INTERACTION OF DIETARY FAT TYPES AND GUT MICROBIOME ON MODULATION OF WHOLE BODY ENERGY BALANCE

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ABSTRACT

Xiaomeng You: Interaction of dietary fat types and gut microbiome on modulation of whole body energy balance
(Under the direction of Steven H Zeisel)

Dietary fats and gut microbes are regarded as environmental factors for the onset of obesity. However, whether there is a direct association between dietary fat type and gut microbiome that promotes obesity remains unclear. In this study, we tested the effect of modulation of the gut microbiome by antibiotics on energy balance in Sprague Dawley rats fed a 45% high fat diet containing primarily saturated fatty acids (SFA) vs. polyunsaturated fatty acids (PUFA). Antibiotic treatment successfully decreased the gut microbiome as evidenced by decreased microbiome α-diversity and β-diversity. We found that food intake was decreased by antibiotic treatment irrespective diet. PUFA-fed rats gained less weight and consumed less food than those fed SFA independent of microbiome composition. No differences were seen in energy expenditure among the 4 groups. Gut hormone and adipokine gene and protein expression was measured in ileum, colon, white adipose tissue (WAT) and blood serum. Compared with SFA, PUFA fed rats had less ileum peptide YY , colon glucagon-like peptide-1, WAT sterol regulatory element binding transcription factor 1 and more ileum β-defensins, WAT adiponectin gene expression. However, no differences were seen in serum protein expression among the 4 groups. In conclusion, SFA are more obesogenic and promote food intake as compared to PUFA and this positive energy balance is independent of the gut microbiome. The mechanisms by which SFA modulate body weight and food intake warrant further investigation.

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α-MSH	Alpha-melanocyte-stimulating hormone
AA	Arachidonic acid
Adipor1	Adiponectin receptor 1
Adipor2	Adiponectin receptor 2
ADM	Antimicrobial defence molecule
AgRP	Agouti-related protein
ALA	Alpha-linolenic acid
AMPK	AMP-activated protein kinase
ANGPTL4	Angiopoitetin-like 4
Antibiotic treatment	Abx
AP	Area postrema
ARC	Arcuate nucleus
CART	Cocaine and amphetamine regulated transcript
CaSR	Calcium-sensing receptor
CCK	Cholecystokinin
CNS	Central nervous system
CO2	Carbon dioxide
DIT	Diet-induced thermogenesis
DHA	Docosahexaenoic acid
DMH	Dorsomedial hypothalamus
EE	Energy expenditure
EFA	Essential fatty acids

ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosaentaenoic acid
FFAR1/ GPR40	Free fatty acid receptor 1
FFAR2/ GPR43	Free fatty acid receptor 2
FFAR3/GPR41	free fatty acid receptor 3
Fiaf	Fasting-induced adipose factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GHS-R	Growth hormone secretagogue receptor
GI track	Gastrointestinal tract
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
GLUT4	Glucose transporter 4
GPCR	G protein coupled receptor
GPR119	G protein-coupled receptor 119
GPR120	G protein-coupled receptor 120
H	Heat production
HBD3	Human β-defensin 3
LA	Linoleic acid
LH/LHA	Lateral hypothalamus
LPAR5/GPR92/GPR93	Lysophosphatidic acid receptor 5
LPS	lipopolysaccharides
MC4R	Melanocortin 4 receptor
ME	median eminence

MUFA	Monounsatureated fatty acids
NAc	Nucleus accumbens
NOD	Nucleotide-binding oligomerization domain receptor
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
O2	Oxygen
OXM	Oxyntomodulin
PFA	Perifornical area
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PPAR-α	Peroxisome proliferator-activated receptors alpha
PPAR-γ	Peroxisome proliferator-activated receptors gamma
PPAR-δ	Peroxisome proliferator-activated receptors delta
PUFA	Polyunsaturated fatty acids
PVN	Paraventricular nucleus
PYY	Peptide YY
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RER	Respiratory exchange ratio
SCFA	Short-chain fatty acid
SFA	Saturated fatty acids
SREBP-1	Sterol regulatory element-binding protein 1
T1R1-T1R3	Amino acids, the umami (savoury) receptor
T1R2-T1R3	Simple sugars and artificial sweeteners, the sweet taste receptor
T2Rs	The bitter receptor family

TLR	Toll-like receptor
TRPA1	Transient receptor potential cation channel, subfamily A, member 1
VMH	Ventromedial hypothalamus
WHO	World Health Organization
Y1/5	Neuropeptide Y receptor type 1/type 5
Y2	Neuropeptide Y receptor type 2

CHAPTER 1: BACKGROUND

Introduction

Obesity is a serious public health concern all over the world. According to a World Health Organization (WHO) report, the incidence of obesity has doubled or even tripled in many countries over the past 30 years [1]. Obesity lies at the root of many prevalent metabolic disorders and diseases such as type 2 diabetes, cardiovascular disease, high blood pressure, dyslipidemia and cancer. Despite the fact that more than 300,000 deaths every year in the United States are due to obesity or its related metabolic disease, obesity is still one of the 10 most preventable health risks according to WHO [2]. Obesity results from energy imbalance and involves various mechanisms and factors including brain control, peripheral signals, gut immune system, and as well as dietary fats, discussed as followed.

Central control of energy balance

The core principle causing obesity is energy imbalance because food intake is greater than energy expenditure. Physiologically, food intake and energy expenditure are tightly regulated through a multi-level system that connects the brain, peripheral tissues (i.e gut, fat tissue, liver, pancreas), and hormonal and neural signals. The brain integrates neural afferents and hormonal signals from the periphery received by hypothalamus and brainstem with energetic needs or anticipated needs, physical and social environmental factors, memory for past experiences, and many other factors to regulate appetite and energy expenditure (Figure 1) [3, 4].

The hypothalamus has been a research hotspot in obesity, as this area contains numerous interacting systems that regulate feeding, satiety, and other motivational states [5]. Electrical lesion and

electrical stimulation studies have demonstrated that the lateral hypothalamus (LH) is a feeding center, the ventromedial hypothalamus (VMH) is a satiety center, and the arcuate nucleus (ARC) is an integrated center for feeding regulation [6]. The ARC contains two populations of neurons with opposing effects on food intake: orexigenic and anorexigenic neurons. Orexigenic neurons express neuropeptide Y (NPY) and Agouti-related protein (AgRP). Anorexigenic neurons express alpha-melanocyte-stimulating hormone (α-MSH) which is derived from pro-opiomelanocortin (POMC), and cocaine and amphetamine regulated transcript (CART) [3]. Circulating signals related to energy status are able to influence the activity of the ARC neurons directly via the underlying median eminence, as this region of the brain is not protected by the blood–brain barrier [3]. These ARC neurons then in turn project to a number of extra-hypothalamic and intra-hypothalamic regions. These areas contain secondary neurons which process the information regarding energy homeostasis.

The melanocortin 4 receptor (MC4R) plays an important role in the downstream pathway of hypothalamus required to modulate short-term and long-term energy homeostasis by integrating signals provided by α-MSH and AgRP [7, 8]. MC4R is expressed primarily in the central nervous system (CNS). Its mRNA was found in multiple sites in virtually every brain region, including the hypothalamus, brainstem, cortex, thalamus, and spinal cord [9]. Particularly, MC4R is expressed in ARC, VMH and PVN which are implicated in energy homeostasis [4]. Rodents studies showed that functional loss of MC4R increased body weight, food intake and white adipose mass [8]. Administration of agonists to the hypothalamic MC4R suppressed food intake, stimulated the thyroid axis and increased energy expenditure. Therefore, MC4R agonists are regarded as a target for obesity therapy.

Peripheral signals of energy balance

To date about 100 unique molecules have been identified to have effects on energy balance [5]. These include satiation signals that are released from the gastrointestinal tract and related organs, and

adiposity signals whose secretion is proportional to body fat [10]. Leptin, adiponectin, insulin, glucagon and gut hormones will be discussed in detail as followed.

Leptin is a hormone discovered in 1994 that is predominantly produced by adipose tissue [11]. Dietary factors and other hormones are thought to influence its secretion. In vitro, insulin is shown to simulate leptin secretion in adipocytes [12]. In addition, the rate of glucose uptake into adipose tissue determines changes in circulating leptin [13]. Other nurients might also influence leptin production. Rodent studies showed that diets rich in n-6 PUFA increase leptin production [14, 15]. Lack of leptin or leptin receptor bioactivity is responsible for a phenotype characterized by hyperphagia, reduced energy expenditure, and severe obesity in both rodents (ob/ob mice and db/db mice) and human [16-18]. Leptin is expressed and secreted in proportion to adipose mass and circulates in plasma in a concentration highly correlated to body fat mass [19]. The circulating leptin crosses the blood brain barrier, binds to leptin receptors on neurons throughout the hypothalamus and provides a negative feedback signal to inhibit food intake and to stimulate energy expenditure [11]. However, the rise in endogenous leptin, or exogenous leptin given as treatment is unable to prevent weight gain in most obese humans, a process called “leptin resistance” [20]. Leptin resistance may result from a decrease in brain transport or attenuation of leptin signaling in the hypothalamus and other central nervous system targets [21]. Further studies will need to focus on developing therapies aimed at reversing leptin resistance.

Adiponectin is specifically and abundantly expressed in adipocytes. Epidemiological evidence has indicated that circulating adiponectin levels are reduced in obese patients [22]. Adiponectin acts in an autocrine/paracrine manner within adipose tissue, and in an endocrine manner on distal tissues [23]. As an autocrine/paracrine factor in adipose tissue, adiponectin has shown beneficial effects on insulin sensitivity by evidence that adiponectin increased insulin's ability to maximally stimulate glucose uptake

by 78% through increased glucose transporter 4 (GLUT4) gene expression and increased GLUT4 recruitment to the plasma membrane. [24] Adiponectin also functions as an endocrine factor, influencing whole-body metabolism via effects on target organs [23]. Adiponectin receptor 1 (Adipor1) expression is ubiquitous in the rat brain and adiponectin receptor 2 (Adipor2) expression is more limited to hypothalamus [25]. By binding to the adiponectin receptors in hypothalamus, adiponectin increases hypothalamic AMP-activated protein kinase (AMPK) phosphorylation to stimulate food intake and suppress energy expenditure [26]. However, it should be noted that intracerebroventricular administration of adiponectin decreased body weight with increasing energy expenditure suggesting a negative regulation pathway for adiponectin effect on energy balance [27].

Insulin is secreted from β-cells of the pancreas. The best known action of insulin is to suppress the synthesis and secretion of glucose by the liver and as a result, reduce the blood glucose level. Levels of insulin are determined to a great extent by peripheral insulin sensitivity, and this is related to total body fat stores and fat distribution, with visceral fat being a key determinant of insulin sensitivity [28, 29]. Therefore, insulin may convey a signal indicating the degree of adiposity to any insulin-sensitive tissue, providing a key negative feedback signal in the regulation of body fat. It is now generally accepted that some plasma insulin can be transported into the brain and especially to hypothalamus to reduce food intake and decrease body weight [30]. Insulin may cross the blood–brain barrier via a saturable, receptor-mediated process, at levels which are proportional to the circulating insulin [31]. Selective decrease in hypothalamic insulin receptor protein by antisense oligodeoxynucleotide against the insulin receptor precursor protein results in hyperphagia and increased fat mass indicating that peripheral insulin acts on hypothalamic nuclei to control energy homeostasis [32].

Glucagon is a pancreatic hormone produced from the preproglucagon precursor molecule by the α cells of the pancreas. Its main effect is to maintain blood glucose levels during fasting and exercise

by promoting hepatic glycogenolysis and gluconeogenesis. Thus, the major metabolic functions of glucagon are opposite to those of insulin. In addition, glucagon has anorectic properties and promotes satiety. Peripherally administered glucagon decreased food intake and increased c-fos expression in the brainstem and amygdala indicating that neurons in the brainstem and amygdala are activated in order to reduce food intake by glucagon [33]. However, the underlying mechanism for how glucagon activates neurons in brain to reduce food intake is unknown. The anorectic effect following glucagon administration is blunted in vagotomized animals suggesting this effect is influenced by vagal input [34, 35]. Also, low levels of glucagon receptor mRNA are found in the hypothalamus and brainstem in rodents indicating the possibility of direct glucagon action in these areas to reduce food intake [36].

Gut hormones are peptides that are synthesized and released from the gastrointestinal tract [37]. There are at least 15 different types of enteroendocreine cells diffusely distributed throughout the gastrointestinal epithelium making the gut the most largest endocrine organ in the body [38]. Contrary to long-acting adiposity signals of leptin and adiponectin, the appetite hormones from gut interact with receptors at various points in the "gut-brain axis" to affect short-term and intermediate-term feelings of hunger and satiety [39]. Evidence showed that endocrine cells can directly sense luminal contents primarily by G protein coupled receptors (GPCRs) [40] (see Table 1). For example, L cells express the complete range of fatty acid receptors that can sense fats in the intestine lumen and release peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) into circulation with effect on the hypothalamus to regulate energy balance [41]. To date, the gastrointestinal (GI) tract releases at least 20 different regulatory peptide hormones at the same time depending on the quality and quantity of the diet and influences various physiological processes [40, 42]. A number of gut hormones have been identified as being involved in appetite control and energy balance. Among them, ghrelin is called “hunger hormone” which is the only known circulating orexigen principally synthesized in endocrine A(X-like) cells (or ghrelin cells) in the stomach [43, 44]. On the contrary, other gut appetite hormones are believed to reduce food

intake by decreasing hypothalamic orexigenic signaling and increasing anorectic signaling (Listed in Table 1). As gut hormones have been shown to have a fundamental role in energy homeostasis, the use of gut hormones as anti-obesity treatments is an attractive option and shows considerable promise.

Endocrine-Immune interaction in gut

Intestinal epithelial cells act as the interface between the external environment and the internal milieu. The content of the intestine is rich in nutrients, chemicals and microorganisms. The gut processes a range of sensory systems to detect nutrients and defend against pathogens and injurious chemicals [40]. As discussed previously, the gut functions as an endocrine organ to release gut hormones by nutrients receptors that can act on other cells locally, or organs at remote sites including pancreatic islets and the CNS to regulate food intake, gastric empting and intestinal transit, release of digestive enzymes, induction of nutrient transporters and digestive enzymes, pancreatic insulin secretion [40].

Also, the gut functions to defend against pathogens and injurious chemicals. In addition to be a physical barrier against infection by the wealth of opportunistic pathogens that can invade through the oral-enteric route, intestine serves as an active immune site including adaptive immunity that lymphocyte mediated secretion of specific antibodies and innate immune response of secretion of non-antigen specific compounds [45]. Intestinal epithelium constantly monitors both the luminal and mucosal environments through receptor mediated interactions with the contents therein [46]. In response to the activation of Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain receptors (NOD) expressed by intestinal epithelial cells, Paneth cells, and neutrophils, antimicrobial defence molecules (ADMs) are released as endogenous antibiotics, which are central to both the innate and adaptive arm of mucosal host defense within the gut [46]. Defensins are one of the major classes of ADMs in the intestine, which are small (2-6kDa), cationic peptides that kill bacteria primarily by

disruption of cell walls. Defensins are classified as α-defensins and β-defensins based on their molecular distribution of cysteine amino acids and the resulting disulfide bonds [47]. Recent work showed that β-defensins can bind to the MC4R to control feeding and body weight [48]. Intracerebroventricular injection of human β-defensin 3 (HBD3) to male Wistar rats had an inhibitory effect on both the food intake and body weight gain with the potential mechanism of blocking AgRP [49]. As the secretion in response to bacteria in the intestine, β-defensins link the gut microbiome, intestine immunity and energy balance together and provide a crosstalk between gut microbiome and obesity.

The potential mechanism linking gut microbiome to obesity

The gut microbiome presents more than 1,000 different molecular species or phylotypes and 90% of the species belong to Firmicutes and Bacteroidetes [50]. Recent work has identified a role for microbiota in the onset of obesity. The link between microbiota and obesity was first uncovered based on the observation that germ-free mice contained 42% less body fat than conventional mice regardless of more food intake [51]. Similarly, pseudo-germ-free mice treated with an ampicillin-neomycin cocktail are prevented from the development of obesity when fed on a high fat diet, indicating an essential role for gut microbiota in obesity development [52]. Today the gut microbiota is considered a "microbial organ" and many studies explore the potential mechanisms of the effect of gut microbiome on host metabolism and its contribution to obesity.

Low-grade inflammation is a common comorbidity of obesity and its related metabolic disorder. Lipopolysaccharides (LPS) originating from gram-negative bacteria in the gut is believed to induce subclinical inflammation and insulin resistance and contribute to obesity [53]. In a study of genetically identical male rats, infusing a low level of LPS for 4 weeks caused the same amount of weight gain as a high-fat diet [53]. Also, rats lacking CD14, which is necessary to cause an inflammatory reaction

to LPS, are resistant weight gain [53]. Gut microbiota modulated by antibiotics can reduce circulating LPS levels, decrease inflammatory signaling and improve insulin signaling [54].

Increasing energy harvest from dietary fibers by gut microbiota is also believed to affect body weight. It is calculated that the intestinal microbiota breaks down indigestible polysaccharides (i.e., fiber) to short-chain fatty acids (SCFAs) providing 80 to 200 kcal per day or about 4–10% of daily energy intake in normal adults [55]. Also, it is proposed that the microbiota of obese individuals may be more efficient at extracting energy from a given diet than the microbiota of lean individuals. Lean mice have less short-chain fatty acids in the caeca and excrete more energy by feces, compared with obese mice [56].

Fasting-induced adipose factor (Fiaf, also referred as Angiopoietin-like 4; ANGPTL4) is a secreted factor involved in regulation of lipid homeostasis by inhibiting lipoprotein lipase [57]. Germ-free mice express excessive Fiaf in the intestine[51], which blocked the disassociation of fatty acids from triglycerides for uptake into tissues and upregulated fatty acid oxidation and uncoupling proteins, and reduced the amount of fat storage [57]. Also, germ-free mice lacking Fiaf respond to a high fat diet with excessive weight gain [58]. Therefore, Fiaf might serve as a circulating mediator between the gut microbiota and fat storage in adipose tissue.

The activation of AMPK is also thought to be involved in linking the gut microbiome and obesity, with evidence showing that germ-free mice increased skeletal muscle and liver levels of phosphorylated AMPK and its downstream targets involved in fatty acid oxidation [58].

These complementary but independent mechanisms indicate that gut microbiota may be a target for treating obesity. However, the effect of the gut microbiome on food intake and energy expenditure, the two key factors determine energy balance, are inconclusive with controversial results due to the different types of animals, the quality of the diet, the period of observation and the sample size in different studies [55].

The effect of dietary fat types on obesity

The effect of dietary fats on human health depends not only on the quantity of ingested fats, but also on the composition and nature of the fatty acids. Depending on the presence of double bonds, fatty acids are classified into three main groups: 1) saturated fatty acids (SFA) that do not contain double bonds 2) monounsaturated fatty acids (MUFA) that contain only one double bond, and 3) polyunsaturated fatty acids (PUFA) that contain at least two double bonds. Among PUFA, linoleic acid (LA: 18:2 n-6) and alpha-linolenic acid (ALA: 18:3 n-3), are known as essential fatty acids (EFA). They are fundamental for the organism, but they cannot be synthesized in the human body and must be obtained from the diet. Essential biological functions have been attributed to EFA-derivatives, such as arachidonic acid (AA, 20:4 n-6), docosahexaenoic acid (DHA, 22: 6 n-3) and eicosapentaenoic acid (EPA, 20:5 n-3) [59].

SFA derive primarily from animal sources, such as fatty meats (pork, beef, veal, lamb, ham), eggs and dairy products (whole milk, cream, butter and cheese) [60]. Some vegetable products (such as coconut oil, and palm kernel oil) are also rich in SFA [60]. PUFA are present in many species of nuts, vegetable oils and fish oil. In particular, n-6 PUFA are abundantly present in corn oil, cotton seed oil, peanut oil, soybean oil, sunflower oil and safflower oil, while canola oil, linseed oil and fish oil are rich in n-3 PUFA [59].

Generally, saturated fat of mammalian origin seems to be far more harmful to human health than unsaturated fat from plants and fish. SFA are associated with an increased risk various diseases including dyslipidemia, coronary heart disease, and cancer [61-63]. In contrast, a considerable number of studies have demonstrated the beneficial effects of PUFA on health [60, 64-68].

In many cases, n-3 PUFA and n-6 PUFA can compensate each other's function in ameliorating pathological conditions such as growth retardation [59] and in the regulation of lipid raft function [59].

In other situations, n-3 PUFA and n-6 PUFA have competitive functions. For example, ingestion of EPA and DHA from fish or fish oil replaces AA from membrane phospholipids in practically all cells, especially those of platelets, erythrocytes, neutrophils, monocytes and liver cells [69].

The balance of n-6 PUFA and n-3 PUFA is very important for homeostasis and normal development. Because of the increased amount of n-6 PUFA in the Western diet (n-6 PUFA to n-3 PUFA is about 15/1 to 16.7/1) [70], the eicosanoid metabolic products from AA are formed in larger quantities than those formed from n-3 PUFA. The eicosanoids from AA are biologically active in small quantities. However, if formed in large amounts, they will contribute to the formation of thrombi and atherosclerosis, the development of allergic and inflammatory disorders, and cell proliferation [69]. On the contrary, ingestion of EPA and DHA leads to a more physiologic state characterized by the production of prostanoids and leukotrienes that have antithrombotic, antichemotactic, antivasoconstrictive and anti-inflammatory properties [69].

A high-fat diet (≥40 % of energy from fat sources) is regarded as one of the factors associated with the current obesity epidemic as it contributes to a positive energy balance and a positive fat balance as well [71]. In the meantime, it is well established that the consumption of different types of fat is associated with different rates of weight gain in obese animals [72]. It is believed that SFA are more obesogenic than PUFA [73-75], although there are controversies [76]. Early studies showed that animals fed with PUFA (from corn oil) have lower weight gain than SFA (from beef tallow) [77]. The observed difference in weight gain might be explained by the differences in capacity to control appetite or effects on energy expenditure. Human studies demonstrated that PUFA may exert a relatively stronger control over appetite than SFA [74]. A diet rich in PUFA also results in increased energy expenditure with preferential stimulation of the thermogenic activity of brown adipose tissue [77] and diet-induced thermogenesis (DIT) [72].

In addition, PUFA are oxidized more rapidly than SFA, and as a result, decrease serum triacylglycerol level and fat accumulation [78, 79]. In rats, the consumption of a PUFA rich (from safflower oil) diet results in a higher lipoprotein lipase activity in heart and skeletal muscle than does a SFA diet (from beef tallow) [79]. Compared to SFA, PUFA (both the *n*-6 and *n*-3 series, but *n-3* PUFA are more potent ligands for nuclear receptors than n–*6* PUFA) are regarded as the preferred activator of peroxisome proliferator-activated receptors delta, gamma and alpha (PPAR-δ, PPAR-γ and PPAR-α) [59, 80]. More PPAR signaling is able to up-regulate the expression of enzymes involved in conversion of fatty acids to acyl-coenzyme A esters, fatty acid entry into mitochondria and peroxisomal and mitochondrial fatty acid catabolism [81]. In addition, PUFA (both the *n*-6 and *n*-3 series, but *n-3* PUFA are more potent ligands for nuclear receptors than *n*−6 PUFA) selectively decreases sterol regulatory element-binding protein 1 (SREBP-1), which is involved in the transcriptional regulation of lipogenic enzymes [59, 80, 82].

Recent studies show that dietary fats also affect the populations of gut microbes and their metabolic end products. A high-fat-fed animal displays a significant shift in both bacterial and metagenomic profiles as compared to an animal on a normal, chow diet [83]. Western diet-associated cecal microbiota are characterized by a reduction in Bacteroidetes and an increase in Firmicutes [83]. Animal studies also showed that the composition of the murine gut microbiome was determined by a high-fat diet independent of obesity [84]. Hildebrandt et al. found that a high-fat diet was associated with murine gut microbiome alterations characterized by a decrease in Bacteroidetes and an increase in both Firmicutes and Proteobacteria in the presence and absence of obesity [84]. Thus, gut microbiome provides another mechanism that connects dietary fats and obesity. However, a direct connection between a specific type of fats and gut microbes contributing to obesity remains unclear.

Hypothesis

In this study, we tested the effect of modulation of the gut microbiome by antibiotics on energy balance in Sprague Dawley rats fed a 45% high fat diet containing primarily saturated fatty acids SFA vs. PUFA as the main source of lipids. The rats within these two dietary groups were then treated with either an antibiotics or a vehicle to modulate the microbiome. We hypothesized that rats fed with a PUFA diet would have less weight gain than those fed a SFA diet due to less food intake and more energy expenditure due to the modulation of peripheral energy balance signaling from the gut, adipose tissue, and pancreas to the brain independent of the gut microbiome. The difference of weight gain would be seen between SFA diet and PUFA diet when gut microbiome is modulated by antibiotics.

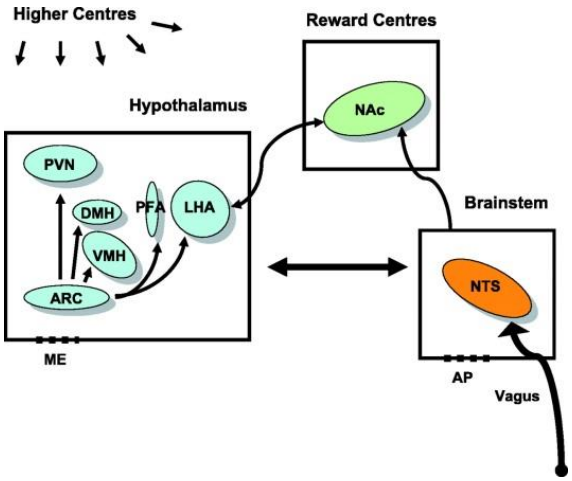

Figure 1 Brain control of energy balance overview. (Adapted from [4])
The brain integrates neural afferents and hormonal signals from the periphery received by hypothalamus and brainstem with energetic needs or anticipated needs, physical and social environmental factors, memory for past experiences, and many others to regulate appetite and energy expenditure.
Abbreviation: ME, median eminence; ARC, arcuate nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; PVN, paraventricular nucleus; PFA, perifornical area; LHA, lateral hypothalamus; NAc, nucleus accumbens; AP, area postrema; NTS, nucleus of the solitary tract

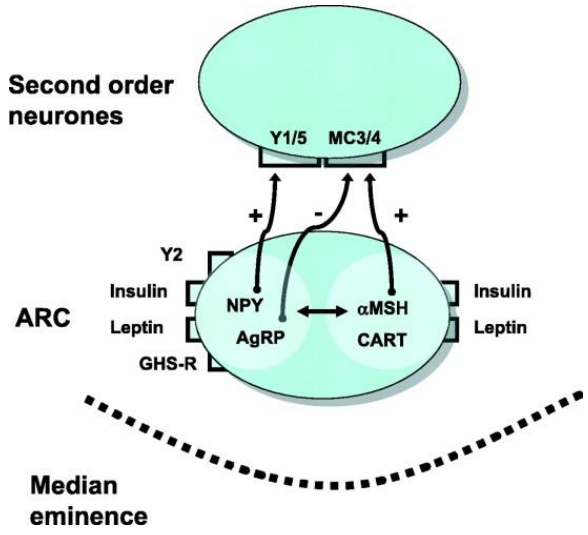

Figure 2 The ARC and the control of energy balance. (Adapted from [4])
The ARC contains two populations of neurons with opposing effects on food intake: orexigenic and anorexigenic neurons. Orexigenic neurons express neuropeptide Y (NPY) and Agouti-related protein (AgRP). Anorexigenic neurons express alpha-melanocyte-stimulating hormone (α-MSH) which is derived from pro-opiomelanocortin (POMC), and cocaine and amphetamine regulated transcript (CART). Circulating signals of energy status come across the median eminence to influence the activity of the ARC neurons directly. The ARC neurons then in turn project to a number of extra-hypothalamic and intra-hypothalamic regions. These areas contain secondary neurons which process the information regarding energy homeostasis.
Abbreviation: α-MSH, α-melanocyte-stimulating hormone; GHS-R, growth hormone secretagogue receptor; CART, cocaine and amphetamine regulated transcript; NPY, neuropeptide Y; AgRP, agouti-related protein; Y2, neuropeptide Y receptor type 2; MC3/4, Melanocortin 3/4 receptor; Y1/5, neuropeptide Y receptor type 1/type 5

Table 1 Gut hormones on appetite control (Summarized from [37, 40, 42, 85])

Gut hormone	Cell	Luminal receptors*	Locations	Effect on food intake
Ghrelin	A(X)-like cells	T1R1-T1R3; T2Rs	Stomach	Increase
Cholecystokinin (CCK)	I cells	T2Rs; FFAR1; GPR120; LPAR5; CaSR; TRPA1; TLRs	Proximal small intestine	Decrease
Glucose-dependent insulinotropic polypeptide (GIP)	K cells	GPR119; GPR120; FFAR1	Proximal small intestine	Decrease
Peptide YY (PYY)	L cells	T2Rs; T1R2-T1R3; FFARs 1-3; GPR119, LPAR5, GPR120; CaSR	Distal small intestine and colon	Decrease
Glucagon-like peptide-1 (GLP-1)	L cells	T2Rs; T1R2-T1R3; FFARs 1-3; GPR119, LPAR5, GPR120; CaSR	Distal small intestine and colon	Decrease
Glucagon-like peptide-2 (GLP-2)	L cells	T2Rs; T1R2-T1R3; FFARs 1-3; GPR119, LPAR5, GPR120; CaSR	Distal small intestine and colon	Decrease
Oxyntomodulin (OXM)	L cells	T2Rs; T1R2-T1R3; FFARs 1-3; GPR119, LPAR5, GPR120; CaSR	Distal small intestine and colon	Decrease

* Luminal receptors: T1R1-T1R3, amino acids, the umami (savoury) receptor; T1R2-T1R3, simple sugars and artificial sweeteners, the sweet taste receptor; T2Rs, the bitter receptor family; FFAR1 (also known as GPR40), free fatty acid receptor 1; FFAR2(also known as GPR43), free fatty acid receptor 2; FFAR3 (also known as GPR41), free fatty acid receptor 3; GPR120, G protein-coupled receptor 120; GPR119, G protein-coupled receptor 119; LPAR5(also known as GPR92 and GPR93), lysophosphatidic acid receptor 5; CaSR, calcium-sensing receptor; TRPA1, transient receptor potential cation channel, subfamily A, member 1; TLRs, toll-like receptors;

CHAPTER 2: MANUSCRIPT[1]

Introduction

Obesity is a serious public health concern. According to a 2012 World Health Organization (WHO) report, the incidence of obesity has doubled or even tripled in many countries over the past 30 years [1]. Obesity lies at the root of many prevalent metabolic disorders and diseases such as type 2 diabetes, cardiovascular disease, high blood pressure, dyslipidemia and cancer. Despite the fact that more than 300,000 deaths every year in the United States are due to obesity or its related metabolic disease, obesity is still one of the 10 most preventable health risks according to WHO [2].

Obesity results from energy imbalance. The control of energy balance relies upon the brain to detect and integrate of peripheral signals from gut, adipose tissues, pancreas of energy homeostasis and social, emotional, circadian, habitual and other situational factors [3, 4].

Recent work has identified a role for gut microbiota in the onset of obesity. The potential mechanisms of the effect of gut microbiota on the host metabolism, and its contribution to obesity, include lipopolysaccharides (LPS)- induced low grade inflammation[54], increased energy harvesting from dietary fibres [56], decreased fasting-induced adipose factor expression [51], changed lipid metabolism [51], and/or decreased AMP-activated protein kinase (AMPK) activation [58]. However, the effect of the gut microbiome on food intake and energy expenditure, the two key factors determine energy balance, are inconclusive with controversial results due to the different type of animals, the quality of the diet, the period of observation and the sample size in different studies [55].

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In response to bacterial, antimicrobial defence molecules (ADMs) are released as endogenous antibiotics, which are central to both the innate and adaptive arm of mucosal host defense within the gut. β-defensins are one of major classes of ADMs. Recent work showed that β-defensins can bind to the melanocortin 4 receptor (MC4R) to control feeding and body weight providing another mechanism linking gut microbiome and energy balance [48].

The current obesity epidemic is associated with the change of diet and sedentary lifestyle in modern life. A high-fat diet is regarded as a risk factor for obesity as it contributes to a positive energy balance and a short-term positive fat balance [71]. In the meantime, it is well established that the consumption of different types of fat is associated with different rates of weight gain in obese animals [72]. Although there are controversies, it is believed that SFA are more obesigenic than PUFA [73, 74, 76]. Early studies showed that animals fed with PUFA have lower weight gain than SFA [77]. The observed difference in weight gain might be explained by the differences in capacity to control appetite or effects on energy expenditure. Human studies demonstrated that PUFA may exert a relatively stronger control over appetite than SFA [74]. A diet rich in PUFAs also results in increased energy expenditure with preferential stimulation of the thermogenic activity of brown adipose tissue [77] and diet-induced thermogenesis (DIT) [72].

In addition, PUFA are oxidized more rapidly than SFA, and as a result, decrease serum triacylglycerol level and fat accumulation [78, 79]. In rats, the consumption of a PUFA rich (from safflower oil) diet results in a higher lipoprotein lipase (LPL) activity in heart and skeletal muscle than does a SFA diet. Compared to SFA, PUFA is regarded as the preferred activator of peroxisome proliferator-activated receptors delta, gamma and alpha (PPAR-δ, PPAR-γ and PPAR-α). PPAR signaling is able to up-regulate the expression of enzymes involved in conversion of fatty acids to acyl-coenzyme A esters, fatty acid entry into mitochondria and peroxisomal and mitochondrial fatty acid catabolism [81].

In addition, PUFA selectively decrease sterol regulatory element-binding protein 1 (SREBP-1), which is considered to be strongly involved in the transcriptional regulation of lipogenic enzymes [82].

Recent studies also show that dietary fats also affect populations of gut microbes and their metabolic end products. A high-fat-fed animal displays a significant shift in both bacterial and metagenomic profiles as compared to an animal on a normal, chow diet [83]. Western diet-associated cecal microbiota are characterized by a reduction in Bacteroidetes and an increase in Firmicutes [83]. Also, it has shown that the composition of the murine gut microbiome was determined by a high-fat diet independent of obesity [84]. Hildebrandt et al. found that a high-fat diet was associated with murine gut microbiome alterations characterized by a decrease in Bacteroidetes and an increase in both Firmicutes and Proteobacteria in the presence and absence of obesity. Thus, gut microbiome provides another mechanism that connects dietary fats and obesity.

In this study, we tested the effect of modulation of the gut microbiome by antibiotics on energy balance in Sprague Dawley rats fed a 45% high fat diet containing primarily saturated fatty acids saturated fatty acids (SFA) vs. polyunsaturated fatty acids (PUFA) as the main source of lipids. The rats within these two dietary groups were then treated with either an antibiotics or a vehicle to modulate the microbiome. We hypothesized that rats fed with a PUFA diet would have less weight gain than those fed a SFA diet by less food intake and more energy expenditure due to the modulation of peripheral energy balance signaling from the gut, adipose tissue, pancreas to the brain independent of the gut microbiome. The difference of weight change would be seen between SFA diet and PUFA diet when gut microbiome is modulated by antibiotics

Methods

Animals, Diet and Tissue Collection

This study was approved by the University of North Carolina Institutional Animal Care and Use Committee. 32 Sprague Dawley male rats (aged 7-week, Charles River Laboratories, USA) were randomized to one of two diet groups (n=16 per group, phase 1) as follows: 1) SFA-rich diet (45% energy from fat, predominant source butter and lard), and; 2) PUFA-rich diet (45% energy from fat, predominant source safflower oil rich in n-6 PUFA) (both Research Diets, New Brunskwick, NJ, USA). The composition of each diet is indicated in Table 2 and Table 3. Animals were housed singly for accurate measurement of food intake and energy expenditure. Following 36-day of diet intervention, feces were collected and stored at -80^{o}C for gut microbiome analysis. Then animals were further randomized to either antibiotic treatment (Abx) or control group (n=8/ group, phase 2) as follows: 1) Imipenem/cilastatin sodium at 50 mg/kg of body weight/day, or; 2) vehicle control (distilled water), for a treatment period of 7 days. Treatment and control were given via water. On the morning following the final treatment day, food was removed from the animals at the end of the dark cycle. Feces were collected, stored at -80^{o}C for gut microbiome analysis. Animals were euthanized via exsanguination under isoflurane and death confirmed via bilateral pneumothorax. Whole blood was collected via cardiac puncture under anesthesia and centrifuged at 3000 RPM for 15 minutes. Serum was collected after centrifugation, stored at -80 ^{o}C. Small intestine and colon were collected and stored in RNAlater (Life Technologies, Carlsbad, CA, USA) at -20 ^{o}C. White adipose tissue, brown adipose tissue, liver were collected, snap frozen in liquid nitrogen, stored at -80 ^{o}C. (see Figure 3 for study design)

Assessment of body weight, food intake and energy expenditure

Body weight and food intake were measured 3 times a week (Monday, Wednesday, Friday 9:00 am) during phase 1 of the study and measured daily during phase 2 and in real-time over the 24-hour

metabolic rate assessment. As much as possible, spillage was accounted for by collecting and measuring smaller pellets and crumbs from the cage floor. Metabolic rate was assessed on 2 occasions: on the final day of the 36-day diet intervention period and the 7-day diet and antibiotic treatment period. During the energy expenditure measurements, animals were housed in individual metabolic cages (TSE systems, St Louis, MO, USA) for a period of 24-hours. The difference between the oxygen (O_2) and carbon dioxide (CO_2) concentrations in the cages were compared with a reference environment, and used to calculate oxygen consumption volume (VO_2), carbon dioxide production volume (VCO_2), respiratory exchange ratio (RER) and heat production (H). Animals were acclimated to the metabolic cages for at least one day prior to the measurement period. As there were only 8 metabolic cages, 32 animals were divided into 4 groups to measure energy expenditure (EE) for 4 consecutive days (see Table 4). Each group contained 2 rats from each treatment (SFA_NonAbx, SFA_Abx, PUFA_NonAbx and PUFA_Abx treatment). As it took about 3 hours to calibrate the machine and change animals in acclimated cage and metabolic cage, 21 hours' data were collected to calculate the EE.

Assessment of microbiome

Fecal pellets were collected on 2 occasions: on the final day of the 36-day diet intervention period and the 7-day diet and antibiotic treatment period. Between-group differences in microbial richness and diversity were measured. DNA was extracted from fecal pellets using QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), according to manufacturer's protocol. After the concentration and quality were determined with NanoPhotometer (Implen GmbH, Munich, Germany), sequencing of the 16S, V6 region was performed on Illumina sequencing system by Dr. Mike Wang's lab at David H. Murdock Research Institute (DHMRI, Kannapolis, NC, USA). The bacterial and bioinformatic analysis was performed by Dr. Raad Gharaibeh (UNCC, NC, USA).

Quantitative reverse transcriptase PCR

RNA was extracted from the ileum, colon, white adipose tissue using PureLink® RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's protocol. After the concentration and quality were determined with NanoPhotometer (Implen GmbH, Munich, Germany), quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) method was used for the assessment of gene expression. First, cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) on an Eppendorf Mastercycle ProS (Eppendorf, Hamburg, Germany). The second part was performed on the LightCycler® 480 Real-Time PCR System (Roche, Penzberg, Upper Bavaria, Germany) using LightCycler® 480 Probes Master (Roche, Penzberg, Upper Bavaria, Germany). TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) were purchased for the following genes: Defb1 (β-defensin 1), Defb2(β-defensin 2), Defb3 (β-defensin 3), Defb4 (β-defensin 4), Pyy (PYY), Gcg (GLP-1), Adipoq (adiponectin), Lep (leptin), Pparg (PPARγ), Srebf1 (SREBP1) and Gapdh (glyceraldehyde 3-phosphate dehydrogenase). The real-time PCR reactions were run in triplicate, and data were retrieved as CT values normalized to Gapdh and log2 transformed for subsequent statistical analysis. Final data were expressed as ratios between sample, and the average of the SFA_NonAbx group for each gene.

Plasma metabolic hormones and β-defensins assessment

Insulin, leptin, GIP, PYY, PP, amylin and ghrelin were analyzed using bead-based multiplex assays (Millipore, Billerica, MA) on a Luminex 100 TM (LUminex, Austin, TX). Total β-defenins, β-defensin 14, adiponectin were measured by enzyme-linked immunosorbent assay ELISA (Millipore or MyBioSource, Inc, San Diego, California, USA). Procedures are according to manufacturer's protocol.

Bioinformatics analysis

The bioinformatics analysis of gut microbiome was performed by Dr. Raad Gharaibeh (UNCC, USA) described as follows.

A single Illumina HiSeq2000 sequencing lane was used to generate 74,502,452 paired-end reads, 100 bases long, for a total of 73 multiplexed samples targeting the V6 region of the 16S rRNA gene. The raw reads were subjected to a QC check using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then processed as described previously [86, 87] except that a minimum of 70 continuous matching nucleotides at 97% similarity across the length of the ungapped alignment was required to produce each merged sequence. For OTU clustering, we used the program AbundantOTU+ v.0.93b (http://omics.informatics.indiana.edu/AbundantOTU/otu+.php) with the "-abundantonly" option and reads from the each comparisons group were clustered together, i.e. if the comparison was between SFA and PUFA, reads belonging to those samples were clusters alone. Sequences that were not clustered into an OTU (singletons) were excluded from further analyses. We employed UCHIME (http://www.drive5.com/uchime/) and the Gold reference database to screen for the presence of chimeras in our OUT sequences and chimeric sequences were removed.

To facilitate the taxonomic classification and to compensate for the short read length of the generated OTUs, BLASTn v. 2.2.26+ was used with an expectation value of e-5 to align the OTU sequences to the Silva database (release 108, http://www.arb-silva.de/). After that, the standalone version of the RDP classifier [88] v. 2.5 was used to classify the full-length Silva sequences with the best BLASTn match to the OTU sequence requiring an RDP confidence score ≥ 80%. A pivot table was generated where each row represents a sample and each column contains the raw counts for each OTU. Those raw counts were normalized and log transformed according to the following equation:

$$\log_{10}\left(\left(\frac{\text{OTU raw count}}{\text{Number of sequences in sample}} \times \text{Average number of sequences per sample}\right) + 1\right)$$

Normalized and log transformed OTU counts were used to produce a Bray-Curtis dissimilarity matrix that was fed into mothur [89] v.1.25.0 for Principle Co-ordinate Analysis (PCoA).

To ensure the results are not pipeline-dependent, a parallel analysis using QIIME v.1.7.0 [90] was also conducted, utilizing both de novo (at 97% similarity level) and close-reference OTU picking approaches (at 97% similarity level using the Greengenes 97% reference dataset, release of May 2013) and yielded similar results.

Statistical analysis

All statistical analysis was carried out using SPSS 15.0 software (IBM). All values were expressed as mean and standard error of the mean. For body weight and food intake data, results were analyzed for equal variances, followed by one-way repeated ANOVA in phase 1 and two-way repeated ANOVA in phase 2. In phase 1, energy expenditure data was analyzed by Student's t-test. In phase 2, gene expression data, protein expression data, and energy expenditure data were analyzed by two-way ANOVA with equal variance or Friedman test with unequal variance. Tukey or Mann Whitney U was used as post hoc analysis. $p<0.05$ was considered statistically significant.

Results

The effect of dietary fat type on body weight, food intake and energy expenditure

To determine the effect of different types of high-fat diet with varying saturated and polyunsaturated fatty acid content on energy balance, body weight and food intake were analyzed with one-way repeated ANOVA. We found a significantly higher ($p=0.016$) body weight gain on the SFA diet compared with the PUFA diet (Figure 4). Student's t-test analysis showed that the significant weight

difference between groups began at day 10 and continued throughout the diet intervention ($p<0.05$, Figure 4). After 36 days of diet intervention, the body weight of rats fed with the SFA diet was 8.68% higher than the PUFA diet ($p<0.05$, Figure 4). Similarly, we found a significantly higher ($p<0.05$) food intake on the SFA diet compared with the PUFA diet (Figure 5). The total food intake per rat on the SFA diet was 9.12% more than on PUFA diet throughout 36 day diet intervention ($p<0.05$, Figure 6). However, when food intake was normalized to body weight (calorie intake per kg body weight of rat), no difference between groups was observed (Figure 7). Energy expenditure was measured at the end of 36 day diet intervention. No significant difference was seen between groups (Table 5).

Gut microbiome in response to diet intervention and antibiotic treatment

To determine the effect of dietary fatty acids and antibiotic treatment on gut microbiota richness and diversity, 16S rRNA of fecal microbiota was analyzed. We found that the 36 day different FA diet intervention did not change gut microbiota α-diversity and β-diversity (Figure 8a and Figure 8b). The 7-day antibiotic treatments changed rat gut microbiota α-diversity ($p=0.01$) (decreased about 50%) and β-diversity on SFA diet (Figure 9a and Figure 9b). The effect of antibiotic treatment on gut microbiota of rats on the PUFA diet was smaller than that seen on the SFA diet. There was a trend that 7-day antibiotic treatments decreased the gut microbiota richness 28.6% ($p=0.06$) and changed the β-diversity on the PUFA diet (Figure 9a and Figure 9b).

Antibiotic treatment on body weight, food intake and energy expenditure

During the 7-day antibiotic treatment, daily body weight and food intake were recorded. We used two-way repeated ANOVA to see the effect of dietary fat type and antibiotic or their interaction with energy balance. We found that rats on the SFA diet were heavier ($p=0.04$) than those on the PUFA diet (Figure 10). However, no significant difference was seen between antibiotic and vehicle treatment

on body weight gain during the 7 days (Figure 10). Regarding food intake, we found that antibiotic treatment significantly decreased total daily food intake per rat ($p=0.006$) and daily food intake per kg of body weight in rats irrespective of dietary fat types ($p<0.001$) (Figure 11 and Figure 12). Daily food intake per rat and daily food intake per kg of body weight were significantly lower with antibiotic treatment than vehicle on Day 1, Day 2, Day 3 and Day 5 ($p<0.05$, Figure 11 and $p<0.01$, Figure 12). However, there is no significant difference between SFA diet and PUFA diet on daily food intake per rat and food intake per kg of body weight during the 7 day of antibiotic or vehicle treatment (Figure 11 and Figure 12). Energy expenditure was measured at the end of the 7 day antibiotic or vehicle treatment (Table 6). No significant differences were seen among the treatments.

Gut β-defensins gene expression and serum protein expressions in response to dietary fat types and antibiotic treatment

To investigate whether the expression of gut β-defensins changed in response to different dietary fat types and antibiotic treatment, qPCR was used to measure gene expression of β-defensin-1, β-defensin-2, β-defensin-3, β-defensin-4 , and β-defensin-14 in ileum and colon which are the major colonization sites of gut microbiota. The gene expression of β-defensin-1 and β-defensin-14 was not detected in ileum and colon or the gene expression is too low to be detected by the machine (data not shown). The gene expression of β-defensin-2, β-defensin-3, β-defensin-4 were detectable but at very low levels. Rats on the PUFA diet increased β-defensin-2 ($p=0.009$), and β-defensin-3 gene expression ($p=0.012$) in ileum compared of rats on the SFA diet with and without antibiotic treatment (Figure 13). No significant differences between antibiotic and vehicle treatment were seen in β-defensins gene expression in both ileum and colon (Figure 13 and Figure 14). As it was found β-defensins are the new potential ligands to MC4R in brain, which might enter into blood and pass through blood brain barrier, serum total β-defensins and β-defensin 14 protein expression were measured by ELISA. No significant differences were noted due to diet or antibiotics (Figure 15 and Figure 16).

Gut hormone PYY and GLP-1 gene expression in response to dietary fat types and antibiotic treatment

PYY and GLP-1 are secreted by L cells which are classically thought to be located in the distal gut, predominantly the ileum and colon. Therefore, we investigated PYY (Pyy) and GLP-1 (Gcg) gene expression in ileum and colon by qPCR. We found that rats on the SFA diet increased ileum Pyy ($p=0.041$) and colon Gcg ($p=0.036$) compared of rats on the PUFA diet with and without antibiotic treatment (Figure 17). No significant differences were seen in PYY and GLP-1 gene expression between antibiotic and vehicle treatment in ileum and colon (Figure 17).

White adipose tissue leptin, adiponectin, PPARγ, and SREBP1 gene expression in response to dietary fat types and antibiotic treatment

Gene expression of leptin, adiponectin, PPARγ and SREBP1 in white adipose tissue (WAT) were determined by qPCR. We found that rats on the PUFA diet increased adiponectin ($p=0.019$) and decreased SREBP1 ($p=0.010$) gene expression in WAT with and without antibiotic treatment (Figure 18). Also, two way ANOVA analyses showed that there is an interaction between dietary fats and antibiotics treatment on leptin ($p=0.029$), adiponectin ($p=0.027$) and PPARγ ($p=0.013$) gene expression (Figure 18).

Serum peripheral energy balance signals in response to dietary fat types and antibiotics treatment

We measured serum ghrelin, pancreatic polypeptide, PYY, insulin, glucagon, leptin and adiponectin. No significant differences were seen among different treatments (Figure 19-Figure 26).

Discussion

Not only the quantity of ingested fats, but also the composition the fatty acids have an effect on obesity development. The results from our study supported the hypothesis that SFA diet is more obesogenic than PUFA diet due to the increased food intake. However, we failed to observe an association between dietary FA and gut microbiota on energy balance as the knockdown of gut microbiota decreased rats' food intake regardless of different dietary FA.

This study failed to find the potential mechanism for rats to increase food intake as no differences were seen in serum protein expression of ghrelin, pancreatic polypeptide, PYY, insulin, glucagon, leptin and adiponectin. However, the secretion and circulating levels of these peripheral signals are all influenced by recent energy intake and dietary macronutrient content as well as were time-dependent after a single meal [91, 92], it may be necessary to investigate these peripheral hormone levels at different time points after a meal during the study to get the true whole picture of peripheral signals in response to different dietary fat types. This is a limitation of our study to be improved in future.

In this study, there was no effect of dietary fat types on energy expenditure. Total energy expenditure was measured including obligatory energy expenditure which is required for performance of cellular and organ functions (also called as resting energy expenditure), physical activity and adaptive thermogenesis providing a comprehensive picture of the physiological effect of dietary fatty acid composition on energy expenditure. Thus, data suggests that there is no significant difference between SFA diet and PUFA diet on long term total energy expenditure.

36-day diet intervention did not change gut microbiota α-diversity and β-diversity. However, we cannot exclude the possible effect of dietary fatty acids on compositional pattern of gut microbiota.

It was reported that lard-based high fat diet increased the the abundance of the Mollicutes class of the Firmicutes phylum and reduced the abundance of Bacteroidetes. [83] But little has been reported on effects of different fatty acid diet on gut microbiota compositional pattern, which remains to be clarified in a future study.

Here in, qPCR analysis showed that PUFA diet increased ileum β-defensin 2 and β-defensin 3 gene expression with and without antibiotic treatment. B-defensins are components of endogenous antimicrobials which provide a first of line of defence against potentially pathogenic microbes at the body's mucosal frontiers [46]. However, as the secretion of β-defensins is also in response to bacteria in the intestine, the real relationship between β-defensins, dietary fatty acids and gut microbiota remains to be clarified in a future study.

A 7-day imipenem/cilastatin treatment successfully decreased gut microbiota. Interestingly, the effect of antibiotics treatment on gut microbiota of rats with the PUFA diet was smaller than those on the SFA diet. The 7-day antibiotic treatment significantly decreased rats' gut microbiota richness about 50% on the SFA diet but only decreased 28.6% on the PUFA diet. The result indicated that PUFA diet might protect gut microbiota from antibiotics due to the proinflammatory property of n-6 PUFA as our PUFA diet was safflower oil based high fat diet which was composed mainly of n-6 PUFA.

The blunting of gut microbiota significantly decreased the food intake regardless of different dietary fatty acids. Thus, based on this result, no association was evident between dietary fatty acids on food intake.

To discover potential mechanisms linking gut microbiota and food intake, we examined potential mediators. However, no differences between antibiotic and vehicle treatment were seen in serum protein expression of ghrelin, pancreatic polypeptide, PYY, insulin, glucagon, leptin and adiponectin. As previously discussed, only one study time point might not reflect the dynamics of these

peripheral hormones in response to gut microbiota change. Thus, how gut microbiota influenced food intake remains to be clarified in a future study.

Recent work showed that β-defensins can bind to the MC4R to control feeding and body weight [48]. Intracerebroventricular injection of human β-defensin 3 (HBD3) to male Wistar rats has an inhibitory effect on both the food intake and body weight gain with the potential mechanism of blocking AgRP [49]. Thus, we measured serum total β-defensins level and β-defensin 14 (homologues of HBD3) [93]. No differences were seen between PUFA diet and SFA diet or between antibiotic and vehicle treatment. Thus, β-defensins were not involved in mediating the different effects of SFA and PUFA diet or gut microbiota effects on energy balance regulation.

In summary, SFA are more obesogenic than PUFA due to increased food intake. Decreased gut microbiota decreased rats' food intake regardless of dietary fatty acid composition. There is no association between dietary fat types and gut microbiome on energy balance. The potential mechanisms for how rats gained more weight with increased food intake and how the ablation of gut micrflora by antibiotics resulted in decreased food intake remain to be clarified in a future study.

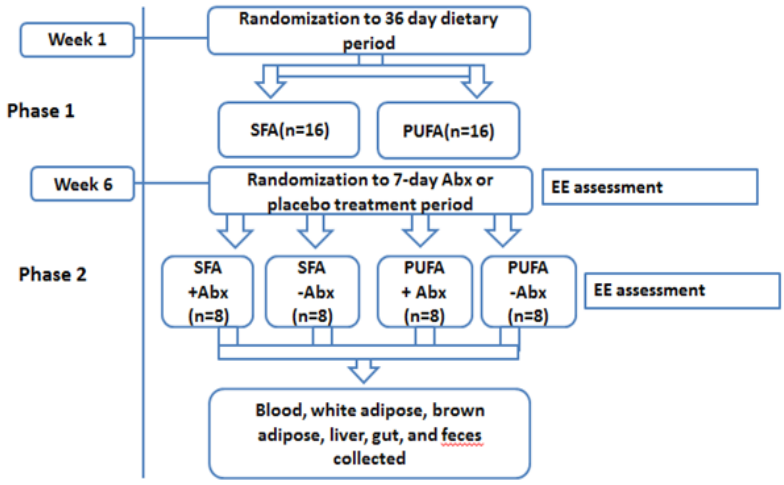

Figure 3 Study Design

32 7-week-old Sprague Dawley male rats were randomized to one of two diet groups (n=16 per group) as follows: 1) SFA-rich diet (45% energy from fat, predominant source butter and lard), and; 2) PUFA-rich diet (45% energy from fat, predominant source safflower oil). Animals were housed singly for accurate measurement of food intake and energy expenditure. Body weight and food intake were measured 3 times a week. Following 36-day of diet intervention, feces were collected and energy expenditure was measured. Then animals were further randomized to either antibiotic treatment or control group (n=8/ group) as follows: 1) Imipenem/cilastatin sodium at 50 mg/kg of body weight/day, or; 2) vehicle control (distilled water), for a treatment period of 7 days. Treatment and placebo were given via water. On the final day of 7-day antibiotic or vehicle treatment, feces were collected and energy expenditure was measured. Animals were euthanized via exsanguination under isoflurane and death confirmed via bilateral pneumothorax. Whole blood, white adipose tissue, brown adipose tissue, and liver were collected for further analysis.

Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; Abx, Antibiotics; EE: energy expenditure; +, with; -, without

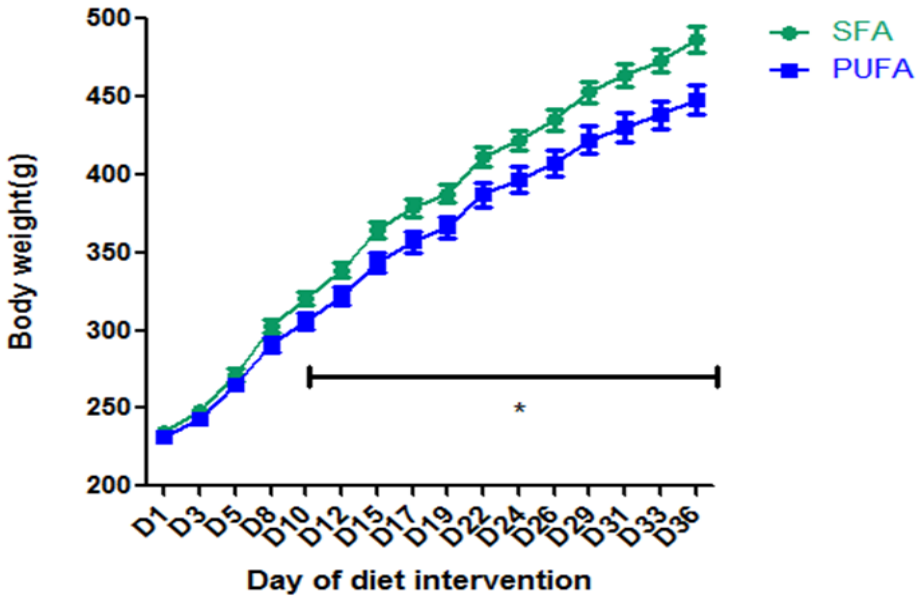

Figure 4 The effect of diet intervention on body weight:
During phase 1 of the study, 32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16). Rats' body weight was measured 3 times a week. Mean ± SEM is shown. One-way repeated ANOVA was used. Rats treated with SFA diet gained more body weight compared with those with PUFA diet ($p=0.016$). *T-test was used. The weight of rats treated with SFA diet is significantly higher than those with PUFA diet from D10 to D36 ($p<0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

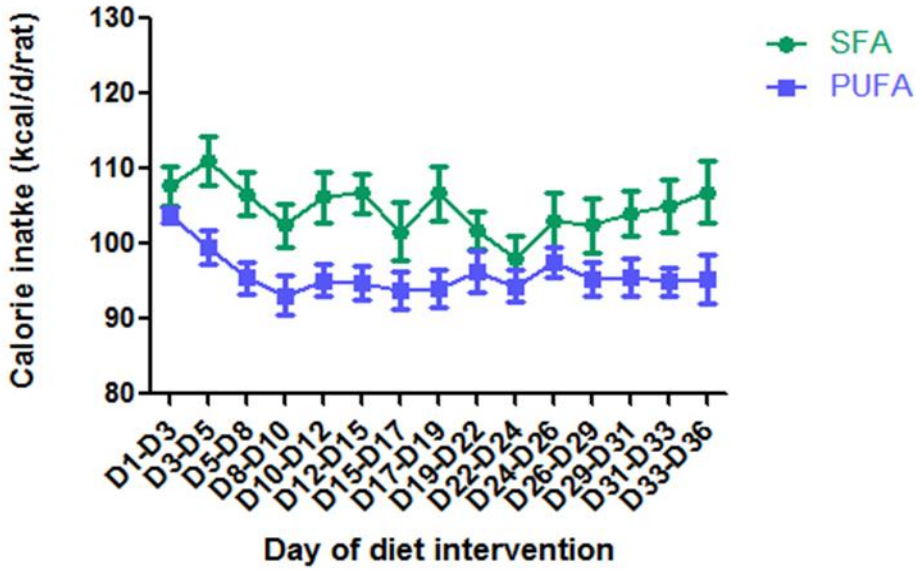

Figure 5 The effect of diet intervention on food intake:
During phase 1 of the study, 32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16). Rats' food intake was measured 3 times a week. Mean ± SEM is shown. One-way repeated ANOVA was used. Rats treated with SFA diet took in more food than those with PUFA diet. ($P<0.05$)
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

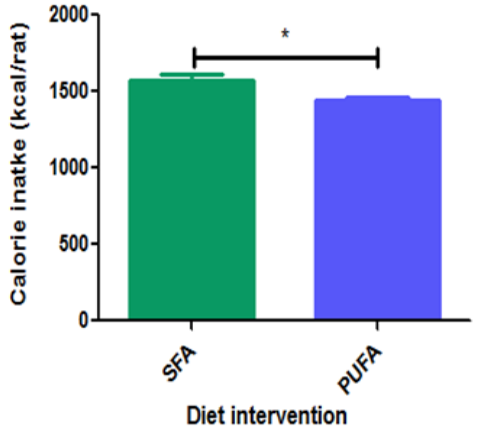

Figure 6 The effect of diet intervention on total food intake for 36 days:
During phase 1 of the study, 32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16). 36 days' of food intake was added up. Mean ± SEM is shown. Student's t test was used. Rats treated with SFA diet took in more food than those with PUFA diet (*P<0.05).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

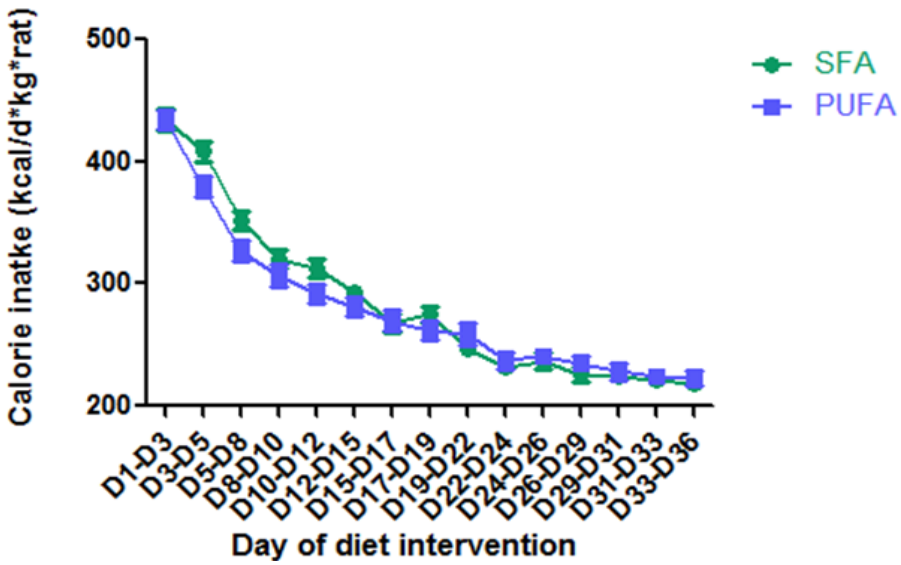

Figure 7 Effect of diet intervention on food intake normalized to body weight
During phase 1 of the study, 32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16). Rats' food intake and body weight were measured 3 times a week. Rats' food intake was normalized to their body weight. Mean ± SEM is shown. One-way repeated ANOVA was used. No significant differences were seen.
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

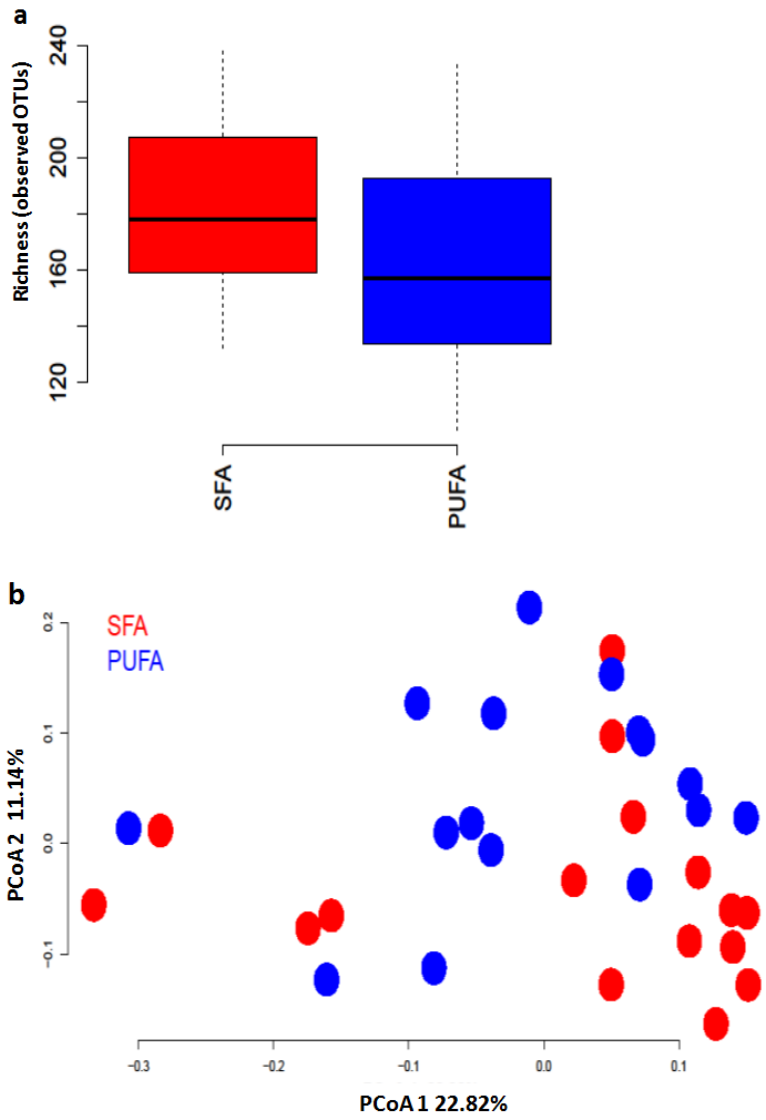

Figure 8 Effect of diet intervention on gut microbiome richness (α-diversity) and β-diversity
32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16) for 36 days. At the end of 36 day diet intervention, rats' feces were collected. DNA was extracted from feces. Sequencing of the 16S, V6 region was performed on Illumina sequencing system to determine the effect of difference diet on gut microbiome richness (α-diversity) and β-diversity. Figure 8a: Mann-Whitney U test was used to compare α-diversity between groups. Figure 8b: PCoA was used to compare β-diversity betw een groups. No significant differences were seen.
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

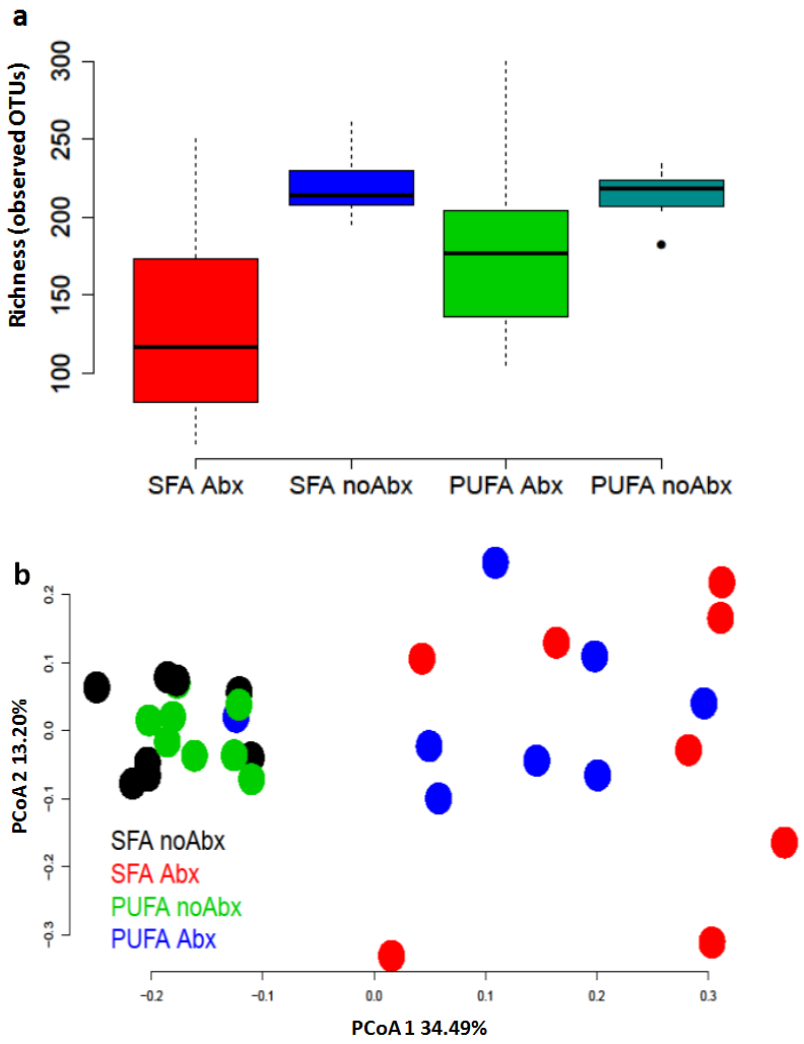

Figure 9 Effect of different diet intervention ± antibiotic treatment on rats' gut microbiome richness (α-diversity) and β-diversity

32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16) for 36 days. At the end of 36-day diet intervention, animals were further randomized to either antibiotic treatment or control group (n=8/ group) as follows: 1) Imipenem/cilastatin sodium at 50 mg/kg of body weight/day, or; 2) vehicle control (distilled water), for a treatment period of 7 days. At the end of 7 day antibiotic or vehicle treatment, rats' feces were collected. DNA was extracted from feces. Sequencing of the 16S, V6 region was performed on Illumina sequencing system to determine the effect of antibiotic treatment on rats' gut microbiome richness (α-diversity) and β-diversity on the SFA diet or the PUFA diet. Figure 9a: Mann-Whitney U test was used to compare α-diversity between groups. Figure 9b: PCoA was used to compare β-diversity between groups. Antibiotic treatment decreased α-diversity compared with vehicle treatment (p=0.01 on the SFA diet and p=0.06 on the PUFA diet). Abbreviation: SFA Abx, saturated fatty acids diet with antibiotic treatment; SFA noAbx, saturated fatty acids diet without antibiotic treatment; PUFA Abx, polyunsaturated fatty acids diet with antibiotic treatment; PUFA noAbx, polyunsaturated fatty acids diet without antibiotic treatment.

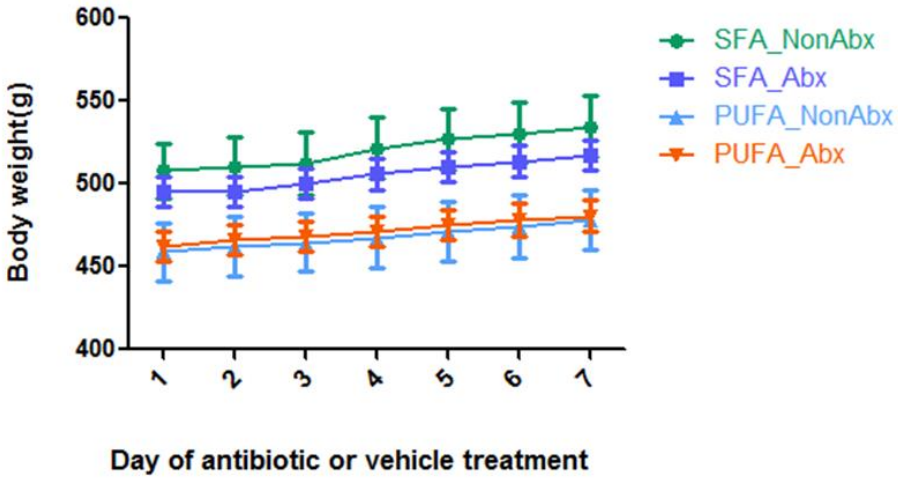

Figure 10 Effect of different diet intervention ± antibiotic treatment on rats' body weight
32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16) for 36 days. At the end of 36-day diet intervention, animals were further randomized to either antibiotic treatment or control group (n=8/ group) as follows: 1) Imipenem/cilastatin sodium at 50 mg/kg of body weight/day, or; 2) vehicle control (distilled water), for a treatment period of 7 days. Daily body weight was measured. Mean ± SEM is shown. Two-way repeated ANOVA was used. Rats on SFA-rich diet had higher body weight than those on the PUFA (p=0.006). No significant differences were seen between antibiotics and vehicle treatment on body weight during 7 days.
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics.

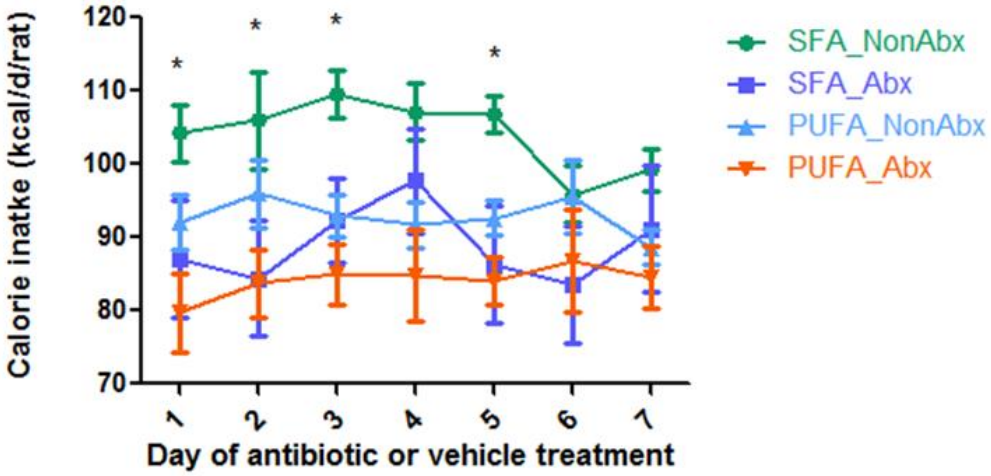

Figure 11 Effect of different diet intervention ± antibiotic treatment on rats' food intake
32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16) for 36 days. At the end of 36-day diet intervention, animals were further randomized to either antibiotic treatment or control group (n=8/ group) as follows: 1) Imipenem/cilastatin sodium at 50 mg/kg of body weight/day, or; 2) vehicle control (distilled water), for a treatment period of 7 days. Daily food intake was measured. Mean ± SEM is shown. Two-way repeated ANOVA was used. Antibiotic treatment had an effect on rats' food intake independently (P=0.006). There is no significant difference between SFA-rich diet and PUFA-rich diet on daily energy intake during the 7 days. *Antibiotic treatment significantly decreased daily food intake on D1, D2, D3, D5 irrespective of fat types (P<0.05).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics.

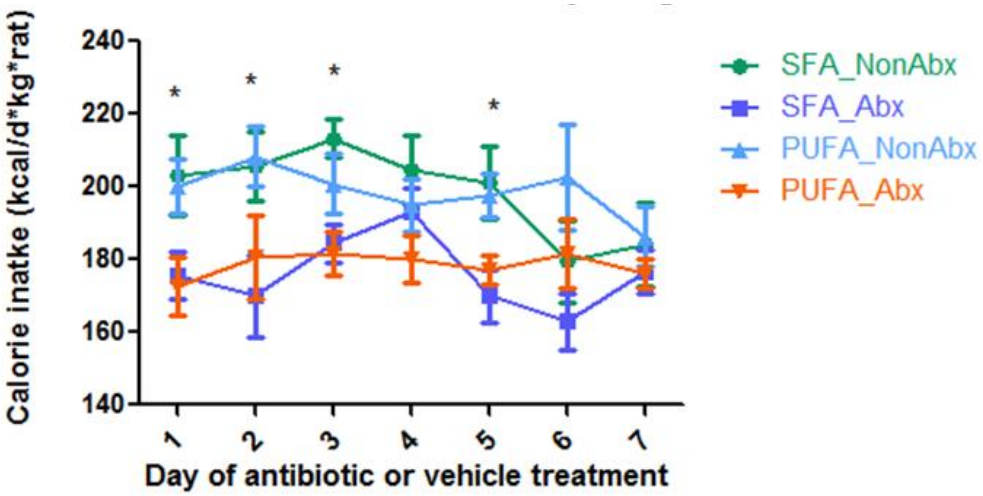

Figure 12 Effect of different diet intervention ± antibiotic treatment on rats' food intake normalized to body weight

32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16) for 36 days. At the end of 36-day diet intervention, animals were further randomized to either antibiotic treatment or control group (n=8/ group) as follows: 1) Imipenem/cilastatin sodium at 50 mg/kg of body weight/day, or; 2) vehicle control (distilled water), for a treatment period of 7 days. Daily rats' body weight and food intake were measured. Food intake was normalized to body weight. Mean ± SEM is shown. Two-way repeated ANOVA was used. Antibiotics or vehicle had an effect on rats' food intake normalized to body weight ($p=0.000$). No significant differences were seen between SFA diet and PUFA diet during the 7 days. *Antibiotic treatment significantly decreased food intake normalized to body weight on D1, D2, D3, D5 irrespective of fat types ($P<0.05$).

Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics.

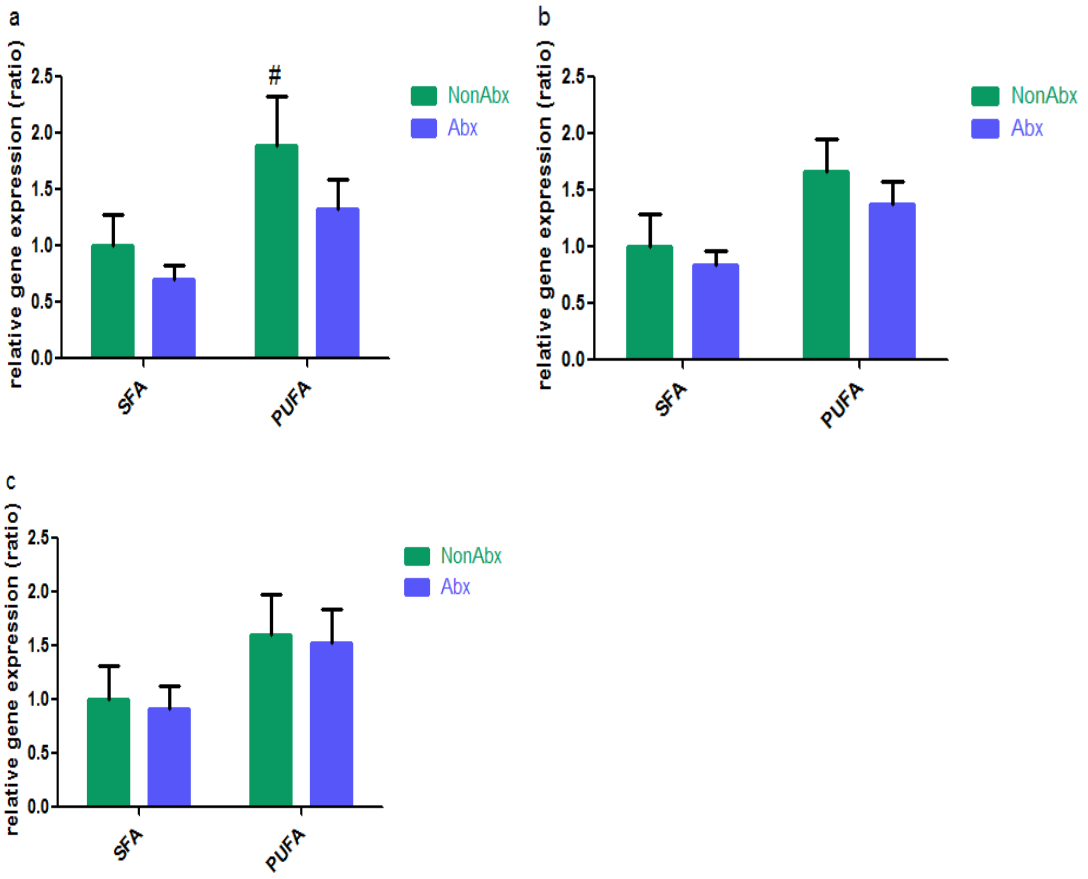

Figure 13 Effect of different diet intervention ± antibiotic treatment on ileum β-defensin-2 (a), β-defensin-3 (b) and β-defensin-4 (c) gene expression

At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and tissues were collected. RNA was extracted from the ileum. qPCR was used to assess β-defensins gene expression. Data were retrieved as CT values normalized to Gapdh and log2 transformed for subsequent statistical analysis. Final data were expressed as ratios between sample, and the average of the SFA_NonAbx group for each gene. Mean ± SEM is shown. Friedman test was used to compare ileum Defb2 expression among groups. Two-way ANOVA was used to compare ileum Defb3 and ileum Defb4 expression among groups. PUFA diet increased ileum Defb2 ($p=0.009$) and Defb3 ($p=0.012$) gene expression compared with SFA diet with and without antibiotic treatment. No significant differences were seen in ileum Defb2, Defb3, Defb4 gene expression between antibiotics and vehicle treatment ($P>0.05$). Tukey was used as post hoc analysis. #$p<0.05$ compared to SFA_Abx group.

Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics, Defb, β-defensin.

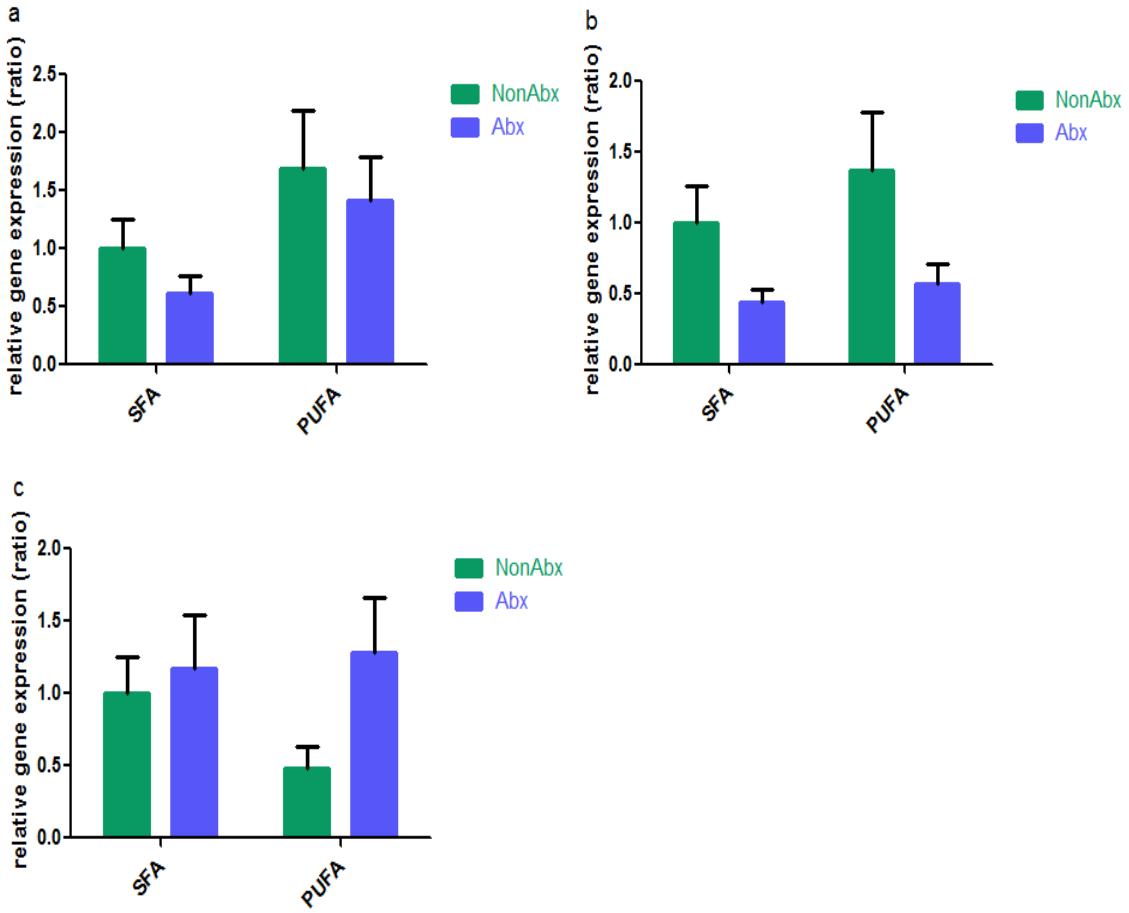

Figure 14 Effect of different diet intervention ± antibiotic treatment on colonβ-defensin-2 (a), β-defensin-3 (b) and β-defensin-4 (c) gene expression

At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and tissues were collected. RNA was extracted from the ileum. qPCR was used to assess β-defensins gene expression. Data were retrieved as CT values normalized to Gapdh and log2 transformed for subsequent statistical analysis. Final data were expressed as ratios between sample, and the average of the SFA_NonAbx group for each gene. Mean ± SEM is shown. Two-way ANOVA was used. No significant differences were seen due to diet intervention or antibiotic treatment. Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics, Defb, β-defensin.

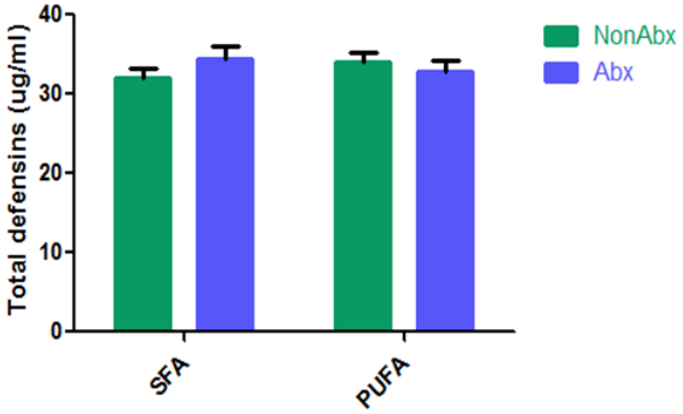

Figure 15 Effect of different diet intervention ± antibiotic treatment on serum total β-defensins protein expression

At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and blood was collected. ELISA was used to measure serum total β-defensins protein expression. Mean ± SEM is shown. Two-way ANOVA was used. No differences were seen due to diet intervention or antibiotic treatment.

Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics.

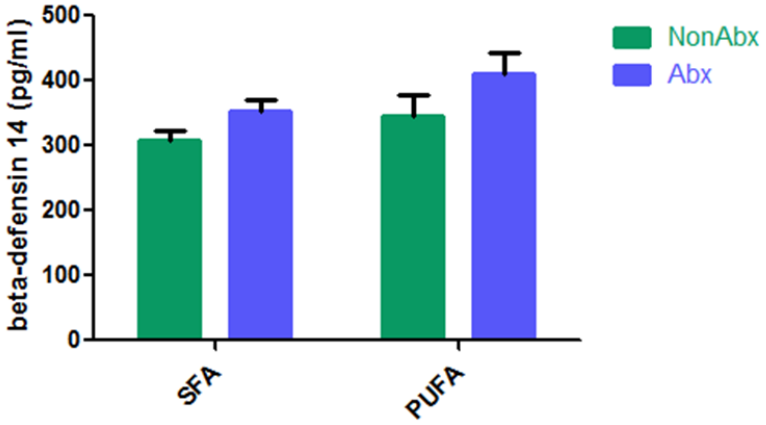

Figure 16 Effect of different diet intervention ± antibiotic treatment on serum β-defensin 14 protein expression

At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and blood was collected. ELISA was used to measure serum β-defensin 14 protein expression. Mean ± SEM is shown. Friedman test was used. No differences were seen due to diet intervention or antibiotic treatment.

Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics.

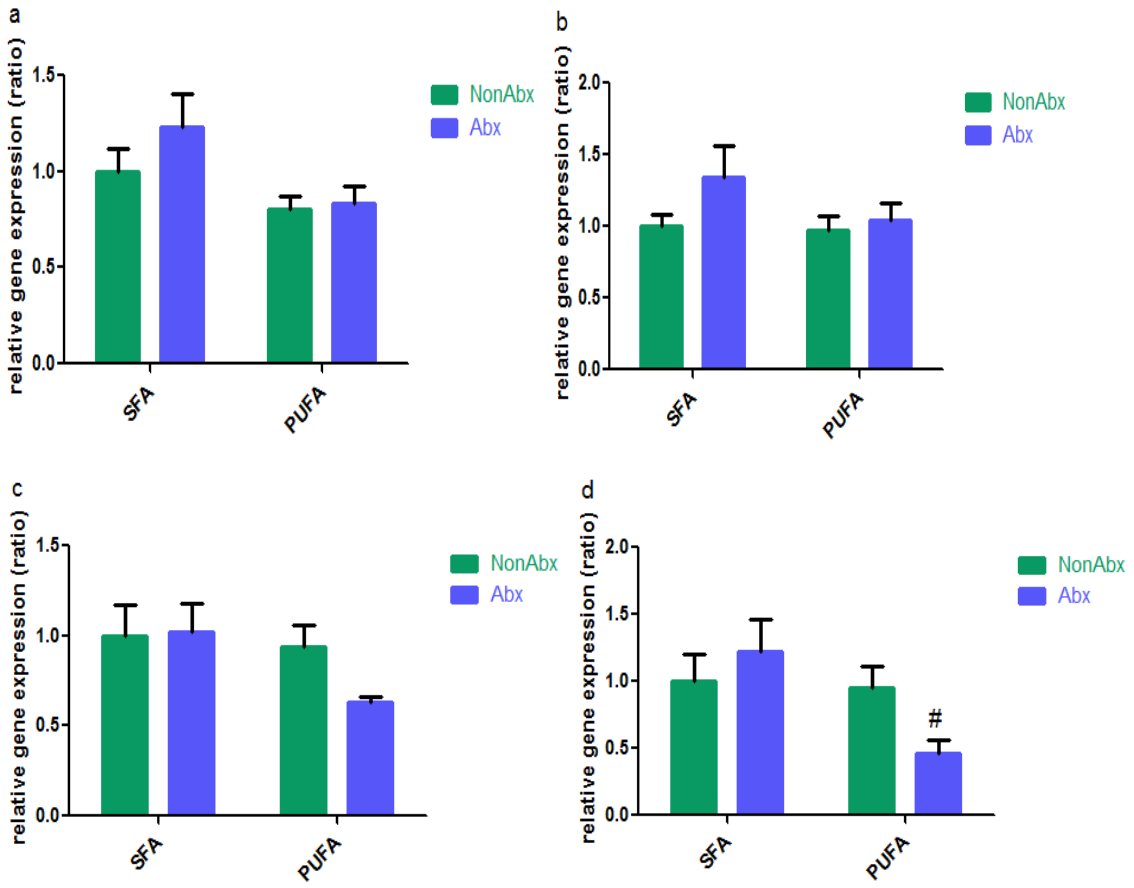

Figure 17 Effect of diet intervention ± Abx on ileum PYY (a), ileum GLP-1 (b), colon PYY (c) and colon GLP-1 (d) gene expression

At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and tissues were collected. RNA was extracted from the ileum and colon. qPCR was used to assess gut hormone PYY and GLP-1 gene expression. Data were retrieved as CT values normalized to Gapdh and log2 transformed for subsequent statistical analysis. Final data were expressed as ratios between sample, and the average of the SFA_NonAbx group for each gene. Friedman test was used to compare ileum Pyy and Gcg gene expression among groups. Two-way ANOVA was used to compare colon PYY and Gcg gene expression among groups. SFA diet increased ileum PYY ($p=0.041$) and colon GLP-1 ($p=0.036$) gene expression compared to PUFA diet with and without antibiotic treatment. No significant differences were seen in PYY and GLP-1 gene expression due to antibiotic treatment in both ileum and colon tissues ($p>0.05$). Tukey was used as post hoc analysis. # $p<0.05$ compared to SFA_Abx group.

Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics, Pyy, gene name for peptide YY (PYY); Gcg, gene name for glucagon-like peptide-1 (GLP-1).

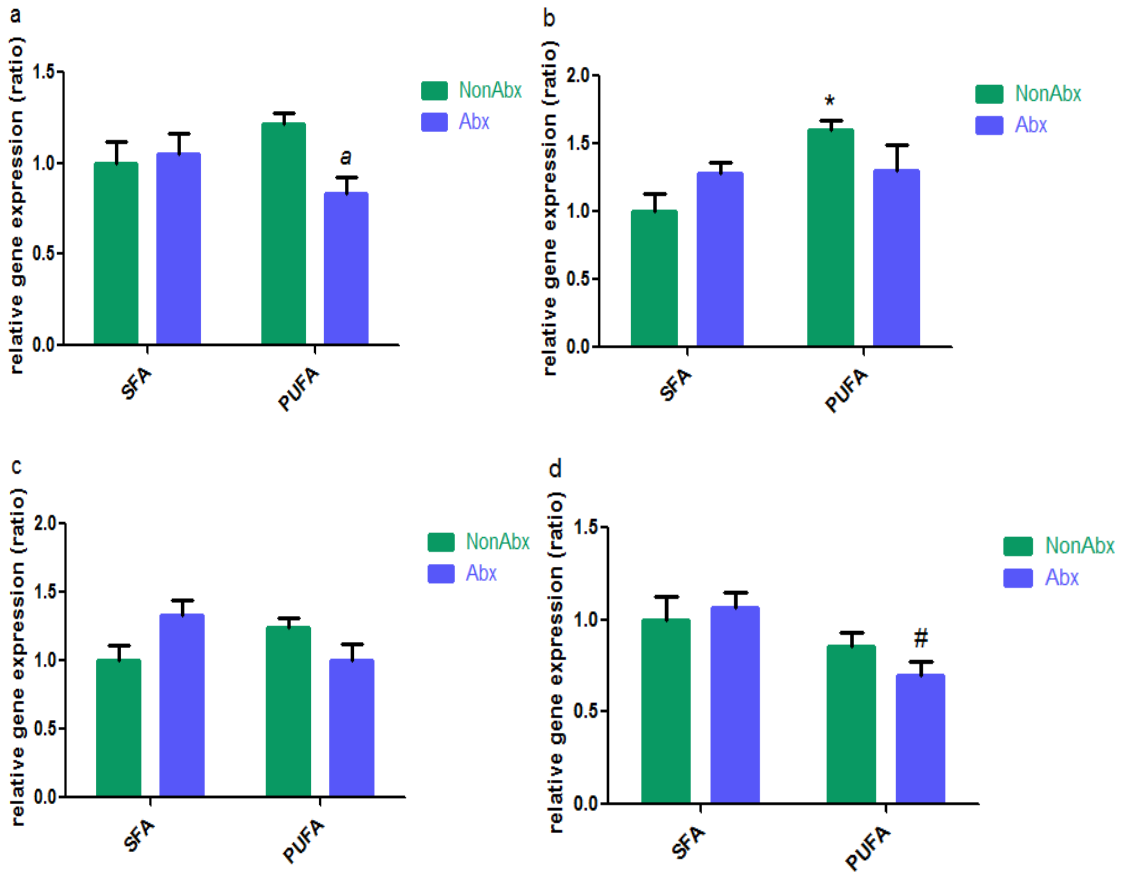

Figure 18 Effect of diet intervention ± Abx on leptin (a), adiponectin (b), PPARγ (c) and SREBP1 (d) gene expression

At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and tissues were collected. RNA was extracted from the white adipose tissue. qPCR was used to assess white adipose tissue Lep (leptin), Adipoq (adiponectin), Pparg (PPARγ) and Srebf1 (SREBP1) gene expression. Data were retrieved as CT values normalized to Gapdh and log2 transformed for subsequent statistical analysis. Final data were expressed as ratios between sample, and the average of the SFA_NonAbx group for each gene. Mean ± SEM is shown. Two-way ANOVA was used. SFA diet decreased adiponectin ($p=0.019$) and increased SREBP1 (0.010) gene expression compared to PUFA diet with and without antibiotic treatment. Diet intervention and antibiotics treatment had an interaction on leptin ($p=0.029$), adiponectin ($p=0.027$) and PPARγ ($p=0.013$) gene expression. No significant differences were seen on WAT leptin, adiponectin, PPARγ and SREBP1c gene expression due to antibiotic treatment ($P>0.05$). Tukey was used as post hoc analysis. * $p<0.05$ compared to SFA_NonAbx group, # $p<0.05$ compared to SFA_Abx group, a $p<0.05$ compared to PUFA_NonAbx group.

Abbreviation: WAT, white adipose tissue; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics; Lep, gene name for leptin; Adipoq, gene name for adiponectin; Pparg, gene name for PPARγ; Srebf1, gene name for SREBP1

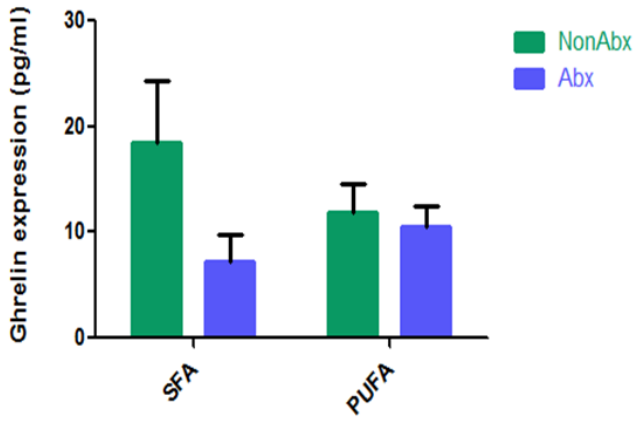

Figure 19 Effect of diet intervention ± Abx on serum ghrelin expression
At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and blood was collected. Ghrelin was analyzed using bead-based multiplex assay. Mean ± SEM is shown. Friedman test was used. No significant differences were seen in there serum peripheral energy balance signals protein expression due to diet intervention and antibiotic treatment ($p>0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics

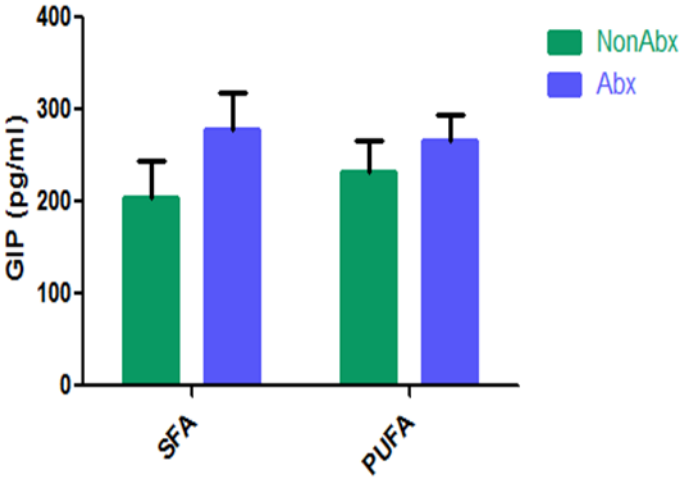

Figure 20 Effect of diet intervention ± Abx on serum GIP expression
At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and blood was collected. GIP was analyzed using bead-based multiplex assay. Mean ± SEM is shown. Two way ANOVA was used. No significant differences were seen in there serum peripheral energy balance signals protein expression due to diet intervention and antibiotic treatment ($p>0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics; GIP, glucose-dependent insulinotropic polypeptide

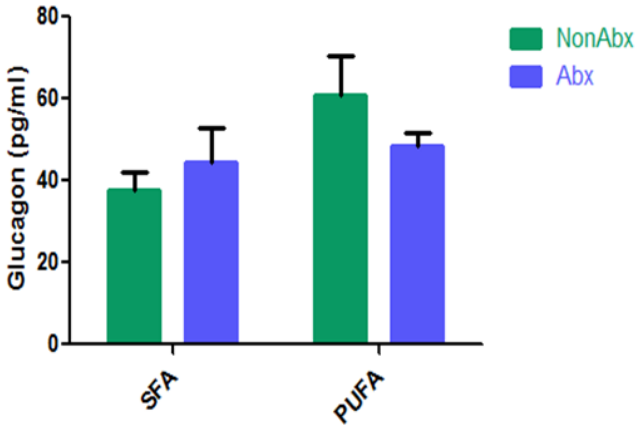

Figure 21 Effect of diet intervention ± Abx on serum glucagon expression
At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and blood was collected. Glucagon was analyzed using bead-based multiplex assay. Mean ± SEM is shown. Two way ANOVA was used. No significant differences were seen in there serum peripheral energy balance signals protein expression due to diet intervention and antibiotic treatment ($p>0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics

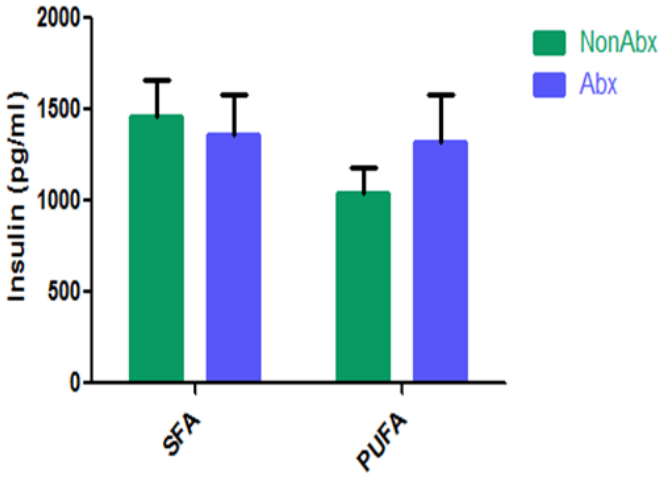

Figure 22 Effect of diet intervention ± Abx on serum insulin expression
At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and blood was collected. Insulin was analyzed using bead-based multiplex assay. Mean ± SEM is shown. Two way ANOVA was used. No significant differences were seen in there serum peripheral energy balance signals protein expression due to diet intervention and antibiotic treatment ($p>0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics

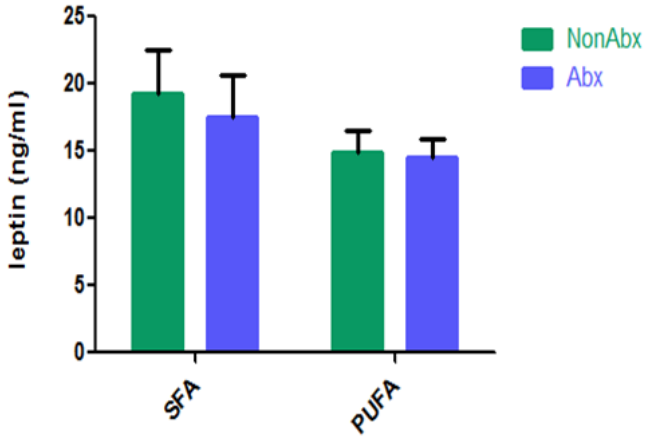

Figure 23 Effect of diet intervention ± Abx on serum leptin expression
At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and blood was collected. Leptin was analyzed using bead-based multiplex assay. Mean ± SEM is shown. Two way ANOVA was used. No significant differences were seen in there serum peripheral energy balance signals protein expression due to diet intervention and antibiotic treatment ($p>0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics

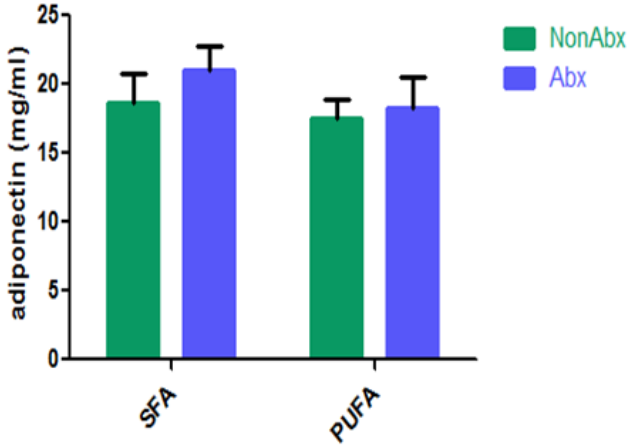

Figure 24 Effect of diet intervention ± Abx on serum adiponectin expression
At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and blood was collected. Adiponectin was measured by ELISA. Mean ± SEM is shown. Two way ANOVA was used. No significant differences were seen in there serum peripheral energy balance signals protein expression due to diet intervention and antibiotic treatment ($p>0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics

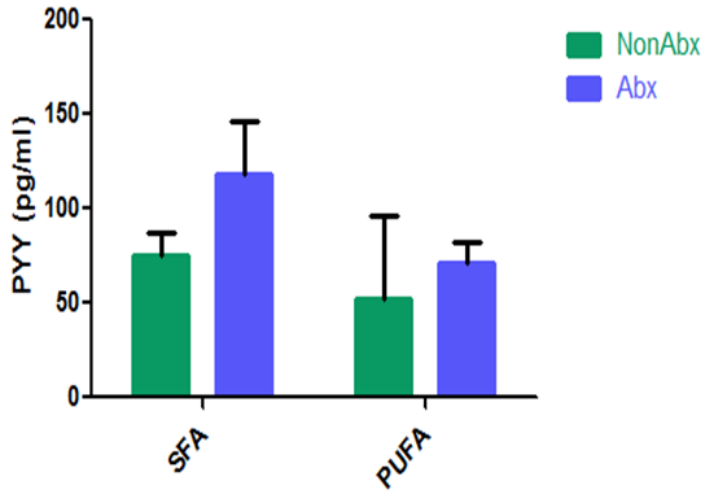

Figure 25 Effect of diet intervention ± Abx on serum PYY expression
At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and blood was collected. PYY was analyzed using bead-based multiplex assay. Mean ± SEM is shown. Two way ANOVA was used. No significant differences were seen in there serum peripheral energy balance signals protein expression due to diet intervention and antibiotic treatment ($p>0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics; PYY, peptide YY

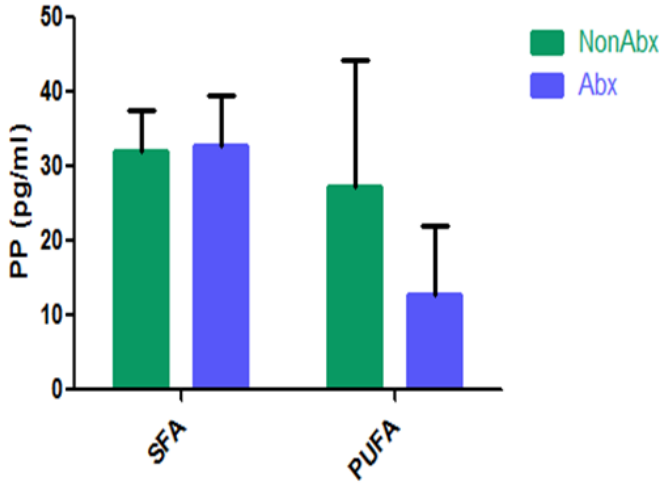

Figure 26 Effect of diet intervention ± Abx on serum PP expression
At the end of 7-day antibiotic or vehicle treatment, rats were euthanized and blood was collected. Mean ± SEM is shown. Friedman test was used. PP was analyzed using bead-based multiplex assay. Mean ± SEM is shown. Two way ANOVA was used. No significant differences were seen in there serum peripheral energy balance signals protein expression due to diet intervention and antibiotic treatment ($p>0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids, Abx, antibiotics; GIP, glucose-dependent insulinotropic polypeptide; PYY, peptide YY; PP, pancreatic polypeptide

Table 2: Macronutrients Composition of the SFA and PUFA Diets

Diets	SFA diet		PUFA diet	
%	gm	kcal	gm	kcal
Protein	24	20	24	20
Carbohydrate	41	35	41	35
Fat	24	45	24	45
Total		100		100
Kcal/gm	4.7		4.7	

Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids

Table 3: Detailed nutrients ingredients of SFA and PUFA diets

Ingredient	gm	kcal	gm	kcal
Casein	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	72.8	291	72.8	291
Maltodextrin 10	100	400	100	400
Surcrose	172.8	691	172.8	691
Cellulose, BW 200	50	0	50	0
Soybean oil	25	225	25	225
Safflower Oil	0	0	177.5	1598
Butter	88.75	799	0	0
Lard	88.75	799	0	0
Mineral Mix S 10025	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H_2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
Total	858.15	4057	858.15	4057

Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids

Table 4 Energy Expenditure Measurement Flowchart

	Acclimated cage	Metabolic cage
Prior to D1	Group 1	No animals
D1 EE measurement	Group 2	Group 1
D2 EE measurement	Group 3	Group 2
D3 EE measurement	Group 4	Group 3
D4 EE measurement	No animals	Group 4

Prior to the first day of energy expenditure measurement, Rats in Group 1 entered into acclimated cage. On the first day, rats in Group 1 were moved out from acclimated cage and entered into metabolic cage, and rats in Group 2 entered into acclimated cage. On the second, rats in Group 1 were moved out from metabolic cage. Rats in Group 2 were moved out from acclimated cage and entered into metabolic cage. Rats in Group 3 entered into acclimated cage. On the third day, rats in Group 3 were moved out from metabolic cage. Rats in Group 3 were moved out from acclimated cage and entered into metabolic cage. Rats in Group 4 entered into acclimated cage. On the final day, rats in Group 4 were moved out from acclimated cage and entered into metabolic cage.
Abbreviation: EE, energy expenditure.

Table 5 Effect of Diet Intervention on Energy Expenditure (Mean ± SEM)

	SFA group	PUFA group
VO_2/BW(ml/h/kg)	2298.16±40.88	2371.99±60.14
VCO_2/BW(ml/h/kg)	1660.84±29.37	1720.025±43.68
RER	0.72±0.0046	0.72±0.0072
H(kcal/h/kg)	10.89±0.19	11.25±0.28

During phase 1 of the study, 32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16). After 36-day diet intervention, rats' energy expenditure was measured by metabolic cage for 24 hours. The difference between the oxygen and carbon dioxide concentrations in the cages were compared with a reference environment, and used to calculate oxygen consumption volume, carbon dioxide production volume, respiratory exchange ratio and heat. 21 hours' data was collected. Mean ± SEM is shown. Student's t test was used. No significant differences were seen in energy expenditure due to diet intervention ($p>0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; BW, body weight; VO2, oxygen consumption volume; VCO2, carbon dioxide production volume; RER, respiratory exchange ratio; H, heat production

Table 6 Effect of Diet Intervention ± Abx on Energy Expenditure (Mean±SEM)

	SFA_NonAbx	SFA_Abx	PUFA_NonAbx	PUFA_Abx
VO2/BW (ml/h/kg)	2176.76 ± 31.34	2144.91 ± 46.58	2269.80 ± 55.31	2224.70 ± 48.44
VCO2/BW (ml/h/kg)	1548.16 ± 23.15	1503.36 ± 29.21	1602.26 ± 37.62	1545.88 ± 42.61
RER	0.71 ± 0.0080	0.70 ± 0.0045	0.70 ± 0.0051	0.69 ± 0.0051
H (kcal/h/kg)	10.29 ± 0.14	10.12 ± 0.21	10.72 ± 0.26	10.48 ± 0.24

32 7-week-old Sprague Dawley male rats were randomized to two diet groups: SFA diet group (n=16) and PUFA diet group (n=16). At the end of 36-day diet intervention, animals were further randomized to either antibiotic treatment or control group (n=8/ group) as follows: 1) Imipenem/cilastatin sodium at 50 mg/kg of body weight/day, or; 2) vehicle control (distilled water), for a treatment period of 7 days. At the end of 7 day antibiotic or vehicle treatment, rats' energy expenditure was measured by metabolic cage for 24 hours. The difference between the oxygen and carbon dioxide concentrations in the cages were compared with a reference environment, and used to calculate oxygen consumption volume, carbon dioxide production volume, respiratory exchange ratio and heat. 21 hours' data was collected. Mean ± SEM is shown. Two way ANOVA was used. No significants differences were seen in energy expenditure due to diet intervention or antibiotic treatment ($p>0.05$).
Abbreviation: SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids; Abx, antibiotics; BW, body weight; VO2, oxygen consumption volume; VCO2, carbon dioxide production volume; RER, respiratory exchange ratio; H, heat production

CHAPTER 3: DISCUSSION

Not only the quantity of ingested fats, but also the composition the fatty acids have an effect on obesity development. In both human and animal studies, it showed that SFA are more obesigenic than are PUFA due to their different effects on appetite control, energy expenditure, and oxidation rate. Recent work also has identified a role for gut microbiota in the onset of obesity. In addition, a high-fat-fed animal displays a significant shift in both bacterial and metagenomic profiles as compared to an animal on a normal, chow diet [83]. However, a direct connection between a specific type of fats and gut microbes contributing to obesity should be further investigated.

In this study, we were interested in the effect of modulation of gut microbiome by antibiotics on food intake and energy expenditure in Sprague Dawley rats treated with 45% high fat diet containing different dietary fat types. We confirmed that SFA are more obesogenic than PUFA with increased food intake. However, no association between dietary fat types and gut microbiome was observed on energy balance. In this chapter, we would discuss the pertinent and research questions, as well as the limitations in the field of study.

The effect of different dietary fat types on body weight, food intake and energy expenditure

Recent evidence suggests that the overall amount of fat intake is not the only factor determining weight gain and fat store. Fat quality is important as well [72, 73, 76]. In this study, we investigated the effect of a high fat diet (45% calorie from fat) containing different fatty acids on rats' body weight, food intake and energy expenditure. The SFA diet is lard and butter based high fat diet

which is main source of dietary SFA in human diet. The PUFA diet is safflower oil based diet which is mainly composed of n-6 PUFA. Therefore, our study was to compare SFA with n-6 PUFA on the whole body energy balance modulation.

We found that rats on the SFA diet had a significantly higher weight gain than did those on the PUFA diet starting from Day 10 to the end of the study (Figure 4). This result is accordance with others' reported results [94]. The daily food intake of rats on the SFA diet was higher than in those on the PUFA diet during the 36-day diet intervention (Figure 5). No significant differences were seen in energy expenditure due to different diet intervention (Table 5). Based on these results, we conclude that the rats on the SFA diet had a higher weight gain mainly due to the increased food intake compared with those on the PUFA diet.

However, it is interesting that when food intake was normalized to rats' body weight, no significant differences were seen between the two groups, although during the first 10 days, there was a slight increase in food intake per kg body weight for rats on the SFA diet (Figure 7). Body weight is an important determinant of energy requirement as energy requirement is defined by the amount of food energy needed to balance energy expenditure in order to maintain body size, body composition and a level of necessary and desirable physical activity consistent with long-term good health. Based on the result that there were no differences on food intake per kg body weight between the two groups, we speculate that there is no food preference of rats between the SFA diet and the PUFA diet. The SFA diet didn't cause rats' overeating during the 36-day diet intervention.

If the rats took in the food amount according to the body weight (that is to say, their energy requirement), then the reasons that the SFA diet caused rats to gain more weight than did the PUFA diet might be due to the following two assumptions.

First, we still can see that during the first 10 days of diet intervention, there was a slight increase in food intake per body weight (Figure 7). It might be that the first 10 days' overeating determined the rats treated with the SFA diet gained more weight than those on the PUFA diet. This assumption can be confirmed that rats treated with SFA diet were significantly heavier than those with PUFA diet starting at day 10 (Figure 4). Second, energy absorption efficiency might be different between the SFA diet and the PUFA diet. In fact, fatty-acid chain length and number of double bonds can influence fat absorption [95]. SFA might have higher absorption efficiency than PUFA. However, on the contrary to our assumption, Nicole de Wit et al showed that compared with the diet rich in SFA, the net energy absorption was higher for PUFA-rich diet in their study.[94]

Therefore, we speculate the first 10 days after starting high fat diet containing different fatty acids is the key period causing the weight difference between the two groups.

Our study results showed no effect of dietary fat types on energy expenditure (Table 5). However, Mercer et al showed in their study that compared with a diet rich in SFA, a diet rich in PUFA results in increased energy expenditure with preferential stimulation of the thermogenic activity of brown adipose tissue [77]. We must point out here that although PUFA has more potential to stimulate brown adipose tissue, mice fed with the PUFA diet had less brown adipose tissue than did those fed with the SFA diet in their study [77]. That is to say, the total capacity to stimulate energy expenditure by brown adipose tissue might be equal between a SFA diet and a PUFA diet. Our results did not contradict their findings.

Casas-Agustench et al proposed that a PUFA diet increases energy expenditure due to that a higher diet-induced thermogenesis (DIT) than caused by a SFA diet [72]. In fact, in their study, diet induced thermogenesis reflected an acute effect of dietary fatty acids on energy expenditure 5 hours' after the meal [72]. In our studies, we measured total energy expenditure including obligatory energy

expenditure which is required for performance of cellular and organ functions (also called as resting energy expenditure), physical activity and adaptive thermogenesis [96] after a 36-day diet intervention. This involves a long term metabolic adaptation to dietary fatty acids, providing a more comprehensive picture of the physiological effect of dietary fatty acid composition on energy expenditure. Actually, our results for energy expenditure are supported by Wongsuthavas et al as they also proved no enhanced energy expenditure on a PUFA diet compared with a SFA diet in in broiler chickens [97].

Peripheral signals of energy balance in response to dietary fat type

Hunger and satiety endocrine hormones play a vital role in controlling energy balance as they act in many different sensing and signaling pathways. While chain length of a dietary fatty acid has been shown to have a significant effect on satiety hormone release [98, 99], less is known about the hormonal responses based on the degree of saturation in fatty acids. In this study, we investigated PYY, GLP-1 gene expression in intestine and leptin, adiponectin gene expression in white adipose tissue.

We found that compared with the PUFA diet, the SFA diet increased ileum PYY and colon GLP-1 gene expression (Figure 17). PYY and GLP-1 are often considered as co-stored or co-released by L cells in distal gut [41]. L-cells exhibit a polarized morphology with an apical surface contacting the gut lumen and a basolateral membrane in close proximity to the circulatory system. This so-called “open-type” morphology should enable them to sense dietary nutrients and non-nutrient substances, present in the intestinal lumen [100]. As lipid reaches the distal portion of the gut, GLP-1 and PYY are secreted in response to increasing lipid calories [101, 102]. The gene expression PYY and GLP-1 was consistent with the food intake as we found that rats fed with the SFA diet increased food intake compared with the PUFA diet (Figure 5).

Our results also showed that compared with the SFA diet, the gene expression of WAT adiponectin was increased in rats on the PUFA diet with and without antibiotic treatment (Figure 18).

The secretion of adiponectin is negatively related to body adipocytes as circulating adiponectin concentrations are reduced in obese animals [103] and humans [104]. The physiological effect of adiponectin on food intake and energy expenditure is controversial. It is found that adiponectin could increases hypothalamic AMPK phosphorylation to stimulate food intake and suppress energy expenditure by binding to adiponectin receptors in hypothalamus [26]. Interestingly, it is also found that intracerebroventricular administration of adiponectin decreased body weight with increasing energy expenditure suggesting a negative regulating pathway for adiponectin effect on energy balance [27].

Treatment with PPAR γ agonists resulted in increased adiponectin levels [105]. Fatty acids from the n−6 and n−3 families of fatty acids and their respective eicosanoid products are the physiologic ligands for PPAR γ [106]. We measured PPAR γ gene expression in WAT and found no difference in gene expression between the two diets (Figure 18). We speculate that the PUFA diet up regulated adiponectin gene expression independent from PPAR γ .

SREBP1 positively regulates adiponectin gene transcription in vitro [107]. In our results, we found that compared with the SFA diet, the PUFA diet decreased Srebf1 expression with and without antibiotic treatment (Figure 18). This result is consistent with previous findings [108]. Based on our results, we can't speculate on any relationship between SREBP1 and adiponectin in vivo. In fact, physiological effect between SREBP1 and adiponectin is opposite. Overexpression of SREBP1c in white adipose tissue is associated with insulin resistance and diabetes [109] while adiponectin has been shown to have beneficial effects on insulin sensitivity [24]. Thus, the relationship between SREBP1 and adiponectin remains to be elucidated in a future study. One study showed that the inhibition of SREBP-1c by pioglitazone is adiponectin dependent [110] suggests that adiponectin might negatively regulate SREBP-1c.

Also, we measured serum protein expression of ghrelin, pancreatic polypeptide, PYY, insulin, glucagon, leptin and adiponectin (Figure 19-Figure 26). No differences were seen in serum protein expression of ghrelin, pancreatic polypeptide, PYY, insulin, glucagon, leptin and adiponectin.

One limitation of our study is that we only measured serum protein level of these peripheral signals for one time point. The secretion and circulating levels of these peripheral signals are all influenced by recent energy intake and dietary macronutrient content, although circulating insulin and leptin concentrations are mainly proportional to body fat content [91]. Also, the circulating levels of many short term regulators of energy balance are time-dependent after a single meal. For example, post-prandially, the circulating PYY levels rise rapidly to a plateau after 1–2 h and remain elevated for up to 6 h [92].

One time point can't reflect the whole picture of peripheral signals in response to different dietary fat types. Therefore, our data do not conclusively show that there is no effect of dietary fat type on peripheral signals of energy balance.

The effect of dietary fat types on gut microbiota

In this study, we were interested the effect of dietary fatty acids on gut microbiota richness and β-diversity. We collected feces from rats and ran 16S rRNA of fecal microbiota analysis at the end of phase 1. We found that 36-day different fatty acid diet intervention did not change gut microbiota richness and β-diversity (Figure 8). However, we cannot exclude the possible effect of dietary fatty acids on the compositional pattern of gut microbiota. It was reported that lard-based high fat diet increased the abundance of the Mollicutes class of the Firmicutes phylum and reduced the abundance of Bacteroidetes [83]. But little has been reported of different fatty acids diet on gut microbiota compositional pattern, which remains to be clarified in a future study.

In our study, qPCR analysis showed the PUFA diet increased ileum β-defensin 2 and β-defensin 3 gene expression with and without antibiotic treatment (Figure 13). B-defensins are components of endogenous antimicrobials which provide a first of line of defence against potentially pathogenic microbes at the body's mucosal frontiers [46]. Thus, it is possible that the increased β-defensins associated with the PUFA diet is involved in modulate gut microbiota. However, as the secretion of β-defensins is also in response to bacteria in the intestine, the real relationship between β-defensins, dietary fatty acids and gut microbiota remains to be clarified in a future study.

Recent work showed that β-defensins can bind to the MC4R to control feeding and body weight [48]. Intracerebroventricular injection of human β-defensin 3 to male Wistar rats has an inhibitory effect on both the food intake and body weight gain with the potential mechanism of blocking AgRP [49]. Thus, we measured serum total β-defensins level and β-defensin 14 (homologues of HBD3) [93]. No differences were seen between PUFA diet and SFA diet. Thus, on the basis of our study result, we speculate that different gene expression induced by different fatty acid diet didn't have an effect on the circulating levels of β-defensins and these were not involved in the different effects of SFA diet and PUFA diet on energy balance.

The effect of antibiotics on gut microbiota

Antibiotics are commonly used to modulate gut microbiota, providing an approach to investigate the causative role of the whole gut microbiota in the body energy balance in addition to germ-free mice [111]. In our study, we choose imipenem/cilastatin to modulate gut microtioa. Imipenem is usually administered intravenously in combination with cilastatin [112]. In vitro it has been demonstrated that imipenem has an extremely wide spectrum of antibacterial activity against Gram-negative and Gram-positive aerobic and anaerobic bacteria, even against many multiresistant strains of bacteria [112]. On the basis of its poor systemic bioavailability, imipenem has a direct impact on the gut

microbiota, instead of host metabolic pathways [113]. Previously, it was reported that Wistar rats treated with imipenem/cilastatin 50 mg/kg body weight for 4 days had altered metabolite patterns in urine and feces [113]. In our pilot study, we compared 4-day antibiotic treatment and 7-day antibiotic treatment with two different methods: oral gavage or given in water. The results showed that 7-day antibiotic treatment given in water most successfully decreased gut microbiota in Sprague Dawley rats. Therefore, we chose to treat rats with 7-day imipenem/cilastatin given in water. As we expected, the results of 16s RNA gene sequencing showed that 7-day imipenem/cilastatin successfully knocked down gut microbiota (Figure 9).

However, the effect of antibiotic treatment on gut microbiota of rats with PUFA diet was smaller than with the SFA diet. The 7-day antibiotic treatment significantly decreased gut microbiota richness about 50% on the SFA diet but only decreased 28.6% on the PUFA diet (Figure 9). The result indicated that the PUFA diet might protect gut microbiota from antibiotics due to the proinflammatory property of n-6 PUFA as our PUFA diet was safflower oil based high fat diet which was composed mainly of n-6 PUFA.

The effect of knockdown of gut microbiota on body weight, food intake and energy expenditure

We were interested in the effect of knockdown of gut microbiota on energy balance. We recorded rats' daily body weight and food intake during 7-day antibiotic or vehicle treatment. It showed that the knockdown of gut microbiota significantly decreased the food intake regardless of different dietary fatty acids (Figure 11 and Figure 12). Thus, based on this result, we speculated no association between dietary fatty acids and gut microbiota on food intake.

The result that decreasing gut microbiota decreased food intake in our study is consistent with many other studies [52, 114]. The effect of gut microbiota on food intake might be mediated by gut

hormones. Thus, we measured PYY and GLP-1 gene expression in ileum and colon, where the major colonization sites of gut microbiota are. However, we didn't detect any difference between antibiotic or vehicle treatment on expression of these two genes in the two parts of the intestine. Also, no differences were seen in serum protein expression of ghrelin, PP, PYY, insulin, glucagon, leptin and adiponectin between antibiotic or vehicle treatment. As the secretion and circulating levels of these peripheral signals are all influenced by recent energy intake and dietary macronutrient content as well as time-dependent after a single meal [91, 92], we cannot get the whole picture of these peripheral signals in response to antibiotic or vehicle treatment. This is a limitation of our study. In a future study, we will focus on these peripheral hormones levels at different time point after a meal during the study to investigate the effect of gut microbiota on peripheral signals to control food intake.

Also, we compared gene expression of β-defensins in ileum and colon as well as protein expression of total β-defensins and β-defensin 14 in serum between antibiotic and vehicle treatment. No significant differences were seen (Figure 15 and Figure 16). We speculate that β-defensins were not involved in the effect of gut microbiota on energy balance. In fact, β-defensins gene expression is very low in intestine [115]. Thus, the effect of gut microbiota on β-defensins might be too small to be detected. Also, the physiological levels of β-defensins might not have an effect on the regulation of energy balance.

As the knockdown of gut microbiota decreased food intake, we expected a less weight gain in rats treated with antibiotics than in those treated with vehicle. However, the knockdown of gut microbiota didn't have an effect on body weight change (Figure 8).

One possible explanation is that the observation period was too short to see a significant weight change between antibiotic and vehicle treatment. Previously, it was reported that weight

difference was observed 28 days after the gut microbiota modified by berberine [116]. Another possible explanation is the adaptive thermogenesis change in response to the decreased food intake [117].

Indications of current study and future directions

The results from our study supported the hypothesis that a SFA diet is more obesigenic than a PUFA diet due to the increased food intake. However, our study failed to discover the potential mechanism for rats to increase food intake on SFA diet as no differences were seen in serum protein expression of ghrelin, pancreatic polypeptide, PYY, insulin, glucagon, leptin and adiponectin. One limitation of our study is that we fail to look at different time points of the peripheral signals in response to the two different fatty acid diets, which should be improved in a future study.

The knockdown of gut microbiota reduced rats' food intake regardless of dietary fatty acid indicates no association between gut microbiota and dietary fatty acid on energy balance. We tried to discover the potential mechanism linking gut microbiota and food intake. But no differences between antibiotic and vehicle treatment were seen in serum protein expression of ghrelin, pancreatic polypeptide, PYY, insulin, glucagon, leptin and adiponectin. As previously discussed, the only one time point measurement can't reflect the whole picture of these peripheral hormones in response to gut microbiota change. Thus, how gut microbiota influenced food intake remains to be clarified in a future study.

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