, some of which include higher levels of hippuric acid, trigonelline, 2-hydroxybutyrate and xanthine (241).

In summary, obesity and not the cause of obesity classify participants into separate clusters and this might be attributed to the distinct dietary, lifestyle, and environmental factors to which the obese and lean phenotypes are exposed. However, our dietary data did not show any effect of diet on the community composition possibly due to under-reporting, limitation related to the method of dietary assessment, and other unknown host-related factors that might contribute to determine this relationship with community composition.

6.7 Conclusion

In conclusion, simple and hypothalamic obese group are not different in terms of relative abundance of the major phyla such as Firmicutes and Bacteroidetes, although differences in Proteobacteria were observed. Obesity and not the cause of obesity explain variations in the gut microbial diversity. The presence of similar metabolic profile in hypothalamic vs. simple obese as seen in previous chapters is not fully translated into their structural diversity at all taxonomic levels which suggest that composition of the gut microbiota may not be related to the similarity in functional diversity and the absence of compositional differences at phylum level may not indicate absence of lower taxonomic level differences. Similar metabolic function may suggest identical or comparable dietary patterns in obese people regardless of the aetiology of obesity.

Analysis of gut microbiota composition in our participants has generated important research questions:

Whether gut microbiota composition and their metabolic products (SCFA, lactate, ammonia, and H_2S) in our cohort are related to the expression of functional genes in metabolic pathways? Whether the gut metabolome in obesity of different aetiology is distinct from metabolome in lean groups? And whether the metabolome of the obese and lean participants is related to their diet?

These are future steps for us to investigate. This has encouraged us to prepare metagenomic libraries for the analysis of 96 samples from our participants, although their analysis could not be completed till the end of this PhD due to time restraints.

Chapter 7: General Discussion and Conclusions

Obesity has nearly doubled in the past 30 years and is growing in pandemic proportions worldwide. It is the leading cause of many preventable causes of death such as type 2 diabetes, coronary heart disease, hypertension, osteoarthritis, and cancer (19). Amongst several other factors related to the aetiology of obesity such as diet, lifestyle, environment, and genetic makeup; the gut microbiota has been implicated in the aetiology of obesity by increasing energy harvest from the diet (via SCFA production), altering gut barrier function, releasing certain hormones that positively affect host hepatic and adipose tissue lipogenesis (Table 1.7, chapter 1). However, in contrast, other studies suggested that diet might be the principal factor driving these changes in gut microbiota and hence increased energy harvest. The evidence largely remains controversial and there is a need to determine the "cause or effect" relationship between gut microbiota and obesity.

Work done towards the completion of this PhD aimed at addressing the potential reverse causality by studying gut microbiota diversity and metabolic activity in children and young adults with obesity of known (Prader-Willi syndrome & Craniopharyngioma) and unknown aetiology (simple or classical obesity). If the gut microbiota were the cause of obesity, we could expect to see differences in gut microbiota diversity and metabolic activity between obesity of known versus unknown causes. Similarity in gut microbiota diversity and metabolic activity would implicate that altered gut microbiota reported in previous studies are a consequence of altered dietary, environmental, lifestyle, and other factors.

The main aim of the study in this PhD was "to compare gut microbial metabolic activity (in the form of SCFA, hydrogen sulphide, D & L lactate, and ammonia), fermentation capacity and microbiota composition between obesity of different aetiology and their relationship with diet and weight loss".

The research content of this thesis was divided into the following four main sections:

1. Subject characteristics including demographic, anthropometric, and dietary analysis
2. Differences in gut microbial metabolites (SCFA, BCFA, H_2S, D & L-lactate, and NH_3) in simple and hypothalamic obesity
3. *In vitro* fermentation capacity of gut microbiota from children with simple and hypothalamic obesity
4. Gut microbiota diversity in simple and hypothalamic obesity

Due to the difference in the nature of the groups, the analysis for determining the difference between the groups was done using Mann-Whitney U test (comparing only two

groups at a time) instead of Kruskal-Wallis test followed by Bonferroni post-hoc test or any other corrections for multiple comparison. Using Mann-Whitney U test for testing multiple hypotheses might have resulted in nominally significant differences which may not have remained significant after multiple testing adjustment or using other tests for false discovery rate.

7.1 Subject characteristics: differences in body composition and dietary intake

In accordance with published data, this study described lower height for age in the hypothalamic obese versus simple obese group. Height SDS of participants with pathology (hypothalamic lean and hypothalamic obese group) was significantly lower than those with no pathology(healthy lean and simple obese group). Patients with Prader Willi syndrome are genetically prone to growth hormone deficiency while patients with craniopharyngioma develop growth hormone deficiency due to the invasion and destruction of anterior pituitary gland and hypothalamus by the tumour. Owing to hyperphagia, we would expect a higher weight SDS in hypothalamic obese patients, however in contrast, weight SDS of hypothalamic obese children was significantly lower than the simple obese patients and interestingly these differences did not translate into the BMI SDS which suggests that PWS and simple obese participants were proportionate or equally obese for their height.

Although not all children with Prader Willi syndrome in our study were obese, the median BMI SDS of PWS children in lean category was higher (median BMI SDS >1 SDS) compared to healthy lean control (median BMI SDS <0 SDS). Whether this represents the transitional phase between already-started metabolic changes (i.e. reduced energy expenditure and increased fat mass) and hyperphagia towards an obese status is a possible subject for future investigation and warrants a long term follow-up of these participants. It would also be interesting to explore changes in weight/BMI gain/velocity with a longer follow up. This also suggests that the clinician should focus not only on weight management in the obese hypothalamic but also “prevention” of weight gain in the lean hypothalamic group as they might likely progress to become overweight and obese in future. This was partially indicated by the higher weight velocity in hypothalamic obese and hypothalamic lean group compared to the healthy lean and simple obese group. Sustained increase in weight velocity despite weight management suggest that, weight velocity of the simple obese group is reduced but the hypothalamic obese and lean group continue to have an increased weight gain under the effect of the pathology.

Measurement of body composition was based on foot-to-foot bioelectrical impedance analysis. Although this has been validated for use in children and adults,

several studies have found that it can over estimate fat-free mass and underestimate percentage body fat (319, 320). Moreover, large variation between measurements has been reported that vary with gender (321). Body composition could not be measured in all participants due to the young age of some and hence the results of the TANITA® need to be interpreted with caution. However this is a simple, cheap bedside approach and might be useful to explore sequential changes where more precise and accurate methods are limited.

Analysis of the dietary data in our study showed that the proportional intake of dietary fat and proteins was higher while that of carbohydrate was lower in the obese group, particularly the simple obese group. Our data suggest a clear downward trend in the percentage recommended intake of dietary fibre from the lean phenotype towards an obese phenotype. The lower percentage of average recommended intake of energy in the hypothalamic lean, hypothalamic obese and simple obese groups could be attributed to either reduced energy requirements, reduced energy expenditure, or to the effect of dietetic management, or it may represent under-reporting of dietary habits. A trend of decreasing energy intake with increasing BMI SDS suggests major underreporting in our obese group. It still remains to be determined how far this reported lower intake of energy in hypothalamic obese group is attributable to the lower energy expenditure. Physical activity and health related quality of life measurements are therefore recommended to correlate reported energy intake with body composition.

Our method of dietary assessment (24 h food diary) has some limitations(430). It was not a weighed diary, therefore potential variation due to the perception of portion sizes by parents or the participants may have contributed to the differences recorded in energy intake. Moreover, a single day diary may not represent the typical diet of participants on other days not recorded in the diary. Parents completing the food diary on behalf of a young child may have missed foods taken at school. Furthermore, the food diary does not give an indication of the frequency of the type of foods consumed and should therefore be supported by a food frequency questionnaire (431). We tried to minimise these potential variables by explaining the food diary in detail to the participant and/or the parent and asking them to preferentially choose a weekday to fill the diary. Food frequency questionnaires (FFQs) were included as part of the dietary assessment for all participants, but unfortunately, certain cereals taken as part of the daily breakfast were missed and not included in the FFQs, which made the extrapolation of any conclusion from that data biased and incomplete. These are therefore not included in the thesis. Multiple pass food diaries are designed to address issues related to memory bias and under-reporting by

participants (339), and thus may give a more accurate measure of food intake in future studies involving overweight and obese groups.

7.2 Differences in gut microbial metabolites (SCFA, BCFA, H_2S, D & L Lactate, and NH_3) in faeces of children with simple and hypothalamic obesity

Several animal and human studies have investigated the relationship of faecal gut microbial metabolites in obese versus lean animals and humans (table 1.7, chapter 1). However, there is no good data to rule out reverse causality i.e. whether gut microbiota are a cause or they are a consequence of obesity.

To the best of our knowledge, this is the first human study attempting to prove reverse causality by comparing gut microbial metabolic activity and diversity between two obese groups; one with a known cause of obesity (hypothalamic obesity - due to a genetic disorder or a tumour), and another with an unknown cause of obesity (simple or classical obesity).

Overall, there was no difference in the faecal metabolites including SCFA, BCFA, H_2S (free, bound, and total), lactate (D &L), and ammonia between simple and hypothalamic obesity and thus our results did not support the pioneering studies by Jeffry Gordon's group suggesting a causative role of the gut microbiota in the aetiology of obesity. All previous human studies have attempted to address the cause or effect relationship of gut microbiota with obesity by focusing only on simple obese children and adults compared with healthy lean populations. However, we have controlled for the most important variable, "obesity" and then compared the gut microbial diversity and metabolic activity between simple and hypothalamic obese group. Furthermore, we also included hypothalamic lean group which acted as a control group for the hypothalamic obese group. Thus, we were able to control both for obesity and cause of obesity (pathology) in our analysis enabling us to draw more confident conclusions with a reasonable number of participants given the rarity of hypothalamic obesity.

Inconsistent differences in the proportion of acetate and the percentage of faecal water between simple and hypothalamic obese groups were seen only at recruitment, which suggested different cross-feeding pathways in individual bacterial groups to harvest energy from the diet while maintaining a functional symbiotic relationship. A higher proportion of acetate in the hypothalamic obese group suggested preferential utilization of lactate as a substrate for butyrate production. This was supported by the lower lactate to butyrate ratio and significantly higher rate of change in D and total lactate over the period

of study in hypothalamic obese than all other groups suggesting higher lactate production. Higher production of lactate was also supported by the presence of significantly higher relative abundance of class Bacilli which amongst other *Bacillus sp*. also include *Lactobacilli*. Further metagenomic analysis of genes involved in bacterial metabolic cross-feeding pathways is suggested to confirm and establish this finding with larger number of participants.

Reduced gastric emptying and prolonged intestinal transit time due to hormonal disturbances has been reported to cause constipation in PWS patients (356, 357). However, in contrast, we observed a significantly higher percentage of faecal water in the hypothalamic obese group which may suggest that increased water content was a consequence of higher SCFA production and absorption, which drives bicarbonate ions to the gut lumen in exchange. Although we do not have data on the gut transit time of our patients, increased faecal water could be due to a reduced transit time leading to lesser absorption of water and salts from the gut lumen.

In our study, the obese groups (simple and hypoth. obese) had a higher faecal SCFA concentration than the lean (healthy and hypoth. lean) groups. Published data suggest that availability and/or changes in the type and amount of substrate in the gut lumen determine the concentration and pattern of SCFA in obese individuals (166, 217). However, changes in the gut microbiota due to different pattern of diets in obese individuals, irrespective of the cause of obesity, might then acquire the capacity to harvest energy from the available substrates in the gut lumen as suggested in mice (159) and human(164)studies. However, this will not be implicated in the primary onset but possibly in the propagation of adiposity and increase in cardiovascular risk. SCFA in faeces are a net difference of what is being produced and what is being absorbed. Whether higher faecal SCFA in the obese group represents higher SCFA production or is due to a state of malabsorption in the gut is unknown. This question was addressed by *in-vitro* batch culture fermentation the results of which suggested no difference in the energy harvesting capability of gut microbiota of the two obese groups.

One interesting finding was the paradoxically higher faecal propionate in the obese phenotype compared to the lean phenotype which was consistent between the two time points and in batch cultures. As discussed in chapter 4, higher levels of propionate have been associated with many beneficial effects on host metabolism whereas obesity is generally thought to be associated with metabolic dysfunction such as hypercholesterolemia and hepatic and adipose tissue lipogenesis. Propionate is a potential anti-obesity agent but our results are opposite to this notion. This paradoxical increase in “beneficial” propionate in our obese groups, which ameliorates these metabolic

abnormalities, has also been reported by other studies (95, 172, 176, 350). Bacteroidetes producing propionate express a wide variety of glycosyl hydrolases capable of degrading host mucus glycans during situation of low-fibre intake. Population based studies in obese humans have reported a reduced intake of dietary fibre and higher amounts of the readily available sources of digestible carbohydrates (327). In accordance with this, the simple obese participants in our study had a significantly lower fibre intake and the intake of dietary fibre was significantly negatively correlated with BMI SDS. This might explain the abundance of Bacteroidetes and the consequently higher concentration of propionate reported in obese people. An increase in "beneficial" propionate might be an indication of a high intake of digestible carbohydrate but less dietary fibre-rich food (172). A high propionate should therefore be seen in the context of its source and not just its increased level in faeces. A comparative study of simple and hypothalamic obese population with higher intake of fibre with that of patients with low fibre intake would help further increase our understanding of this propionate paradox in obesity.

Although studies have suggested alteration in the gut microbial diversity and metabolic activity with changes in weight, changes in weight were not significantly different between any of the groups in our study and were not associated with changes in gut bacterial metabolites. This suggests functional resilience of the gut microbiota diversity and metabolic activity in our participants which does not vary significantly with obesity or pathology, at least within a period of 2-3 months. It is however very hard to determine whether differences in weight gain/loss over an extended period of follow-up would cause differences in metabolism and diversity of gut microbiota in our cohort. This would require long term close follow-up of these participants and measurable changes in weight. Furthermore, future research is suggested to determine if the changes with weight loss or weight gain are due to changes in gut microbiota or due to changes in diet that usually happen with weight management, or due to changes in the endocrine and neuronal pathways of metabolism.

Finally, the lack of association of dietary intake with major gut microbial metabolites in multivariate analysis with and without adjustment for pathology indicate that other factors might be playing more important role in determining the metabolic potential of the gut microbiota such as lifestyle and environment, host genetic makeup. However, analysis of the dietary data from our participant strongly suggest under-reporting and call for a more robust dietary assessment method in combination with FFQ as suggested above.

7.3 *In vitro* fermentation capacity of gut microbiota from children with simple and hypothalamic obesity

SCFA measured in faecal samples represent only the difference of what is produced and what is absorbed into the gut epithelium. The fermentation capacity of the gut microbiota represent their capability to degrade complex polysaccharides and proteins to SCFA and BCFA and is a true representation of the energy harvesting capability of the gut microbiota from the diet.

In vitro fermentation studies conducted on faecal samples of our participants confirmed our findings from faecal bacterial metabolites. No significant differences were observed in energy harvesting capability of gut microbiota between obesity of different aetiology contradicting the causal relationship of gut microbiota in the aetiology of obesity. On the other hand, significant differences in the rate of production of SCFA between obese (simple and hypothalamic obese) and lean groups (healthy lean and hypothalamic lean) indicated that obesity, and not the aetiology of obesity, determine differences between lean and obese population.

Thus far, only three human studies have investigated the differences in fermentation capacity between obese vs. lean human subjects (95, 172, 395). This is the first *in vitro* batch culture fermentation study conducted for the measurement of the energy harvesting capability to investigate the cause or effect relationship of gut microbiota fermentation capacity in obesity of different aetiology. Furthermore, we used a range of different commonly consumed dietary fibres as substrates to assess if the response of gut microbiota to individual fibres was "general" (i.e. fermenting all fibres uniformly; as a proxy for a general increase in the capacity) or whether any differences in microbiota were "substrate-selective" (i.e. specific to predominant components of the diet of the participants). Moreover, in contrast to other studies (172), we worked hard to preserve the colonic relative abundance of faecal gut microbiota by keeping those in anaerobic media, by keeping the sample cold at approximately 4^{o}C, and processing majority of the samples within 4 h after being produced. This was achieved for majority of the samples. Additionally, the number of participants is this study was higher than previous human studies.

Several findings in this chapter correlated very well with findings from previous chapters. Of particular note was the high inter individual variation in fermentation capacity, across all dietary substrates and at both time points, particularly in the two obese groups. High inter-individual variations only in the obese group may indicate higher inter-individual variations in the gut microbiota communities in obese but not in lean hosts.

Furthermore it also indicated that a much larger cohort of participants is needed to bring up or establish a significant difference that is otherwise blunted due to small sample size. Similar to changes in faecal SCFA in chapter 4, the fermentation capacity of gut microbiota did not significantly change with weight management. Although there was no significant weight loss and the concentration of SCFA did not change significantly with weight management, significant changes in the proportion of SCFA within the groups over the period of study suggested that weight management might beneficially affect the pattern of SCFA production (such as propionate and butyrate).

One of the important findings was the significantly higher rate of production of propionate in obese (simple and hypoth. obese) compared to lean (healthy and hypoth. lean) phenotype which was consistent across most of the dietary fibres. This consistent response in the obese group once again indicates adaptation of the obese participants' microbiota to low fermentable carbohydrate availability in the colon. This may have allowed Bacteroidetes to produce more propionate with the help of their abundant glycosyl-hydrolases. Relative abundance of the phylum Bacteroidetes was however not significantly different between the obese and lean groups as discussed in chapter 6. This prompts us to investigate whether or not these differences in the rate of propionate production are due to the differences in the expression of functional genes in Bacteroidetes in obese vs. lean groups. This can be tested by metagenomic sequencing of the bacterial DNA extracted from the fermentation supernatant.

Presence of trends but lack of significant differences between the lean and obese might be due to limitations related to the *in-vitro* batch culture fermentation studies. Accessing the colon in ideal conditions by conducting *in-vivo* studies or obtaining caecal samples could reduce variations related to the *in-vitro* procedures. However, both of these procedures carry practical and ethical issues making it difficult to put in practice. Another suggestion could be to use *in-vitro* continuous culture fermentation which allows the fermentation products to be removed from the system while maintaining the pH. Although this facility was available in the department, it was not chosen for practical reasons: a) due to multiple samples collected at a given time which would require more than one continuous culture set-up, b) the time sample was produced by the patient, which varied between 7am till 8pm, and c) it was labour intensive, given the time constraints to carry processing of faecal sample for other analyses and the total number of samples to be incubated (n=151). *In-vitro*batch culture studies were therefore chosen for their convenience, proven reproducibility, repeatability, and inter- and intra-laboratory validity in many centres across different countries.

Another possible reason for the presence of trends but lack of significant results could be related to the small study numbers, particularly in the obese groups. Post hoc power analysis based on our results suggested that recruitment of at least 20 participants in each group would give sufficient power to see a significant difference between the groups. However, having mentioned these issues, the numbers of participants in our study are still far greater than the number of participants in previous studies. We used five different dietary substrates which were also expected to provide the element of consistency to any significant difference found between the two obese groups or between the obese and lean phenotypes. Moreover, despite meticulous sample collection process, rigorous *in-vitro* incubation, and sample analysis; inter-individual variation might be an idiosyncratic response of the participants' microbiota. This has been a feature of most *in-vitro* fermentation studies.

7.4 Gut microbiota diversity in simple and hypothalamic obesity

Our study did not support a causal relationship of the gut microbiota composition with obesity as we did not find any significant difference in the relative abundance of the major bacterial phyla i.e. Firmicutes and Bacteroidetes, between obesity of different aetiology and also between obese (simple & hypoth. obese) and lean (healthy & hypoth. lean) phenotypes.

The hypothalamic obese group showed two significant differences from the simple obese group; significantly higher relative abundance of a) phylum Proteobacteria and b) class Bacilli. This suggests that the similarity in metabolic potential may not fully translate into the composition of gut microbiota at all taxonomic levels. Therefore, analysis of composition should be accompanied with the analysis of metabolic function to better elucidate this relationship.

Members of phylum Proteobacteria, particularly Gammaproteobacteria (dominated by family Enterobacteriaceae) produce endotoxin which might contribute to the chronic low-grade inflammation and metabolic endotoxemia. Children with PWS have been shown to have higher prevalence of non-alcoholic fatty liver disease (NAFLD) and inflammation compared to simple obese patients (419, 420). However, the link between gut microbiota, chronic low grade inflammation, and NAFLD has not been studied. Here we proposed for the first time that higher relative abundance of Gammaproteobacteria in hypothalamic obese group might link with increased incidence of NAFLD in PWS through endotoxin-induced chronic low-grade inflammation (figure 6.7, chapter 6). Gathering data on the systemic inflammatory markers and hepatic health status could prove this explanation and

is therefore a potential new area for us to investigate in near future. Furthermore, studying systemic chronic low-grade inflammation and development of NAFLD in a genetic germ-free animal model of PWS colonised with Enterobacteria could be another future study to establish this link. Moreover, higher levels of Enterobacteria (containing pathogenic strains such as *Shigella, Klebsiella, Salmonella, E.coli*, *Enterobacter,* and *Citrobacter*) have been associated with frequent hospitalisation and repeated infections(432). Establishing a link between Enterobacteria, inflammation, and NAFLD in PWS would potentially enable researchers in future to introduce strategies to prevent factors related to the colonisation of pathogenic strains of Enterobacteria as a step to prevent the development of NAFLD and cardiovascular disease.

Several taxonomic features were found to be distinct between the lean and obese phenotype and were mildly influenced by the presence of pathology. Genus level and OTU level composition in obese phenotype clustered together and distinctly from lean phenotype. Although this was shown in previous studies (409, 410), our study gives a novel insight indicating that adiposity, but not the cause of adiposity, is more closely related to the structure of gut microbiota which further strengthens our assumption that gut microbiota may not be causally related to the aetiology of obesity. Similarly, the obese phenotype had significantly lower richness of operational taxonomic units than the lean phenotype as suggested by previous studies. However, to the best of our knowledge, this is the first evidence from human studies to suggest that lower gut microbiota richness is independent of the cause of obesity and most likely is the effect rather than the result of the latter. These findings implicate that factors other than gut microbiota such as dietary, environmental, lifestyle, and genetic factors may be much stronger determinants of the energy harvesting capability of gut microbiota and should therefore be considered while studying gut microbiota in relation to obesity.

Differences between the obese (simple & hypoth. obese) and lean (healthy & hypoth. lean) phenotype were also seen at different taxonomic levels. Of particular importance was the absence of methanogenic archaea and reduced relative abundance of Verrucomicrobia in obese compared to lean phenotype. The presence of methanogenic archaea has been paradoxically associated both with leanness (236, 237, 241) and obesity (167). Additionally, levels of methanogens have been inversely associated with the levels of sulphate reducing bacteria (SRBs). Complete absence of methanogens in the simple obese or reduced numbers in hypothalamic obese compared to healthy lean individual therefore suggests that SRBs may have out-competed methanogens to generate hydrogen sulphide. On the other hand, the presence of methanogens in the lean group suggest that methanogens might have accepted molecular hydrogen produced by members of

Prevotellaceae, *Ruminococci*, *Dorea*, and Enterobacteriaceae. Relative abundance of the members of Prevotellaceae have been positively correlated with methanogenic archaea in previous studies (167). However, in contrast to our expectation, an inverse relationship of methanogens with SRBs and a positive correlation with Prevotellaceae was not found in our study as there was no significant difference in the relative abundance of Desulfovibrionaceae and family Prevotellaceae between lean and obese phenotype. This suggests that the association of methanogenic archaea with SRBs, members of family Prevotellaceae, or with leanness and obesity is dependent on the interaction of gut microbial communities with each other that varies between individuals and may be dependent on the dietary pattern of individual.

Higher levels of Verrucomicrobia (*Akkermansia muciniphila)* in lean participants in our study agreed with previous studies (247, 248). However, gathering data regarding inflammation and markers of metabolic syndrome that are associated with reduced levels of Verrucomicrobia would further help in establishing whether this group of bacteria are actively playing a protective role in the gut.

Our dietary data did not show any effect on the community composition, however, under-reporting in the obese groups and a small sample size may have limited our ability to identify any link that may exist in this group. Other unknown environmental or genetic host-related factors might also contribute to community composition making it difficult to identify links with diet.

Measurement of the gut microbiota composition using high throughput sequencing is efficient and informative. However, it measures relative abundance of species and a quantitative shift in the population of one species may result in a change in the relative abundance of another related species resulting in a "secondary effect" of change in another species. Similarly, quantitative changes in two groups may not translate into changes in their relative abundance.

7.5 Challenges in recruitment of the participants

Although the overall prevalence of obesity has reached up to ~64% in men and women of all ages in Scotland, the dietetic and weight management clinic in this tertiary care hospital receives children/young adults referred from general practitioners surgeries or community weight management programs. Recruiting from these clinics was a challenge for the researcher for several reasons. In addition to the management of simple obese patients, this clinic also manages obese and overweight children associated with other disease states. Despite weekly clinics, only 2-4 attendants in the list were usually eligible to be informed about the study and asked for participation. Simple obese patients often have a low level of

motivation and can be resistant to changes in behaviour by weight loss programmes. More importantly, among those patients who were potential for recruitment, they had a very low rate of attending their appointments. Moreover, one third of patients who gave their signed informed consent for the study did not give any faecal sample or refused after an informed consent. Another difficulty in recruitment was in our hypothalamic obese and lean group where the number of eligible participants for the study was limited by several factors including severe co-morbidities due to the disease, age of the patient (>25 years), resistance of the child to provide a sample, and distance of the hospital (recruitment centre) from the patient's residence. Another challenge was demographically matching our control group with our two obese groups as patients attending the endocrine and dietetic clinics were referred from whole of West of Scotland and some patients from Northern England.

Despite these challenges, the researcher successfully organised travel efficiently to recruit as many participants by visiting homes (at participants' convenience), covering distances up to 120 miles, collecting samples at evenings and weekends, and travel efficiently to bring the samples to the lab and process them within 4 h after being produced. The end result of travelling for more than 2000 miles and working out of hours in the lab on a regular basis greatly increased the researcher's ability to collect a good sample size despite these difficulties. The period of recruitment was limited to 16 months due to the set funding and time constraints of the study.

7.6 Aspirations for future study

Keeping in view the high inter-individual variability, particularly in the two obese groups, it is strongly recommended to conduct studies with larger numbers of subjects and under better controlled conditions in terms of dietary intake and weight loss management. Due to small number of participants in this "pilot" study, the results obtained should be considered exploratory. This study however provides a base for designing a future study that address only specific primary outcomes to avoid testing multiple hypotheses. Moreover, testing each outcome should be appropriately adjusted for multiple comparisons or false discovery rates. As mentioned earlier, post hoc power analysis for future study would need at least 20 participants in each group. Sufficient power coupled with appropriate adjustment for multiple testing would enable the researcher finding statistically significant differences between the groups.

Two years since the recruitment of the first participant for this study, follow-up anthropometric data and faecal samples are also recommended to assess changes in gut microbiota in relation to weight loss or weight gain. Additionally, hypothalamic lean

patients, who had median BMI SDS scores >1SDS at the time of recruitment, may represent a transition from lean towards obese status as suggested in the literature. Their follow-up is therefore of particular interest to study the changes in gut microbiota composition and function from leanness towards obesity.

Results from our study have encouraged us to investigate the link of gut microbiome of our participants further by sequencing their gut microbial metagenome to explore if similar metabolic profile and microbial communities in simple and hypothalamic obese groups translate into the functional genes. This is another exciting aspect to this study and we have sent 96 genomic bacterial DNA samples (hypoth. obese n=10, hypoth. lean, n=12, simple obese, n=13, healthy lean, n=13 each for recruitment and after 2-3 months)for shotgun illumina Hiseq® sequencing. However, this will not constitute a part of this PhD.

We also suggest further studies into gut microbial metabolic activity, gut microbiome, and gut microbial metagenome by transplantation of gut microbiota of our obese groups into germ free animals to explore if characteristics associated with obesity are transmissible in terms of microbial composition and functionality.

Furthermore, characterisation of microbial community composition and expression of microbial genes with different dietary substrates in *in vitro* fermentation studies is also suggested to explore how gut microbiota of simple and hypothalamic obese patients respond to dietary fibre challenge over a period of 24 h. This will help in identifying potential prebiotics that could beneficially affect gut microbiota that produce beneficial metabolites (such as butyrate) and express functional genes related to metabolic health (such as genes related to SCFA production). As a first step to achieve this aim, we have modified and established our genomic bacterial DNA extraction method for the fermentation samples. This was deemed essential, as these incubated faecal samples are a diluted mixture of fermentation medium (containing phosphate and carbonate buffer, trypton, and microminerals), reducing solution (sodium sulphide, cysteine hydrochloride, and NaOH), one of the five dietary fibre substrates, and sodium hydroxide.

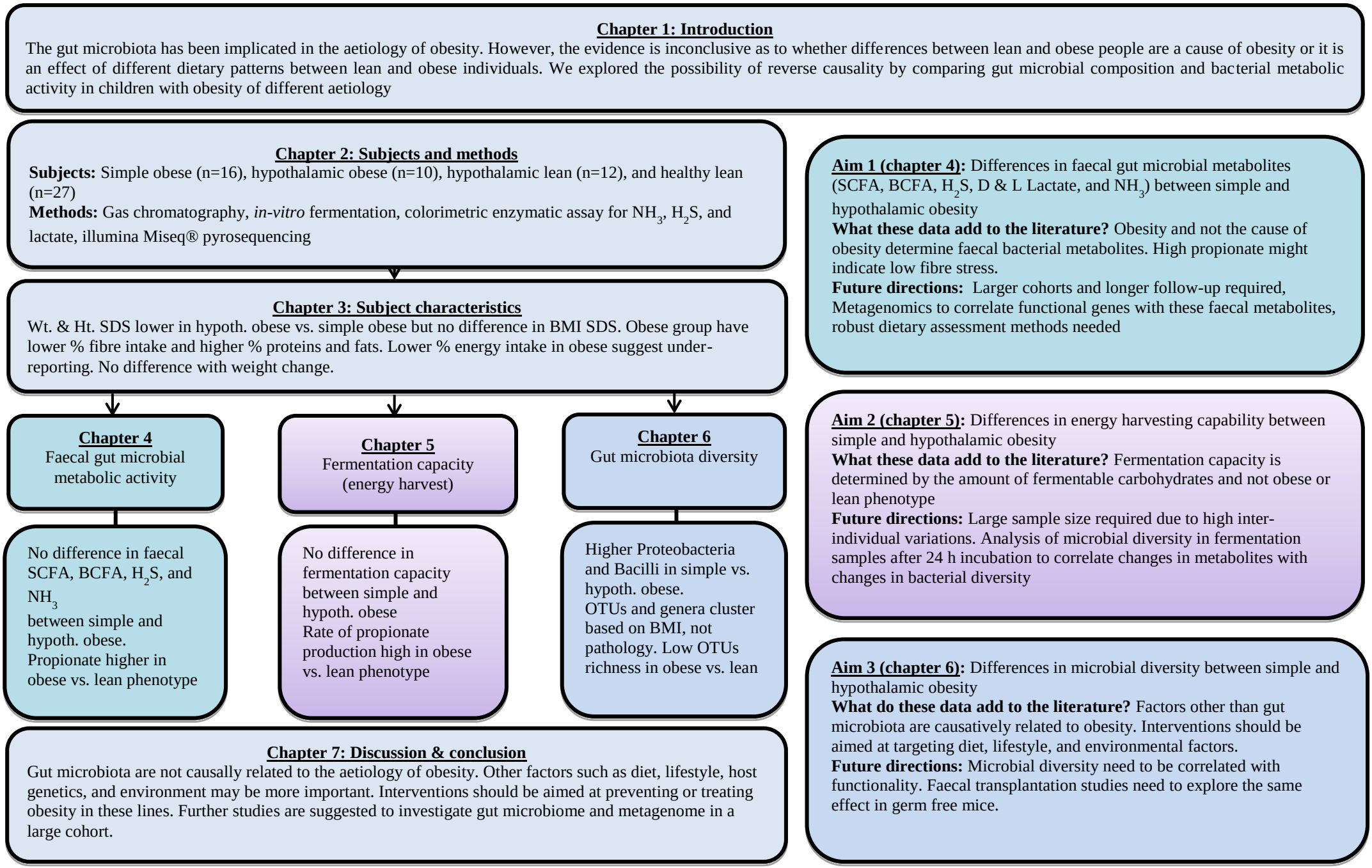
Since our simple obese group had a higher proportional intake of proteins, the measurement of faecal phenol and cresol, which are potentially harmful degradation products of dietary and endogenous proteins, would also give an indication of the risk to gut health in the two obese groups. Initial attempts by a Masters (MRes) research student were not successful in optimising a simple and quick colorimetric assay for the determination of total faecal phenol. Therefore a more sensitive and specific method based on high performance liquid chromatography is under consideration for future analysis of these samples.

Whether obese participants share similarities in gut microbiota composition and metabolic activity with their parents is another interesting subject for us to investigate. Since we also collected anthropometric data and faecal samples from parents (n= 30) who agreed to take part, analysis of their faecal samples for bacterial metabolic activity, fermentation capacity, and microbial diversity would further help in establishing the influence of parents on the gut microbiota of their offspring's in determining their lean or obese phenotype.

7.7 Conclusion

In the final analysis, this PhD provided several novel insights into the "cause or effect" relationship of the gut microbiota with obesity (Figure 7.1). We explored the relationship of gut microbiota with obesity by studying three dimensions: a) gut microbial metabolic activity, b) energy harvesting capability, and c) gut microbial diversity. Based on our results we conclude that gut microbiota are not causally related to the aetiology of obesity as there was no difference in the metabolic activity, energy harvesting capability, and microbial diversity between obesity of different aetiology. Furthermore, obesity, and not the cause of obesity was explaining differences in metabolites and microbial diversity between the groups.

Figure 7.1: Schematic flow-chart of this PhD



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Appendices

1. Approval letter from Research and Development department, NHS Greater Glasgow and Clyde for conducting the study.
2. List of information sheets and consent forms developed for this study
3. List of Golay barcoded reverse fusion primers used for the amplification of 16S rRNA gene for all the study samples

Appendix 1: R&D management approval for the study

Research & Development
R&D Management Office
1st Floor, Tennent Institute
Western Infirmary
GLASGOW G11 6NT

NHS Greater Glasgow and Clyde

Our Ref: MB/LR
Enquiries to: Dr Michael Barber
Direct Line: 0141 211 8548
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14th Sept 2011

Dr Mohammed G Shaikh
Consultant Paediatric Endocrinologist
Paediatric Endocrinology
R H S C
Dalnair Street
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R&D Management Approval

Dear Dr Shaikh

R&D Reference: GN11KH307
REC Ref: 11/WS/0032
Chief Investigator: Dr Mohammed G Shaikh
Academic Supervisor: Dr Mohammed G Shaikh
Student: Dr Muhammad J Khan
Project Title: Diet, gut microflora and energy from the colonic fermentation of carbohydrates in children with simple and pathological obesity; cause or effect?
Protocol Ref: V2 dated 13/07/11

I am pleased to confirm that Greater Glasgow & Clyde Health Board is now able to grant **Management Approval** for the above study.

Conditions of Approval

1. **For Clinical Trials** as defined by the Medicines for Human Use Clinical Trial Regulations, 2004
   a. During the life span of the study GGHB requires the following information related solely to this site
      i. Notification of any potential serious breaches.
      ii. Notification of any regulatory inspections.

It is your responsibility to ensure that all staff involved in the study at this site have the appropriate GCP training according to the GGHB GCP policy (www.nhsggc.org.uk/content/default.asp?page=s1411), evidence of such training to be filed in the site file.

2. **For all studies** the following information is required during their lifespan.
   a. Recruitment Numbers on a quarterly basis
   b. Any change of staff named on the original SSI form
   c. Any amendments – Substantial or Non Substantial
   d. Notification of Trial/study end including final recruitment figures
   e. Final Report & Copies of Publications/Abstracts

Please add this approval to your study file as this letter may be subject to audit and monitoring.

Your personal information will be held on a secure national web-based NHS database.

I wish you every success with this research study

Yours sincerely

Dr Michael Barber
Research Co-ordinator

Cc: Dr Muhammad J Khan, Student, University of Glasgow

Appendix 2: List of information sheets and consent forms developed for this study

List of information sheets developed for this study

1. Information sheet for children younger than 7 years; healthy normal weight children
2. Information sheet for children younger than 7 years attending Yorkhill endocrine/weight management clinic
3. Information sheet for children 8-13 years; healthy normal weight children
4. Information sheet for children 8-13 years; attending the Yorkhill outpatient endocrine/weight management clinic
5. Information sheet for children/young adults older than 13 years; healthy children/young adults
6. Information sheet for children older than 13 years children/young adults attending the Yorkhill outpatient endocrine/weight management clinic
7. Information sheet for the carers of healthy children
8. Information sheet for the carers of children/young adults attending Yorkhill outpatient endocrine/weight management clinic

List of consent forms developed for the study

1. Assent form for children (To be completed by the child and their parent/guardian): Do the bugs inside the bellies of different kinds of children with increased weight, act differently?
1. Consent form (young persons and adults): Do the bugs (bacteria) normally present in human guts affect body weight?
2. Consent form (carer/parent of participant): Diet, gut microbiota and energy from colonic fermentation of carbohydrates in children with simple and pathological obesity, cause or effect?

Appendix 3: List of Golay bar-coded reverse fusion primers used for amplification of 16S rRNA gene in the study samples

sample ID	Concentration (ng/µl)	Name	RC of Illumina 3' Adapter	Golay Barcode
PWS001	3.02	806rcbc302	CAAGCAGAAGACGGCATACGAGAT	TGTATCTTCACC
PWS002	2.96	806rcbc198	CAAGCAGAAGACGGCATACGAGAT	CGAGCTGTTACC
PWS003	2.52	806rcbc328	CAAGCAGAAGACGGCATACGAGAT	ACTTTGCTTTGC
PWS004	4.98	806rcbc235	CAAGCAGAAGACGGCATACGAGAT	AGGTGAGTTCTA
PWS005	2.86	806rcbc300	CAAGCAGAAGACGGCATACGAGAT	TCCAACTGCAGA
PWS006	4.40	806rcbc220	CAAGCAGAAGACGGCATACGAGAT	GTCGTCCAAATG
PWS007	5.04	806rcbc248	CAAGCAGAAGACGGCATACGAGAT	ACCGTGCTCACA
PWS008	6.68	806rcbc245	CAAGCAGAAGACGGCATACGAGAT	TATCACCGGCAC
PWS009	6.12	806rcbc291	CAAGCAGAAGACGGCATACGAGAT	GATCCTCATGCG
PWS010	4.74	806rcbc331	CAAGCAGAAGACGGCATACGAGAT	GAGGACCAGCAA
PWS011	14.3	806rcbc301	CAAGCAGAAGACGGCATACGAGAT	TAAAGACCCGTA
PWS012	31.2	806rcbc259	CAAGCAGAAGACGGCATACGAGAT	GAACGGGACGTA
PWS013	7.32	806rcbc304	CAAGCAGAAGACGGCATACGAGAT	TCGTGGATAGCT
PWS014	9.54	806rcbc242	CAAGCAGAAGACGGCATACGAGAT	GCAGATTTCCAG
PWS015	17.9	806rcbc266	CAAGCAGAAGACGGCATACGAGAT	TGGCTTTCTATC
PWS016	4.10	806rcbc268	CAAGCAGAAGACGGCATACGAGAT	GAGCGTATCCAT
PWS017	26.4	806rcbc241	CAAGCAGAAGACGGCATACGAGAT	GCCGTAAACTTG
PWS018	4.96	806rcbc238	CAAGCAGAAGACGGCATACGAGAT	AGACAGTAGGAG
PWS019	3.14	806rcbc314	CAAGCAGAAGACGGCATACGAGAT	AGGCACAGTAGG
PWS020	20.8	806rcbc316	CAAGCAGAAGACGGCATACGAGAT	CTCTTCTGATCA
PWS021	7.26	806rcbc300	CAAGCAGAAGACGGCATACGAGAT	TCCAACTGCAGA
PWS022	3.32	806rcbc327	CAAGCAGAAGACGGCATACGAGAT	ACGGCGTTATGT
PWS023	13.3	806rcbc303	CAAGCAGAAGACGGCATACGAGAT	GACTGACTCGTC
PWS025	3.76	806rcbc360	CAAGCAGAAGACGGCATACGAGAT	AACCGATGTACC
PWS026	6.42	806rcbc366	CAAGCAGAAGACGGCATACGAGAT	CGCCACGTGTAT
PWS027	8.96	806rcbc274	CAAGCAGAAGACGGCATACGAGAT	TAACGCTGTGTG
PWS028	3.84	806rcbc302	CAAGCAGAAGACGGCATACGAGAT	TGTATCTTCACC
PWS029	5.52	806rcbc319	CAAGCAGAAGACGGCATACGAGAT	TATCCAAGCGCA
PWS030	20.2	806rcbc348	CAAGCAGAAGACGGCATACGAGAT	CTTGACGAGGTT
PWS031	2.78	806rcbc261	CAAGCAGAAGACGGCATACGAGAT	GGTCTCCTACAG
PWS032	3.74	806rcbc204	CAAGCAGAAGACGGCATACGAGAT	AGGGTGACTTTA
PWS033	5.24	806rcbc303	CAAGCAGAAGACGGCATACGAGAT	GACTGACTCGTC
PWS034	2.90	806rcbc244	CAAGCAGAAGACGGCATACGAGAT	GAGACGTGTTCT
PWS035	6.32	806rcbc322	CAAGCAGAAGACGGCATACGAGAT	AACTGCGATATG
PWS036	3.92	806rcbc315	CAAGCAGAAGACGGCATACGAGAT	CTACTTACATCC
PWS037	10.8	806rcbc236	CAAGCAGAAGACGGCATACGAGAT	CCTGTCCTATCT
PWS038	4.38	806rcbc304	CAAGCAGAAGACGGCATACGAGAT	TCGTGGATAGCT
PWS039	15.8	806rcbc311	CAAGCAGAAGACGGCATACGAGAT	CTACGAAAGCCT
PWS040	3.32	806rcbc251	CAAGCAGAAGACGGCATACGAGAT	CCTTGACCGATG
PWS041	24.0	806rcbc249	CAAGCAGAAGACGGCATACGAGAT	CTCCCTTTGTGT
PWS042	15.3	806rcbc305	CAAGCAGAAGACGGCATACGAGAT	GACGCACTAACT
PWS043	19.2	806rcbc306	CAAGCAGAAGACGGCATACGAGAT	GGCGATTTACGT
PWS044	3.00	806rcbc269	CAAGCAGAAGACGGCATACGAGAT	ATGGGCGAATGG
PWS045	8.28	806rcbc364	CAAGCAGAAGACGGCATACGAGAT	CACACAAAGTCA
PWS046	4.50	806rcbc240	CAAGCAGAAGACGGCATACGAGAT	ATTGTTCCTACC

PWS047	8.82	806rcbc307	CAAGCAGAAGACGGCATACGAGAT	TAAGGCATCGCT
PWS048	4.84	806rcbc258	CAAGCAGAAGACGGCATACGAGAT	GAGAGTCCACTT
PWS049	21.0	806rcbc332	CAAGCAGAAGACGGCATACGAGAT	AATAGCATGTCG
PWS050	14.5	806rcbc308	CAAGCAGAAGACGGCATACGAGAT	ACCCATACAGCC
PWS051	7.44	806rcbc268	CAAGCAGAAGACGGCATACGAGAT	GAGCGTATCCAT
PWS052	14.8	806rcbc336	CAAGCAGAAGACGGCATACGAGAT	ATAGGCTGTAGT
PWS053	6.82	806rcbc309	CAAGCAGAAGACGGCATACGAGAT	CGCACTACGCAT
PWS054	6.60	806rcbc289	CAAGCAGAAGACGGCATACGAGAT	GATCATTCTCTC
PWS055	4.14	806rcbc358	CAAGCAGAAGACGGCATACGAGAT	TTGGTAAAGTGC
PWS056	66.0	806rcbc239	CAAGCAGAAGACGGCATACGAGAT	GCCACGACTTAC
PWS057	20.2	806rcbc275	CAAGCAGAAGACGGCATACGAGAT	AACCAAACTCGA
PWS058	18.70	806rcbc370	CAAGCAGAAGACGGCATACGAGAT	ACCTGTCCTTTC
PWS059	3.12	806rcbc246	CAAGCAGAAGACGGCATACGAGAT	TATGCCAGAGAT
PWS060	7.34	806rcbc333	CAAGCAGAAGACGGCATACGAGAT	CGGAGTAATCCT
PWS061	21.2	806rcbc368	CAAGCAGAAGACGGCATACGAGAT	CATGTGCTTAGG
PWS062	4.14	806rcbc273	CAAGCAGAAGACGGCATACGAGAT	ATAGCGAACTCA
PWS063	4.54	806rcbc342	CAAGCAGAAGACGGCATACGAGAT	TATGAACGTCCG
PWS064	2.78	806rcbc295	CAAGCAGAAGACGGCATACGAGAT	TCCATCGACGTG
PWS065	9.78	806rcbc363	CAAGCAGAAGACGGCATACGAGAT	TTGGGCCACATA
PWS066	10.3	806rcbc317	CAAGCAGAAGACGGCATACGAGAT	ATGCTAACCACG
PWS067	27.6	806rcbc227	CAAGCAGAAGACGGCATACGAGAT	CACCCGATGGTT
PWS068	3.16	806rcbc260	CAAGCAGAAGACGGCATACGAGAT	ACGTGTAGGCTT
PWS069	5.89	806rcbc221	CAAGCAGAAGACGGCATACGAGAT	CAACGTGCTCCA
PWS070	5.66	806rcbc340	CAAGCAGAAGACGGCATACGAGAT	AGGAACCAGACG
PWS071	9.14	806rcbc347	CAAGCAGAAGACGGCATACGAGAT	CTAGCTATGGAC
PWS072	4.8	806rcbc225	CAAGCAGAAGACGGCATACGAGAT	GTCGCCGTACAT
PWS073	4.22	806rcbc270	CAAGCAGAAGACGGCATACGAGAT	GATCTCTGGGTA
PWS074	13.4	806rcbc230	CAAGCAGAAGACGGCATACGAGAT	GGTTCCATTAGG
PWS075	3.18	806rcbc323	CAAGCAGAAGACGGCATACGAGAT	CTTCCAACTCAT
PWS076	11.4	806rcbc310	CAAGCAGAAGACGGCATACGAGAT	CAGTCGTTAAGA
PWS077	24.2	806rcbc350	CAAGCAGAAGACGGCATACGAGAT	CTCTGCCTAATT
PWS078	2.86	806rcbc283	CAAGCAGAAGACGGCATACGAGAT	CGCATTTGGATG
PWS079	7.00	806rcbc260	CAAGCAGAAGACGGCATACGAGAT	ACGTGTAGGCTT
PWS080	15.1	806rcbc358	CAAGCAGAAGACGGCATACGAGAT	TTGGTAAAGTGC
PWS081	4.58	806rcbc301	CAAGCAGAAGACGGCATACGAGAT	TAAAGACCCGTA
PWS082	9.94	806rcbc207	CAAGCAGAAGACGGCATACGAGAT	ACTGATGGCCTC
PWS083	3.60	806rcbc307	CAAGCAGAAGACGGCATACGAGAT	TAAGGCATCGCT
PWS084	6.22	806rcbc309	CAAGCAGAAGACGGCATACGAGAT	CGCACTACGCAT
PWS085	4.12	806rcbc362	CAAGCAGAAGACGGCATACGAGAT	GCATTACTGGAC
PWS086	8.52(1/20 dilution)	806rcbc319	CAAGCAGAAGACGGCATACGAGAT	TATCCAAGCGCA
PWS087	4.90	806rcbc252	CAAGCAGAAGACGGCATACGAGAT	CTATCATCCTCA
PWS088	55.8	806rcbc367	CAAGCAGAAGACGGCATACGAGAT	GCAACCGATTGT
PWS089	5.24	806rcbc290	CAAGCAGAAGACGGCATACGAGAT	AGACATACCGTA
PWS090	3.30	806rcbc321	CAAGCAGAAGACGGCATACGAGAT	TCGCCGTGTACA
PWS091	10.5	806rcbc330	CAAGCAGAAGACGGCATACGAGAT	CGAAACTACGTA
PWS092	3.22	806rcbc312	CAAGCAGAAGACGGCATACGAGAT	ATAATTGCCGAG
PWS093	102	806rcbc322	CAAGCAGAAGACGGCATACGAGAT	AACTGCGATATG
PWS094	17.7	806rcbc212	CAAGCAGAAGACGGCATACGAGAT	GTTCGGTGTCCA

PWS095	23.0	806rcbc357	CAAGCAGAAGACGGCATACGAGAT	GGCTGCATACTC
PWS096	4.44	806rcbc294	CAAGCAGAAGACGGCATACGAGAT	AGCTCTAGAAAC
PWS097	9.04	806rcbc282	CAAGCAGAAGACGGCATACGAGAT	GTAGGTGCTTAC
PWS098	3.22	806rcbc271	CAAGCAGAAGACGGCATACGAGAT	CATCATACGGGT
PWS099	5.78	806rcbc325	CAAGCAGAAGACGGCATACGAGAT	TGTACATCGCCG
PWS100	4.60	806rcbc343	CAAGCAGAAGACGGCATACGAGAT	CCACATTGGGTC
PWS101	8.10	806rcbc196	CAAGCAGAAGACGGCATACGAGAT	CGCTCACAGAAT
PWS102	9.56	806rcbc272	CAAGCAGAAGACGGCATACGAGAT	TACGGATTATGG
PWS103	5.48	806rcbc263	CAAGCAGAAGACGGCATACGAGAT	GATGCTGCCGTT
PWS104	5.34	806rcbc325	CAAGCAGAAGACGGCATACGAGAT	TGTACATCGCCG
PWS105	2.50	806rcbc296	CAAGCAGAAGACGGCATACGAGAT	CGATGTGTGGTT
PWS106	20.4	806rcbc365	CAAGCAGAAGACGGCATACGAGAT	GCCAAGGATAGG
PWS107	13.6	806rcbc326	CAAGCAGAAGACGGCATACGAGAT	TGTTAAGCAGCA
PWS108	6.48	806rcbc327	CAAGCAGAAGACGGCATACGAGAT	ACGGCGTTATGT
PWS109	14.4	806rcbc329	CAAGCAGAAGACGGCATACGAGAT	CAAAGCGGTATT
PWS110	2.50	806rcbc334	CAAGCAGAAGACGGCATACGAGAT	CTGTGTCCATGG
PWS111	10.9	806rcbc284	CAAGCAGAAGACGGCATACGAGAT	ATAACATGTGCG
PWS112	3.60	806rcbc318	CAAGCAGAAGACGGCATACGAGAT	ACCAATCTCGGC
PWS113	15.7	806rcbc235	CAAGCAGAAGACGGCATACGAGAT	AGGTGAGTTCTA
PWS114	4.92	806rcbc199	CAAGCAGAAGACGGCATACGAGAT	CAACACATGCTG
PWS115	3.96	806rcbc256	CAAGCAGAAGACGGCATACGAGAT	CTTAGGCATGTG
PWS116	3.44	806rcbc217	CAAGCAGAAGACGGCATACGAGAT	ATTTAGGACGAC
PWS117	4.34	806rcbc219	CAAGCAGAAGACGGCATACGAGAT	TGGTTGGTTACG
PWS118	9.36	806rcbc208	CAAGCAGAAGACGGCATACGAGAT	TTCGATGCCGCA
PWS119	19.5	806rcbc361	CAAGCAGAAGACGGCATACGAGAT	TCGATTGGCCGT
PWS120	5.12(1/20 dilution)	806rcbc328	CAAGCAGAAGACGGCATACGAGAT	ACTTTGCTTTGC
PWS120	22.2	806rcbc208	CAAGCAGAAGACGGCATACGAGAT	TTCGATGCCGCA
PWS121	15.2	806rcbc336	CAAGCAGAAGACGGCATACGAGAT	ATAGGCTGTAGT
PWS122	5.46	806rcbc277	CAAGCAGAAGACGGCATACGAGAT	CTGGGTATCTCG
PWS123	10.3	806rcbc265	CAAGCAGAAGACGGCATACGAGAT	ATTAAGCCTGGA
PWS124	2.52	806rcbc251	CAAGCAGAAGACGGCATACGAGAT	CCTTGACCGATG
PWS125	42.6	806rcbc285	CAAGCAGAAGACGGCATACGAGAT	CTTGAGAAATCG
PWS126	3.20	806rcbc313	CAAGCAGAAGACGGCATACGAGAT	GGCATGTTATCG
PWS127	8.70	806rcbc337	CAAGCAGAAGACGGCATACGAGAT	TGTGTAGCCATG
PWS128	9.82	806rcbc350	CAAGCAGAAGACGGCATACGAGAT	CTCTGCCTAATT
PWS129	84.4	806rcbc337	CAAGCAGAAGACGGCATACGAGAT	TGTGTAGCCATG
PWS130	9.86	806rcbc338	CAAGCAGAAGACGGCATACGAGAT	AAGGGCGCTGAA
PWS131	4.32	806rcbc339	CAAGCAGAAGACGGCATACGAGAT	GTTTCCGTGGTG
PWS132	5.04	806rcbc341	CAAGCAGAAGACGGCATACGAGAT	TAATGCCCAGGT
PWS133	4.44	806rcbc243	CAAGCAGAAGACGGCATACGAGAT	AGATGATCAGTC
PWS134	22.80	806rcbc245	CAAGCAGAAGACGGCATACGAGAT	TATCACCGGCAC
PWS135	18.8	806rcbc218	CAAGCAGAAGACGGCATACGAGAT	GGATAGCCAAGG
PWS136	3.30	806rcbc310	CAAGCAGAAGACGGCATACGAGAT	CAGTCGTTAAGA
PWS137	11.9	806rcbc308	CAAGCAGAAGACGGCATACGAGAT	ACCCATACAGCC
PWS138	3.06	806rcbc338	CAAGCAGAAGACGGCATACGAGAT	AAGGGCGCTGAA
PWS139	4.98	806rcbc342	CAAGCAGAAGACGGCATACGAGAT	TATGAACGTCCG
PWS140	5.44	806rcbc233	CAAGCAGAAGACGGCATACGAGAT	AGCGTAATTAGC
PWS140	3.18	806rcbc324	CAAGCAGAAGACGGCATACGAGAT	GAGATCGCCTAT

PWS141	16.1	806rcbc306	CAAGCAGAAGACGGCATACGAGAT	GGCGATTTACGT
PWS142	3.90	806rcbc343	CAAGCAGAAGACGGCATACGAGAT	CCACATTGGGTC
PWS143	11.6	806rcbc209	CAAGCAGAAGACGGCATACGAGAT	TGTGGCTCGTGT
PWS144	14.9	806rcbc267	CAAGCAGAAGACGGCATACGAGAT	ACAGCTCAAACA
PWS145	10.4	806rcbc351	CAAGCAGAAGACGGCATACGAGAT	ATATGACCCAGC
PWS146	6.84	806rcbc347	CAAGCAGAAGACGGCATACGAGAT	CTAGCTATGGAC
PWS147	18.3	806rcbc262	CAAGCAGAAGACGGCATACGAGAT	ACTGACTTAAGG
PWS148	5.00	806rcbc353	CAAGCAGAAGACGGCATACGAGAT	ATTGAGTGAGTC
PWS149	5.04	806rcbc210	CAAGCAGAAGACGGCATACGAGAT	AACTTTCAGGAG
PWS150	4.16	806rcbc263	CAAGCAGAAGACGGCATACGAGAT	GATGCTGCCGTT
PWS151	2.50	806rcbc266	CAAGCAGAAGACGGCATACGAGAT	TGGCTTTCTATC