Investigating the effects of a high carbohydrate, high fat diet on the ghrelin-serotonin 2C receptor pathway in the brain

Kara Stuart

Declaration

I certify that the work reported in this thesis is entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not previously been submitted for assessment in any other course of study, at any other University.

Signature of candidate Kara Stuart

Date 10/03/17

Endorsement

Supervisor signature

Date 10/3/17.

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Abbreviations

5-HT - 5-hydroxytryptamine, serotonin

5-HT_{2C}R - serotonin 2C receptor

AG - acylated ghrelin

AgRP - agouti related peptide

AOM – Azoxymethane

ARC – arcuate nucleus

BCA – bicinchonic acid

BMI - body mass index

CS – corn starch

CTR – control

CTRW - control plus tryptophan

DIO – diet-induced obesity

DMEM - Dulbecco's Modified Eagle's Mdium

DSS – dextran sodium/sulfate

DZIP3 – Deleted in azoospermia (DAZ) interacting zinc finger protein 3

ECL – enhanced chemiluminescent

ER- α – oestrogen receptor α

GAM – gifu anaerobe media

GHRL – ghrelin

GHSR - Growth hormone secretagogue receptor

GIT - Gastrointestinal tract

GOAT - Ghrelin-O-acyltransferase

H8C8 – HCHF eight weeks, CS eight weeks

HCHF - High carbohydrate and high fat diet

HDACi – histone deactylase inhibitor

HED – high energy diet

HEDW – high energy plus tryptophan

HGC – high gene count

HPA – hypothalamic-pituitary-adrenal

Hprt1- Hypoxanthine phosphoribosyltransferase 1

HRP – horseradish peroxidase

KAT5 - Lysine acetyltransferase 5

KAT6A - Lysine acetyltransferase 6A

LGC – low gene count

mCPP – (m-chlorophenylpiperazine)

MUC2 – mucin 2

MYSM1 - Myb Like, SWIRM And MPN Domains 1

NCOA6 - Nuclear receptor coactivator 6

NEK6 - NIMA related kinase 6

NPY – neuropeptide Y

NSPs – non-starch polysaccharides

OF – oligofructose

PAK1 - P21 (RAC1) activated kinase 1

PBS – phosphate buffered saline

POMC – proopiomelanocortin

PRMT 1, 2, 5, 7- Protein arginine methyltransferase 1, 2, 5, 7

RIPA – radioimmunoprecipitation assay

RPLP0 - Ribosomal protein lateral stalk subunit P0

RS – resistant starch

SCFA – short chain fatty acid

SUV420H1 - Histone-lysine N-methyltransferase

TBST – tris-buffered saline with Tween 20

Tlr4 – Toll like receptor 4

Tph1 - Tryptophan hydroxylase 1

UAG – unacylated ghrelin

UBE2A - Ubiquitin conjugating enzyme E2 A

VTA – ventral tegmental area

WL – weight loss

α -MSH - α -melanocyte stimulating hormone

βME- β-mercapto-ethanol

Abstract

Obesity is a global pandemic with more than 13% of the adult population classified as obese and 39% overweight. Strategies to treat and prevent the energy imbalance that causes obesity could be developed by understanding the gene expression and epigenetic changes that drive appetite and energy regulation. Ghrelin is a stomach and brain-secreted peptide hormone that stimulates appetite and energy balance. It is activated by the enzyme ghrelin-O-acyltransferase (GOAT) and is mediated by the ghrelin receptor (GHSR1a). Serotonin (5-HT) is another stomach-secreted hormone that regulates appetite by stimulating post-meal satiety and reducing food intake through binding to the 2C serotonin receptor (5-HT_{2C}R) in the brain. Plasma ghrelin levels are increased and plasma 5-HT levels are decreased by a high fat diet in rats, however the expression of ghrelin, GOAT, GHSR1a and 5-HT_{2C}R by the brain in response to a long term high carbohydrate and high fat diet (HCHF), representative of the 'Western' diet, has not been explored. This study investigated the effects of diet and diet composition change (reverting from a HCHF diet back to a standard corn starch (CS) diet) on the expression of the ghrelin and 5-HT_{2C}R pathways in the rat brain using real time reverse transcriptase polymerase chain reaction (RT-PCR) and Western Blot (Immunoblot). This study also investigated whether the HCHF diet could influence the epigenome of human colon cells via changes in the gut microbiome. There was not a consistent upregulation or down-regulation of ghrelin, GOAT or GHSR in response to the HCHF diet however there was a consistent and significant decrease in the expression of both GOAT and GHSR when the diet was reverted back to the standard diet. The expression of 5-HT_{2C}R was more consistent with all rats fed the HCHF diet demonstrating a non-significant decrease in the expression of the 5-HT_{2C}R and three out of four rats demonstrating a non-significant increase in

response to reverting back to the standard diet. In order to investigate the effects of the HCHF-induced microbiome population change on colon cells (independent of other diet-induced changes), colon contents from rats fed the HCHF and CS were cultured under anaerobic conditions and then *in vitro* co-cultured with human colon cells. RT-PCR analysis of the expression of epigenetic modifying enzymes in both groups demonstrated a twelve-fold increase in the expression of deleted in azoospermia (DAZ) interacting zinc finger protein 3 (DZIP3) in response to the HCHF. DZIP3 is a chromatin modifying enzyme and thus may influence changes to the epigenome. In summary, these findings support a role for brain-expressed GOAT and GHSR1a in reducing the ability of ghrelin to stimulate appetite and for brain-expressed 5-HT_{2C}R to induce satiety in the brain when HCHF food is removed. This study also provides pilot data that HCHF can modify the epigenome of colon cells via changes in the gut microbiome.

CHAPTER 1
INTRODUCTION

Obesity

As of 2014, there were more than 1.9 billion overweight and 600 million obese adults worldwide, translating to 39% of the adult population being overweight and 13% obese (World Health Organisation 2015). The obesity 'pandemic' has been attributed to factors such as a sedentary lifestyle, insufficient physical activity, the Western diet and genetics (Le Chatelier *et al.* 2013). Diet is an important modifiable factor especially when considering energy intake versus energy expenditure. Appetite, or the motivation to eat, is also an integral factor in obesity. The ghrelin axis is a short term regulator of appetite and satiety and it appears that this pathway is dysregulated in obese individuals, with obese individuals having paradoxically low levels of ghrelin. Emerging evidence suggests that serotonin (5-HT), acting via the serotonin 2C receptor (5-HT_{2C}R), induces satiety and thus is also a key regulator of appetite. Whether this pathway is still intact in obese individuals has not been explored. The link between diet, obesity and the ghrelin and serotonergic pathways in the brain has not yet been reported. Additionally, obese individuals exhibit characteristic changes in their gut microbiome, with overall less bacterial diversity and disrupted ratio of the two dominant bacterial phyla, *Bacteroides* and *Firmicutes (*Mishra *et al.* 2016). Obese humans and rats characteristically have increased numbers of *Firmicutes* and lowered numbers of *Bacteroides* in comparison to healthy weight individuals. These differences can lead to epigenetic changes in the colon that can also influence gut and overall health.

The Ghrelin Axis

Ghrelin

Ghrelin is an orexigenic peptide hormone that stimulates appetite by transmitting a 'hunger signal' to the brain (Mishra *et al.* 2016). Ghrelin has a significant role in regulating food intake and energy balance, therefore changes in the expression of ghrelin and other factors regulating the ghrelin axis may contribute to the development of obesity. Ghrelin levels increase with body mass index (BMI), however obese individuals exhibit paradoxically low levels of ghrelin. Ghrelin is activated by the enzyme ghrelin O-acyltransferase (GOAT) and functions via binding to the growth hormone secretagogue receptor (GHSR). GOAT also increases with BMI, but unlike ghrelin, circulating levels of GOAT are also increased in obesity (Goebel-Stengel *et al.* 2013).

Ghrelin was initially discovered in the gastrointestinal tract (GIT) and is most well-known for its involvement in the regulation of energy metabolism and stimulation of growth hormone (Kojima *et al.* 1999). However, ghrelin has many additional physiological functions, making it more than simply a growth hormone stimulating peptide, as indicated by its widespread expression throughout the body including a sup-population of cells in the hypothalamus (Watterson *et al*. 2012). Ghrelin functions in metabolism, appetite regulation and gut motility, and is also involved in the function of the immune, cardiovascular and reproductive systems (Kojima & Kangawa 2010).

There are multiple steps involved in the production of ghrelin. After cleavage from preproghrelin, the enzyme GOAT facilitates the addition of an octanoyl group to the hydroxyl group of the serine residue, forming acylated ghrelin (AG) (Figure 1) (Gutierrez *et al.* 2008; Khatib *et al.* 2015).

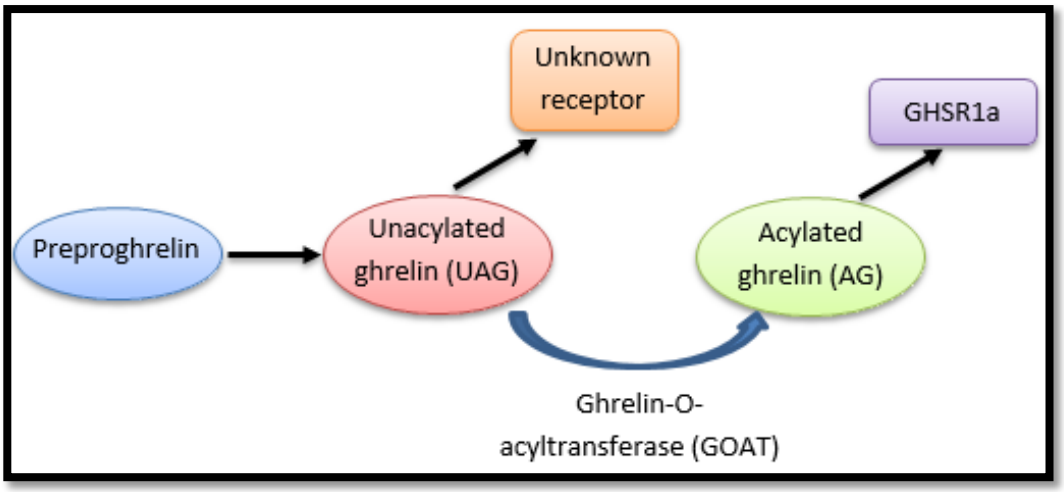


Figure 1: A schematic diagram of the ghrelin axis illustrating the process in which acylated ghrelin (AG) is synthesised. Unacylated ghrelin (UAG) is cleaved from its precursor, preproghrelin. Ghrelin-O-acyltransferase (GOAT) facilitates the addition of an octanoyl group to unacylated ghrelin to form AG. AG is able to bind to the ghrelin secretagogue receptor 1a (GHSR1a), while it is hypothesised that UAG binds to an unknown receptor.

It is only the acylated form of ghrelin which can bind to, and activate its receptor, the GHSR, specifically via the 1a subunit (GHSR1a) (Kojima *et al.* 1999). Results regarding the role of unacylated ghrelin (UAG) have been inconsistent. It was thought that due to its inability to bind to GHSR1a, UAG may not have any biological function, however a study by Toshinai and colleagues (2006) found that UAG facilitates biological effects similar to AG. The central administration of UAG to Wistar rats increased feeding through the activation of the orexin pathway in the hypothalamus. Interestingly, central administration of UAG to rats lacking GHSR still induced an orexigenic response, indicating that UAG binds to a currently unknown receptor to produce its biological actions. A more recent study by

Fernandez *et al.* (2016), reported that UAG acts on a subset of neurons in arcuate nucleus (ARC) in the hypothalamus (a region known for its involvement in appetite regulation) through a GHSR-independent pathway. The study found that centrally administered UAG impaired the action of peripherally administered AG, at least in part by activation of proopiomelanocortin (POMC) neurons, having an antagonistic effect on AG (Fernandez *et al.* 2016). AG makes up only a small proportion of circulating ghrelin, with UAG contributing to approximately 80-90% of circulating total ghrelin (Pacifico *et al.* 2009). This is possibly due to the shorter half-life of AG (Toshinai *et al*. 2006; Fernandez *et al.* 2016). Changes in the ratio of circulating forms of ghrelin, which is partly regulated by the level of GOAT, may affect energy metabolism and feeding.

Ghrelin plasma levels increase prior to a meal to induce hunger and decrease rapidly at the end of a meal, indicating satiety (Mishra *et al.* 2016). Ghrelin induces food intake centrally via neurons of the ARC, specifically through activation of neuropeptide Y (NPY) and agouti related peptide (AgRP) neurons (Sato *et al.* 2005; Ferrini *et al*. 2009). Plasma ghrelin levels are positively correlated with body mass index (BMI), except in obese individuals which have paradoxically low levels of ghrelin (Tschöp *et al.* 2001). However, the decrease in total plasma ghrelin in obesity has been attributed to a decrease in circulating UAG concentrations, whereas AG concentrations remain at a similar level (Barazzoni *et al*. 2007; Pacifico *et al.* 2009). It is hypothesised that UAG is more quickly degraded in obese individuals, possibly due to increased peptidase activity (François *et al.* 2015). Despite the lower circulating total ghrelin levels in obesity, it is hypothesised that a greater threshold must be reached in obese individuals in order to suppress the orexigenic signalling of ghrelin and induce satiety (Roux *et al.* 2009). Therefore, obese individuals may continually have an increased appetite which is more

difficult to satisfy. This is supported by evidence which suggests that obese individuals need to consume a greater number of calories in order to suppress ghrelin levels compared to non-obese individuals (Roux *et al.* 2009).

Ghrelin O-acyltransferase (GOAT)

GOAT is expressed at higher levels in the stomach, the major site of ghrelin acylation, and is co-expressed with ghrelin in the pancreas (Gutierrez *et al.* 2008). GOAT is not only expressed in gastric mucosal cells, but has also been found to circulate in the plasma (Goebel-Stengel *et al.* 2013). Plasma levels of GOAT are increased in obese individuals compared to healthy weight individuals. In fact there is a positive correlation between circulating levels of GOAT and BMI, while GOAT has a negative correlation with ghrelin in obese individuals. This suggests GOAT may have a role in long term energy metabolism, possibly influencing the development or state of obesity (Goebel-Stengel *et al.* 2013).

GOAT is regulated by a number of growth factors and hormones, including growth hormone releasing hormone (GHRH), leptin, somatostatin and AG itself, however not UAG (Gahete *et al.* 2010). Interestingly, AG may be able to influence its own production by stimulating the expression of GOAT. Gahete and colleagues (2010) found that total ghrelin levels were decreased, with no change in AG levels, in diet-induced obese mice. This was accompanied by reduced total ghrelin levels in the stomach but no change in GOAT mRNA expression. It should be noted that this study did not report the expression of GOAT protein. Therefore, there may be additional factors which influence circulating AG levels, such as the rate of AG degradation or GOAT enzyme and/or UAG levels (Gahete *et al.* 2010).

Serotonin 2C Receptor (5-HT_{2C}R)

5-HT is also involved in appetite and food incentive, and consequently, weight regulation. 5-HT is largely known for its psychological effects that are elicited via binding to one of a large family of receptors categorised into subtypes. It is through the serotonin 2C receptor (5-HT_{2c}R) that 5-HT induces satiety (Tecott *et al.* 1995; Werry *et al.* 2008) via the central melanocortin system (Garfield *et al.* 2016) which makes 5-HT_{2c}R a potential target for controlling obesity. 5-HT_{2C}R is involved in the regulation of appetite and food intake through homeostatic feeding mechanisms in the hypothalamus along with reward and food incentive in the ventral tegmental area (VTA) (Garfield *et al*. 2016; Valencia-Torres *et al.* 2016).

5-HT_{2c}R is one of a family of seven transmembrane domain receptors which produce inter-cellular signals through G-proteins (Werry *et al.* 2008). Experiments by Tecott *et al.* (1995) and Nonogaki *et al.* (2002) illustrate the role of 5-HT_{2c}R in appetite regulation, through the use of mice lacking functional 5-HT_{2c}R which resulted in obesity induced by hyperphagia (excessive eating). Other research has shown that 5-HT_{2c}R can form heterodimers with the GHSR-1a in the brain suggesting that there could be a link between the ghrelin axis and serotonin pathways (Schellekens *et al.* 2013). A recent study investigated the role of GHSR in food intake, particularly focusing on the role of central GHSR in food intake in a mouse model. Neuronal deletion of GHSR almost completely prevented diet-induced obesity (Lee *et al.* 2016). This suggests that central GHSR, most likely due to activation by ghrelin, affects energy metabolism and may be a potential anti-obesity target (Lee *et al*. 2016).

Two populations of neurons are located in the ARC of the hypothalamus which exhibit opposite effects on feeding and energy metabolism. POMC neurons express

α-melanocyte stimulating hormone (α-MSH) while the other neurons express AgRP (Heisler *et al.* 2002). Both α-MSH and AgRP act on the melanocortin 4 receptor (MC4R), with agonistic and antagonistic abilities to illicit an anorexigenic or orexigenic response respectively (Kawahara *et al.* 2008; Heisler *et al.* 2002). 5-HT_{2C}R is expressed by, and regulates the activation of, ARC POMC neurons (Heisler *et al*. 2002). 5-HT acts on the MC4R pathway, by binding to 5-HT_{2C}R on POMC neurons which act on α-MSH upstream of MC4R to produce an anorexigenic effect, therefore inducing satiety (Kawahara *et al.* 2008; Berglund *et al.* 2013).

Serotonin agonists in the treatment of obesity

Both circulating 5-HT levels in the periphery, and central 5-HT levels in the brain, decrease in response to high-fat diet induced obesity in animal models (Kim *et al.* 2013; Zemdegs *et al.* 2016) with a negative correlation shown between 5-HT levels and weight gain (Kim *et al.* 2013). A study by Derkach *et al*. (2015) demonstrated that long-term intranasal 5-HT treatment of a high fat diet and low dose streptozotocin-induced diabetic rat model led to improved metabolic parameters with a decrease in body weight and food intake compared to the diabetic group without 5-HT treatment (Derkach *et al.* 2015). This supports the role of 5-HT in metabolism and appetite and food intake regulation.

With the increasing obesity pandemic, 5-HT_{2C}R gained interest as a potential target for reducing obesity. Put simply obesity occurs due to a surplus of energy compared to energy expenditure. Thus diet and physical activity are seen as the two most important factors in energy regulation. Considering the role of 5-HT_{2C}R in appetite regulation and satiety, a selective 5-HT_{2C}R agonist may be an effective therapeutic approach against obesity. D-fenfluramine, an indirect 5-HT agonist, was marketed as an effective weight-loss drug (Heisler *et al.* 2002). Due to the

lack of selectivity for specific 5-HT receptors, cardiovascular complications occurred in some patients and the drug was withdrawn from use (Heisler *et al.* 2002). A selective 5-HT_{2C}R agonist, Lorcaserin hydrochloride, was approved for use as an adjunct therapy to a reduced-calorie diet and increased physical activity for chronic weight management for obese or overweight individuals with at least one weight-related comorbidity (Boge *et al.* 2014). A recent study by Valencia *et al.* (2016) showed that neurons in the VTA which express 5-HT_{2C}R further modulate food intake by influencing incentive and motivation behaviours. It was shown that chemogenic activation of VTA 5-HT_{2C}Rs in rats significantly reduced *ad libitum* food intake by 59%, which was comparable to the 60% reduction of food intake achieved by administration of Lorcaserin.

Expression of 5-HT_{2C}R in the brain in response to diet

It is known that plasma 5-HT levels are decreased in animal diet-induced obesity models due to a high fat diet. However, it is unknown whether a HCHF diet, representative of the Western diet, leads to changes in the expression of 5-HT_{2C}R in the brain thereby influencing the brain's response to 5-HT.

Lopez-Esparza *et al.* (2015) investigated the effect of the addition of tryptophan supplementation to a high energy diet, on the expression of the serotonin receptors 5-HT_{2C}R and 5-HT_{2A}R in the rodent brain. The amino acid L-tryptophan is of particular significance as it a precursor in the synthesis of 5-HT and it has been shown that manipulation of plasma tryptophan levels via dietary supplementation affects plasma 5-HT levels (van der Stelt *et al.* 2004). However, it was unknown whether tryptophan levels also affected 5-HT receptor expression. A diet-induced obesity (DIO) rodent model was used to compare the expression of the serotonin receptors between control (CTR) rats fed a normal rodent chow

diet, high energy diet (HED) (8% corn oil, 44% sweetened condensed milk and 48% purina rodent chow), control plus tryptophan (CTRW) or high energy plus tryptophan (HEDW). Immunohistochemistry experiments showed a significant difference in the expression of 5-HT_{2C}R between CTR and HED rats, with HED having decreased expression. Expression of 5-HT_{2C}R was also decreased in the HEDW compared to CTRW, however the reduction was not as great as seen between the HED and CTR groups. Western blot analysis showed the HEDW had the weakest expression of 5-HT_{2C}R, however, the HEDW rats did not develop DIO. As demonstrated by immunohistochemistry techniques, the expression of 5-HT_{2C}R was still reduced in the HEDW but potentially the addition of tryptophan prevented the onset of DIO by increasing the synthesis and storage of 5-HT, therefore leading to increased activation of 5-HT_{2C}R (Lopez-Esparza *et al.* 2015).

Link between ghrelin axis and serotonergic pathway

Yamada *et al.* (2015) explored the link between stress and food intake in mice. In aged male mice, exposure to a novel stressor resulted in a significant increase in corticosterone and decreased food intake that was seen for 24 hours following exposure. Aged female mice were not affected in the same way as male mice. This is most likely due to the fact that the aged female mice had a higher basal level of corticosterone and this level was not significantly affected by exposure to stress. Plasma levels of AG significantly decreased in aged male mice following exposure to the novel stressor, suggesting that decreased feeding is due to a stress-induced decrease in AG. The decrease in AG was completely reversed by administration of 5-HT_{2C}R agonist CP-809101 (3.0mg/kg). This not only supports the role of 5-HT_{2C}R in the regulation of food intake, but suggests that activation of 5-HT_{2C}R may decrease food intake through, at least in part, inhibition of AG release. Yamada *et*

al. (2015) explored the role of oestrogen receptor α (ERα) on the food intake of aged animals following exposure to a novel stressor. Administration of a low dose of the ERα agonist propyl pyrazole triol (PPT) to aged male rats produced an anorexigenic effect. This effect was decreased by administration of the 5-HT_{2C}R antagonist SB242084, suggesting that the anorexigenic effect of ERα is achieved through an increase in 5-HT_{2C}R activation. This is in agreeance with previous studies which found that oestradiol increases 5-HT_{2C}R protein synthesis in the dorsal raphe region, caudal brainstem and hypothalamus (Henderson & Bethea 2008; Rivera *et al.* 2012; Santollo *et al.* 2012). The authors concluded that exposure to a novel stressor results in ERα activation, leading to 5-HT_{2C}R hypersensitivity and ultimately activation of the hypothalamic-pituitary-adrenal (HPA) axis and decreased food intake.

A study by Nonogaki *et al.* (2006) investigated the effect of feeding and fasting on hypothalamic 5-HT_{2C}R expression and plasma ghrelin levels in mice. 24 hours fasting induced an increase in hypothalamic 5-HT_{2C}R expression along with an increase in circulating AG levels compared to the fed state. Treatment with 5-HT_{2C}R agonists m-chlorophenylpiperazine (mCPP) or fenfluramine significantly reduced the increase in circulating AG levels seen with fasting and increased the expression of hypothalamic POMC. These results support the notion of a negative feedback mechanism between the central serotonergic pathway and ghrelin axis in the regulation of energy homeostasis (Nonogaki *et al.* 2006). A study by Schellekens *et al* (2013) further supports the link between the serotonin and ghrelin pathways. This study provided evidence for a novel heterodimer between GHSR1a and 5-HT_{2C}R and that this dimerisation attenuates ghrelin-mediated signalling through 5-HT_{2C}R. Administration of RS102221, a specific 5-HT_{2C}R antagonist, restored GHSR1a mediated calcium signalling. This supports that 5-

$HT_{2C}R$ can attenuate GHSR1a potentially through negative feedback upon dimerisation (Schellekens *et al*. 2013).

The Gut Microbiome

Evidence is emerging to suggest that the composition of the gut microbiome can have a profound effect on various physiological functions. Obese individuals exhibit characteristic changes in their gut microbiome, with overall less bacterial diversity and disrupted ratio of the two dominant bacterial phyla, *Bacteroides* and *Firmicutes (*Mishra *et al.* 2016). Obese individuals characteristically have increased numbers of *Firmicutes* and lowered numbers of *Bacteroides* in comparison to healthy weight individuals. External factors can greatly influence the composition of the gut microbiome. Diet is a significant determiner of the gut microbiome composition, altering the residing microorganisms and the proportions in which they are found. It has been suggested that the gut microbiome should be viewed as an endocrine organ, due to the dynamic feedback which occurs between the microflora and the host, and vice versa (Clarke *et al.* 2014). This concept is supported by evidence which shows the plasticity and responsiveness of the gut microbiome in response to dietary changes (Cotillard *et al.* 2013; O'Keefe *et al.* 2015; Salonen *et al.* 2014.

Studies in mice have shown greater microbial response to dietary changes, with diet accounting for approximately 60% of variation in the gut microbiome, compared to approximately 10% in humans (Faith *et al.* 2011; Salonen *et al.* 2014; Zhang *et al.* 2010). However, it should be noted that there is much less inter-individual variation seen in the gut microbiome composition of laboratory mice than humans. Walker *et al.* (2011) also demonstrated how rapidly the gut

microbiome adjusts to dietary changes, with changes in the microflora evident in around 3-4 days. However, these changes were reversed just as quickly.

Diet has also been shown to affect the cell biology of the colon. An innovative study by O'Keefe and colleagues (2014) involved a two-week food exchange between African American and African subjects, who generally consume a high-fat, low-fibre Western style diet and high-fibre, low-fat African-style diet respectively. Colonoscopy prior to exchange revealed adenomatous polyps in nine African Americans (n=20), but no Africans (n=20). African Americans also had significantly greater mucosal epithelial proliferation than the Africans. It has been suggested that greater epithelial proliferation is an indicator of neoplasmic (tumour-forming) change (O'Keefe *et al.* 2014). The risk of DNA mutations occurring is increased due to a greater number of proliferating cells, which are especially vulnerable to carcinogens and thus more susceptible to carcinogenesis. It was suggested that the lower mucosal proliferation in Africans was associated with greater butyrogenesis, with increased butyrate synthesising genes and higher faecal butyrate concentrations also evident. Changes in the fibre and fat content resulted in significant changes to the gut microbiome composition, with notable changes in mucosal inflammation and proliferation. Following the dietary intervention faecal butyrate concentrations increased on average by two and a half times in the African Americans, while concentrations decreased by approximately half in the Africans. Overall, the findings suggest that diet and the gut microbiome are involved in colorectal carcinogenesis potentially through epigenetic changes (modifications to DNA and chromatin without altering genetic sequence) via butyrate (Paparo *et al*. 2014). Increasing fibre consumption may offer a protective effect against colorectal cancer by increasing butyrogenesis through modification of the gut microbiome.

Short Chain Fatty Acids

Short chain fatty acids (SCFA) are the major metabolites produced from bacterial fermentation of carbohydrate and protein in the GIT (Flint *et al.* 2015). Bacteria in the GIT have direct access to nutrients and thus can alter host energy balance by enhancing the calorie utilising capacity of the host. As such the production of SCFAs is determined by host dietary intake, with SCFA production increasing with greater intake of non-digestible carbohydrates, proteins and fats (Flint *et al.* 2015). It is estimated that more than 95% of SCFAs are absorbed by the colon (Topping & Clifton 2001) with the major SCFAs, acetate, proprionate and butyrate found at a molar ratio of approximately 60:20:20 (Cummings 1981). Approximately 60-70% of the energy requirements of colonocytes (the epithelial cells that line the colon) is acquired from fermentation products and butyrate is the predominant energy substrate (Clarke *et al.* 2014; Topping & Clifton 2001).

The role of butyrate in intestinal health and epigenetics

Butyrate plays an important role in gut health and is integral to many areas including: the maintenance of the intestinal barrier, regulation of intestinal motility and visceral perception, immune regulation, ion absorption, amelioration of mucosal inflammation and oxidation, and cell growth and differentiation (Canani *et al*. 2011). Butyrate promotes colonocyte proliferation and cell differentiation in the basal crypt, therefore decreasing the number and size of aberrant crypt focus and reducing the risk of neoplastic lesions in the colon (Canani *et al.* 2011). As butyrate is integral for maintaining colonocyte health, increasing the production of butyrate could potentially protect against colorectal cancer. Not only is butyrate an important energy source for colonocytes, but it has been demonstrated to have anti-carcinogenic properties. Butyrate has opposing effects on the cell growth and differentiation of healthy colonocytes compared to malignant cells (Canini *et al.*

2011). This seemingly contradictory response is known as the 'butyrate paradox' (Canini *et al*. 2011). Butyrate can act as a histone deacetylase inhibitor (HDACi) and may stimulate epigenetic changes, regulate gene expression and promote cell cycle arrest, differentiation and/or apoptosis (Lazarova *et al*. 2004; Mátis et al. 2013). Butyrate influences inflammation and cell proliferation through regulation of the NF-κB pathway, a key inflammatory pathway (Kumar *et al.* 2009). Activation of the NF-κB pathway is regulated by the phosphorylation, ubiquitination and degradation of its inhibitor IκB-α (Kumar *et al.* 2009). IκB-α is ubiquitinated by E3-$^{SCF\beta\text{-}}$TrCP, a specific ubiquitin ligase complex (Kumar *et al.* 2009). It is through the ubiquitination and degradation of E3-$^{SCF\beta\text{-}}$TrCP that butyrate attenuates activation of the NF-κB pathway (Kumar *et al.* 2009). Butyrate can also hyperactivate the WNT/catenin signalling pathway (Lazarova *et al.* 2013) with the butyrate-induced increase in WNT activity resulting in apoptosis in colorectal cancer cell lines (Lazarova *et al*. 2004). Recently, Hu *et al.* (2015) demonstrated another pathway through which butyrate inhibits colon cancer cell proliferation and induces apoptosis. By down-regulating the proto-oncogene c-Myc-induced expression of the oncogenic microRNA miR-17-92a, the expression of miR-92a was also diminished (Hu *et al.* 2015). Furthermore, the addition of exogenous miR-92a to colon cancer cells reversed the beneficial effect of butyrate in inhibiting colon cancer cell proliferation.

Butyrate may also influence the gut microbiome composition through regulating mucin secretion. A study by Jung *et al.* (2015) found that treatment of LS174T human colorectal cells with butyrate resulted in a dose-dependent increase in mucin protein. This decreased adherence of the bacteria *Escherichia coli*, while increasing adherence of more beneficial *Lactobacillus* and *Bifidobacterium*

bacterial strains. Thus, butyrate also maintains the health of colonocytes through modulation of the mucosal layer.

One approach to increasing butyrate production is via dietary changes; by increasing the intake of foods which are metabolised to produce butyrate. All non-digestible carbohydrates and unabsorbed monosaccharides which reach the large intestine can theoretically produce butyrate through their fermentation (Morrison *et al.* 2006) however, there are some particular groups which have a greater butyrogenic capacity, such as RS and oligofructose (OF) (Klessen *et al.* 2001). It has been reported that OF compounds, in particular inulin- type fructans, increase the production of butyrate and also increase the numbers of *Bifidobacteria spp.,* which are thought to be particularly beneficial to the host. Given that inulin-type fructans promote both a butyrogenic and bifidogenic effect, it is hypothesised that there is a relationship between *Bifidobacterium* and butyrate production. *Bifidobacterium* do have the ability to metabolise inulin-type fructans, and in fact, have been reported to have a preference for OF. However, *Bifidobacterium* do not produce butyrate (De Vuyst & Leroy 2011). It is believed that the butyrogenic characteristic of *Bifidobacterium* is due to cross-feeding with other microorganisms (De Vuyst & Leroy 2011). It is hypothesised that the metabolic products of *Bifidobacterium* may be utilised by butyrate producing bacteria, resulting in an overall increase in butyrogenesis (Belenguer *et al*. 2006).

Belenguer *et al.* (2006) investigated the possibility of a metabolic cross-feeding mechanism between lactate-producing *Bifidobacterium adolescentis* and butyrate-producing *Eucbacterium hallii* and *Anaerostipes caccae*, two members of the *Firmicutes* phyla. Co-culture of *E. hallii* and *B. Adolescentis* on starch resulted in butyrogenesis, which did not occur when each bacterial species were grown

separately in pure culture (Belenguer *et al.* 2006). Investigations of the carbon flow between the two bacterial species confirmed that the fermentation of starch by *B. Adolescentis produces* lactate which is utilised by *E. hallii* to produce butyrate (Belenguer *et al.* 2006). Similar findings were evident when *B. Adolescentis* was co-cultured with *A.caccae*. Microorganisms in the *Firmicutes* phyla are generally good producers of butyrate (Belenguer *et al.* 2006). Thus it may seem counterintuitive that *Firmicutes* numbers are increased in obese individuals compared to lean individuals given that obesity is a risk factor for colon cancer, and the important role that butyrate plays in maintaining colonocyte health. However, it is hypothesised that due to the metabolic cross-feeding relationship *Firmicutes* has shown with *Bifidobacteria,* the butyrogenic characteristic of *Firmicutes* may be reliant on the presence of other microorganisms such as *Bifidobacteria.* Unpublished data has shown that during the gut microbiome characterisation, the *Bifidiobacterium* were completely removed by the feeding of HCHF diet against a 14% population in the CS diet in Wistar rats (unpublished data from USQ Functional Foods Research Group – Panchal et al.). This suggests that diet-induced obesity may deplete *Bifidobacterium spp.,* consequently causing a decrease in butyrate production and increased risk of cancer potentially through epigenetic changes. Therefore measures which increase *Bifidobacterium* such as low fat and low carbohydrate diet or through probiotic (with *Bifidobacteria spp*.) and prebiotic supplementation, offer a potential mechanism of increasing butyrate production and ultimately reducing the risk of carcinogenesis.

Hypotheses

The hypotheses of this study were that:

1. The HCHF diet would lead to a decrease in the expression of the satiety signal 5-HT_{2C}R and an increase in the expression of the ghrelin appetite signals in comparison to the CS diet;

2. The H8C8 diet would change the gene expression of the HCHF diet back to the expression of the CS diet rats;

3. The microbiome in the HCHF diet rats would induce gene expression changes in chromatin modifying enzymes in colon cells that were different to those induced by the microbiome of CS diet rats.

Aims and objectives

The objective of this study was to use a male Wistar diet-induced obese rat model to investigate the effect of a HCHF diet on the gene and protein expression patterns of ghrelin, GOAT, GHSR and 5-HT_{2C}R in the brain.

The aims were to:

1. Measure the mRNA gene expression of ghrelin, GOAT GHSR and 5-HT_{2C}R in the brain tissue of rats fed a control diet based on CS or treatment diets of either 16 weeks of HCHF or eight weeks of HCHF followed by eight weeks of the CS diet.
2. Determine whether any changes in gene expression were also evident at the protein level.
3. Investigate whether the gut microbiome from rats fed a HCHF diet could stimulate epigenetic changes in colon cells by stimulating the gene expression levels of chromatin modifying enzymes.

CHAPTER 2
EXPERIMENTAL PROCEDURES

Tissue collection

Male Wistar rats were fed either a corn starch (CS) (n=3), high carbohydrate and high fat (HCHF) diet (n=4), a high carbohydrate and high fat for eight weeks then corn starch for eight weeks (H8C8) (n=4) or modified HCHF (n=3) or CS (n=3) diets (Refer to Appendix A for detailed descriptions of diet). Collection of brain tissue samples was performed during the scheduled termination for each rat following 16 weeks on the diets. Tissue collection was mostly outsourced, being completed by other individuals. Tissue samples were collected directly following sacrifice and placed on disposable weigh boats on ice. Bench space, mortar and pestles and spatulas were RNase-zapped to reduce RNA degradation. This was done at the beginning of experimentation and then in between the use of the mortar and pestle for each different tissue sample. Samples were snap-frozen in liquid nitrogen and homogenised to a fine powder using liquid nitrogen and a mortar and pestle. For each RNA sample, 600µl of RLT buffer (containing 1% β mercapto-ethanol) was added. A 5 gauge syringe and needle was then used to further homogenise each sample into solution. All samples were then stored at -80°C until use.

Protein extraction

Brain tissue samples were weighed to be approximately 0.075g per 500µl radioimmunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors added. Tissue samples were homogenised in RIPA buffer using a 3mL syringe and 19 gauge needle. The lysates were then placed in a test-tube rack in an esky with ice and water to achieve a 4°C environment, on a plate shaker for

two hours. Samples were then centrifuged for 20 minutes at 12 000 rpm at 4°C. The supernatant was then carefully removed and placed in a microfuge tube, the pellet was discarded. The extracted protein was then aliquoted into 100µl and 25µl aliquots and stored at -80°C. A bicinchonic acid (BCA) assay was performed on the protein extracts to determine concentrations.

mRNA extraction

30mg of brain tissue (animals: CS1, CS2, CS3, HCHF1, HCHF2, HCHF3, HCHF4, H8C81, H8C82, H8C83, H8C84) was weighed and placed in a microfuge tube. Favorgen RNA extraction kit was used to perform RNA extractions (FavorPrep Tissue Total RNA Mini Kit 2009). 350µl of FARB Buffer and 3.5µl of β mercapto-ethanol was added to the microfuge tube. The tissue samples were homogenised by passing the lysate through a 25 gauge needle syringe 10 times. The homogenised sample was then transferred to a filter column in a collection tube, then centrifuged for two minutes at 18000 x g. The clarified supernatant was transferred from the collection tube to a new microfuge tube. One volume of 70% RNase-free ethanol was added to the microfuge and mixed by vortex. The sample mixture was then added to a FARB Mini Column in a collection tube and centrifuged for one minute at 18000 x g. The flow through was discarded and FARB Mini Column returned to the collection tube. 500µl of Wash Buffer 1 was added to the FARB Mini Column and centrifuged at 18000 x g for one minute. Flow through discarded. 750µl of Wash Buffer 2 was added to the FARB Mini Column and centrifuged for one minute at 18 000 x g. This step was repeated with another 750µl of Wash Buffer 2. The FARB Mini Column was the centrifuged for another three minutes to dry the column. The FARB Mini Column was placed in an elution tube and 40µl of RNase-free double-distilled water added. The FARB Mini Column

was allowed to stand for one minute before centrifuging for one minute at 18 000 x g to elute the RNA.

cDNA synthesis

Following quantification by the NanoDrop (Implen), the samples used for mRNA extraction were converted to cDNA by using a BIO-RAD Reverse Transcription Kit (iScript cDNA Synthesis Kit 2017). A master mix was made for each sample with a one volume reaction made with 4µl 5 x iScript Reaction Mix, 1µl iScript Reverse Transcriptase with appropriate amounts of RNA and RNase-free H_2O.

Analysis of gene expression by real-time quantitative polymerase chain reaction

Preliminary experiments investigated the gene expression of various targets including ghrelin (GHRL), growth hormone secretagogue receptor (GHSR), ghrelin-o-acetyltransferase (GOAT), mucin 2 (MUC2), serotonin 2C receptor (5-HT_{2C}R), tryptophan 5-hydroxylase 1 (Tph1) and toll-like receptor 4 precursor (Tlr4). Based on the findings from the preliminary screening, further experiments were conducted investigating the gene expression of GHRL, GHSR, GOAT and 5-HT_{2C}R, specifically focusing on the effect of a HCHF diet on the ghrelin and 5-HT pathways in the gut-brain axis. Brain tissue samples were obtained from male Wistar rats representing three different dietary conditions: CS (n=3), HCHF (n=4) and H8C8 (n=4). The expression of the genes of interest were analysed by RT-PCR. RT-PCR was performed using validated PrimePCR primers (BIO-RAD Laboratories Inc., USA). Analysis of gene expression was achieved using the $2^{-\Delta\Delta CT}$ method, giving the fold change in gene expression due to the treatment diet compared to the normal diet, normalising to polyubiquitin-C precursor (UBC) as a stable reference/housekeeping gene (Silver *et al*. 2008). As seen below, firstly ΔΔCT is calculated by subtracting ΔCTC from ΔCTE. ΔCTE is the difference between

the average cycle threshold for the gene target (i.e. ghrelin, GOAT, etc) and the reference/ housekeeping gene (Ubiquitin C) using cDNA from the treatment diet (experimental) rats. While ΔCTC is the difference between the average cycle threshold for the gene target (i.e. ghrelin, GOAT, etc) and the reference/ housekeeping gene (Ubiquitin C) using cDNA from the normal diet rats.

$$\Delta CTE - \Delta CTC = \Delta\Delta CT$$

$2^{-\Delta\Delta Ct}$= Fold change in expression compared to control diet

Western Blot (Immunoblots)

Protein lysates from brain tissue of rats fed CS (n=2) (animals 1 and 3), HCHF (n=2) (animals 3 and 4), H8C8 (n=2) (animals 1 and 3) and pooled samples from modified corn starch (n=3) (mCS) and modified high fat high carbohydrate (n=3) (mHCHF) fed rats were used to analyse 5-HT_{2c}R protein expression. Protein lysates were separated by denaturation on a SDS-PAGE on a 12% Amersham ECL Gel. The proteins on the gel were electro transferred to nitrocellulose membrane by using Life Technologies Mini Blot. The membrane was blocked with 10% skim-milk powder diluted in Tris-buffered saline with Tween 20 (TBST) for one hour at room temperature. The membrane was then incubated with mouse monoclonal IgG antibody for 5-HT_{2C}R (1:100, SR-2C (D-R): sc 17797, Santa Cruz Biotechnology, USA) or β-actin (1:3000, BIO-RAD Laboratories Inc., USA) diluted in 5% blocking buffer. The membrane was washed in TBST six times for five minute intervals. The primary antibody was detected using goat-anti mouse horseradish peroxidase (HRP) conjugate (1:1000, Santa Cruz Biotechnology, USA). The membrane was incubated in the secondary antibody for one hour at room temperature. The membrane was washed in TBST six times for five minute intervals. The

immunocomplexes were visualised by enhanced chemiluminescence (ECL) by combining 1mL each of Detection Reagent 1 Peroxide Solution and Detection Reagent 2 Luminol Enhancer Solution (Thermo Fisher Scientific). The ECL solution was mixed by inversion and poured onto cling film, then the membrane placed protein side down on the solution, wrapped up in cling film then allowed to develop for two minutes. Serial images were taken using a Fusion FX Vilber Lourmat (Fisher Biotec) from one minute exposure, with the exposure time increasing 30 seconds between each image. Imaging continued until saturation was reached.

Co-culture of colon microbiome with human colon cancer cells (SW620)

The co-culture protocol was adapted from the Human oxygen-Bacteria anaerobic (HoxBan) coculturing system developed by Sadabad *et al.* (2015). Two pellets were collected from the colon of the rats following termination, and immediately placed in pre-prepared gifu anaerobe media (GAM). The cultures were placed in a shaking incubator overnight at 37°C. Following 18 hours incubation, 500µl of colon content culture inoculum was combined with 500µl of GAM agar mixture then pipetted gently on top of pre-prepared semi-solidified GAM agar mixture in a falcon tube. The falcon tube was then placed in an anaerobic culture chamber with GENbox anaerobic sachet at 37°C for two and a half hours. The falcon tubes were then removed and a scaffold with pre-cultured SW620 cells was added to the falcon tube, along with fresh DMEM media to create an interface between the colon content inoculum culture and the SW620 cells (Figure 2).

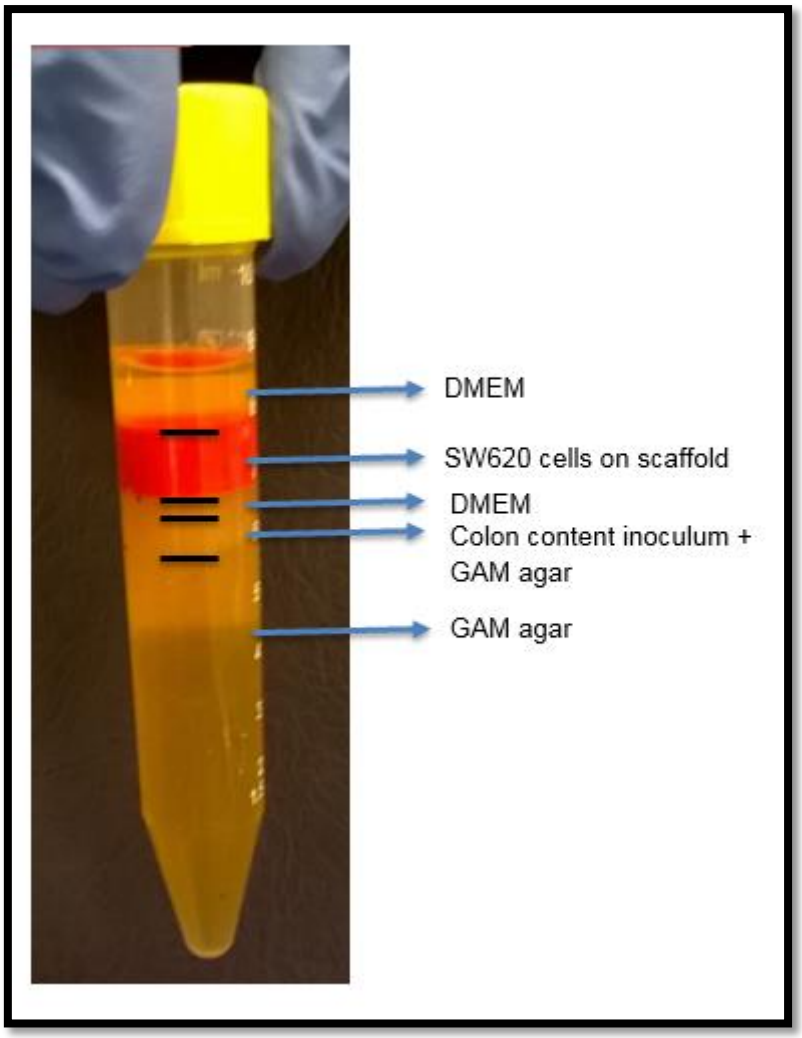


Figure 2: Labelled photograph of the co-culture, illustrating the different sections of the co-culture including the red scaffold with SW620 human colon cells.

The co-culture was then incubated at 37°C overnight. Scaffolds were then removed and placed in 24 well plate and washed with trypsin to detach cells from the scaffold. Cells were resuspended in phosphate-buffered saline (PBS) and centrifuged to form a pellet. The remaining contents of the falcon tube was added to a 50mL falcon tube with 25mL of PBS and centrifuged for five minutes at 4000 rpm to form a bacterial pellet. The pellet was then resuspended in PBS. (Refer to Appendix B for detailed co-culture protocol).

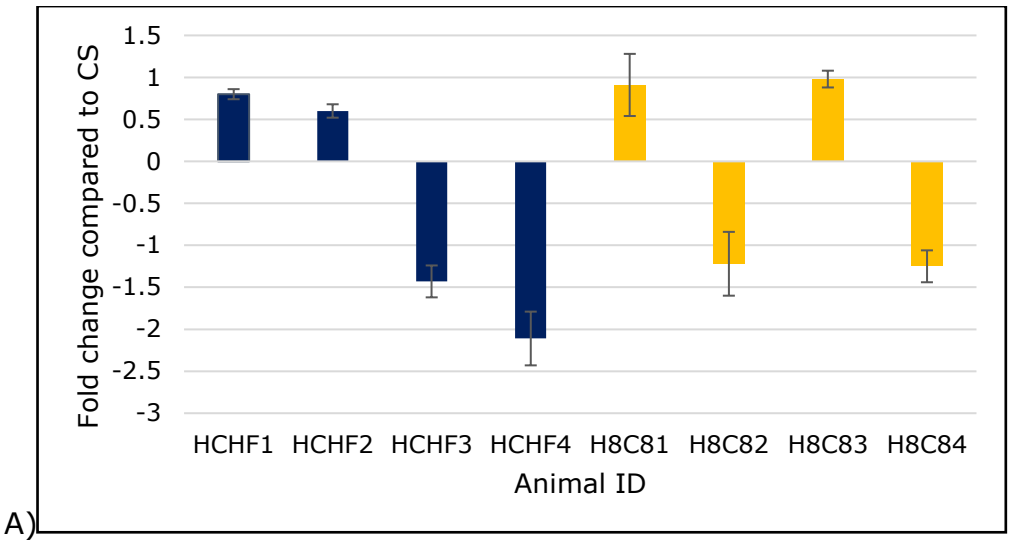
Epigenetic Chromatin Modification Enzyme Plate

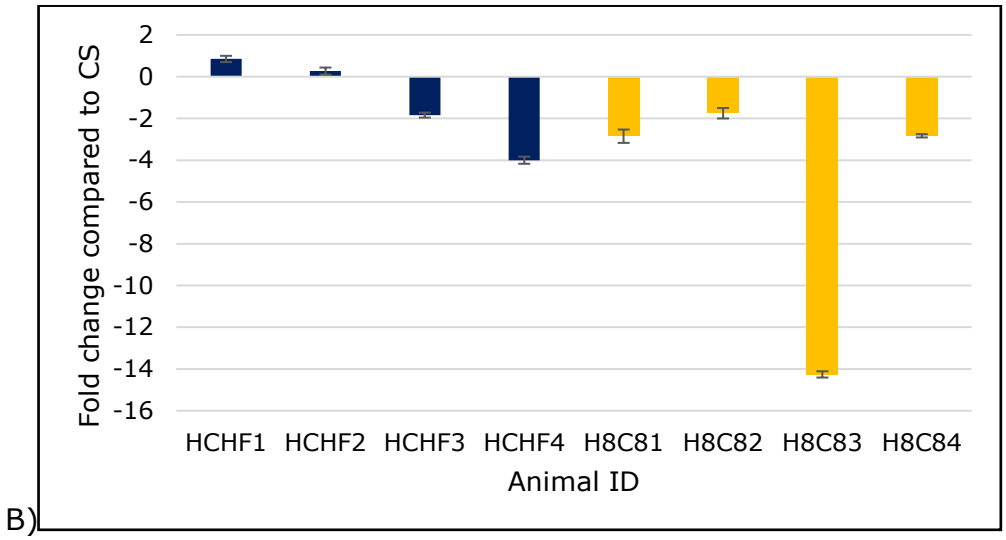
RNA extraction was performed on the SW620 cell lysate as per Qiagen RNeasy mini-prep kit instructions (*RNeasy® Mini Handbook* 2012). RNA was quantified using the Nanodrop (Implen) and reverse transcription was performed using the BIORAD iScript cDNA Synthesis kit to convert the RNA to cDNA as described above. Master mixes were made for the pooled cDNA sample of the SW620 co-culture lysate representing the modified corn starch (mCS) and modified high carbohydrate high fat (mHCHF) diets. (Refer to Appendix A for detailed comparison of diets). Samples and master mix were pipetted into each quadrant of the epigenetic chromatin modification enzyme H384 plate (BIO-RAD Laboratories Inc., USA) which RT-PCR used to measure the change in expression of 86 genes associated with chromatin remodelling as well as five housekeeping genes and five controls. ΔΔCT method was used to determine the fold change in any targets that had a CT value of less than 35 as per the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Bustin *et al.* 2009). Hypoxanthine phosphoribosyltransferase 1 (HPRT1) as a reference gene.

CHAPTER 3
RESULTS

Messenger RNA (mRNA) expression analysis by RT-PCR

Figure 3 demonstrates the pattern of expression exhibited in the HCHF and H8C8 diet group animals. For the HCHF diet, if ghrelin expression was increased in comparison to rats fed a normal, corn-starch based diet (CS) as seen in animals 1 and 2, then GOAT and GHSR expression were also increased. Conversely, if ghrelin expression was decreased compared to CS, as seen in animals 3 and 4, GOAT and GHSR expression were also decreased. The effect of reverting back to a CS diet for 8 weeks after consuming a HCHF diet for 8 weeks (H8C8) was also not consistent for ghrelin expression amongst the rats. There was a consistent and significant ($p = 0.001$) decrease in GOAT expression in all animals compared to CS fed animals (Figure 4). All animals showed a decrease in GHSR expression compared to CS fed rats. This decrease in GHSR expression in the H8C8 diet compared to CS diet was statistically significant with a p value of 0.0153 (Figure 4). (Animal H8C83 was deemed an outlier for GHSR expression and excluded from statistical calculations). There was no statistically significant mean change in gene expression in ghrelin in the HCHF or H8C8 diet groups.





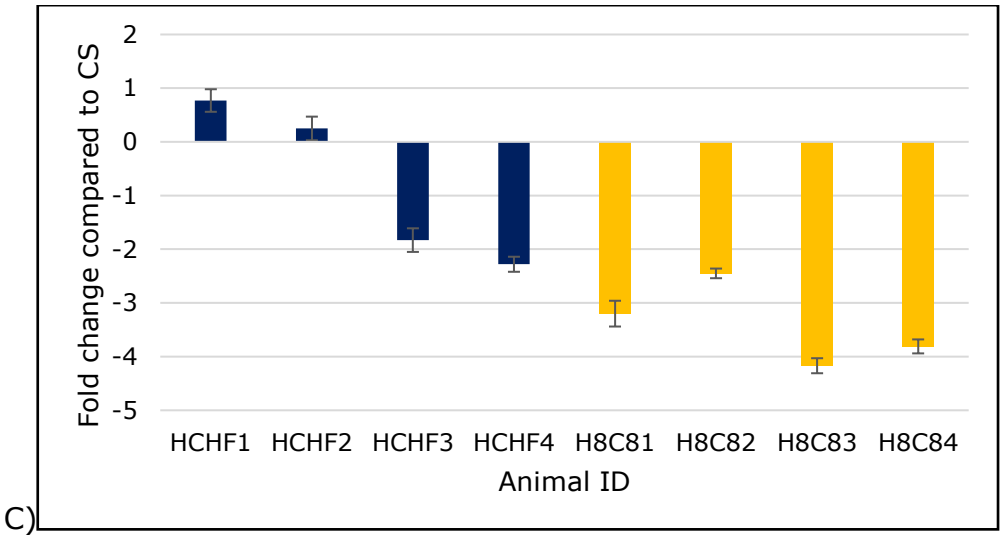


Figure 3: Illustrates the fold change of expression of A) ghrelin (GHRL), B) growth hormone secretagogue receptor (GHSR) and C) ghrelin-O-acetyltransferase (GOAT) compared to CS in each individual HCHF (1-4) and H8C8 animal (1-4). Standard error values were calculated from at least three technical replicates within each individual animal.

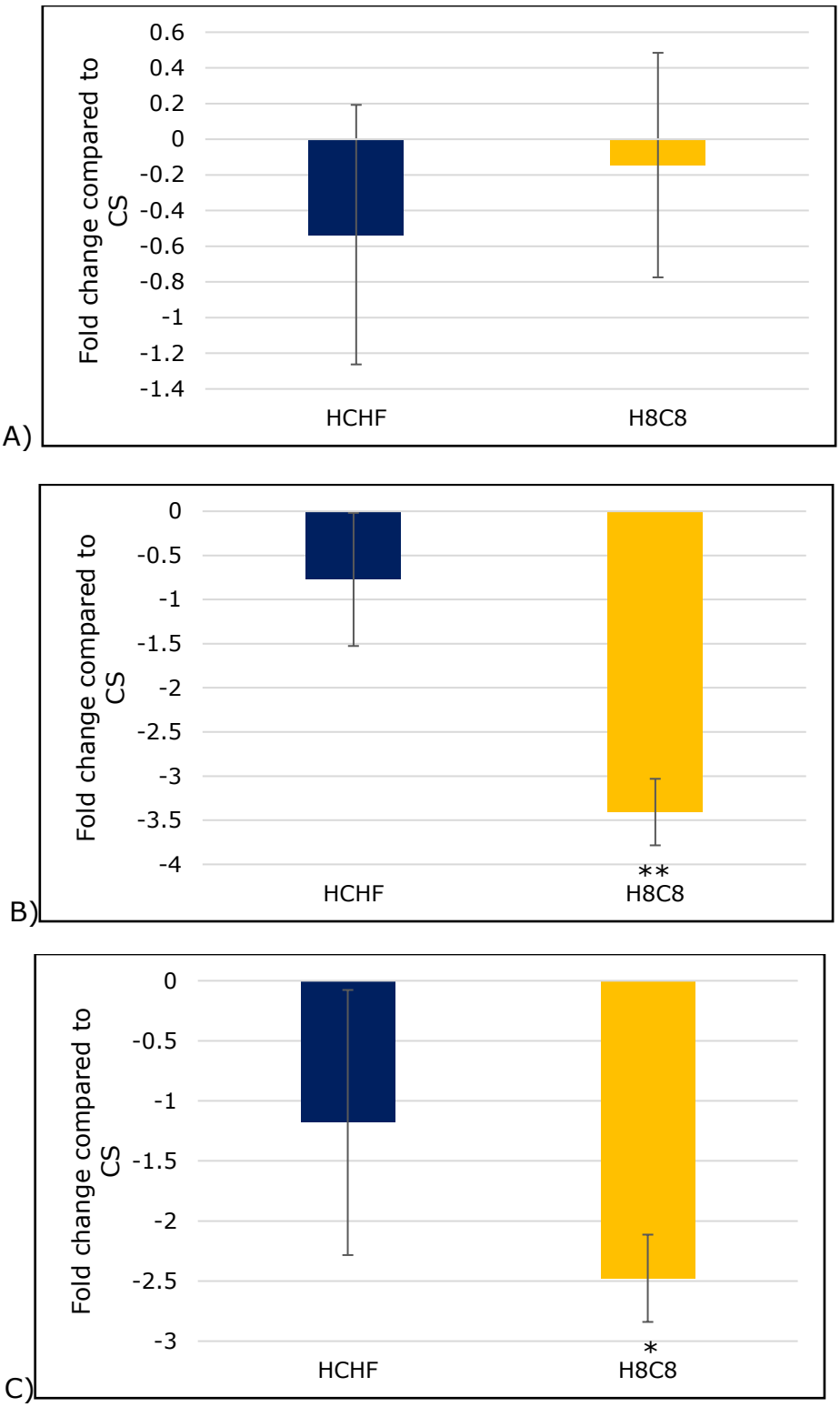


Figure 4: Shows the mean A) Ghrelin, B) GOAT and C) GHSR expression change. A) One-way ANOVA with post-hoc Tukey statistics showed no significant difference in ghrelin expression in the HCHF or H8C8 diets. B) Statistics showed a significant (p= 0.001) difference in the mean expression of GOAT compared to CS fed animals in H8C8 (n=4) animals but not HCHF (n=4). C) Statistics showed a significant (p=0.0153) difference in the mean GHSR expression in the H8C8 (n=3) animals, but not in HCHF (n=4) compared to CS.

Figure 5 shows the expression of 5-HT2CR was more consistent with all rats fed the HCHF diet demonstrating a decrease in the expression of the 5-HT_{2C}R and three out of four rats demonstrating an increase in response to the H8C8 diet.

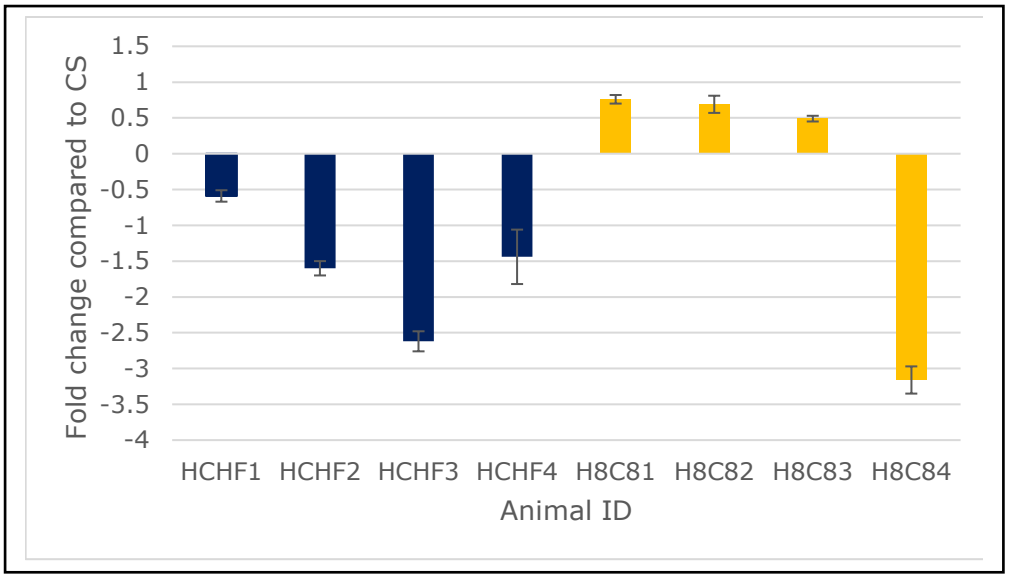


Figure 5: Fold change in 5-HT_{2c}R expression in HCHF diet and H8C8 animals compared to CS fed animals. Standard error values were calculated from at least three technical replicates within each individual animal

Western (Immunoblot) Analysis of 5-HT_{2C}R protein expression

Protein lysates from brain tissue of rats fed CS, HCHF, H8C8, mCS and mHCHF were used to analyse 5-HT_{2c}R expression. β-actin was used as a loading control and was consistent between samples and the HeLa control lysate, however β-actin signalling appeared at approximately 85 kDa (expected size is 42 kDa). Western blot analysis showed no consistently significant difference in 5-HT_{2c}R protein levels between diet groups, indicated by varied the intensity of the bands (Figure 6).

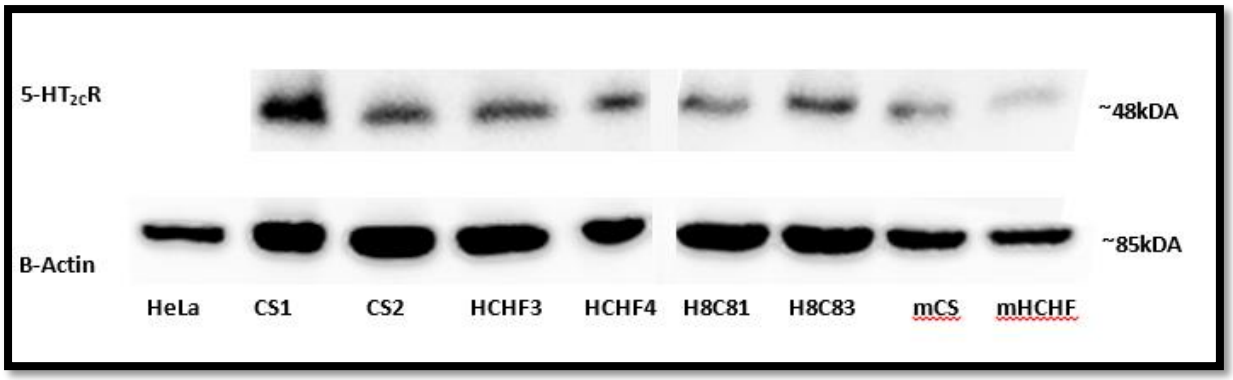


Figure 6: Illustrates the Western blot for 5-HT_{2C}R expression using protein lysates from CS (n=2), HCHF (n=2), H8C8 (n=2), modified CS (pooled samples n=3) and modified HCHF (pooled samples n=3) animals. β-actin was used as a loading control.

Epigenetic chromatin modification enzyme H384 plate

There was a significant difference in the expression of the chromatin remodelling enzyme DZIP3 (Deleted in azoospermia (DAZ) interacting zinc finger protein 3) in the SW620 cell line in response to the mHCHF diet rat gut microbiome. The SW620 cell line co-culture exhibited a 12.55 fold increase in DZIP3 expression in mHCHF compared to mCS samples, following use of the ΔΔCT method. This should be further investigated as this target was considerably upregulated in response to the high fat diet and DZIP3 is involved in chromatin remodelling, thus could lead to epigenetic changes (Inoue *et al.* 2015).

CHAPTER 4
DISCUSSION

This study supported a role for the ghrelin-serotonergic pathway in the brain in response to a high fat and high carbohydrate diet. There was a significant change in the brain expression of GHSR and GOAT between the H8C8 and CS groups (Figure 4). There was also a trending decrease in expression of 5-HT_{2C}R in response to the HCHF diet compared to CS (Figure 5). This supports the hypothesis that 5-HT_{2C}R expression would be decreased in the HCHF group compared to CS. Expression in the H8C8 group was comparable to CS, except for animal H8C8 4 which appears to be an outlier in this case. This outlier is most likely explained by variation during tissue sample collection. This supports the hypothesis that converting back to a CS diet after eight weeks on a HCHF diet, would return expression of genes involved in the ghrelin-serotonin axis to a level comparable to CS fed animals. It was expected that there would be a significant difference in the gene expression of the H8C8 diet compared to HCHF, as it was hypothesised that eight weeks on the CS could reverse the effects of eight weeks on the HCHF diet. It appears that the change in diet from HCHF to CS after eight weeks on a HCHF diet is enough to reverse the trending decrease in 5-HT_{2C}R that was seen in the HCHF group. The decrease in 5-HT_{2C}R in the HCHF group is supported by the literature which gives evidence for the involvement of 5-HT_{2C}R in appetite and weight regulation and metabolism and suggests that diet may affect the expression of brain 5-HT_{2C}R (Garfield *et al.* 2016; Valencia-Torres *et al.* 2016). The decrease in 5-HT_{2C}R expression in HCHF animals would decrease the receptor availability for 5-HT binding and therefore attenuate the anorexigenic pathway.

There were differing patterns of mRNA expression in individual rats. For example, in the HCHF diet, animals 1 and 2, expression of ghrelin, GOAT and GHSR increased while the expression of 5-HT_{2C}R decreased. This pattern of expression is logical, and supports the hypothesis that the expression of the orexigenic ghrelin axis would be increased in the HCHF diet, consequently resulting in an increase in body weight (Appendix C). Furthermore, animals 1 and 2 have decreased expression of 5-HT_{2C}R, suggesting that the anorexigenic serotonergic pathway is downregulated, which would further exacerbate the increase in bodyweight seen with the HCHF diet. This also supports the hypothesis. Conversely, HCHF animals 3 and 4 had decreased expression of all target genes. This may be due to inter-individual variation (Jasinska et al. 2009), however as the animals were all littermates and genetically identical this is not likely. It is more likely that the differences in trends between HCHF animals is due to variation in sample collection from different regions of the brain. Further investigation is needed to fully understand the role of the ghrelin-serotonin 2C receptor pathway in the brain in appetite regulation and weight gain. As previously mentioned, plasma ghrelin and GOAT levels increase with BMI (Goebel-Stengel *et al.* 2013). However, if GHSR levels are decreased (as exhibited by HCHF animals 3 and 4), the orexigenic affect will be attenuated due to decreased availability of GHSR. As the stomach is the main site of synthesis of targets of the ghrelin axis and also 5-HT (Jenkins *et al.* 2016), it should be investigated whether similar expression changes are seen in the stomach to those observed in the brain.

It is well documented that the level of gene expression of both ghrelin and 5-HT_{2C}R varies greatly between areas of the brain (Burns *et al.* 1997). The main source of central ghrelin is the hypothalamus, however ghrelin expression has also been reported at lower levels in the midbrain, hindbrain, hippocampus and spinal cord

(Kojima *et al.* 1999; Ferrini *et al.* 2009). GHSR is also most highly expressed in the hypothalamus (Wang *et al.* 2015). Ghrelin is also found in the pituitary where it binds to GHSR to promote the release of growth hormone (Wang *et al.* 2015). Ghrelin mRNA and protein expression is decreased in the hypothalamus after 24 and 48 hours fasting in rats (Sato *et al.* 2005). It is clear that the hypothalamus, specifically the ARC, is integral in the regulation of food intake through the expression of orexigenic and anorexigenic neurons, NPY and AgRP and POMC. It is reasonable to expect that the hypothalamus would have greater expression of genes related to appetite and metabolism, such as the ghrelin axis and serotonergic genes. As previously mentioned, higher expression of these genes in the hypothalamus and, also the pituitary as part of the growth hormone axis, is well documented. It is possible, and likely, that the samples in this project were collected from different areas within the brain. In order to improve the experimental design and the integrity of the results it is recommended that a conscious effort be made to collect tissue from the same region within the brain, ideally the hypothalamus as it is a key location involved in the appetite regulation. This is common practice within this area of research as demonstrated by Chiu *et al.* (2014) and Garfield *et al.* (2016), who collected samples from the medial prefrontal cortex and whole hypothalamus respectively. The use of microdissection methods would increase the accuracy of sample collection. Additionally, the use of immunohistochemical staining could be used to determine the location of gene targets in a cross section of the brain. Neither of these techniques were a feasible option for this project due to time and financial constraints. Alternatively, a punch biopsy could be performed in order to compare the difference in expression of the ghrelin axis and 5-HT_{2C}R genes in various areas of the brain. This was not possible

during this project due to time constraints and limitations on the availability of tissue samples.

Additionally, it should be noted that due to the highly degradable nature of RNA it is important that tissue collection be performed under optimal conditions to ensure that a real representative of RNA and thus gene expression be achieved. This includes using cleaning agents such as RN-ase Zap on all equipment (mortar and pestle, microfuge tubes and other extraction equipment), changing gloves frequently, working quickly as possible when handling samples and storing samples at -80°C.

Western blot analysis indicated that there was no significant difference in the protein expression of 5-HT_{2C}R between rats fed a CS, HCHF, H8C8, mCS or mHCHF (Figure 6). While this does not consistently reflect the results of mRNA expression, it should be noted that mRNA expression is not always an accurate representation of the protein which is being translated. Pooled sampling of protein for mCS (n=3) and mHCHF (n=3) does support the hypothesis. With less intense banding seen for mHCHF compared to mCS, suggesting decreased expression of 5-HT_{2C}R protein. It is recommended that this experiment be repeated to conclusively establish that there is no significant difference in 5-HT_{2C}R protein expression due to diet. A larger sample size within each diet group and consistent tissue sampling from the same area of the brain would increase the integrity of the results. Furthermore, additional quantification of the bands would enhance analysis of the results.

Loading control was confirmed with consistent banding with a putative β-actin. β-actin appeared at a molecular weight of approximately 85kDa, instead of 42kDa which would usually be expected. Interestingly, the control lysate also appeared

at this molecular weight. The blot was repeated with another β-actin antibody from Santa Cruz, resulting in the same banding at around 85kDa. Other variables such as primary and secondary antibody concentration were altered to confirm that the unusual banding was not simply due to non-specific binding. Changing these variables did not change the result, suggesting that the signal around 85kDa is in fact some form of β-actin. This may be due to incomplete denaturation, however standard protocol was followed, including the heating of samples and use of β-mercaptoethanol (BME) in the loading buffer to denature the protein. Furthermore, 5-HT_{2C}R was successfully denatured as it appeared at the correct molecular weight. Therefore, it is unlikely that the issue was incomplete denaturation of protein. It is also unlikely that it was a problem with protein migration through the gel, as previously stated, 5-HT_{2C}R which is a similar molecular weight (48kDa) to β-actin successfully migrated through the gel. It is hypothesised that the larger molecular weight of β-actin is due to dimerisation with another protein, however this has not been documented in the literature.

There was only one difference in the gene expression of colon cells in response to the high fat diet which was DZIP3. DZIP3 is involved in epigenetic regulation via its ubiquitination of HDACs (Inoue *et al.* 2015). It is hypothesised that diet-induced changes in the microbiome resulted in a decrease in butyrate, a known HDACi (Lazarova *et al.* 2004), through a decrease in *Bifidobacterium.* The upregulation of DZIP3 expression by 12.55 fold in the HCHF assay was quite considerable. DZIP3 ubiquitinates HDACs and butyrate inhibits HDACs. It is possible that the increase in DZIP3 expression is the result of a compensatory mechanism to account for the absence of butyrate due to changes in microbiome composition as a result of HCHF diet. Potentially in the absence of butyrate, DZIP3 is involved in removing HDACs and therefore regulation of chromatin remodelling

(Frank *et al.* 2016). Chromatin remodelling enzymes have an integral role in DNA organisation and epigenetic modifications and include genes involved in post-translational histone modifications (Marfella & Imbalzano 2007).

It would also be of interest to sequence samples from the rat gut microbiome culture in order to compare the difference in microbiome composition of the rats fed CS and HCHF diets or to perform qPCRs using primers specific to the 16s rDNA of known butyrogenic bacteria. This would be advisable particularly as unpublished data has shown that *Bifidiobacterium* were completely removed by the HCHF diet against a 14% population in the CS diet in Wistar rats (unpublished data from USQ Functional Foods Research Group – Panchal et al.). This is supported by evidence from O'Keefe *et al*. (2014) who found a considerable increase in faecal butyrate concentration due to diet changes. As previously mentioned, *Bifidiobacterium* have butyregenic characteristics and butyrate is a known HDAC inhibitor (HDACis) and may stimulate epigenetic changes, regulate gene expression and promote cell cycle arrest, differentiation and/or apoptosis (Lazarova *et al*. 2004). Due to time and financial constraints it was not possible to establish the composition of the colon content culture microflora. It should also be noted that the co-culture protocol used was novel in nature, thus there are other factors, such as length of incubation that could be adjusted in order to improve the experiment.

Conclusion

In conclusion, this study investigated the effects of diet and diet composition change on the expression of the ghrelin and the serotonin receptor 5-HT_{2C}R pathway in the rat brain. The HCHF diet caused a trending decrease in the expression of the 5-HT_{2C}R and then a trending increase in response to the H8C8

diet. The effect of reverting back to a CS diet for eight weeks after consuming a HCHF diet for eight weeks (H8C8) led to a consistent and significant decrease in expression of both GOAT and GHSR. Overall, these findings support the role of the ghrelin-serotonin 2C pathway as a target for obesity. This is supported by the emergence of 5-HT_{2C}R agonists as adjunct therapy for obesity. These findings also provided previously unreported evidence that targeting GOAT and/or GHSR along with 5-HT_{2C}R centrally in the brain may be a target for treating obesity and should be further investigated.

Colon contents from rats fed on the HCHF and CS diets that were co-cultured with human colon cells led to an increase in the expression of the chromatin modifying enzyme DZIP3. This provided preliminary evidence of an epigenetic mechanism whereby diet can influence the gut microbiome which can in turn alter the gene expression of colon cells.

This study supports a role for diet composition in driving appetite regulation in the brain through the expression of 5-HT_{2C}R, GOAT and GHSR however this requires further investigation. The use of microdissection to collect samples from different parts of the brain involved in appetite regulation is recommended and may explain inconsistent results. It is also recommended that Western immunoblot be performed for both GHSR and GOAT to establish any changes in these genes at the protein level. Future directions would be to investigate the use of small molecule inhibitors of GOAT in reducing appetite and body weight in the obese rat model (Garner & Janda 2011). This study also supports the further investigation of the gut microbiome as a target for regulating the epigenome of colon cells.

REFERENCES

Barazzoni, R, Zanetti, M, Ferreira, C, Vinci, P, Pirulli, A, Mucci, M, Dore, F, Fonda, M, Ciocchi, B, Cattin, L & Guarnieri, G 2007, 'Relationships between Desacylated and Acylated Ghrelin and Insulin Sensitivity in the Metabolic Syndrome', *The Journal of Clinical Endocrinology & Metabolism*, vol. 92, no. 10, pp. 3935-3940.

Belenguer, A, Duncan, SH, Calder, AG, Holtrop, G, Louis, P, Lobley, GE & Flint, HJ 2006, 'Two Routes of Metabolic Cross-Feeding between Bifidobacterium adolescentis and Butyrate-Producing Anaerobes from the Human Gut', *Applied and Environmental Microbiology*, vol. 72, no. 5, pp. 3593-3599.

Berglund, ED, Liu, C, Sohn, J-W, Liu, T, Kim, MH, Lee, CE, Vianna, CR, Williams, KW, Xu, Y & Elmquist, JK 2013, 'Serotonin 2C receptors in pro-opiomelanocortin neurons regulate energy and glucose homeostasis', *The Journal of Clinical Investigation*, vol. 123, no. 12, pp. 5061-5070.

Boge, R, Tummala, AK, Peddy, V, Rangineni, S, Dahanukar, VH, Rakeshwar, B, Srinivas, A, Prabhakar, M & Ganesh, V 2014, *Lorcaserin hydrochloride*, Google Patents, <http://www.google.com/patents/US20140187538>

Burns, CM, Chu, H, Rueter, SM, Hutchinson, LK, Canton, H, Sanders-Bush, E & Emeson, RB 1997, 'Regulation of serotonin-2C receptor G-protein coupling by RNA editing', *Nature*, vol. 387, no. 6630, pp. 303-308.

Bustin, SA, Benes, V, Garson, JA, Hellemans, J, Huggett, J, Kubista, M, Mueller, R, Nolan, T, Pfaffl, MW, Shipley, GL, Vandesompele, J & Wittwer, CT 2009, 'The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments', *Clinical Chemistry*, vol. 55, no. 4, p. 611.

Canani, RB, Costanzo, MD, Leone, L, Pedata, M, Meli, R & Calignano, A 2011, 'Potential beneficial effects of butyrate in intestinal and extraintestinal diseases', *World Journal of Gastroenterology : WJG*, vol. 17, no. 12, pp. 1519-1528.

Chiu, H-Y, Chan, M-H, Lee, M-Y, Chen, S-T, Zhan, Z-Y & Chen, H-H 2014, 'Long-lasting alterations in 5-HT_{2A} receptor after a binge regimen of methamphetamine in mice', *International Journal of Neuropsychopharmacology*, vol. 17, no. 10, p. 1647.

Clarke, G, Stilling, RM, Kennedy, PJ, Stanton, C, Cryan, JF & Dinan, TG 2014, 'Minireview: Gut Microbiota: The Neglected Endocrine Organ', *Molecular Endocrinology*, vol. 28, no. 8, pp. 1221-1238.

Cummings, JH 1981, 'Short chain fatty acids in the human colon', *Gut*, vol. 22, no. 9, pp. 763-779.

De Vuyst, L & Leroy, F 2011, 'Cross-feeding between bifidobacteria and butyrate-producing colon bacteria explains bifdobacterial competitiveness, butyrate production, and gas production', *International Journal of Food Microbiology*, vol. 149, no. 1, pp. 73-80.

Derkach, KV, Bondareva, VM, Chistyakova, OV, Berstein, LM & Shpakov, AO 2015, 'The Effect of Long-Term Intranasal Serotonin Treatment on Metabolic Parameters and Hormonal Signaling in Rats with High-Fat Diet/Low-Dose Streptozotocin-Induced Type 2 Diabetes', *International Journal of Endocrinology*, vol. 2015, p. 17.

Faith, JJ, McNulty, NP, Rey, FE & Gordon, JI 2011, 'Predicting a Human Gut Microbiota's Response to Diet in Gnotobiotic Mice', *Science*, vol. 333, no. 6038, pp. 101-104.

FavorPrep Tissue Total RNA Mini Kit 2009, User Manual, Favorgen, viewed 02 March 2017, http://www.favorgen.com/pdf/DataSheet/Nucleic_Acid_Extraction/Total_RNA/FATRK%20000%20001%20001-1%20001-2.pdf.

Fernandez, G, Cabral, A, Cornejo, MP, De Francesco, PN, Garcia-Romero, G, Reynaldo, M & Perello, M 2016, 'Des-Acyl Ghrelin Directly Targets the Arcuate Nucleus in a Ghrelin-Receptor Independent Manner and Impairs the Orexigenic Effect of Ghrelin', *Journal of Neuroendocrinology*, vol. 28, no. 2.

Ferrini, F, Salio, C, Lossi, L & Merighi, A 2009, 'Ghrelin in Central Neurons', *Current Neuropharmacology*, vol. 7, no. 1, pp. 37-49.

Flint, HJ, Duncan, SH, Scott, KP & Louis, P 2015, 'Links between diet, gut microbiota composition and gut metabolism', *Proceedings of the Nutrition Society*, vol. 74, no. 01, pp. 13-22.

François, M, Barde, S, Legrand, R, Lucas, N, Azhar, S, Dhaybi, Me, Guerin, C, Hökfelt, T, Déchelotte, P, Coëffier, M & Fetissov, SO 'High-fat diet increases ghrelin-expressing cells in stomach, contributing to obesity', *Nutrition*.

Frank, CL, Manandhar, D, Gordân, R & Crawford, GE 2016, 'HDAC inhibitors cause site-specific chromatin remodeling at PU.1-bound enhancers in K562 cells', *Epigenetics & Chromatin*, vol. 9, p. 15.

Gahete, MD, Córdoba-Chacón, J, Salvatori, R, Castaño, JP, Kineman, RD & Luque, RM 2010, 'Metabolic regulation of ghrelin O-acyl transferase (GOAT) expression in the mouse hypothalamus, pituitary, and stomach', *Molecular and Cellular Endocrinology*, vol. 317, no. 1–2, pp. 154-160.

Garfield, AS, Davies, JR, Burke, LK, Furby, HV, Wilkinson, LS, Heisler, LK & Isles, AR 2016, 'Increased alternate splicing of Htr2c in a mouse model for Prader-Willi syndrome leads disruption of 5HT2C receptor mediated appetite', *Molecular Brain*, vol. 9, no. 1, p. 95.

Garner, AL & Janda, KD 2011, 'A small molecule antagonist of ghrelin O-acyltransferase (GOAT)', *Chemical Communications*, vol. 47, no. 26, pp. 7512-7514.

Goebel-Stengel, M, Hofmann, T, Elbelt, U, Teuffel, P, Ahnis, A, Kobelt, P, Lambrecht, NWG, Klapp, BF & Stengel, A 2013, 'The ghrelin activating enzyme ghrelin-O-acyltransferase (GOAT) is present in human plasma and expressed dependent on body mass index', *Peptides*, vol. 43, pp. 13-19.

Gutierrez, JA, Solenberg, PJ, Perkins, DR, Willency, JA, Knierman, MD, Jin, Z, Witcher, DR, Luo, S, Onyia, JE & Hale, JE 2008, 'Ghrelin octanoylation mediated by an orphan lipid transferase', *Proceedings of the National Academy of Sciences*, vol. 105, no. 17, pp. 6320-6325.

Heisler, LK, Cowley, MA, Tecott, LH, Fan, W, Low, MJ, Smart, JL, Rubinstein, M, Tatro, JB, Marcus, JN, Holstege, H, Lee, CE, Cone, RD & Elmquist, JK 2002, 'Activation of Central Melanocortin Pathways by Fenfluramine', *Science*, vol. 297, no. 5581, pp. 609-611,

Hosoda, H, Kojima, M, Matsuo, H & Kangawa, K 2000, 'Ghrelin and Des-acyl Ghrelin: Two Major Forms of Rat Ghrelin Peptide in Gastrointestinal Tissue', *Biochemical and Biophysical Research Communications*, vol. 279, no. 3, pp. 909-913.

Hu, S, Liu, L, Chang, EB, Wang, J-Y & Raufman, J-P 2015, 'Butyrate inhibits pro-proliferative miR-92a by diminishing c-Myc-induced miR-17-92a cluster transcription in human colon cancer cells', *Molecular Cancer*, vol. 14, p. 180.

Inoue, D, Aihara, H, Sato, T, Mizusaki, H, Doiguchi, M, Higashi, M, Imamura, Y, Yoneda, M, Miyanishi, T, Fujii, S, Okuda, A, Nakagawa, T & Ito, T 2015, 'Dzip3 regulates developmental genes in mouse embryonic stem cells by reorganizing 3D chromatin conformation', *Scientific Reports*, vol. 5, p. 16567.

iScript cDNA Synthesis Kit 2017, BIO-RAD, viewed 3rd March 2017, < http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4106228.pdf>.

Jasinska, AJ, Service, S, Choi, O-w, DeYoung, J, Grujic, O, Kong, S-y, Jorgensen, MJ, Bailey, J, Breidenthal, S, Fairbanks, LA, Woods, RP, Jentsch, JD & Freimer, NB 2009, 'Identification of brain transcriptional variation reproduced in peripheral blood: an approach for mapping brain expression traits', *Human Molecular Genetics*, vol. 18, no. 22, pp. 4415-4427.

Jenkins, TA, Nguyen, JCD, Polglaze, KE & Bertrand, PP 2016, 'Influence of Tryptophan and Serotonin on Mood and Cognition with a Possible Role of the Gut-Brain Axis', *Nutrients*, vol. 8, no. 1, p. 56.

Jung, T-H, Park, JH, Jeon, W-M & Han, K-S 2015, 'Butyrate modulates bacterial adherence on LS174T human colorectal cells by stimulating mucin secretion and MAPK signaling pathway', *Nutrition Research and Practice*, vol. 9, no. 4, pp. 343-349.

Kawahara, Y, Grimberg, A, Teegarden, S, Mombereau, C, Liu, S, Bale, TL, Blendy, JA & Nishikura, K 2008, 'Dysregulated Editing of Serotonin 2C Receptor mRNAs Results in Energy Dissipation and Loss of Fat Mass', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, vol. 28, no. 48, pp. 12834-12844.

Khatib, MN, Gaidhane, S, Gaidhane, AM, Simkhada, P & Zahiruddin, QS 2015, 'Ghrelin O Acyl Transferase (GOAT) as a Novel Metabolic Regulatory Enzyme', *Journal of Clinical and Diagnostic Research : JCDR*, vol. 9, no. 2, pp. LE01-LE05.

Kim, M, Bae, S & Lim, K-M 2013, 'Impact of High Fat Diet-induced Obesity on the Plasma Levels of Monoamine Neurotransmitters in C57BL/6 Mice', *Biomolecules & Therapeutics*, vol. 21, no. 6, pp. 476-480.

Kleessen, B, Hartmann, L & Blaut, M 2001, 'Oligofructose and long-chain inulin: influence on the gut microbial ecology of rats associated with a human faecal flora', *British Journal of Nutrition*, vol. 86, no. 02, pp. 291-300.

Kojima, M & Kangawa, K 2010, 'Ghrelin: more than endogenous growth hormone secretagogue', *Annals of the New York Academy of Sciences*, vol. 1200, no. 1, pp. 140-148.

Kojima, M, Hosoda, H, Date, Y, Nakazato, M, Matsuo, H & Kangawa, K 1999, 'Ghrelin is a growth-hormone-releasing acylated peptide from stomach', *Nature*, vol. 402, no. 6762, pp. 656-660.

Kumar, A, Wu, H, Collier-Hyams, LS, Kwon, Y-M, Hanson, JM & Neish, AS 2009, 'The Bacterial Fermentation Product Butyrate Influences Epithelial Signaling via

Reactive Oxygen Species-Mediated Changes in Cullin-1 Neddylation', *Journal of immunology (Baltimore, Md. : 1950)*, vol. 182, no. 1, pp. 538-546.

Lazarova, DL, Bordonaro, M, Carbone, R & Sartorelli, AC 2004, 'Linear relationship between Wnt activity levels and apoptosis in colorectal carcinoma cells exposed to butyrate', *International Journal of Cancer*, vol. 110, no. 4, pp. 523-531.

Lazarova, DL, Chiaro, C, Wong, T, Drago, E, Rainey, A, O'Malley, S & Bordonaro, M 2013, 'CBP Activity Mediates Effects of the Histone Deacetylase Inhibitor Butyrate on WNT Activity and Apoptosis in Colon Cancer Cells', *Journal of Cancer*, vol. 4, no. 6, pp. 481-490.

Le Chatelier, E, Nielsen, T, Qin, J, Prifti, E, Hildebrand, F, Falony, G, Almeida, M, Arumugam, M, Batto, J-M, Kennedy, S, Leonard, P, Li, J, Burgdorf, K, Grarup, N, Jorgensen, T, Brandslund, I, Nielsen, HB, Juncker, AS, Bertalan, M, Levenez, F, Pons, N, Rasmussen, S, Sunagawa, S, Tap, J, Tims, S, Zoetendal, EG, Brunak, S, Clement, K, Dore, J, Kleerebezem, M, Kristiansen, K, Renault, P, Sicheritz-Ponten, T, de Vos, WM, Zucker, J-D, Raes, J, Hansen, T, Meta, HITc, Bork, P, Wang, J, Ehrlich, SD & Pedersen, O 2013, 'Richness of human gut microbiome correlates with metabolic markers', *Nature*, vol. 500, no. 7464, pp. 541-546.

Lee, JH, Lin, L, Xu, P, Saito, K, Wei, Q, Meadows, AG, Bongmba, OYN, Pradhan, G, Zheng, H, Xu, Y & Sun, Y 2016, 'Neuronal Deletion of Ghrelin Receptor Almost Completely Prevents Diet-Induced Obesity', *Diabetes*, vol. 65, no. 8, p. 2169.

Lopez-Esparza, S, Berumen, LC, Padilla, K, Miledi, R & García-Alcocer, G 2015, 'Expression of hippocampal serotonin receptors 5-HT2C and 5-HT5A in a rat model of diet-induced obesity supplemented with tryptophan', *International Journal of Developmental Neuroscience*, vol. 42, pp. 80-85.

Marfella, CGA & Imbalzano, AN 2007, 'The Chd Family of Chromatin Remodelers', *Mutation research*, vol. 618, no. 1-2, pp. 30-40.

Mátis, G, Neogrády, Z, Csikó, G, Gálfi, P, Fébel, H, Jemnitz, K, Veres, Z, Kulcsár, A, Kenéz, Á & Huber, K 2013, 'Epigenetic effects of dietary butyrate on hepatic histone acetylation and enzymes of biotransformation in chicken', *Acta Veterinaria Hungarica*, vol. 61, no. 4, pp. 477-490.

Mishra, AK, Dubey, V & Ghosh, AR 2016, 'Obesity: An overview of possible role(s) of gut hormones, lipid sensing and gut microbiota', *Metabolism*, vol. 65, no. 1, pp. 48-65.

Morrison, DJ, Mackay, WG, Edwards, CA, Preston, T, Dodson, B & Weaver, LT 2006, 'Butyrate production from oligofructose fermentation by the human faecal

flora: what is the contribution of extracellular acetate and lactate?', *British Journal of Nutrition*, vol. 96, no. 03, pp. 570-577.

Nonogaki, K, Ohashi-Nozue, K & Oka, Y 2006, 'A negative feedback system between brain serotonin systems and plasma active ghrelin levels in mice', *Biochemical and Biophysical Research Communications*, vol. 341, no. 3, pp. 703-707.

O'Keefe, SJD, Li, JV, Lahti, L, Ou, J, Carbonero, F, Mohammed, K, Posma, JM, Kinross, J, Wahl, E, Ruder, E, Vipperla, K, Naidoo, V, Mtshali, L, Tims, S, Puylaert, PGB, DeLany, J, Krasinskas, A, Benefiel, AC, Kaseb, HO, Newton, K, Nicholson, JK, de Vos, WM, Gaskins, HR & Zoetendal, EG 2015, 'Fat, fibre and cancer risk in African Americans and rural Africans', *Nat Commun*, vol. 6.

Pacifico, L, Poggiogalle, E, Costantino, F, Anania, C, Ferraro, F, Chiarelli, F & Chiesa, C 2009, 'Acylated and nonacylated ghrelin levels and their associations with insulin resistance in obese and normal weight children with metabolic syndrome', *European Journal of Endocrinology*, vol. 161, no. 6, pp. 861-870.

Paparo, L, di Costanzo, M, di Scala, C, Cosenza, L, Leone, L, Nocerino, R & Berni Canani, R 2014, 'The Influence of Early Life Nutrition on Epigenetic Regulatory Mechanisms of the Immune System', *Nutrients*, vol. 6, no. 11, pp. 4706-4719.

RNeasy® Mini Handbook 2012, Qiagen, viewed 02 March 2017, < https://www.qiagen.com/au/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en>.

Roux, CWl, Patterson, M, Vincent, RP, Hunt, C, Ghatei, MA & Bloom, SR 2005, 'Postprandial Plasma Ghrelin Is Suppressed Proportional to Meal Calorie Content in Normal-Weight But Not Obese Subjects', *The Journal of Clinical Endocrinology & Metabolism*, vol. 90, no. 2, pp. 1068-1071.>

Sadaghian Sadabad, M, von Martels, JZH, Khan, MT, Blokzijl, T, Paglia, G, Dijkstra, G, Harmsen, HJM & Faber, KN 2015, 'A simple coculture system shows mutualism between anaerobic faecalibacteria and epithelial Caco-2 cells', *Scientific Reports*, vol. 5, p. 17906.

Santollo, J, Yao, D, Neal-Perry, G & Etgen, AM 2012, 'Middle-aged female rats retain sensitivity to the anorexigenic effect of exogenous estradiol', *Behavioural Brain Research*, vol. 232, no. 1, pp. 159-164.

Sato, T, Fukue, Y, Teranishi, H, Yoshida, Y & Kojima, M 2005, 'Molecular Forms of Hypothalamic Ghrelin and Its Regulation by Fasting and 2-Deoxy-d-Glucose Administration', *Endocrinology*, vol. 146, no. 6, pp. 2510-2516.

Schellekens, H, van Oeffelen, WEPA, Dinan, TG & Cryan, JF 2013, 'Promiscuous Dimerization of the Growth Hormone Secretagogue Receptor (GHS-R1a) Attenuates Ghrelin-mediated Signaling', *The Journal of Biological Chemistry*, vol. 288, no. 1, pp. 181-191.

Silver, N, Cotroneo, E, Proctor, G, Osailan, S, Paterson, KL & Carpenter, GH 2008, 'Selection of housekeeping genes for gene expression studies in the adult rat submandibular gland under normal, inflamed, atrophic and regenerative states', *BMC Molecular Biology*, vol. 9, pp. 64-64.

Tecott, LH, Sun, LM, Akana, SF, Strack, AM, Lowenstein, DH, Dallman, MF & Julius, D 1995, 'Eating disorder and epilepsy in mice lacking 5-HT2C serotonin receptors', *Nature*, vol. 374, no. 6522, pp. 542-546.

Topping, DL & Clifton, PM 2001, 'Short-Chain Fatty Acids and Human Colonic Function: Roles of Resistant Starch and Nonstarch Polysaccharides', *Physiological Reviews*, vol. 81, no. 3, pp. 1031-1064.

Toshinai, K, Yamaguchi, H, Sun, Y, Smith, RG, Yamanaka, A, Sakurai, T, Date, Y, Mondal, MS, Shimbara, T, Kawagoe, T, Murakami, N, Miyazato, M, Kangawa, K & Nakazato, M 2006, 'Des-Acyl Ghrelin Induces Food Intake by a Mechanism Independent of the Growth Hormone Secretagogue Receptor', *Endocrinology*, vol. 147, no. 5, pp. 2306-2314.

Tschöp, M, Weyer, C, Tataranni, PA, Devanarayan, V, Ravussin, E & Heiman, ML 2001, 'Circulating Ghrelin Levels Are Decreased in Human Obesity', *Diabetes*, vol. 50, no. 4, pp. 707-709.

Valencia-Torres, L, Olarte-Sanchez, CM, Lyons, DJ, Georgescu, T, Greenwald-Yarnell, M, Myers Jr, MG, Bradshaw, CM & Heisler, LK 2016, 'Activation of Ventral Tegmental Area 5-HT2C Receptors Reduces Incentive Motivation', *Neuropsychopharmacology*.

van der Stelt, HM, Broersen, LM, Olivier, B & Westenberg, HGM 2004, 'Effects of dietary tryptophan variations on extracellular serotonin in the dorsal hippocampus of rats', *Psychopharmacology*, vol. 172, no. 2, pp. 137-144.

Walker, AW, Ince, J, Duncan, SH, Webster, LM, Holtrop, G, Ze, X, Brown, D, Stares, MD, Scott, P, Bergerat, A, Louis, P, McIntosh, F, Johnstone, AM, Lobley, GE, Parkhill, J & Flint, HJ 2011, 'Dominant and diet-responsive groups of bacteria within the human colonic microbiota', *The ISME journal*, vol. 5, no. 2, pp. 220-230.

Wang, J, Guo, S, Han, LIN, Fang, M, Wang, LEI, Bartsch, JW & Li, JUN 2015, 'Correlation of ghrelin and growth hormone secretagogue receptor expression

with clinical features in human pituitary adenomas', *Experimental and Therapeutic Medicine*, vol. 9, no. 5, pp. 1909-1914.

Watterson, KR, Bestow, D, Gallagher, J, Hamilton, DL, Ashford, FB, Meakin, PJ & Ashford, MLJ 2012, 'Anorexigenic and Orexigenic Hormone Modulation of Mammalian Target of Rapamycin Complex 1 Activity and the Regulation of Hypothalamic Agouti-Related Protein mRNA Expression', *Neuro-Signals*, vol. 21, no. 1-2, pp. 28-41.

Werry, TD, Loiacono, R, Sexton, PM & Christopoulos, A 2008, 'RNA editing of the serotonin 5HT2C receptor and its effects on cell signalling, pharmacology and brain function', *Pharmacology & Therapeutics*, vol. 119, no. 1, pp. 7-23.

World Health Organisation (WHO) 2015, World Health Organisation, viewed 2nd April 2016, <http://www.who.int/mediacentre/factsheets/fs311/en/>.

Yamada, C, Sadakane, C, Nahata, M, Saegusa, Y, Nakagawa, K, Okubo, N, Ohnishi, S, Hattori, T & Takeda, H 2015, 'Serotonin 2C receptor contributes to gender differences in stress-induced hypophagia in aged mice', *Psychoneuroendocrinology*, vol. 55, pp. 81-93.

Zemdegs, J, Quesseveur, G, Jarriault, D, Pénicaud, L, Fioramonti, X & Guiard, BP 2016, 'High-fat diet-induced metabolic disorders impairs 5-HT function and anxiety-like behavior in mice', *British Journal of Pharmacology*, vol. 173, no. 13, pp. 2095-2110.

Zhang, C, Zhang, M, Wang, S, Han, R, Cao, Y, Hua, W, Mao, Y, Zhang, X, Pang, X, Wei, C, Zhao, G, Chen, Y & Zhao, L 2009, 'Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice', *ISME J*, vol. 4, no. 2, pp. 232-241.

APPENDIX

Appendix A

Modified corn starch and high carbohydrate, high fat diet break-down

Components (g/kg)	mCS	CS	mHCHF	HCHF
Corn starch (g)	570	570	-	-
Fructose (g)	-	-	175	175
Condensed milk (mL)	-	-	395	395
Beef tallow (g)	-	-	150	200
Salt mixture (g)	25	25	25	25
Powered food (g)	-	155		155
Skim milk powder (g)	45	-	45	-
Vitamin mixture from MP Biomedicals (g)	5	-	5	-
Canola oil (mL)	5	-	5	-
Water (mL)	350	250	200	50
Drinking water (mL)	No additives	No additives	25% fructose	25% fructose

Appendix B

Gifu anaerobe media (GAM) preparation and rat colon content culture

1. Autoclaved GAM was retrieved from 4°C overnight storage.

2. Aseptic technique was followed, working around a Bunsen burner. 1.5g dextrose and 0.6g L-cysteine were dissolved in 50ml of distilled water. A filtered syringe was used to add the dextrose and L-cysteine to the media, then pipetted up and down.

3. A pH meter was used to measure the pH of the media, which was 5.8 at first. NaOH was added until pH was 7.2.

4. 20ml of media was pipetted into 6 autoclaved McCartney jars, which were then labelled (MCS28, MSC29, MCS30, MHCHF28, MCHF29, MCHF30).

5. During the tissue collection, approximately two pellets of colon contents were placed into each appropriate jar of media, which was then incubated at 37°C. The terminations were staggered throughout the day, with MCS28 and MHCHF28 at approximately 8:30am, MCS29 and MHCHF29 at approximately 12:30pm and MCS30 and MHCHF30 at approximately 3:30pm.

6. At approximately 7:00pm (10 hrs) all cultures were placed in a shaking incubator at 150RPM and 37°C, for overnight storage.

SW620 and colon content culture preparation

1. 1.5g of agar was added to 100ml of GAM (without dextrose and L-cysteine) was autoclaved.

2. The autoclaved media was left for about 5min to cool before pipetting 6ml of media into 8 labelled 10ml falcon tubes (MCS28, MCS29, MCS30, MHCHF28, MCHF29, MHCHF30 and two controls) in a biosafety cabinet. The agar was left to semi-solidify at room temperature for 30min. The remaining GAM agar was placed on a heating block in order to remain molten.

3. The GAM and colon content cultures were retrieved from 37°C incubation. 1ml from each culture was pipetted into 6 labelled 1.6ml cyrovials, then stored at -80°C.

4. 500µl of each rat colon content culture was added to labelled microfuge, along with 500µl of molten GAM agar. This mixture was pipetted up and down before transferring this to the corresponding falcon tube, pipetting very slowly in order to preserve the integrity of the semi-solidified agar.

5. The falcon tubes were placed in an anaerobic culture chamber with a GENbox anaer sachet. The anaerobic culture chamber was placed in the incubator at 37°C for 2 ½ hours.

6. After 2 ½ hours the falcon tubes were removed from incubation. SW620 cell scaffolds were also removed from incubation.

7. 500µl of fresh DMEM media (without antibiotics or foetal calf serum) was very slowly, drop by drop pipetted onto the GAM agar inoculum in one of the control falcon tubes, to create an interfaced between the bacterial and cell culture.

8. A SW620 inoculated scaffold was removed from the well containing foetal calf serum, using two forceps (care was taken to only touch the outside of the scaffold, not the cell-containing sides). The scaffold was then very carefully placed onto the interface, with the most cell populated side facing down.

9. 500µl of DMEM media was the pipetted on top of the scaffold.

10. The lids of the co-cultures were then loosened, and the falcon tubes incubated overnight 37°C.

Tissue collection and sample homogenisation

1. Tissue samples were collected (see table) and homogenised. The terminations were staggered throughout the day, with MCS28 and MHCHF28 at approximately 8:30am, MCS29 and MHCHF29 at approximately 12:30pm and MCS30 and MHCHF30 at approximately 3:30pm.

2. Tissue samples were collected directly following sacrifice and placed on disposable weigh boats on ice.

3. Bench space, mortar and pestles and spatulas were RNase-zapped to reduce RNA degradation originally then in between homogenisation of each sample. Liquid nitrogen was used to cryogenically homogenised the tissue samples with the mortar and pestle being used to grind to a fine powder. Some tissues were easier to homogenise more finely than others. After homogenisation, the spatula was used to divide the sample into two labelled microfuge tubes, one for RNA and the other DNA, this was then placed in ice.

4. For each RNA sample, 600µl of RLT buffer (50% mercapto ethanol) was added. A syringe and needle was then used to further homogenise each sample into solution. Again, some tissues went into solution easier than others.

5. All samples were then stored at -80°C.

Appendix C
Animal weights

Rat	Body weight (g)	Total abdominal fat mass (g) *	Total abdominal fat mass (mg/mm)#
HCHF 1	553	46.76	954.31
HCHF 2	538	54.26	1064.00
HCHF 3	554	53.47	1091.22
HCHF 4	499	52.16	1043.10
Mean ± SEM	530 ± 12	51.13 ± 1.41	1030.51 ± 24.20
H8C8 1	438	20.42	416.69
H8C8 2	442	21.77	426.92
H8C8 3	432	29.98	611.84
H8C8 4	435	24.73	480.19
Mean ± SEM	437 ± 2	24.23 ± 2.12	483.91 ± 44.86

*The abdominal fat consists of retroperitoneal, omental and epididymal fat pads. #The fourth column is abdominal fat (mg) normalised to tibial length (mm).