Engineering and characterization of probiotics to tackle fructose-induced metabolic syndrome

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**Engineering and characterization of probiotics to tackle
fructose-induced metabolic syndrome**

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Engineering and characterization of probiotics to tackle fructose-induced metabolic syndrome

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**Interdisciplinary Graduate School
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A thesis submitted to the Nanyang Technological University in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2020

Statement of Originality

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A handwritten signature in black ink, appearing to read 'RAY' with a stylized flourish at the end.

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Benjamin Au-Yeung

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To God be the glory, the best is yet to be

ABSTRACT

The twin epidemics of obesity and diabetes are the primary metabolic disorders fuelled by processed food availability and modern lifestyles. No current medical interventions and treatments have been successful in reversing their rising trends. Thus far, multiple causal factors have been identified with fructose being singled out by many as the major driver for this upward trajectory. Apart from the liver, most body cells lack sufficient GLUT5 transporters for fructose uptake which results in the bulk of fructose metabolism being concentrated in the liver; the immense stress placed on that single organ would lead to hepatic de novo lipogenesis and hepatic insulin resistance. Besides excess fructose, Saturated Fatty Acids (SFA) are also macronutrients to watch out for since they raise cholesterol levels and increase risk of cardiovascular diseases; both fructose and SFA are detrimental macronutrients that health authorities/bodies recommend the population to consume less of. With the advancement in synthetic biology, engineering probiotics could offer new means to tackle these metabolic diseases. A potential solution could be to have fructophilic probiotic (e.g. *Lactobacillus kunkeei*) metabolize the excess dietary fructose in the gut, and have an engineered probiotic SFA genetic biosensor to detect high dietary SFA content for appetite regulation. However, there are apparent gaps towards achieving this goal: (1) current mice models for fructose-induced metabolic syndrome give a myriad of findings that do not agree with each other; (2) there is a lack of characterization studies measuring fructose consumption amounts for *L. kunkeei*; (3) current fatty acid biosensors detect fatty acids in general but are not specific enough to sense and discriminate SFA, and the biosensors are not developed using probiotics as the host.

This thesis presents a number of studies to overcome abovementioned gaps. First, the thesis presents a study on a mice model which shows fructose-induced hepatic steatosis with low

bodyweights, very similar to the Non-Alcoholic Fatty Liver Disease (NAFLD) phenotype in non-obese populations within society. Findings from the mice study imply that liver fibrosis along with increased hepatic triglyceride and lipid storage are the very possible outcomes from excessive consumption of added fructose in the diet. Second, this thesis also provides a better understanding of *L. kunkeei*, its fructophilic nature and basic genetic tools to engineer it. The results show that *L. kunkeei* outperforms commonly used probiotics, *Lactobacillus rhamnosus* and *Escherichia coli* Nissle, in fructose metabolism/uptake rate. Last but not least, a genetic fatty acid biosensor was developed in *E. coli* Nissle to be able to detect SFA with up to ~8-fold change in readings. This biosensor could eventually work alongside with the probiotic *L. kunkeei* as a co-culture treatment; both probiotics address metabolic syndrome via restricting calorie consumption of detrimental macronutrients. Ultimately, the findings from this thesis form a foundation to enable future development of probiotic treatment which could improve overall state of health and significantly reduce the risk of obesity and type 2 diabetes mellitus.

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Benjamin AY, CL Poh. Saturated fatty acid genetic biosensor in probiotic *E. coli* Nissle. *In preparation*

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AgRP	Agouti-Related Peptide
ARC	Hypothalamic Arcuate Nucleus
ATP	Adenosine Triphosphate
AUC	Area Under Curve
CNS	Central Nervous System
CVD	Cardiovascular Diseases
DALYs	Disability-Adjusted Life-Years
FabR	Fatty Acid Biosynthesis Repressor
FadR	Fatty Acid Degradation Repressor
FAS	Fatty Acid Synthesis
FLAB	Fructophilic Lactic Acid Bacteria
GI	Gastrointestinal
GLP-1	Glucagon-Like Peptide 1
GTT	Glucose Tolerance Test
H&E	Hematoxylin and Eosin
HCl	Hydrochloric Acid
HFCS	High-Fructose Corn Syrup
HMDS	Hexamethyldisilazane
IACUC	Institutional Animal Care and Use Committee
IDF	International Diabetes Federation
IGTT	Intraperitoneal Glucose Tolerance Test

IPTG	Isopropyl β -d-1-thiogalactopyranoside
iRFP	infrared RFP
LB	Luria-Bertani
LDL	Low-Density Lipoprotein
LSR	Lipid-Sensing Receptor
MRS	De Man-Rogosa-Sharpe
NAEs	N-acylethanolamides
NAFLD	Non-Alcoholic Fatty Liver Disease
NAPE-PLD	NAPE-hydrolyzing Phospholipase D
NAPEs	N-acylphosphatidylethanolamines
NASH	Non-Alcoholic SteatoHepatitis
NBF	Neutral Buffered Formaldehyde
NCBI	National Center for Biotechnology Information
NHG	National Healthcare Group
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NPY	Neuropeptide Y
OCT	Optimal Cutting Temperature compound
PE	Phosphatidylethanolamine
POMC	Proopiomelanocortin
RB	Rich Broth
RBS	Ribosome Binding Site
RFP	Red Fluorescence Protein
RNAP	RNA Polymerase

SD	Standard Deviation
SEM	Scanning Electron Microscopy
SFA	Saturated Fatty Acid
TCA	Tricarboxylic Acid
TEE	Total Energy Expenditure
TEM	Transmission Electron Microscopy
UFA	Unsaturated Fatty Acid
UKPDS	UK Prospective Diabetes Study
W.H.O.	World Health Organization
WAT	White Adipose Tissue

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Chapter 1. Introduction

1.1. Obesity and Diabetes

The fundamental cause of weight gain and obesity is an imbalance between dietary intake and energy expenditure output. Additionally, studies have indicated that obesity is highly heritable, either by family environment or genetic contribution [1]. The World Health Organization (W.H.O.) concluded in 1998 that the root source of obesity epidemics are the high-fat and high-energy diets along with sedentary lifestyles [2]. With such causes identified as the driving force of the epidemic, it is little wonder why obesity was initially classified as a “first-world problem”. Fortunately W.H.O. is joined by many others in dispelling this misconception [3-6]; they put the blame of weight gain in developing nations squarely on the high-fat, high-sugar and micronutrient-poor western diet structure that have pervaded the global food market. The low price of western-inspired processed food is a honey trap for low and middle income nations who are also dealing with population-wide undernutrition.

Obesity is not just a standalone metabolic disorder; it's also a precursor of other metabolic syndromes to come. Research has demonstrated a strong link between obesity and non-communicable diseases like diabetes mellitus. A mere bodyweight increment of 1kg raises cardiovascular risk by 3.1% and diabetes risk by 4.5% to 9% [7]. Furthermore, 80% of obese adults suffer from at least one or more comorbidities such as type 2 diabetes mellitus, hyperlipidemia, hypertension, cardiovascular disease and some types of cancer [1, 8]. Evidently, majority of patients with type 2 diabetes are obese and people with severe obesity are at greater risk of type 2 diabetes than obese people with a lower BMI.

Obesity and diabetes are challenging worldwide health problems and they continue to rise by epidemic proportions. They are major health concerns that pose a considerable burden to people and public health all over the world. Overall, more than 1.9 billion adults, 18 years and older, were overweight, and over 600 million from this group were obese in 2014 [3]. A local study forecasted that the worrying trend of rising obesity in Singapore will see this nation face obesity prevalence of 15.9% in 2050, up from 4.3% in 1990 [9]. With increasing affluence, overweight and obesity have become the health risks that drive the ascent of type 2 diabetes [10, 11]. In 2010 alone, overweight and obesity were the causes of an estimated 3.4 million deaths, 3.9% of years of life lost, and 3.8% of disability-adjusted life-years (DALYs) globally [4]. Till date, no country has successfully reverse its obesity epidemic [12].

According to the American Diabetes Association [13], diabetes mellitus is a term to describe a “group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.” The pathogenic mechanisms of diabetic condition ranges from autoimmune destruction of pancreatic β -cells, with ensuing insulin deficiency, to resistance towards insulin action [14]. Of particular interest is the type 2 subclass or non-insulin-dependent diabetes mellitus (NIDDM). Type 2 diabetes mellitus is a category with higher prevalence, comprising at least 90% of diabetic patients; its aetiology is a combination of insulin resistance and inadequate compensatory insulin secretory response which gives rise to varying degrees of hyperglycemia [13].

The global prevalence of diabetes among adults (aged 20–79 years) was estimated to be 6.4% in 2010 and this percentage is projected to grow to 7.7% in 2030. This is equivalent to 285 million adults in 2010 and 439 million by 2030. Between 2010 and 2030, developing countries will see a 69% increase in number of diabetic adults while developed countries will see a 20%

increment [15]. Right here in Singapore, sedentary lifestyles and aging population have resulted in her having the highest proportion of young people with type 2 diabetes across nine Asian territories [16]. The percentage of Singapore residents with diabetes aged between 18 and 69 years old has increased from 8.2% in year 2004 to 11.3% in year 2010 [17]. It has even been forecasted that the prevalence of type 2 diabetes among Singapore adults in that same age group will double from 7.3% in 1990 to 15% in 2050 [9]

According to the W.H.O. [18] and the International Diabetes Federation (IDF) [16], diabetes will be the seventh leading cause of death by year 2030 [19]. 1.5 million deaths in 2012 were a direct result from diabetes [20] and deaths from diabetic complications are expected to rise by more than 50% within the next 10 years [18]. Diabetic patients face greatly increased risks of cardiovascular disease; half of whom will die from this diabetic complication [21]. Diabetes is also one of the leading causes for kidney failure [22], blindness and amputation [18].

Beyond the toll they take on the health and wellbeing of individuals, the twin epidemics of obesity and diabetes impose heavy economic burden on societies. IDF estimates the average per capita health expenditure for diabetes worldwide in 2014 to be between US\$1583 and US\$2842; they estimate annual global health expenditure towards diabetes lies between US\$612 billion and US\$1099 billion [23]. The direct costs of diabetes mellitus take up anywhere from 2.5% to 15% of national annual health budgets [24]. Zooming in on Singapore, a study on the National Healthcare Group (NHG) healthcare system puts the local mean annual direct cost of diabetes per patient at S\$2,034.6 which is above that of other Asian countries [25]. With the imminent prospect of a greying population, Singapore should strive keenly to minimize this long-term economic burden.

1.2. Current Obesity Treatments

The goal of obesity treatments is to reach a healthy weight range by decreasing absorption and consumption of food or increasing energy expenditure [26]. There are two categories of medical interventions: pharmacologic therapy and surgery. Few drugs exist to combat obesity and their results are limited to palliation instead of addressing the actual cause [27]. These medications do not appear to promote long-term thermogenesis; instead they accelerate weight loss by increasing satiety, decreasing hunger or by reducing caloric absorption [28]. Current approved drugs, such as sibutramine, orlistat and amphetamine derivatives, are hampered by unintended adverse effects and achieve only moderate results, with weight loss persisting only for as long as treatment is continued [26, 27, 29]. Alternatively, patients can also opt for the more drastic route of bariatric surgery which has been proven to be more efficacious in weight loss, comorbidity and mortality reduction [30, 31]; however, surgical complications and the need for repeated operating procedures relegate this treatment option as a last resort for the morbidly obese [26]. Needless to say, not everyone qualifies as a suitable candidate for this gastrointestinal surgery.

The last category of commonly prescribed treatments is lifestyle modifications which is essentially the balance of energy input and output. This category of healthy eating and exercise is included in the weight loss plan of every obese person but its effect is counterbalanced by the convenience of technology and availability of processed foods (i.e. snacks) [11]. With processed food products dominating world food markets [32] and long working hours, physical inactivity and poor diet choices are gradually becoming the norm. Unfortunately, a combination of dieting and increased physical activity is effective only for as long as it is

adhered to [33]. In fact, any weight lost will be regained the moment one deviates from diet plans [34].

From the aspect of total energy expenditure (TEE), physical activities barely makes up 10 to 30 percent of it while basal metabolic rate, the uncontrollable portion of TEE, accounts for 60 to 80 percent [35, 36]. Adding to that, research has revealed that the body has adaptive mechanisms (e.g. hormonal regulation, adaptive thermogenesis) diminishing the energy expenditure in response to reduced energy intake [34]; these could be the prime reasons why lifestyle changes have failed to produce significant or sustainable weight loss on the population level [33, 37].

With few therapeutic agents emerging from the long history of drug development, and current anti-obesity therapies being mostly ineffectual, new treatments with greater efficacy are urgently needed [38, 39].

1.3. Current Diabetes Treatments

The best approach towards diabetes and its related complications is the prevention, treatment and proper management of diabetes. For type 2 diabetes, most medicinal therapies are targeted at either raising sensitivity of tissues to insulin or enhancing insulin secretion; Metformin is the first medication commonly prescribed [40, 41]. Sulfonylureas, Meglitinides and Thiazolidinediones are some of the other medications that may also be prescribed but they come with possible side effects such as low blood sugar and weight gain [28, 41]. Insulin therapy can also be administered as a standalone or in conjunction with the above-mentioned therapeutic drugs [42].

Non pharmacological means are currently the most effective measure in the prevention and treatment of obesity and even type 2 Diabetes. Exercise has been proven to induce GLUT4 glucose transporter translocation to plasma membrane surface and therefore enhance skeletal muscle glucose uptake [43, 44]. In fact, the UK Prospective Diabetes Study (UKPDS) even labeled diet as the conventional treatment for type 2 [40] though questions were raised as to whether improvements in blood glucose levels were a result of weight loss or simply the decrease in energy intake [34]. Nevertheless, positive lifestyle modifications has been shown to raise the body's insulin sensitivity and also reduce the risk factors for diabetic complications; these lifestyle changes include regular physical activity, healthy diet, weight reduction, and abstinence from alcohol and smoking [13, 14, 16].

Similar to the case of anti-obesity therapy, global development and rising affluence in many countries have made it increasingly harder to maintain a healthy lifestyle which is, to date, the best treatment for type 2 diabetes. Furthermore, current pharmacologic therapies have no preventive element. They are more focused on treatment of diabetes and its complications after the disease is diagnosed; these drugs display a wide variation in efficacy with some patients even having adverse pharmacological response [41, 45]. Last but not least, the common insulin administration that patients survive on involves daily injections, making the treatment a painful process.

1.4. Probiotics as Treatment Option

In view of the limitations present in available medical options and the worrying trend of the twin metabolic disorders, there exists a great need to expand the existing repertoire of treatment

choices. The ideal treatment should be easy to administrate and be able to limit weight gain or sustain weight loss. Besides having high efficacy, the new addition will need to have little to no disruption of lifestyle and minimal side effects. These desired attributes call for an organic, natural intervention that is non-invasive in nature; gut microbiome manipulation. The gut microbiota is the vast microbial community in the gastrointestinal tract which consists of bacterial genus like gram-positive *Lactobacilli* and *Bifidobacteria*; it has a composition specific to each individual that may even vary over the course of the individual's life span [46]. It serves key functions such as dietary fiber fermentation, nutrient breakdown and absorption, vitamin synthesis, pathogen suppression, healthy bowel movement, development of immunity system, metabolic pathway regulation, gastrointestinal hormone regulation and nerve function [46-49]. Interestingly, the gut microbiota is also shown to have massive influence on the metabolic state of the host and studies have demonstrated that it can be readily modified through a series of interventions; one of which is the introduction of probiotics to reshape the gut bacterial flora composition [46, 49, 50].

Probiotics are live microorganisms that confer a positive effect on the health of the host when ingested in adequate amounts [51]. Their mechanisms of action include modification of the gut environment (in terms of pH, nutrient availability etc.), competitive adherence to the surface of gastrointestinal cells, improvement of epithelium barrier function, strengthen the innate immune system of the body and inhibition of pathogenic bacterial growth while promoting growth of beneficial microbes [46, 50-52]. For this particular project, the main mechanism of interest is the metabolism of excess nutrients i.e. dietary fructose. Probiotics for such purpose have been in use for lactose metabolism to benefit the lactose intolerant; and also for galactose metabolism to combat the colony collapse disorder of honeybees [53].

Probiotics are already a common sight in the health industry. They are widely sold as commercial dietary supplements in the form of cultured drinks, yogurt and other fermented products. Probiotic products have become so popular that the global market for these products is estimated to be worth US\$32.6 billion in 2014 [54]; this figure is forecasted to reach US\$52.34 billion by 2020 according to a recent report by Grand View Research, Inc. [55]. At the same time, the number of clinical trials to investigate novel uses of probiotics has multiplied by tenfold from 2002 to 2012 [56]. These researches and reports no doubt promote probiotics as an effectual and highly profitable avenue towards improving the state of human health.

1.5. Aim of Project

The aim of this project is to study and develop the foundations (including mice model, new genetic biosensors and better understanding of probiotics with special traits) to enable the eventual development of co-culture treatment to address the on-going challenges of fructose-induced metabolic syndrome. The long-term goal is to introduce a cocktail consisting of natural Fructophilic Lactic Acid Bacteria (FLAB) and an engineered probiotic (e.g. *Escherichia coli* Nissle strain) to significantly reduce the risk of obesity and type 2 diabetes mellitus; while improving the state of health of those in the obese and diabetic category. Figure 1 shows a potential system design of the co-culture.

Figure 1: Envisioned project system design

NAPEs synthase catalyzes the N-acylation of phosphatidylethanolamine(PE) to form N-acylphosphatidylethanolamines (NAPEs), an anorexigenic plasma phospholipid naturally synthesized in the small intestine.

The proposed strategy is to substantiate the detrimental effects of fructose with a murine study and work towards a co-culture treatment with dual functionalities when present in the human gut. The idea is to have *Lactobacillus kunkeei* in the human gut as the natural FLAB strain to metabolize excess dietary fructose that is the primary driver of diabetes and diabetes-related metabolic abnormalities. The second strain is *E. coli* Nissle engineered with Saturated Fatty Acid (SFA) genetic sensor for diet-dependent production of an appetite suppressant; NAPEs synthase is released in response to high concentrations of SFA in the diet content. Considering that excess fructose will be metabolized by *L. kunkeei*, the next prominent unfavourable nutrient to be targeted is SFA since it is known for raising cholesterol levels and increasing risk of cardiovascular diseases. The overarching hypothesis is that high-fructose consumption would induce metabolic syndrome in mice models, *L. kunkeei* in the human gut would metabolize most of the excess dietary fructose, and probiotic Nissle biosensor would release an appetite suppressant upon detection of high concentrations of SFA.

To realize the above solution, a number of fundamental gaps need to be first addressed. At the moment, current mice models for fructose-induced metabolic syndrome give a myriad of findings that do not agree with each other. Moreover, there is a lack of microbiology studies measuring fructose consumption amounts for bacteria, and also inadequate tools for *L. kunkeei* transformation and subsequent basic gene expression. Finally, current fatty acid sensors detect fatty acids in general but are not specific enough to sense and discriminate SFA, and the biosensors are not developed using probiotics as the host.

Hence the following specific aims/tasks were performed in this project:

- Established a fructose-induced hepatic steatosis mouse model through performing a mice study that demonstrates the aggravation of metabolic syndrome by high-fructose solution.
- Characterized the growth and fructophilic capacity of *L. kunkeei* using an *in vitro* platform, establishing its unparalleled fructose metabolism proficiency against other probiotic strains.
- Developed plasmid backbones for *L. kunkeei* - transformation with plasmid vectors constructed using a few different origins of replication.
- Engineered probiotic *E. coli* Nissle with first-ever SFA genetic sensor comprising of but not limited to *E. coli* type II Fatty Acid Synthesis (FAS) genes along with their transcriptional regulators FabR and FadR.

Outcomes of this research provide new understanding and fundamental tools that further enable the development of probiotic dietary supplements which will benefit current patients and future generations who are vulnerable to metabolic syndrome.

1.6. Thesis Outline

Chapter 1 gives the introduction and motivation of the research, followed by the aims of the research.

Chapter 2 reviews the literature on the research subject. First, this chapter presents the evidence and justifications to label fructose as one of the root causes of obesity and type 2 diabetes. Next, it describes the establishment of *L. kunkeei* as the fructophilic probiotic of choice. Lastly, it describes the bacterial regulatory systems that make up the fatty acid biosensor genetic circuits of this thesis.

Chapter 3 presents the fructose mice study with high-fructose water.

Chapter 4 presents the characterization study of *L. kunkeei* and demonstrates its fructophilic capacity.

Chapter 5 presents the development of probiotic fatty acid biosensors that have distinctive response to SFA.

Chapter 6 summarizes the observations, achievements and potential applications of the thesis. It also describes the limitations and future works.

Chapter 2. Literature Review

2.1. The Case Against Fructose

According to the American Diabetes Association (ADA) and Diabetes UK, the overconsumption of calories from any source leading to excessive weight gain is one of the major culprits. Yet there is a clearer understanding that not all calories are created equal. Besides the quantity, quality and source of diet/calories have momentous influence on the metabolic health of an individual [57]. There are hedonistic food choices, like cakes, doughnuts and chocolates, that stimulate the brain reward centres, resulting in a greater appetite as compared with less palatable equicaloric dietary options. There are also different macronutrient compositions of meals which give rise to varying levels of metabolizable energy available for use due to thermic effect of food [57-60]. In fact, it is in the recent decades that the concept of fibre, glycemic index, ‘good’ carbohydrates and fats all come into play to contribute to this understanding [61].

Figure 2: Fructose sources

Taken from: <https://i quitsugar.com/sugar-substitute-worse-than-sugar/>

Recent articles by clinical experts identified added fructose as a major driver of type 2 diabetes and raised serious concerns about its consequences [62, 63]. Concurrently, studies have pointed out that the rise in obesity and diabetes have paralleled the increasing use of high-fructose artificial sweeteners especially High-Fructose Corn Syrup (HFCS) [62-65]. Fructose is a simple sugar found in honey, fruit, table sugar (sucrose), dates, syrups, raisins, molasses, figs and HFCS [63, 65]. HFCS is relatively cheaper than the rest and is sweeter than glucose or the common table sugar; therefore it is commonly used as a bulk sweetener. Examples of food items containing HFCS are soft drinks, fruit juices, yogurt, salad dressing, sauces, nutrition bars, cereals and many more.

Fructose provides the same energy content (calories) as the other two carbohydrate macronutrients. The critical difference is the quality of calories available in fructose because of the way fructose is uptaken and metabolized by the human body. Although fructose is an intermediary in the metabolism of glucose, GLUT5 transporter for fructose uptake is not an insulin-dependent receptor. While glucose transporters are ubiquitous in the body, humans have no real biological need for dietary fructose and thus GLUT5 is predominantly expressed only in the liver, intestines and, to a lesser extent, kidney and testes as well [65-67]. As such, dietary fructose is almost entirely removed by the liver after being absorbed from the gastrointestinal tract because most cells have insufficient fructose GLUT5 transporters [65]. Considering that fructose metabolism is concentrated in the liver, fructose-rich diets place immense stress on the organ to shuttle excess fructose towards fatty acid biosynthesis through the TCA cycle. Consequently, large amounts of fructose will increase hepatic de novo lipogenesis and reduce oxidation of hepatic fatty acids; the combined effect of both results in hepatic insulin resistance and fat accumulation in the liver which are both symptomatic of

obesity and type 2 diabetes [62, 64, 65, 68]. Evidently, fructose-induced metabolic syndrome begins with the liver, specifically in the form of a fatty liver condition.

In human and animal studies, high fructose loads reduced adenosine triphosphate (ATP) and intracellular phosphate levels in the liver because fructose metabolism involves phosphorylation that bypasses the rate-limiting phosphofructokinase step central to glucose metabolism [65]. This drop in ATP levels may result in reduced cellular binding of insulin and possibly a decline in insulin receptors [62, 68]; while the bypass means that hepatic fructose metabolism is not subjected to feedback inhibition which will lead to increased glycogen deposition and also bring about further hepatic de novo lipogenesis [69, 70]. Moreover, fructose has been shown to induce gluconeogenesis, raising serum glucose levels and exacerbating the diabetic condition by placing undue stress on the pancreatic β -cells [62, 71]. Worse still, since β -cells of the pancreas lack the GLUT5 transporter, fructose molecules themselves do not acutely stimulate insulin secretion which also cascades to tenuous leptin and ghrelin response to feeding; chronic high fructose diet might eventually lead to leptin resistance [63, 64]. The final condemning piece of evidence is found in the higher serum fructose concentrations in patients with diabetes compared to those in healthy subjects and non-diabetic patients [72]. However, emphasis should be made that fructose found naturally in whole foods such as fruits and vegetables are harmless because whole foods contain a much lower concentration of fructose accompanied with much water and fiber [62, 65].

Figure 3: Impact of added fructose

In the United States, HFCS represents more than 40% of all caloric sweeteners and is the only sweetener added in soft drinks [64]; the mean consumption of fructose for an American rose from 8% of total daily caloric intake in 1977 to over 10% in 2008 [69]. Currently, fructose is abundant in all seasons and present in high concentrations in processed foods [73]. With prices falling due to its rising availability [74], the sale of processed food has been growing exceedingly for the past three decades till now it comprises of three quarters of the world food market [32]. As highlighted in the introduction, these facts prove that food processing has tainted the food industry of almost every nation. From the underdeveloped to the first world countries, none are safe from the far-reaching effects of the expanding processed food market. As such, it is believed that added fructose is one of the root causes for the growing prevalence of obesity and type 2 diabetes that needs to be addressed.

2.2. Fructose Mice Studies

In murine studies, fructose is included in the mice diet through either their solid feed pellets or their drinking water. The fructose composition ranges from 47% to 60% for solid feed pellets, and 10% to 55% for drinking water [75-87]. With the solid fructose diets, mice bodyweights have no significant difference when compared to control even up to ~5.5 months of fructose feeding [75, 77-80], yet their daily caloric intake is substantially higher [78]. Three fructose pellet mice studies have found elevated fasting blood glucose with glucose intolerance from fructose feeding [77, 79, 80], while two reported no change [75, 78]. The findings for fasting insulin levels are equally dissimilar; results from two studies have indicated that fructose solid diet has negligible influence on insulin levels [75, 77], while two other studies have reported raised insulin levels coupled with insulin resistance and suppressed glucose-stimulated insulin secretion [79, 80]. With the exception of a single study, biochemical blood analyses forewarn of an apparent rise in triglyceride and cholesterol levels from chronic intake of fructose via a solid diet [75, 76, 78, 79].

Different trends can be seen when fructose was delivered to mice in the drinking water instead of diet pellets. With an additional source of calories, most mice on fructose water have reduced caloric intake from their chow/control solid feed pellets [81, 84, 86, 87] and only one study claimed otherwise [82]; similarly, their total caloric intake (from feed pellets and fructose water) is greater than control groups [81, 82, 84-86] and only the study using the lowest fructose water concentration (i.e. 10%) reported different [87]. Three studies observed little bodyweight difference between fructose water and control groups [85-87], but unlike the fructose pellet experiments, four others found measurably higher weight gain in the fructose water groups with some manifesting the characteristic in just 8 weeks [81-84]. Conversely, fructose water

and pellets delivered a very similar spectrum of findings for fasting blood glucose and insulin levels. Fructose water can induce a drop in fasting blood glucose readings for some mice [86], glucose intolerance on top of a reading spike for the majority [82, 84, 87], and no change for others [81]. Likewise, fructose water can induce insulin resistance and a rise in fasting blood insulin readings [82, 87], or simply have negligible influence on insulin levels [81, 84]. Interestingly, according to biochemical blood analyses, fructose water is less potent than fructose pellets when considering triglyceride content in blood since only a single experiment yielded elevated triglyceride levels [82]. Be that as it may, when the consideration is broadened to encompass fat content of the mice in general, fructose delivery by drinking water is proven to be as potent as fructose pellets if not more; chronic intake of fructose water can possibly lead to massive gains in cumulative body fat, rapid accumulation of hepatic triglycerides and a steady rise in cholesterol levels [81-86].

Whichever delivery mode is used, current mice models paint a vague picture of the true impact of high dietary fructose. The only resolute trend is the association of fructose with significant liver weight gain, rapid hepatic triglyceride and lipid accumulation, and hepatic steatosis [76, 79, 81-86]. In contribution towards Non-Alcoholic SteatoHepatitis (NASH) and Non-Alcoholic Fatty Liver Disease (NAFLD), chronic fructose feeding is linked to the loss of tight junction proteins; fructose metabolism impairs intestinal gut barrier function, allowing endotoxins and pathogens to enter the portal vein and liver [88, 89]. This induces toll-like receptors in the liver which leads to inflammation and degradation of the mucosal barrier. Since liver is identified as the centerpiece of fructose-induced metabolic syndrome, the organ will be the focal point for this project's mice study.

2.3. Probiotic of Choice: *Lactobacillus kunkeei*

A recent study by Park and his colleagues [90] used *L. curvatus* HY7601 and *L. plantarum* KY1302, isolated from Korean fermented cabbage, in a novel probiotic treatment with the similar purpose of suppressing high-fructose induced metabolic syndrome. Although the treatment was fairly successful in reversing certain risk factors like high plasma glucose and hepatic triglyceride levels; it had little to no effect on food intake, body weight gain, liver mass and epididymal fat mass.

Similar to Park's work, this research would likewise involve lactic acid bacteria but the final probiotic choice has to be fructophilic in nature in order to breakdown excess fructose in the gut efficiently. Amongst the lactic acid bacteria, *Lactobacillus kunkeei* is chosen for two reasons – (i) its fructophilic nature; and (ii) it is isolated from sources belonging or relating to human diet such as commercial grape wine and other fructose-rich niches like flowers, fruits, and even honeybee gut microbiota [91-94]. Type strain YH-15 (= ATCC 700308, = DSM 12361, GenBank accession no.Y11374) is the selected strain of *L. kunkeei* because of its relatively slower growth compared to other strains [93]; thus colonization of the gut by non-resident probiotic bacteria is avoided. This gives the additional benefit of leaving the bulk composition of the gut flora unaltered while allowing the number of eventual microbes per intake to be readily varied via dosage control.

L. kunkeei cells are gram-positive, non-motile rods with length range of 0.7 to 1.3 μm and a diameter ranging from 0.3 to 0.5 μm as determined by transmission electron microscopy (TEM). Colonies grown on De Man-Rogosa-Sharpe (MRS) agar are white and smooth [91, 93, 95]. *L. kunkeei* is considered an obligate FLAB, meaning that cells require fructose to grow well in

the absence of external electron acceptors like oxygen or pyruvate [92, 93]. For the common sugar types, most *L. kunkeei* strains ferment D-fructose the fastest, followed by sucrose and then D-glucose. Although fermentation order for type strain YH-15 is D-glucose first and D-fructose last, the strain is still viable because fructose is taken up as an electron acceptor for glucose fermentation [92, 93]. All three sugar fermentations produce lactic acid [93]. Other than lactic acid, D-glucose fermentation gives acetic acid and traces of ethanol; while D-fructose fermentation gives mannitol in a mannitol production to D-fructose consumption molar ratio ranging from 0.7:1 to 1:1 [92, 93]. Since mannitol is also fermented by *L. kunkeei* [91-93], the mannitol by-product from D-fructose fermentation is being recycled. In any case, mannitol is classified as a sugar alcohol and is being used as a sweetener for diabetic patients due to its low glycemic index.

In 2015 and 2016, human trials were conducted for heat-killed *L. kunkeei* [96, 97]; they yielded positive results like increase in immunoglobulin A production and increased bowel movement. Additionally, Bisson et al. [98] wrote a paper, published in July 2016, which highlighted the role *L. kunkeei* plays in the defense of bees and their hives against pathogens. These recent developments and findings give tremendous support to the idea of using *L. kunkeei* as probiotics. However, on record, there have been only two cases of successful bacterial transformation to insert exogenous plasmid into *L. kunkeei* [53, 94]; this would be one of the major hurdles in working towards creation of an engineered *L. kunkeei* strain.

2.4. Synthetic Biology: Genetic Circuits

One of the project aims is to have the probiotic culture release an appetite suppressant (or anorexigen) under feedback regulation. This added functionality requires the probiotic strain to harbour a genetic circuit that modifies the cell behaviour. Given that bacterial transformation of *L. kunkeei* is a challenge in itself, *E. coli* Nissle will be the host strain to carry the appetite suppressant genetic circuit. Not only are there readily available resources and optimised tools for bacterial transformation of *E. coli*, but the Nissle strain is also a well-established GRAS strain that is already commercially sold as supplementary probiotics.

For this project, synthetic biology framework will be used to develop the genetic system that tackles the twin threats of obesity and type 2 diabetes. Synthetic biology is a rapidly emerging field of research that involves designing biological systems that do not exist in the natural world and re-engineering of existing biological elements; it lies at the intersection of engineering, biological sciences, and computational modelling [99]. Synthetic biology builds on genetic engineering by bringing in standardization, decoupling and abstraction as the three foundational engineering principles that enable researchers to construct novel biological systems in a systematic fashion using standard biological parts [100]. Synthetic biology has demonstrated its applicability through the creation of new metabolic pathways and re-programming of microbial bacteria cells; resulting in medical and environmental advances such as the creation of anti-malarial drug Artemisinin [101], the design of anti-tumour bacteria [102], the removal of herbicide Atrazine from water [103] and the engineering of microbes to produce biodiesel from simple sugars [104].

Before the rise of synthetic biology, there were remarkable breakthroughs in genetic engineering and biotechnology dating a few decades back. Existing genes could be scrutinized and deconstructed while new gene arrangements could be fabricated and analyzed [100]; reconstruction of living organisms following insertion of synthetic genome was already a definite possibility [105]. Even so, most rational designs of useful synthetic biological systems with predictable functions are time-consuming, with unreliable engineering processes that are limited to a number of genes [106]. The most probable reason for such a conundrum is the immense complexity of biological systems [107, 108]; the massive volume of functional components coupled with their myriad interactions with each other hinder any attempts at engineering regular and consistent behaviour/response. Adding on to that is the shortage of predefined parts and refined tools for the construction of synthetic biological systems. While large established labs/enterprises may have a library of these tools and parts along with a systemic approach to circumvent the biological complexity; that is not the case for most regular-sized research groups and individuals in this field. Thus, there exists the need for foundational engineering technologies of standardization, decoupling and abstraction. These three make up the synthetic biology framework that will simplify design and construction of the genetic circuit.

2.4.1. Foundational Engineering Principles

Standardization is the development of technologies and standards that define, describe and characterize basic biological parts [109] including the rules and conditions for the use of those parts in an integrated system [100]. Similar to how all screwdriver heads have standardized designs for unique purposes; genetic parts like promoters, ribosome binding sites and terminators can be standardized and classified based on their basic biological function. A classic example of a collection of standard parts is the Registry of Standard Biological Parts.

Its catalog (<http://parts.igem.org/Catalog>) is an extensive library made open to everyone. Besides the technology aspect, establishment of better legal standards is also necessary to ensure responsible use and sharing of resources [100]. Current legal standards like material transfer agreements lead to lengthy arrangements which can be shortened by sharing in a public domain instead. With proper standards like barcoding, signing and watermarking DNA, more in the community will be encouraged to share genetic information freely because they will receive the due acknowledgement for their contributions.

Decoupling is essentially the breakdown of the engineering challenges into simple tasks that can be tackled singly [100]. The most common type of decoupling is the separation of design from fabrication. This process is driven by improvements in DNA synthesis technologies and the evolution of computational model simulations. The capacity for DNA synthesis has grown exponentially and its price has fallen rapidly within the last decade or so [110]; while computational and mathematical models are becoming widely utilized as more biological system parameter values surface for simulations with better precision [111, 112]. The developments in both domains make it necessary for one to specialize in either instead of working on both. With clearly delineated tasks laid out, each group can focus on advancing the process of design or fabrication of synthetic biological systems.

Abstraction is the technology to mask information and manage biological complexity by organizing biological information into levels of abstraction hierarchies [100]. This creates a platform for tasks to be carried out at each hierarchy level without considering the details belonging to other levels, yet enable the flow of limited information across these levels; the most regularly implemented abstraction levels are ‘DNA’, ‘parts’, ‘devices’ and ‘systems’ (listed in ascending order).

By applying these engineering principles to biology, catalogued and standardized biological components can be easily attainable for systemic and reliable construction of synthetic systems/devices with novel and consistent functionalities [106]. There are a few notable synthetic gene circuit functions and designs that may be relevant to this project. Biosensing genetic modules incorporated into whole synthetic systems can provide the capability of sensing and responding to a wide range of environmental signals [113, 114]; genetic devices can be constructed to emulate electrical circuit components such as switches, oscillators and logic gates [113-115].

2.4.2. Tackling Metabolic Disorders

The field of synthetic biology has managed to put forth several feasible strategies to tackle the twin threats of obesity and type 2 diabetes. One of the response to obesity is the introduction of glyoxylate shunt to mammalian cells [116]. Glyoxylate shunt is a two-enzyme pathway found only in plants and bacteria. This pathway provides the cells a means of conserving carbon by bypassing the oxidative CO₂-producing steps in the tricarboxylic acid (TCA) cycle; the natural pathway avoids complete fatty acid oxidation and channels additional carbon to gluconeogenesis. On the other hand, synthetic glyoxylate shunt in mice resulted in increased free fatty acid oxidation along with resistance to diet-induced obesity without a rise in blood glucose levels [116, 117]. In essence, this method edits the metabolic pathways to counter high-fat diets without aggravating or inducing obesity associated diabetic complications.

Another research group has proposed the use of a unique closed-loop synthetic gene circuit for treatment of diet-induced obesity [118]. The synthetic circuit comprises of a lipid-sensing receptor (LSR) and a LSR-dependent transcription modulation coupled to expression of

pramlintide, which is a clinically licensed synthetic anorectic peptide hormone that is also an amylin analogue. When implanted in mice receiving high fat diet (HF), the circuit continuously monitored blood fatty acid levels; it is activated to release pramlintide at peak fatty acid levels and it switches off at normal concentrations. The result was a reduction of blood fatty acid levels, food consumption and body weight of the HF mice while no significant changes were observed in treated mice on low-fat diet. If necessary, the device circuit can be deactivated any time using skin lotion containing the transdermal cosmetic additive phloretin [119].

The final noteworthy intervention stems from glucagon-like peptide 1 (GLP-1), a potent anti-hyperglycemic hormone that is an emerging pharmacological option for diabetes. Recent studies demonstrated that both rat and human intestinal epithelial cells can be converted to insulin-secreting cells when stimulated by inactive full-length form of glucagon-like peptide-1, GLP-1(1-37) [120, 121]. This peptide is delivered using intestinal commensal bacteria, *E.coli* Nissle and *Lactobacillus gasseri*, transformed with the plasmid encoded for GLP-1(1-37) [121]. Suzuki et al. [122] postulates that the undifferentiated intestinal epithelia develops into β -like cells when stimulated by GLP-1; these β -like cells secrete insulin and respond to glucose. This belief is backed up by tissue homogenization which revealed that the insulin in rat subjects fed GLP-1-secreting bacteria originated from the intestine and not the pancreas where the real β cells reside in [121]. This intervention is a direct treatment for diabetic symptoms. GLP-1 secretion will address the issue of insulin deficiency, reducing patients' reliance on external insulin therapy. In fact, this can be a long-term solution to type I diabetes whereby the metabolic condition is absolute insulin deficiency.

As presented above, synthetic biology opens up new doors in the search of novel strategies to beat the rise of obesity and type 2 diabetes. A common thread among the prominent strategies

listed is the introduction of a synthetic gene circuit into the natural system, albeit they have different objectives and purposes. The first intervention modifies natural metabolic pathways to raise fatty acid oxidation; the second releases a synthetic anorexigen when high levels of fatty acid is detected in blood; and the third releases naturally-occurring GLP-1 to boost insulin secretion levels. Alas, each intervention has its shortcomings. The first two focuses on the high fat content of unhealthy diets but not on the high sugar content present in nearly all processed foods, while GLP-1 does not ameliorate the metabolic state of insulin resistance.

Given that no intervention can be all-encompassing, this project is not seeking to supplant existing ones but rather to venture on a different approach/objective, as stated in the project aims, to better address fructose-induced metabolic disorders. The synthetic gene circuits in this project will be designed and constructed following the rational framework of synthetic biology.

2.5. Fatty Acid Genetic Sensor in *E. coli* Nissle

Production/Release rate of appetite suppressant by this project's genetic circuit will depend on the Saturated Fatty Acids (SFA) content in the diet. In 2015, the U.S. Department of Health and Human Services and U.S. Department of Agriculture recommended the public to switch food choices from those high in SFA to those high in Unsaturated Fatty Acids (UFA). Their guideline is to reduce SFA intake to less than 10% of daily calorie requirement. Similarly, Mayoclinic also urged the population to avoid SFA because SFA leads to elevated low-density lipoprotein (LDL) cholesterol levels, which raises the risk of Cardiovascular Diseases (CVD), and may increase the risk of type 2 diabetes. In fact, the American Dietetic Association advocated for a lower SFA intake of less than 7% of daily calorie requirement for prevention of CVD [123]. Multiple cohort studies corroborate the recommendations listed above, yielding

data that highlights replacement of SFA with polyunsaturated fats as a key strategy in reducing risk of coronary heart disease and other CVD [124, 125]. Considering the published evidence and recommended guidelines, the genetic sensor will have to discriminate between saturated and unsaturated fatty acids.

E. coli Nissle 1917 is the probiotic host strain for the fatty acid genetic sensors. It is advantageous to use a probiotic agent already found in medicines and treats various gastroenterological diseases [126]. One prime example is Mutaflor, typically sold as treatment for constipation; it is a brand of Nissle 1917 marketed by Australian company Natural Therapy Imports. Additionally, Nissle has also been proven to be an effective remedy for irritable bowel syndrome [127]. Above all else, researchers have shown that Nissle 1917 can deliver heterologous therapeutic molecules directly to the human gut [128] which is the precise purpose of the genetic sensors of this thesis.

For bacteria, fatty acid components are among the most energetically intensive resource to produce, which is why fatty acid production is closely regulated to be commensurate with the growth rate of cells [129]. Bacterial cell growth and survival rely on its membrane lipid homeostasis to adapt membrane permeability for diverse environments [130, 131]. In *E. coli*, FabA and FabB are responsible for the fatty acid composition and hence the biophysical properties as well as permeability of the phospholipid membrane; they are the essential genes of type II fatty acid synthesis pathway for UFA biosynthesis in *E. coli*. In turn, fatty acid degradation repressor (FadR) and fatty acid biosynthesis repressor (FabR) are responsible for the proportion of UFA available for the cell and its membrane; they are the transcriptional repressors that regulate the expression of FabA and FabB genes, and thereby ensuring the composition of fatty acids remains balanced at all times [129, 131, 132].

Figure 4: Regulation of fatty acid metabolism in *E. coli* [132]

Genes in green brackets are repressed by FadR; genes in red brackets are activated by FadR; those in blue brackets are repressed by FabR. Adapted from [132] with permission.

2.5.1. Fatty Acid Degradation Repressor (FadR)

Besides FabA and FabB, FadR is involved in the regulation of a host of fatty acid degradation genes in *E. coli* such as FadD, FadE and FadH [133]. FadR is a member of the GntR family of transcription factors that operate as dimers comprising of a ligand-binding domain at the C-terminus and a DNA binding helix-turn-helix domain at the N-terminus [134, 135]. In the absence of fatty acids, FadR binds to its cognate operator site within the promoter region of the fatty acid degradation genes, and thereby acting as steric hindrance to transcription by preventing RNA polymerase (RNAP) from coupling with the promoter. When exogenous fatty acids are present, they are converted to acyl-CoAs as part of the beta-oxidation process in fatty acid metabolism of *E. coli*; the CoA thioesters induce a conformational change that antagonizes

the DNA-binding activity of FadR, releasing FadR from its operator site and thus allowing RNAP to resume transcription [129, 132, 133, 136]. This derepression of gene expression corresponds to the level of fatty acids available to the cell, forming the cornerstone working principle for the genetic sensor of this project.

Lo et al. [137] and Zhang et al. [138] have both incorporated the FadR system into their genetic circuits. Both tested their circuit response to the UFA oleic acid with Zhang et al. achieving up to a 60-fold change relative to uninduced expression levels while Lo et al. managed a ~6-fold change. Zhang et al. tested native/natural and synthetic promoter sequences in their FadR system. *E. coli* native FadBA promoter, consisting of a single FadR operator site, only delivered a 5-fold dynamic range when the system was induced with 5mM oleic acid. To boost the dynamic range up to 60-fold, Zhang et al. integrated the FadR operator sequence, from native FadBA promoter, into a phage lambda promoter at two locations flanking the -10 region of the phage promoter.

Lo et al. selected *E. coli* K12 MG1655 as the host strain for their genetic circuits while Zhang et al. housed theirs in *E. coli* DH1; neither are established probiotic strains like the *E. coli* Nissle chosen for this project. Both abovementioned studies did a FadE gene knockout for their host strains; since FadE gene expresses the acyl-CoA dehydrogenase enzyme that catalyzes fatty acid degradation, FadE deletion will maintain the acyl-CoA concentration in the cell [131, 132, 138]. For the same purpose, acyl-CoA synthase, FadD gene, was included in the FadR system of the two featured studies. FadD is essential for hastening the activation process of exogenous fatty acid to acyl-CoA and thus will be included in this project as well.

2.5.2. Fatty Acid Biosynthesis Repressor (FabR)

FabR is a member of the TetR family of transcriptional repressors and act as a regulation control for type II fatty acid synthesis pathway in *E. coli* [129, 132]. The working mechanism of the FabR system is similar to that of FadR; but unlike FadR, the response of FabR differs between saturated and unsaturated thioesters, resulting in two possible conformational changes. The binding of UFA-CoA (from UFA) to FabR increases the affinity of the repressor to its operator site within the promoter, leading to transcriptional repression of the regulated gene. Whereas the binding of SFA-CoA (from SFA) reduces the affinity of FabR for its operator site, releasing the repressor from the promoter, resulting in unhindered gene expression [131]. Further studies have illuminated a more complex binding mechanism of FabR; unsaturated thioesters like UFA-CoA act to enhance FabR binding but are not essential to that binding [139]. Electrophoretic mobility shift assays demonstrated that FabR cannot bind to its operator sequence alone without any flanking sequences. However, FabR is able to bind to the FabA and FabB gene promoters when there are nucleotide sequences flanking its operator site; flanking sequences for the FabR operator site in FabA promoter have to be specific while operator flanking sequences for FabB can be non-specific for FabR to bind. Consequently, FabR will act as a transcriptional repressor to FabA and FabB genes, even in the absence of fatty acids, since the operator flanking sequences are in the promoter. Presence of UFA enhances FabR binding and thus raises the extent of repression on gene expression.

Figure 5: Mechanism of both FadR & FabR

On the whole, FabR detects the ratio of UFA:SFA rather than the absolute concentration of either UFA or SFA [131]. This discriminatory response of FabR makes it a choice selection for the SFA sensor of this project. Thus far, there is no study that test the response of a synthetic FabR system to exogenous fatty acids.

2.6. Appetite Suppressant: Gut Hormones

Gastrointestinal (GI) surgery is the hitherto sole weight loss solution delivering sustainable results. Remarkably, the abovementioned has been associated with alterations in the physiology of circulating gut hormones and their capabilities in appetite regulation [140, 141]. Gut hormones, in this paper, refers to the various peptide hormones secreted along the GI tract that exhibit anorexigenic or orexigenic properties; Together with neuronal pathways, humoral and nutrient signals, these gut hormones play an important role in generating satiety [31].

At the centre of appetite regulation lies the hypothalamus, specifically the hypothalamic arcuate nucleus (ARC). The ARC is not fully insulated from circulation of appetite modulators by the blood brain barrier hence it is well-suited to detect endocrine and nutrient factors [140]. The ARC has two distinct neuronal populations; orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) co-expressing neurons and anorexigenic proopiomelanocortin (POMC) neurons. They project from the ARC to other key hypothalamic nuclei, communicating with second-order regulatory neurons, creating a signal cascade to eventually modulate feeding behavior [1].

There is a wide range of anorexigenic agents; some naturally existing, others are chemical entities developed by companies. Due to their intrinsic role in daily physiological appetite regulation, natural hormone-based therapies have low risks of adverse side effects compared to a number of commercial drugs already out in the market [140, 142]. Synthetic pharmaceutical options developed to target receptors concerning appetite regulation may have an impact on other Central Nervous System (CNS) functions under the control of the affected receptors; likewise is the case for neural pathway signals with roles in multiple physiological systems and responses. On the other hand, natural gut anorexigens as therapeutic agents would be specific to only the relevant appetite systems [141]. Hence administration of natural gut hormones might be a potent and safe physiological approach to combat obesity in terms of calorie intake.

From the list of anorexigenic gut hormones, N-acylphosphatidylethanolamines (NAPEs) are the tentative focus of this project. NAPEs are plasma lipids naturally produced in the proximal small intestine in response to food intake and fat feeding. They are hydrolyzed by NAPE-hydrolyzing phospholipase D (NAPE-PLD) to active N-acylethanolamides (NAEs) which

modulate adiposity through mechanisms like reduction in food intake and delayed gastric emptying [143]. Whilst other anorexigenic gut hormones are either not that effective, have adverse nauseating side effects, taste aversion or even a short half-life [140, 141]; NAPEs have been shown to be expressed by modified *E. coli* Nissle [143] (similar to project aim) and proven to be effective even in leptin-deficient ob/ob mice [144].

Hypothalamic appetite regulation is a complex mosaic network map made up of signaling pathways within the brain, peripheral signals acting via the brainstem and reward circuitries [1]. Regardless of whichever anorexigenic agent is chosen, it will be a challenge to determine all the interactions that anorexigen has with the other signaling pathways on top of distinguishing between genuine satiation and appetite reduction due to nausea [31, 140].

Chapter 3. Fructose Mice Study

To test the hypothesis that high-fructose consumption would induce metabolic syndrome in mice models, this mice study is carried out to determine the impact of excess added fructose on the state of health and also observe any occurring clinical characteristics of high-fructose induced metabolic syndrome. Since current mice models for fructose-induced metabolic syndrome give a myriad of findings that do not agree with each other, these two objectives will ultimately go towards substantiating the detrimental effects of fructose; while the outcome of this study could even establish a mice model for the future development of probiotic FLAB for dietary fructose removal as a form of treatment or prevention of obesity and diabetes. With fructose and HFCS pervading the food industries of almost every nation of the world, it is of paramount importance that their health impacts be clearly understood and proven.

Fructose can be fed to the mice either through their feed pellets or their drinking water; with different trend of results observed for both approaches. For this study, drinking water was the mode of delivery chosen for excess fructose. In the literature review comparisons, past studies demonstrated that mice on fructose water treatment do experience significant bodyweight gain over a relatively short duration as opposed to mice receiving fructose feed pellets; weight gain is one of the most easily observable traits. More importantly, high fructose water simulates a major source of added fructose in human diet which is sweetened or soft drinks, thus making it a more relatable fructose source.

3.1. Methodology & Materials

3.1.1. Animals and Study Protocol

Table 1: Mice study experimental setup

Fructose Group	Control Group
50 series no. labelling n=10	51 series no. labelling n=10
Control Diet + 30% Fructose Water	Control Diet + Normal Water

Twenty C57BL/6J male mice of 5 weeks (InVivos, Singapore) with mean initial bodyweight of 21.1 ± 1.2 grams were received for this study after a week of acclimatization with standard chow diet and

sterile drinking water. The mice were housed as 5 per cage with a 12:12-hour light-dark schedule and a housing temperature of 30°C, which is the optimal ambient temperature for better translation of study data. The feeding phase began when the mice were separated into their 2 groups, n=10 per group. Both groups were fed control diet (13% protein, 10% fat, 77% carbohydrate) ad-libitum, but one group was provided 30% (w/v) fructose water while the other was provided sterile drinking water. Water was provided in bottles for easy measurement of water consumption. A control bottle was used to quantify the amount of liquid lost through dripping and leakage, thus helping the measurements account for such losses. Diet pellets and water were topped up weekly; bodyweight and food/water consumption measurements were also taken on a weekly basis.

This study spanned across 32 weeks. Mice were euthanized via carbon dioxide chamber on week 21 (first batch) and week 32 (second batch), or if a sick mouse could not live till the next day. White adipose tissue, liver, kidneys and spleen were harvested from each mouse after euthanization. All procedures were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University.

3.1.2. Glucose Tolerance Test

Glucose tolerance test (GTT) was carried out on weeks 5, 13, 20 and 32 of mice study 2. The mice were fasted for 16 hours by being transferred to fresh sterile cages with free access to sterile drinking water, but no diet pellets, at ~5pm in the evening; the GTT was performed on the next day morning around 9am. Sterile 20% (w/v) glucose solution was injected intraperitoneally into the mice using 27-gauge, half inch needles to a glucose concentration of 2g/kg for each mouse. Blood glucose levels were then measured by tail vein sampling with Accu-Check glucometer from Roche (Basel, Switzerland). Measurements were taken at 0, 15, 30, 60, 90, 120 minute after glucose injection.

3.1.3. Histological Analysis

During organ harvesting right after euthanization, the left liver lobes were divided into quarters. Cube pieces of 0.5cm x 0.5cm x 0.5cm were cut out from the middle 2 quarters of the left liver lobe. These cube pieces were immersed in 10% Neutral Buffered Formaldehyde (NBF) at room temperature; some pieces were left to incubate in NBF for one week while others were left for 48 hours. The pieces incubated for one week were dehydrated and embedded into paraffin, while the rest were immersed into cryoprotectants, like sucrose, and frozen in Optimal Cutting Temperature (OCT) compound.

Slices of 5µm paraffin-embedded liver sections were stained with Hematoxylin and Eosin (H&E) to evaluate liver morphology; they were also stained with Sirius Red and Masson's Trichrome to assess for fibrosis. Slices of 8µm frozen liver sections were stained with Oil-Red-O to determine the extent of hepatic steatosis and hepatic lipid accumulation. All stainings were viewed through a slide scanner microscope (Axio Scan.Z1, Zeiss, Oberkochen, Germany). Representative images were captured at 4x zoom magnification.

3.1.4. Statistical Analysis

All data are reported as mean $\pm$ standard deviation. Unpaired t-test was used to determine statistical significance. Results with P -value < 0.05 were considered to be statistically significant. Statistical analyses for the mice studies were performed with GraphPad Prism 7.02 (GraphPad Software, Inc., CA, USA).

3.2. Results & Discussions

3.2.1. Weight, Diet & Water

Weight measurements are the quickest indicator of health status, while diet and water consumption levels uncover any impact fructose might have on the feeding patterns or habits of the mice. Both groups had a general upward rise in bodyweight throughout the entire experiment that mirrored each other in terms of the micro spikes and dips. Prominent dips were the result of stress endured from intraperitoneal glucose tolerance test (IGTT); they were most evident in the weeks of IGTT. As can be seen from the stagnating bodyweight in the first 5 weeks, mice in fructose group took a few weeks to get used to the fructose water. On the other hand, the control group saw accelerated bodyweight gain, $p < 0.01$, right from week 1. By the second week, average bodyweight for the control group was significantly greater than that of fructose group, $p < 0.01$. Contrary to trends in past reports [81-87], the gap between bodyweights of the two groups continued to widen all the way till the end of the experiment when fructose mice weighed in at an average of 25.53 grams while control mice weighed an average of 31.89 grams.

Figure 6: Mice average bodyweight [A] and cumulative weight gain [B]

Both groups had the same upward trend for their bodyweights with most spikes and dips occurring at the same weeks. Control group was significantly heavier from week 2 of feeding phase. Error bars denote standard deviation (SD). Both graphs have the same number of mice and thus share the same mice number labelling at the bottom right of [B].

Initial assumption was that the bodyweight disparity was due to the difference in diet consumption, but overall calorie intake was calculated to be pretty much equal every week for both groups throughout the experiment. As mentioned in the previous chapter, not all calories are created equal and different caloric sources can result in varying outcomes. That said, it was unexpected to obtain lower average bodyweight from having a portion of caloric intake come from fructose water; no other study has mice on fructose water with significantly lower bodyweight than control.

Figure 7: Mice weekly consumption amounts

Diet [A] and water [B] weekly consumption. Drinking water consumption for the control group was recorded from the 14th week onwards. Diet and water raw reads were collected from entire cages; readings were averaged out according to the number of mice in each cage. There were insufficient raw data to obtain standard deviation, hence no error bars. Calorie intake [C] was computed from the weekly diet and fructose water consumptions. **Fructose group, combined** is the combined calorie intake from both diet and fructose water of the fructose group. Sterile drinking water offers 0 calories, thus control group calorie intake is solely from diet. All graphs have the same number of mice and thus share the same mice number labelling at the bottom right of [C]

Once again, fructose and control groups had nearly perfect matches for both their diet and caloric intake plots. Their fluctuations were in synchrony, having identical peaks and troughs. Similar to the findings from Ishimoto et al. [84], the fructose group consumed lesser feed pellets because calories from fructose water contributed towards the amount of calories needed to meet their intake requirement for each week.

3.2.2. Glucose Tolerance Test

Glucose intolerance is widely regarded as the strongest indicator of type 2 diabetes and its related metabolic complications. From the GTT and AUC readings, it was evident that glucose intolerance was not observed. Significant difference was only observed in the first 15 minutes of week 5 and week 13 GTT. By the second half of the study, glucose tolerance levels of both groups were very much equivalent. Contrary to expectations, control group had the remarkably higher fasting blood glucose levels, $p < 0.001$, during the early stages. In the subsequent weeks, fasting glucose level of fructose group rose while that of control group lowered till the disparity in readings were reduced to be inconsequential.

A: GTT

B: GTT 0 minute

C: GTT AUC

D: AUC timeline

Figure 8: Glucose tolerance test (GTT) readings

[A] Fasted GTT interval readings over 120 minutes. [B] GTT initial 0-minute reading. [C] GTT area under curve (AUC) reading. [D] GTT AUC trendline over the duration of the experiment. Error bars denote SD.

3.2.3. Harvesting & Liver Histology

The chief purposes of this section are the comparison of the weights of key organs between the two mice groups and the examination of their livers for hepatic lipid accumulation or any signs of liver fibrosis. Even with a huge difference between their bodyweights, both groups had comparable liver and spleen weights throughout the study with fructose group achieving significantly higher, $p < 0.05$, liver:bodyweight ratio at the week 32 harvesting time point. Following along the same narrative, it is peculiar for the White Adipose Tissue (WAT) weight to be very even despite control mice being much heavier than fructose mice. Keeping in mind that the WAT measured in this project was collected solely from the perigonadal deposit location, it was highly likely that other WAT deposits in the control mice were heavier than those in the fructose mice since the harvested organs (kidney, liver & spleen) from both groupings were of similar weight.

Figure 9: Harvesting measurements by batch

[A] Liver weight & liver weight : bodyweight ratio. [B] Spleen weight & spleen weight : bodyweight ratio. [C] White Adipose Tissue (WAT) weight. [D] Blood glucose, non-fasted. Error bars denote SD.

Effect of fructose on non-fasted blood glucose levels did not paint a clear picture. Fructose mice had higher blood glucose level than control at week 21 but the reverse was true at week 32. Serum measurements taken with Alfamedic profile cassettes revealed very similar ALT/AST/TBIL/BUN/ALB/TCO₂ levels between both groups at both harvesting time points.

A: H&E stain

B: Masson's Trichrome stain

C: Oil Red O stain

D: Sirius Red stain

Figure 10: Liver histology

Scale bar at bottom right of each picture represents 50μm.

Since fructose is mostly metabolized by the liver, emphasis was placed on the histology of the mice livers. Even though fructose group had significantly higher liver:bodyweight ratio only at week 32, histological analysis presented strong evidence of hepatic steatosis in the fructose group at both weeks 21 and 32. For both time points, Oil Red O stainings showed greater hepatic triglyceride and lipid storage for fructose mice as compared to control mice.

Early signs of liver fibrosis for the fructose group were present in the Masson's trichrome and Sirius red stainings of the fructose mice livers. Chronic consumption of 30% fructose water had led to a visible increase in collagenous tissue as can be seen in the liver sections stained with Masson's trichrome. From the Sirius red stain in week 21, the red coloration, highlighting collagen fibres, became more widespread and intense in week 32; a clear indication that prolonged exposure to fructose deteriorated the liver condition to a worse stage of liver fibrosis.

3.2.4. Chapter Discussion

Table 2 shows a comparison of bodyweights, calorie intake and fasting blood glucose levels. Fructose and control mice groups both started out with identical average bodyweights. Over the course of the study, control group gained a lot more weight than fructose group even though average calorie intake for both groups were very much comparable. Fasting blood glucose levels were higher for the control mice during the first half of the study; this disparity was absent in the second half.

Table 2: Comparison summary of the impact of fructose

	FRUCTOSE	CONTROL
Average initial bodyweight (grams)	21.10 ± 1.76	21.11 ± 0.42
Average bodyweight @ week 21 (grams)	24.12 ± 1.64	29.40 ± 1.66***
Average final bodyweight (grams)	25.53 ± 0.62	31.89 ± 0.84***
Average cumulative bodyweight gain (grams)	3.54 ± 0.56	10.64 ± 1.16***
Average calorie Intake per week (kcal)	56.46 ± 7.24	56.38 ± 8.80
Fasting blood glucose @ week 5 (mmol/L)	3.02 ± 0.45	4.75 ± 0.63***
Fasting blood glucose @ week 13 (mmol/L)	3.26 ± 0.29	4.09 ± 0.68**
Fasting blood glucose @ week 20 (mmol/L)	3.45 ± 0.25	3.48 ± 0.60
Fasting blood glucose @ week 32 (mmol/L)	3.33 ± 0.41	3.84 ± 0.47

Week number is with reference to the weeks into feeding phase

*P < 0.05 compared to fructose group

**P < 0.01

***P < 0.001

In a separate early trial (data not shown), high-fructose feed pellet was used as the delivery mode for excess fructose. It was evident from the very beginning that the mice did not take to the fructose feed pellets well. The fructose pellets became sticky and moist within one to two days, losing their rigidity and turning into more of a mushy liquid mix than a solid pellet. Liquefied pellets transformed the cages into humid living environments where the mice were always observed to have damp furs. The fructose pellet mice ate way below their expected daily consumption amount and gradually lost weight. Mid-way into the study, the animal facility vet diagnosed those mice to be sick with the most probable cause of illness being lack of food intake. Seeing as how diet pellet for rodents were not well received by the mice, the fructose feed pellet manufacturer was queried for an answer. Apparently, there exists an adaptation protocol (attached in appendix) for the high-fructose diet to be gradually introduced to the mice over the course of four weeks. According to the manufacturer, following the recommended feeding instructions in the adaptation protocol should get the mice accustomed to the high-fructose diet pellets. Unfortunately, this crucial instruction was not made known earlier and the trial had to be discontinued due to the already-compromised state of health. Overall, fructose feed pellets put the mice off from eating and turn their living spaces into an uncomfortably moist environment. Thus, use of fructose feed pellets should be avoided to avert unnecessary health issues.

Unlike the literature review comparisons, the mice on fructose water treatment (this mice study) followed in the trends of those on fructose feed pellets; they had lower food intake than control and were not gaining much bodyweight. Nonetheless, even existing fructose water murine studies present a wide range of findings that do not align with each other in certain cases. Similarly, this study adds on to the variation spectrum of reported results. The fructose group in this study had way lower final bodyweight and an even total caloric intake when compared

to control group. This is in great contrast with the heavier fructose mice having higher total caloric intake as reported by majority of literature. Conversely, as mentioned in the literature review section, there are groups with findings that match the lower fasting blood glucose levels observed in the fructose mice of this study. With regards to the liver histology, notwithstanding the liver weights, this study is in complete agreement with past research that chronic consumption of fructose leads to rapid hepatic triglyceride and lipid accumulation.

3.3. Conclusion

The results from this murine study closely resembled observations of Non-Alcoholic Fatty Liver Disease (NAFLD). Fatty liver phenotype without weight gain, the growingly common traits of NAFLD, were clearly displayed in the fructose mice of this study. The number of NAFLD cases among the global population has been on the rise; this poses as a major concern because most afflicted with NAFLD are asymptomatic and seek treatment only when the disease has reached a critical stage. If they do show symptoms, one of which could be as subtle as the appetite loss exhibited by the fructose mice. Like the mice drinking 30% fructose water, there are no explicit signs or warnings that point to NAFLD, making it a silent killer. More attention and research are needed for fructose-induced NAFLD especially in the diagnostic front. With the prevalence of fructose in our daily foods, otherwise healthy-looking individuals need to be aware that they could be at risk of fructose-induced NAFLD if they do not make healthy diet choices.

This fructose mice study is a good demonstration of the extent to which fructose-induced metabolic syndrome can develop undetected; liver fibrosis along with increased hepatic

triglyceride and lipid storage are the very possible outcomes from excessive consumption of added fructose.

Chapter 4. Characterization of *Lactobacillus Kunkeei*

From past literature and the fructose mice study in the previous chapter, it was revealed that fructose is a major driver of obesity and diabetes. Excessive consumption of added fructose often results in fatty liver before leading to more pronounced stages of metabolic syndrome. The strategy is to remove the excessive fructose that enter the gut using probiotic FLAB. *L. kunkeei* (a probiotic FLAB) could be a promising candidate. However, it lacks characterization. As such, in this chapter, growth and metabolism of *L. kunkeei* were characterized to decide if its fructophilic nature is strong enough to qualify it to be the chosen probiotic FLAB strain for excess dietary fructose removal; the growth rate and fructose uptake efficiency of *L. kunkeei* were pitted against other common probiotic strains, *L. rhamnosus* and *E. coli* Nissle, for relative comparison. Besides its fructose metabolizing capacity, this chapter also explored other traits of *L. kunkeei* such as adaptation features towards unfavourable media and gastric tolerance to determine its survivability in gastric juice. The last part of this chapter focused on the transformation efficiency and exogenous gene expression of *L. kunkeei* to examine its suitability to be the host strain for the fatty acid biosensor genetic circuits. Since there are inadequate tools for *L. kunkeei* transformation and gene expression, plasmid backbones harbouring different origins of replication are developed for *L. kunkeei*.

4.1. Methodology & Materials

4.1.1. Bacterial Strains and Optimal Growth Conditions

Bacterial strains used were *E. coli* Nissle, *L. rhamnosus* (ATCC 7469, Virginia), *E. coli* TOP10 (Invitrogen, California), *L. kunkeei* type strain YH-15 (ATCC 700308) and *L. casei* Shirota strain from Yakult; bacterial cultures kept for long durations were stored as 50% glycerol stock (v/v) in -80°C. All five strains grew well under aerobic conditions. The two *E. coli* strains were

inoculated in 5ml of Luria-Bertani (LB) broth (Becton Dickinson and company, Franklin Lakes, New Jersey) at 37°C with shaking at 225 rpm. The *E. coli* overnight cultures would grow to saturation after 12 to 16 hours of shaking incubation. *Lactobacilli* were inoculated in 5ml of Oxoid brand De Man-Rogosa-Sharpe (MRS) broth (Basingstoke, UK), supplemented with 1% fructose (w/v), with shaking at 225 rpm; *L. kunkeei* overnight culture was incubated at 30°C and would take 20 to 24 hours to reach saturation, while *L. rhamnosus* overnight culture was incubated at 37°C for up to 20 hours. All bacterial cultures were grown as described in this section unless specified otherwise. For selective media/agar, appropriate antibiotics were added as necessary: ampicillin - 100µg/ml, kanamycin - 50µg/ml, chloramphenicol - 25µg/ml, erythromycin - 10µg/ml for *Lactobacilli* and 200µg/ml for *E. coli*.

4.1.2. Characterization of Natural *L. kunkeei*: Growth Trials

The purpose of growth trials is to observe the growth characteristics and determine the optimal fructose concentration. *L. kunkeei* was first grown for 2 days in 5ml of basal media, with 0.5% glucose & 2% fructose (w/v), 30°C with shaking at 225rpm. Basal media in this report refers to the ATCC-recommended modified Rogosa medium with liver extract and apple juice omitted because they contain variable amounts of fermentable carbohydrates that will interfere with fermentation studies [145], and the trials require known and fixed amounts of simple sugars. 250ml conical flasks were then filled with 100ml of basal media containing varying concentrations of fructose (modified Rogosa media recipe consists of 0.5% glucose). Each flask was inoculated with 1ml of the 2-day culture and placed in either 30°C (optimum) or 37°C (body temperature) with shaking at 225rpm for 6 days. OD₆₀₀ reading for each flask was taken every day of the 6 days. Each fructose concentration of both temperatures was repeated till triplicate results were obtained.

4.1.3. Characterization of Natural *L. kunkeei*: Metabolism Trials

Like the growth trials, *L. kunkeei*, *L. rhamnosus* and *E. coli* Nissle pre-cultures were grown in basal media for the first set of metabolism trials. For the second set of trials, precultures of the three probiotic strains were all grown in their optimal conditions (i.e., *Lactobacilli* in MRS, *E. coli* in LB broth). 250ml conical flasks were filled with 100ml of 0.5% glucose & 2% fructose basal media for the first set of trials; and 100ml MRS broth with 2% fructose for the second set (MRS recipe consists of 2% glucose). Each flask was inoculated with 1ml of preculture from a probiotic species. For trials in basal media, flasks were kept in a 37°C shaking incubator (225 rpm) over a 5 to 6-day period. During the incubation period, the OD₆₀₀ and glucose/fructose concentration readings were measured daily, and the difference in sugar levels each day was calculated to give the daily sugar consumption; both glucose and fructose concentration measurements were taken using MaxSignal® fructose assay kit from Bioo Scientific (Austin, Texas). For trials in MRS broth, flasks were incubated for 16 hours and measurements were taken at 4-hour intervals. Every flask underwent three repetitions.

4.1.4. Characterization of Natural *L. kunkeei*: Gastric Tolerance

An ideal probiotic would be one that can survive the passage through the GI tract, especially the acidic gastric environment, to establish itself in the small intestine and colon and thereby impart their health benefits. The simulated gastric composition and trial procedure was taken and modified from Corcoran et al. [146]. Survivability was measured in terms of probiotic concentration in the cell culture at each time point; concentration in CFU/ml was established by using the spread plate cell counting technique on agar. Gastric juice was prepared in the following steps. NaCl (2.05 g/L), KH₂PO₄ (0.60 g/L), CaCl₂ (0.11 g/L), and KCl (0.37 g/L) were dissolved in 800ml DI water and autoclaved. 18ml of syringe-filtered 1M hydrochloric acid (HCl) and 170ml of autoclaved DI water were added to the solution. Porcine bile (0.05

g/L), lysozyme (0.1 g/L), and pepsin (13.3 mg/L) were all syringe filtered and added as stock solutions to give a final pH of ~2.1. Components were all ordered from MilliporeSigma (Darmstadt, Germany).

Gastric tolerance trial for *L. kunkeei*, *L. rhamnosus* and *E. coli* Nissle followed the procedure below; After overnight incubation, optimally grown cultures of the three probiotic strains were centrifuged at 4,000 rpm and 4°C for 15 minutes (Eppendorf; Hamburg, Germany), supernatant discarded thereafter. Probiotic cell pellets were suspended and washed in 45ml of cold 0.25x Ringer's solution (MilliporeSigma). Starting/initial cell count measurements were taken at this juncture followed by centrifugation at the same settings, supernatant discarded. Cell pellets were then suspended in the simulated gastric juice, 45ml to simulate fasted state which is harsher than fed state [147]. The gastric juice cell cultures were carefully decanted into 125ml conical flasks and placed in a 37°C shaking incubator at 225 rpm for 90 minutes. Additional cell count measurements were taken at 10, 30, 60 & 90 minutes. For Yakult's *L. casei* Shirota, the entire 100ml Yakult bottle was emptied into a 250ml conical flask containing 45ml simulated gastric juice; there was no washing with Ringer's solution.

4.1.5. Characterization of Natural *L. kunkeei*: Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) was used to visualize *L. kunkeei* under higher magnification and with greater resolution to investigate the morphology difference between nascent and seasoned *L. kunkeei*, and analyze the filamentation undergone by the seasoned strain. *E. coli* Nissle was also imaged for reference. SEM sample fixation and dehydration protocol was taken from 'Alternate Protocol 6' of Fisher et al. [148]; it is an alternative protocol that uses chemical dehydrant Hexamethyldisilazane (HMDS) when critical point dryer is unavailable. Subsequently, sputter coating was done using platinum and samples were

visualized under JSM-6700 Field Emission Scanning Electron Microscope from Jeol (Akishima, Tokyo).

4.1.6. *E. coli* Top10 Transformations

All *E. coli* transformations were carried out using heat shock treatment on chemically competent cells. Subsequently, transformed cultures were plated on LB agar plates with selective antibiotics for incubation. Successful transformant colonies were picked from the agar plates and grown in overnight cultures with the appropriate antibiotic concentrations. Plasmids were then extracted from the colony overnight cultures using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and sent for DNA sequencing to verify plasmid DNA. Sequencing service is provided by 1st base (Singapore).

4.1.7. *L. kunkeei* Transformations

L. kunkeei has poor transformation efficiency. Assembled plasmid construct volumes need to be amplified by a first transformation into *E. coli* Top10 strain. The purified plasmids acquired from the first transformations were then transformed into competent *L. kunkeei* cells. Typical electrotransformation methods of *Lactobacillus* spp. all share a similar format. The protocols for competent cell preparation and electroporation in this project were modified from Aukrust et al. [149], Maddaloni et al. [53] and Wei et al. [150] To prepare electrocompetent cells, *L. kunkeei* overnight culture was used to inoculate 50ml of 1% fructose MRS broth and incubated for 5 hours to catch cells in exponential growth phase. After the 5 hours of incubation, OD₆₀₀ value of 50ml culture was checked to be at least 1.25. Subsequently, the 50ml culture was used to inoculate fresh 1% fructose MRS broth, containing 1.5% glycine (w/v), to give 125ml total volume with OD₆₀₀ value of 0.25. This 125ml preculture was incubated for slightly less than 2 hours to give an OD₆₀₀ value of at least 0.6. Moving forward, the 125ml preculture was

centrifuged at 4,000 rpm for 5 minutes at 4°C, supernatant was discarded. The remaining *L. kunkeei* cell pellets were washed four times to remove all traces of ions from the growth media, twice with 90ml of ice-cold DI water and centrifuged at the same settings; the next two washes with 90ml of ice-cold 0.3M sucrose media and centrifuged at 4,000 rpm for 10 minutes at 4°C. Finally, the cells were suspended in 1ml of sucrose media with 20% glycerol (v/v) and aliquoted into microcentrifuge tubes to be stored in -80°C; 100ul of *L. kunkeei* competent cell suspension per tube. Throughout the entire preparation procedure, great care was taken to ensure that the cells were kept at 4°C or on ice at all times.

Right before electroporation, *L. kunkeei* electrocompetent cells, plasmid DNA and 2mm electroporation cuvette were all placed on ice. After the competent cells were thawed, 2-3ul of 0.1-1ug/uL plasmid DNA (DNA to be dissolved in Tris-EDTA buffer) was added to the cell suspension and mixed. The entire cell suspension volume was then transferred to the electroporation cuvette without trapping any air bubbles. Next, an electric pulse was delivered to the cuvette at the following electroporator settings: 2.5kV, 25uF & 400ohms; Gene Pulser Xcell™ Electroporation Systems from Bio-Rad (Hercules, California) was the electroporator in use. Immediately after electroporation, 900ul of MRS broth with 1% fructose & 0.3M sucrose was added to the cell suspension as recovery media and the entire volume was incubated at 30°C for 3 hours in a microcentrifuge thermoshaker (Eppendorf) at 900rpm. Subsequently, the cell suspension was spread onto 1% fructose MRS agar plate with 10ug/ml erythromycin and left in a 30°C incubator for a few days. *L. kunkeei* transformant colonies typically appear within 2 to 3 days of incubation.

4.1.8. Plasmid Construction

For each plasmid construction, Gibson assembly was performed according to plasmid designs modified using Benchling (<https://benchling.com>) [151]. The DNA assemblies were carried out using NEBuilder HiFi DNA assembly reaction (New England Biolabs) following manufacturer's recommendations. Assembly fragments to be ligated together are the PCR amplified products of the target gene sequences with their overhangs; PCR was carried out using Q5 PCR kit by New England Biolabs. The PCR products were put through 1% agarose gel electrophoresis followed by gel extraction using QIAquick gel extraction kit from Qiagen. The fragments then had their concentrations measured using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) to determine the volume of each component to add into the single-tube assembly reaction. After assembly, the ligated product was transformed into TOP10 so that the purified plasmid construct can be isolated and verified by DNA sequencing.

The plasmid backbones used for *L. kunkeei* transformations were pTRKH3-ermGFP & pTRKH3-slpGFP by Addgene (Cambridge, Massachusetts, USA) [152], pMG36e by Dr. Jan Kok from University of Groningen (Groningen, the Netherlands) [153], and pNucA by Geir Mathiesen from Norwegian University of Life Sciences (Norway). pMG36e was used in the first-ever *L. kunkeei* transformation [53], while the second *L. kunkeei* transformation on record used plasmid pNucA [94]. The origins of replication for pMG36e, pNucA and pTRKH3 plasmid backbones are pWV01, pSH71 and pAM β 1 respectively; all confer erythromycin resistance. Foreign genes, which were cloned into the selected plasmid backbones, were taken from pBR322 by New England Biolabs (Ipswich, Massachusetts, United States), pBbE8k & pBbE8a-RFP & pBbA8c-RFP plasmids (Addgene) belonging to the Biobricks vector database [154]. Oligonucleotide primers were supplied by Integrated DNA Technologies (Coralville, Iowa, USA).

To verify workable genetic parts and measure plasmid expression levels, red fluorescence protein (RFP) reporter gene from pBbE8a-RFP was assembled into the open reading frame of pMG36e, just upstream of the terminator sequence, to give pMG-RFP; default Ribosome Binding Site (RBS) from Biobricks database was further inserted into pMG-RFP to create pMG-deRBS-RFP. Subsequently, RBS K3 (BBa_K1841003) & K12 (BBa_K1841012) from iGEM parts registry were also inserted into pMG-RFP. pMG-15A-RFP was constructed by having the whole cassette length of ‘default RBS-RFP-BBa_B0015 terminator-p15A origin’ from pBbA8c-RFP cloned into pMG36e in the place of its native terminator. The additional p15A *E. coli* origin of replication would enable constructs to act as shuttle vectors between *E. coli* and *Lactobacillus*. ColE1 from pBbE8k and pMB1 from pBR322 are the two other alternative *E. coli* origins of replication selected to replace p15A in pMG-15A-RFP, thus creating pMG-E1 and pMG-MB1 respectively.

All the plasmid constructs above hold only pWV01 as the sole gram-positive origin of replication. Construct variants of the other two gram-positive origins were created by replacing pWV01 in the plasmid constructs above with pSH71 and pAM β 1 origins of replication.

4.1.9. Characterization with M9 & MRS Media

All characterization measurements of plasmid constructs in *E. coli* and *L. kunkeei* transformants were carried out in BioTek’s Synergy^{TM} HTX Multi-Mode microplate reader (Winooski, Vermont, United States). Overnight cultures of transformed *E. coli* and *L. kunkeei* strains were used to inoculate fresh M9 medium and MRS broth, with antibiotics, respectively for one to three hours of exponential outgrowth with shaking. M9 medium was prepared by mixing 200ml 5x M9 media with 2ml 1M MgSO₄, 20ml 20% glucose, 100ul 1M CaCl₂ and

100ml of 2% casein hydrolysate, before topping up to 1 liter with distilled water; each component was individually filter sterilized or autoclaved before being added to the mix. When OD₆₀₀ of the outgrowth cultures reached between 0.1 to 0.3, they were aliquoted into 96-well plates for microplate characterization, 300ul per well. Each plasmid construct or sample group had 3 wells allocated to it for triplicate readings. OD₆₀₀, RFP fluorescence reading (excitation 540 nm, emission 600 nm) & RFP/OD were the measurements taken at regular time intervals while the cultures were incubating inside the microplate reader at 30°C for 12 hours for *L. kunkeei*; or 37°C for 8 hours for *E. coli*. In every microplate run, negative controls were used as blank measurements to account for background readings.

4.1.10. Statistical Analysis

All data are reported as mean $\pm$ standard deviation. Unpaired t-test was used to determine statistical significance. Results with *P*-value < 0.05 were considered to be statistically significant.

4.2. Results & Discussions

4.2.1. Growth and Metabolism Characterization of *L. kunkeei*

Referring to Figure 11, there is minor difference in the growth profiles for both temperatures and also between varying fructose concentrations. For the former, the sole difference is the higher initial growth rate that cultures in 30°C experience which supports the understanding of 30°C being the optimal temperature for *L. kunkeei*. With regards to the effect of fructose concentration, the early stagnation of cultures without fructose in both temperatures suggests that fructose is used as an external electron acceptor to improve growth in cases of co-fermentation, as mentioned in Endo et al. [93] However, higher fructose does not necessarily give greater growth seeing that variations in fructose concentration did not give a variety of

growth profiles. In fact, several pre-trials have seen the OD₆₀₀ readings of 4% fructose cultures to be significantly lower than 2% fructose cultures. There is no optimal fructose concentration for *L. kunkeei* growth; fructose availability is the main factor.

Figure 11: Growth trials of *L. kunkeei* YH-15 type strain in varying concentrations of fructose (F)
Error bars denote SD (n = 3). All flask cultures contain 0.5% glucose.

To corroborate the use of *L. kunkeei* as the probiotic FLAB for this project, its fructose metabolism capacity had to be validated. Glucose concentration and fructose consumption were the two additional measurements taken for the sugar metabolism trials which also included *E. coli* Nissle and *L. rhamnosus* for comparison. The incubating temperature was

37°C to simulate conditions in the GI tract; basal media was once again the growth medium because it is not favourable towards any one probiotic strain.

Figure 12: *L. kunkeei* metabolism trials with 0.5% glucose & 2% fructose basal media

Graphs A,B,C&D used seasoned *L. kunkeei* while graph E used nascent *L. kunkeei*. Graphs A,B,C are comparisons of one measurement across all 3 probiotic strains while graphs D&E are compilations of all 3 measurements in *L. kunkeei*. All trials were conducted in 37°C; all error bars denote SD (n = 3). [A] shows daily fructose consumption in grams /litre. [B] shows daily OD₆₀₀ readings. [C] shows daily glucose concentration (not consumption) in grams/litre. [D] & [E] both show glucose and fructose consumption levels following the left vertical axes; and OD₆₀₀ values following the right vertical axes.

Mid-way through the first set of metabolism trials, *L. kunkeei* presented significant discrepancies in measurement readings of certain flasks. Figure 12(D) and Figure 12(E) clearly presented two distinct growth and metabolism profiles of *L. kunkeei*. Figure 12(D) belongs to a strain that had been through several 6-day metabolism trials and thus was so labeled ‘seasoned’. Figure 12(E) belongs to flasks inoculated directly from the overnight culture of the ATCC *L. kunkeei* glycerol stock and therefore labeled ‘nascent’. In basal media, seasoned *L. kunkeei* has higher growth and nearly double the fructose consumption compared to the nascent strain.

Regardless of seasoned or nascent, *L. kunkeei* has the evident lead on fructose consumption by a long shot albeit the poor growth rate compared to *L. rhamnosus* and *E. coli* Nissle. Another stark difference is the sugar preference in the consumption patterns. *L. kunkeei* metabolized both glucose and fructose concurrently even though it is considered fructophilic; probably due to fructose acting as electron acceptors for glucose metabolism. On the other hand, *E. coli* and *L. rhamnosus* showed immense preference for glucose when both finished the 0.5% glucose (5g/L of glucose) but did not reach the same level for fructose consumption; *E. coli* barely consumed any fructose at all.

With impressive fructose consumption levels in a media unfavourable for growth, it would be of great interest to explore the full metabolism potential of *L. kunkeei*. Thus, the growth media was switched to MRS broth for the second set of metabolism trials since it is a medium specifically for *Lactobacillus*. Unfortunately, glucose concentrations cannot be measured in MRS broth; a proper glucose standard curve for MRS broth was unattainable. It is possible that the ‘Lab-Lemco’ powder, a type of meat extract, in the MRS broth formula contains a variable amount of glucose that creates fluctuations in the readings [145].

Figure 13: *L. kunkeei* metabolism trials with 2% fructose MRS broth

All trials were conducted in 37°C; error bars denote SD (n = 3). All graphs show fructose consumption levels following the left vertical axes; and OD₆₀₀ values following the right vertical axes.

For this second set of metabolism trials, both nascent and seasoned *L. kunkeei* took up all the fructose added into the media (~20g/L) and grew well enough to outgrow *E. coli*, all within 16 hours. Both strains of *L. kunkeei* exhibited identical growth and metabolism profiles in MRS broth with the nascent strain edging out just a little in growth. Meanwhile, the other two probiotic strains had zero fructose consumption; probably because glucose provided in the MRS broth formula was sufficient for them.

The two sets of metabolism trials have strongly validated the efficiency of *L. kunkeei*'s fructose metabolism capability regardless of the growth media used. Even so, a new strain of *L. kunkeei* was born out of the basal media metabolism trials with traits that help it adapt better to a harsher basal media environment but confer no advantage when placed in a different growth medium.

4.2.2. Nascent vs. Seasoned *L. kunkeei*

To ascertain if the 'seasoned' strain is indeed *L. kunkeei* and not a random contamination, 16S rRNA sequencing (AITbiotech, Singapore) of the strain was carried out and matched with sequences in National Center for Biotechnology Information (NCBI) genebank database via a BLAST search. The result came back with 100% identity match to *L. kunkeei* MP2 strain (CP012920), YH-15 rRNA gene and a few other *L. kunkeei* strains. It would be safe to infer that the 'seasoned' strain was indeed *L. kunkeei* YH-15 strain that underwent some point mutations.

Careful observation under light microscope revealed seasoned cells having huge variations in length with many longer than 10µm while nascent cells are mostly smaller than 2µm; seasoned cells have also flocculated to form many small clusters in what may be presumed to be a stress response to the unfavourable basal media. Similar behavior was recorded in Lemay et al. [155] and Ingham et al. [156] when cells are faced with environmental stress. The elongation of cells is termed as filamentation whereby there is continuous cell growth but inhibited septation. Interestingly, these elongated seasoned cells can revert back to normal cell length when placed in favourable MRS media; likewise, clustering was also observed to be reduced. Perhaps *L. kunkeei* is able to switch back and forth between strains in response to different environments. Whichever the case, *L. kunkeei* nascent strain will be used for all future experimentation purposes.

Figure 14: SEM images of *L. kunkeei*

[A] is seasoned strain. [B] is nascent strain. Both images are at 7,500x magnification

In the SEM images, the filamentous seasoned cells are seen to be long enough to twist and curl around each other. With the use of the measurement scale at the bottom right hand of each image, the shortest seasoned cell was determined to be 1.41µm and the longest that can be measured is 3.921µm; there are many cells that extend beyond the image and would most certainly be longer than the two lengths recorded. On the other hand, nascent cells in Figure 14B are all smaller than 2µm.

4.2.3. Gastric Tolerance

The simulated gastric juice used in this project is a strong antimicrobial medium that killed off all *L. kunkeei* and *L. rhamnosus* cells before the 10-minute mark; even the robust *E. coli* Nissle completely died off by the 30-minute mark. Once more, visible flocculations were seen in the nascent *L. kunkeei* culture though no survivor colonies appeared on the agar plate. It could very well be that this simulated gastric juice has greater potency than the actual one within the human stomach. Nonetheless, this gastric tolerance trial established the need for encapsulation or at least a protective medium for the probiotic to survive through the real gastric acid.

On the flip side, Yakult's *L. casei* Shirota cells survived very well till the 90-minute mark when buffered by its accompanying milk culture. Unfortunately, the surviving cell count was underestimated and the resulting agar plate was too overcrowded with overlapping colonies. Indeed, this serves to prove the efficacy of the high-sugar Yakult milk culture as a buffer against gastric acid.

Figure 15: Gastric tolerance trial without added sugars
Concentration of surviving cells given in logarithmic value.

4.2.4. *L. kunkeei* Transformations

Starting off with pMG36e plasmid constructs, the visible RFP expression of pMG-deRBS-RFP and negligible expression of pMG-RFP in Top10 are clear indications that promoter P32, native promoter of pMG36e, and deRBS function well in *E. coli*. According to Van et al. [153], there is a native RBS present in the plasmid pMG36e. Also, considering that P32 is working yet pMG-RFP has no expression, it is safe to say that native RBS does not function well in *E. coli*.

Figure 16: pMG36e plasmid constructs characterizations in *L. kunkeei* and *E. coli* Top10

[A] and [B] are characterizations for *L. kunkeei* at 30°C while [C] and [D] are characterizations for Top10 at 37°C. Error bars denote SD (n=3). Even though [A] and [C] both measure RFP production, their vertical axes value ranges are vastly different.

Although p15A origin of replication boosted gene expression in *E. coli*, several attempts to transform pMG-15A-RFP into *L. kunkeei* have failed. It was suspected that p15A, being the second replication origin, interfered with the plasmid replication mechanism in *L. kunkeei* transformants. Unfortunately, the other pMG36e constructs failed to express RFP in *L. kunkeei*, leading to the initial assumption that it was the promoter and RBS that did not function in *L. kunkeei*. Concurrently, a seemingly unrelated issue arose in the form of inconsistent sequencing results of plasmids extracted from *L. kunkeei*. The sequencing reports often yielded low strength readouts accompanied by heavy contamination. When queried, the sequencing service provider, Axil Scientific, had the following in their reply: *Low plasmid concentration is the root cause of early signal loss; and when the software attempts to compensate by boosting up*

the weak signal of sample bands to detectable levels, background noise is also artificially amplified. Thus, giving the impression that the sample is heavily contaminated. Further reading led to a past study [157] that claimed pWV01, the replication origin for pMG36e, as having low copy numbers in gram-positive bacteria. This finding reasonably explains both the low plasmid concentration and lack of RFP expression from pMG36e plasmid constructs in *L. kunkeei*.

To remedy the low copy number of pWV01 and possible p15A incompatibility, alternative gram-positive and *E. coli* origins of replication were sourced out. These replication origins were paired up into varying combinations within the pMG36e plasmid backbone to be transformed into *L. kunkeei*; as can be seen in Table 3, only 4 combinations were successful. Since lack of gene expression is part of the issue and deRBS is originally intended for *E. coli*, another remedy attempted was to improve RBS functionality by replacing deRBS in pMG-deRBS-RFP with K3 and K12 RBS; pMG-K3-RFP and pMG-K12-RFP were both successfully transformed into *L. kunkeei* as well. Despite the best efforts, none of the construct variants in *L. kunkeei* were able to express detectable levels of RFP.

There remains a number of *Lactobacillus*-compatible promoters and RBS [158-160] yet to be tested out. However, it may just be better to switch out *E. coli* Top10 for *Lactococcus lactis* for plasmid purification and amplification before transformation into *L. kunkeei*. Unlike *E. coli*, *L. lactis* and *L. kunkeei* share the same phylum, and are closely related in the phylogenetic tree.

Table 3: Table of all origins of replication used

Gram Positive Origin	<i>E. coli</i> origin	<i>E. coli</i> transformation	<i>L. kunkeei</i> transformation
pWV01	NA	☑	☑
pWV01	p15A	☑	
pAM β 1	NA		
pAM β 1	p15A	☑	
pSH71	NA		
pSH71	p15A	☑	
pWV01	ColE1	☑	☑
pSH71	ColE1	☑	
pAM β 1	ColE1	☑	☑
pWV01	pMB1	☑	☑
pSH71	pMB1		
pAM β 1	pMB1		

4.3. Conclusion

This chapter established the fructophilic nature of *L. kunkeei*, solidifying its place as the probiotic of choice for the purpose of reducing the impact of excess dietary fructose. Furthermore, two other characteristics of *L. kunkeei* give it an additional edge over other probiotic strains: absence of the typical preferential glucose uptake & its adaptation features in an unfavourable growth environment; *L. kunkeei* will always uptake fructose regardless of the glucose content of the diet & this fructose uptake rate will rise if the gut environment becomes adverse for growth.

It was observed that *L. kunkeei* transformants did not have detectable levels of exogenous gene expression. The problem could reside in transformation efficiency or copy number or even any of the operon genetic structures. While it is evident that engineering *L. kunkeei* would be challenging, there are still alternative origins of replication, genetic promoters and RBS that could be more compatible with *L. kunkeei*. Nonetheless, *E. coli* Nissle was chosen as the probiotic host strain for the fatty acid biosensors since it is a well-established probiotic strain that has the proven capability of delivering heterologous therapeutics directly to the human gut. The engineered Nissle could be combined with the natural *L. kunkeei* to make up the co-culture treatment in the proposed long-term strategy; *L. kunkeei* is responsible for removal of excess dietary fructose while engineered Nissle releases appetite suppressant in response to high dietary SFA.

Chapter 5. Development of Fatty Acid Biosensor

This chapter presents the development of the genetic SFA biosensor using probiotic *E. coli* Nissle. With the unresolved issue of gene expression in *L. kunkeei*, *E. coli* Nissle was chosen as the probiotic host strain for the fatty acid biosensor genetic circuits. The final segment of the thesis is to develop a SFA biosensor in the form of a genetic regulation circuit system for the future goal of tethering the release of appetite suppressant to an individual's diet SFA content. The hypothesis for this chapter is that the probiotic Nissle biosensor would release an appetite suppressant upon detection of high concentrations of SFA. To this end, the SFA biosensor was developed by engineering synthetic promoters which utilize the FabR/FadR mechanism. A number of different promoters were explored and characterized in both single-plasmid and split-plasmid designs. Response of the synthetic FabR/FadR sensor systems to exogenous fatty acids were measured over time and their steady-state endpoint expression levels juxtaposed for comparison; impact of FabR/FadR on repression levels was also assessed.

Of the few microbial fatty acid biosensors that have been reported, most are designed for cell factory applications like high-throughput screening of Short- and Medium-Chain Fatty Acid (SMCFA)-producing cell factories [161, 162], or regulating bioconversion circuits and microbial production of biofuels by dynamically controlling the enzyme activity levels [137, 138, 163]. To date, biosensors from past studies detect fatty acids in general, but none so far can respond specifically to SFA and distinguish SFA from a mixture of fatty acids. Additionally, none of the existing fatty acid biosensors are developed using probiotic strains. Even though there was no FadE gene knockout for the Nissle strain used in this thesis, the other experimental conditions were adapted from these past studies as much as possible. The results obtained in this chapter demonstrated that a design of the promoter system is able to detect SFA with up to

~8-fold change in output; system response is equally distinguishable even with the additional presence of UFA. Distinction for this chapter would arise from unique genetic circuit components and a few nuances in characterization methods. Overall, one of the most prominent significance, apart from SFA detection, is the use of the well-established probiotic Nissle strain, rather than laboratory-testing strains, to house the fatty acid biosensor genetic circuit system.

5.1. Methodology & Materials

E. coli Nissle was used as the host strain for the biosensor genetic circuits. Its growth condition for this chapter was 5ml LB broth with shaking incubation at 37°C, 225 rpm for 12 to 16 hours as described in the previous chapter, section 4.1.1; 100µg/ml kanamycin and 50µg/ml chloramphenicol were added for selective purposes.

5.1.1. *E. coli* Nissle Transformations

Similar to *L. kunkeei* transformations, only purified plasmids amplified by *E. coli* Top10 were used for transformation into *E. coli* Nissle, which was also carried out by heat shock treatment. For the split-plasmid genetic sensors, multiple plasmids were introduced into Nissle cells through sequential transformation.

5.1.2. Plasmid Construction

All plasmids were constructed according to the Gibson assembly method described in section 0; PCR amplified fragments with overhangs were assembled using NEBuilder HiFi DNA assembly reaction. Starting with the Biobricks backbone vector pBbE6k-RFP, inducible pLac promoter was replaced with the strongest Anderson constitutive promoter, BBa_J23100. FabR-binding operator sequences from FabA and FabB genes were inserted at two locations; either

between -35 and -10 regions of BBa_J23100, or downstream of the Anderson promoter's transcription start site. The former was assigned the number suffix 35 to its name while the latter were given the number suffix 10. Therefore, there are four construct variants, A10 & A35 plasmids containing FabA gene operator sequences, and B10 & B35 plasmids containing FabB gene sequences.

Figure 17: pLac promoter replaced with FabR promoters

Subsequently, FabR and FadD gene cassettes were cloned into the FabR sensor circuit within a single plasmid as illustrated in the figure below. FabR gene (NCBI Gene ID: 948460) from *E. coli* K12 was placed under the control of pLac promoter; the repressor expression is induced by IPTG. On the other hand, the acyl-coA synthase FadD gene (NCBI Gene ID: 946327) was placed under another constitutive Anderson promoter, BBa_J23101. The final plasmid constructs for the fatty acid biosensor single-plasmid design are FabR-A10D, FabR-B10D, FabR-A35D and FabR-B35D.

Figure 18: FabR sensor single-plasmid design

Purple block(s) are the unmodified elements of the plasmid vector backbone. pBB deRBS and BBa_B0015 are the common RBS and transcriptional terminators used in this design.

The next stage was to split the sensor circuit into two separate plasmids. pBbE6k vector backbone together with the RFP modules under their FabR/FadR promoters as the first plasmid constructs; pBbA2c vector backbone together with the FabR/FadR and FadD gene modules as the second plasmid constructs. LacI gene cassette was removed from pBbE6k vector before the insertion of FabR/FadR promoters. Similar to the single-plasmid design, pLac was swapped out for BBa_J23100 constitutive promoter. However, repressor operator sequences were only cloned downstream of the transcription start site for the RFP modules of this circuit design. A10 and B10 were the chosen FabR promoters, while FadR binding operator sequences came from FadH and FadBA genes. The resulting plasmid constructs with the RFP modules are RFP-A10, RFP-B10, RFP-FadH and RFP-FadBA.

The second plasmids were created by assembling p15A origin of replication and chloramphenicol resistance gene from pBbA2c, together with FabR/FadR and FadD gene cassettes. For the split-plasmid design, FabR/FadR and FadD genes were all placed under

constitutive promoters. FabR/FadR gene modules include BBa_J23101 while FadD module has P26 promoter. Due to assembly constraints, BBa_B0034 RBS was used to replace the common pBb deRBS in FadD cassette. The FadR gene sequence selected is from *E. coli* K12 with NCBI nucleotide accession no. X08087.1. The second plasmid constructs in this design were simply named FabR and FadR.

Figure 19: FabR/FadR sensor split-plasmid design
Purple block(s) are the unmodified elements of the plasmid vector backbone.

5.1.3. Characterization with M9 & Rich Broth (RB) Media

To characterize the fatty acid biosensor circuits, oleic acid (O1008, Sigma) was chosen as the UFA and stearic acid (S4751, Sigma) as the SFA. Both are long-chain fatty acids with each chain made up of 18 carbon atoms. Oleic acid [stearic acid] was dissolved in ethanol to a stock concentration of 1M [500mM]. This stock dilution was neutralized with 1M [500mM] NaOH at 70°C [85°C] to give 500mM [250mM] neutralized oleic acid which was subsequently diluted in 1% [1%] tergitol (NP40S, Sigma) M9 to 0.5mM [0.5mM]. M9 was pre-warmed to 55°C

[80°C] before the addition of neutralized fatty acids; antibiotics were added in after the M9 medium had cooled.

Step by step procedures on M9 medium preparation and the use of microplate reader for Nissle characterizations are similar to section 4.1.9. M9 with fatty acids dissolved was used as the outgrowth medium for characterization of the *E. coli* Nissle strain with the genetic sensor plasmid constructs. IPTG was added to the outgrowth culture, as an inducer for the single plasmid biosensor constructs, right before the culture was spilt into the 96-well plates for microplate characterization. RFP fluorescence reading (excitation 540 nm, emission 600 nm) & RFP/OD were the measurements taken at regular time intervals while the cultures were incubating inside the microplate reader at 37°C for 8 hours.

A second method employed for characterizing the fatty acid biosensor circuits uses RB medium [138]. RB medium consists of 10g tryptone, 5g NaCl and 1g yeast extract in every litre. Fatty acids were dissolved in RB via the same procedure as how it was done for M9. Overnight cultures of transformed *E. coli* Nissle strains were used to inoculate fresh RB medium with dissolved fatty acids. The RB medium subcultures were incubated at 37°C with shaking for 24 hours before an endpoint microplate read. When needed, IPTG was added between the 3 to 4-hour marks of the subculture incubation.

5.1.4. Statistical Analysis

All data are reported as mean $\pm$ standard deviation. Unpaired t-test was used to determine statistical significance. Results with P -value < 0.05 were considered to be statistically significant.

5.2. Results & Discussions

As expounded in the literature review and Figure 5, FadR represses gene expression by binding to its DNA operator site; expression is then derepressed when this binding is antagonized by acyl-CoA from UFA and SFA [129, 132, 133, 136]. On the other hand, presence of UFA-CoA (from UFA) increases the affinity of FabR to its operator site within the target gene, leading to greater transcriptional repression of the regulated gene. Whereas, presence of SFA-CoA (from SFA) reduces the affinity of FabR for its operator site, resulting in derepression of gene expression [131]. According to their working mechanisms, the expected response of the FabR and FadR sensor systems are presented in the logic truth table below.

Table 4: Biosensor system logic truth table

UFA	SFA	FadR promoter	FabR promoter
L	L	L	M
H	L	H	L
L	H	H	H
H	H	H	M (depending on UFA:SFA ratio)

Fatty acid column represents the level of fatty acids available to the sensor system

Promoter column represents the level of RFP expression under the control of that promoter

L – Low; M – Moderate; H – High

5.2.1. FabR Single-Plasmid Characterization

The first sensor constructs were built with all components housed in single plasmids. FabR was the only repressor used for this initial biosensor design because FabR is relatively more novel compared to FadR and has unique responses to both types of fatty acids; making it the ideal candidate to discriminate between UFA and SFA. The aim of this characterization is to study

and compare the performance of each construct in the presence of oleic acid and stearic acid, and under varying levels of FabR repression.

A**FabR-A10D characterization in Nissle**
IPTG: 0, no FabR **FabR-A10D characterization in Nissle**
IPTG: 3uM **FabR-A10D characterization in Nissle**
IPTG: 6uM **B****FabR-B10D characterization in Nissle**
IPTG: 0, no FabR **FabR-B10D characterization in Nissle**
IPTG: 3uM **FabR-B10D characterization in Nissle**
IPTG: 6uM

Figure 20: FabR single-plasmid Nissle characterization in M9 media

Each row belongs to a unique plasmid construct variant of the single-plasmid sensor design. Colour coding is as follows: red – M9 without fatty acids; green – M9 with 0.5mM oleic acid; yellow – M9 with 0.5mM stearic acid; blue – M9 with 0.5mM oleic acid and 0.5mM stearic acid. FabR expression is induced by IPTG. Error bars denote SD (n=3). The value ranges for vertical axes differ for each graph.

Nissle experienced extremely slow initial growth when the M9 media contained only stearic acid; this phenomenon is reflected in the elevated initial RFP/OD readings and slow OD value climb rates for all stearic acid only M9 cultures in Figure 20. Interestingly, this was not seen when the M9 media contained both oleic and stearic acid. One possible explanation is that growth and survival of bacteria relies heavily on the biophysical properties of their membrane phospholipids, which are determined by the proportion of UFA available [130, 131]; having both oleic acid and stearic acid present in the M9 media will not disturb the UFA proportion as much as having just stearic acid in the M9 media. Hence, stearic acid only M9 cultures were the only ones experiencing slow initial growth rates.

Two peculiar observations can be made from the results presented in Figure 20. First is with reference to the graphs under the leftmost column, there was repression of RFP expression even when there was no repressor (IPTG=0). Since pLac is considered to be a tight promoter, any repression activity could be from Nissle's own fatty acid regulatory system in response to exogenous UFA and SFA.

The second observation is made when comparing along the rows, one row at a time. All four sensor constructs have fairly similar values and patterns of RFP/OD readouts regardless of FabR concentration. It seems that FabR has minimal impact on the gene expression of the sensor circuits in M9 characterization media. Presence of FabR resulted in at most a two-fold change in readings. Also, oleic acid did not contribute much to FabR repression while stearic acid did not antagonize FabR binding as expected; in fact, more than half of the graphs presented oleic acid only M9 cultures with RFP/OD readout values close to that of M9 cultures without fatty acids while most stearic acid only M9 cultures had the lowest readout values at the later durations. On the other hand, it could be the growth rather than gene expression that

was affected by FabR and the fatty acids. Either ways, it is highly likely that the issue resides with the characterization media. Therefore, RB media endpoint characterization method was carried out and its result compared alongside that of M9 characterization. FabR-A10D and FabR-B10D sensor constructs were chosen for the comparison because FabR-A35D and FabR-B35D displayed inconsistent results over repeated characterization trials.

Figure 21: FabR-A10D & FabR-B10D Nissle endpoint characterization in M9 & RB media

Graphs in blue gradient on the left represent FabR-A10D readings while those in red gradient on the right represent FabR-B10D readings. [A] to [D] test the 0.5mM oleic acid combinations while [E] to [H] test the 5mM oleic acid combinations. FabR expression is induced by IPTG. Error bars denote SD (n=3). Vertical axes value ranges are same for every graph. Wordings above each set of result are the type of fatty acids that particular sensor construct can sense under that given IPTG concentration, “expected” means result resembles expectations presented in Table 4; those wordings are colour coded to the construct variant they represent.

The steady-state endpoint readings from Figure 20 were used in the results comparison in Figure 21. RB media endpoint characterization method was adapted from Zhang et al. [138]. 5mM stearic acid is not fully soluble in both M9 and RB characterization media, hence 0.5mM stearic acid was the only concentration used. Looking at Figure 21, stearic acid raised RFP/OD endpoint readouts for RB cultures in the absence of FabR and oleic acid had the same effect too when it was increased from 0.5mM to 5mM. This impact from the exogenous fatty acids was too pronounced to be classified as a response from Nissle's internal regulatory system. Thus, it was initially presumed to be the effect of the fatty acid imbalance on bacterial growth. However, that idea too was dispelled with a quick check on the growth of RB cultures revealing their OD₆₀₀ values to be higher without FabR than in the presence of FabR. Overall, RB cultures experienced much greater RFP repression from FabR compared to M9 cultures. Whereas, M9 cultures of FabR-A10D and FabR-B10D had little variation in endpoint values across FabR concentrations (IPTG induction concentrations) for both oleic acid concentration combinations. This could be the outcome of RFP expression that took place before the concentration of FabR could reach a critical level for RFP transcriptional repression to take effect.

Fatty acid was considered detectable by the sensor system if the sensor had a distinct endpoint readout in response to that one fatty acid type; the readout needs to be equally distinguishable even if both UFA and SFA were present. Even though SFA and UFA were detectable at 0.5mM oleic acid combinations, the pattern of results changed at a higher concentration of oleic acid and different FabR concentrations. Furthermore, SFA was only detectable when FabR was supposedly absent (IPTG=0), which meant that the repressor had nothing to do with the sensing of SFA unless there was an overlap of transcriptional processes from other gene cassettes in the sensor circuits.

The sensor gene expression readouts and type of fatty acid detectable by each construct variant vary too much according to the concentration of FabR and fatty acid present in the characterization media. To eliminate the possibility of overlapping transcriptional processes and RFP amassing before repression effect, the development of fatty acid biosensors proceeded to the split-plasmid design stage; involving both FabR and FadR repressors this time round.

5.2.2. FabR & FadR Split-Plasmid Nissle Characterization

In the split-plasmid design, the repressor and RFP expressing gene cassettes were separately housed in a plasmid each, isolating their respective transcriptional processes. Additionally, inducible promoters were replaced with constitutive ones for the purpose of removing the delay in repressor expression, which allows RFP to amass before repressor has any impact. Said delay masks the true extent of repression that the sensor system faces which could very well be the key reason for the inconsistencies in the initial single-plasmid design. Similar to the previous section, this characterization compared the endpoint readouts of sensor constructs in RB media.

Figure 22: FabR & FadR split-plasmid Nissle endpoint characterization in RB media

[A] represents the responses from the FadR circuits while [B] represents the responses from the FabR circuits. Error bars denote SD (n=3).

Split-plasmid Nissle characterization in M9 media yielded low RFP/OD readout values and no distinctive patterns to classify any of the circuit combinations as fatty acid sensors. On the other hand, characterization in RB media presented a clear picture. One glance at Figure 22, one can identify FadR circuit combinations as UFA sensors, and FabR circuits as SFA sensors. For FabR circuits, presence of stearic acid resulted in a very distinctive RFP/OD readout. Endpoint values for stearic acid only RB cultures were significantly greater than RB cultures without fatty acids even when compared to the elevated readouts of oleic acid RB cultures, $p < 0.001$; [stearic acid] had three fold or higher RFP/OD than both [oleic acid] and [no fatty acid] for FabR sensors. The endpoint values were still easily distinguishable when RB cultures had both oleic and stearic acid; [oleic & stearic acid] had more than twice the RFP/OD values of [oleic acid] and [no fatty acid] for FabR sensors. Contrary to the expected results in Table 4, FabR

circuits did not respond to exogenous UFA for this RB characterization could be probably because the *E. coli* type II FAS pathway provided endogenous UFA that already enhanced FabR binding to operator sites.

Proceeding along to FadR circuits, RFP-FadH + FadR circuit combination was ruled out because its raw RFP readings were too weak to qualify it to act as a fatty acid biosensor of any sort. As for RFP-FadBA + FadR, its oleic acid only RB culture and oleic/stearic acid RB culture had endpoint readouts close to each other; [oleic acid] and [oleic & stearic acid] both had nearly double the RFP/OD value of [stearic acid], and more than triple the value of [no fatty acid].

Figure 23: FabR & FadR split-plasmid Nissle endpoint characterization in RB media – dose response [A] is the results for oleic acid RB media; [B] is the results for stearic acid RB media. Error bars denote SD (n=3).

To further corroborate the newly discovered fatty acid biosensors, the split-plasmid circuit combinations were characterized in RB media for their dosage response to oleic and stearic acid. Unlike the single-plasmid design, varying the concentration of exogenous fatty acids did not result in a different pattern of response from the split-plasmid sensor design. FabR circuits remained unresponsive towards all concentrations of oleic acid, while their RFP/OD readings were commensurate with increasing stearic acid concentrations, up to ~8-fold change for RFP-

A10 and ~5-fold change for RFP-B10. Thus, both FabR split-plasmid Nissle strains (RFP-A10 & RFP-B10) have validated status as probiotic SFA biosensors.

Once more, RFP-FadH circuit had fluorescence readings that were too low. However, the classification for RFP-FadBA+FadR circuit is not so clear cut. RFP-FadBA produced the highest peak readings for both the oleic acid and stearic acid dose response in Figure 23 with a fold change of up to ~4 times on addition of oleic acid, and up to ~3 times upon addition of stearic acid. Hence, RFP-FadBA+FadR circuit can only qualify as a generic fatty acid biosensor, as expected from the working mechanism of FadR, because it is highly responsive to both UFA and SFA.

5.3. Conclusion

In this chapter, a first-ever SFA biosensor was developed in probiotic Nissle strain using the *E. coli* FabR regulatory system. The split-plasmid design addressed many of the issues that arose from the initial single-plasmid design. FabR split-plasmid Nissle sensors displayed very distinctive responses to stearic acid. The responses were equally distinguishable even with the additional presence of oleic acid, and they had a consistent pattern throughout all tested concentrations of exogenous oleic and stearic acid. Therefore, it can be concluded that RFP-A10 + FabR and RFP-B10 + FabR are both SFA biosensors. Conversely, FadR circuits can only qualify as generic fatty acid sensors due to FadR's binding mechanism responding to both UFA and SFA without discrimination.

Comparing the fold change from fatty acid induction, the level of response achieved from Zhang et al. [138] surpassed that of this project's biosensors. However, UFA oleic acid was

used for detection/induction experimentations by Zhang and colleagues for biosensors that were housed in *E. coli* DH1. On the other hand, SFA can be detected with the biosensors developed in this project, which are housed in probiotic Nissle, no less. Both abovementioned are progressive accomplishments in the field of genetic biosensors.

Chapter 6. Summary & Future Works

6.1. Summary

Obesity and type 2 diabetes are both multicausal metabolic diseases with contributing factors ranging from genetics and environmental to physical inactivity and unhealthy diets. Natural and engineered probiotics have the potential to serve as viable treatment options for both diseases. In this thesis, the focus is on the studying of the effect of excess fructose on mice, characterization of a FLAB (*L. kunkeei*) and developing a novel SFA biosensor. The long-term goal is to eventually develop a probiotic co-culture that could address obesity and type 2 diabetes.

This project demonstrated the liver condition of hepatic steatosis and liver fibrosis that result from chronic consumption of excessive added fructose. The fructose mice study presents fructose-induced NAFLD as a disease without easily observable symptoms such as increased weight gain and raised fasting blood glucose; NAFLD is commonly thought of as a disease that only overweight individuals suffer from, but the fructose mice model in this thesis exhibited NAFLD as a silent ailment capable of afflicting subjects without excessive bodyweight. As expounded in the literature review, through the years, large amounts of fructose will bring about reduced hepatic fatty acid oxidation and hepatic insulin resistance along with the fatty liver; eventually leading to a full-blown state of obesity and type 2 diabetes. Since added fructose cannot be eliminated from the shelves, the use of probiotic FLAB for dietary fructose removal in the gut may be the most viable option to address the global rise of added fructose availability.

This project also elucidated some of the key growth and metabolism characteristics of the chosen FLAB, *L. kunkeei*; even established its incredible fructose metabolism capacity, showing it to be way higher than the other tested probiotic strains regardless of the growth environment. As such, *L. kunkeei* has potential to be taken as a probiotic treatment/supplementation for dietary fructose removal from the gut. Similar to other obesity and diabetic treatments, the probiotic FLAB is not meant as an absolute cure but as a mitigation of the effects from the high volume of sugar-filled processed food that makes up a large portion of available food choices.

After testing out the circuitry with various replication origins and RBS, *L. kunkeei* transformation still faces unresolved issues related to exogenous gene expression. Consequently, the targetted probiotic co-culture will consist of *L. kunkeei* as a natural strain while engineered *E. coli* Nissle will be the host strain for the SFA biosensor circuit. As opposed to the generic fatty acid sensors reported in earlier literature, the SFA biosensor developed in this project is the first of its kind with three distinctive traits. Firstly, the incorporation of *E. coli* FabR regulatory system offers unique responses to both types of fatty acids, enabling the sensor circuit to discriminate between UFA and SFA. Secondly, the split-plasmid circuit design prevents overlapping of transcriptional processes and also prevents masking of FabR repression effect. Thirdly, the biosensor was developed using an established probiotic as host strain.

In the characterizations of the Nissle biosensors, FabR split-plasmid circuits had distinct fluorescence output readings that were commensurate with the concentration of stearic acid present in the characterization media; yet the very same circuits were unresponsive towards

any concentration of oleic acid present in the characterization media. Therefore, FabR split-plasmid Nissle sensors have been established as probiotic SFA biosensors.

6.2. Future Works

From the envisioned project system design shown in Figure 1, the newly developed Nissle SFA biosensor is to be repurposed to not just detect SFA but also release an appetite suppressant upon detection of high concentrations of SFA. That is the future strategy for the added functionality of appetite suppression to be included alongside dietary fructose removal as the probiotic intervention benefits from the co-culture treatment of natural *L. kunkeei* and engineered Nissle.

In line with the future strategy, the next step will naturally be to tether the production of an appetite suppressant to the Nissle SFA biosensor. This can be accomplished by adding the coding sequence of N-acyltransferase At1g78690 gene to RFP-A10 & RFP-B10 plasmids so as to emulate the success of NAPes-producing bacteria in Chen et al.[143]. N-acyltransferase is a NAPes synthase that catalyzes the N-acylation of phosphatidylethanolamine (PE) to form NAPes which, as elaborated in Chapter 2.6, is a potent appetite suppressant; NAPes production levels can be measured by LC/MS/MS after methylamine hydrolysis.

Additionally, infrared RFP (iRFP) expression should replace RFP because of the near-infrared window in biological tissue that allows for *in vivo* optical imaging in small animals to track bacteria movement and potential colonization. NAPes and iRFP are both outputs under the control of the FabR promoter which will ensure that NAPes production is commensurate with dietary SFA content.

Needless to say, the SFA biosensor gene circuit design is not resolute. Sensitivity analysis can be carried out across the possible design space to source for other suitable genetic promoters (with FabR operator sites added on) and RBS that can help to raise the output levels of NAFEs/RFP in the presence of stearic acid. The entire circuit structure can even be revamped to include additional sensors, for detection of other microenvironmental features, through layering of circuits and logic gate functions. If necessary, genetic modular components like adhesion module or biocontainment module (i.e. kill switch) can be added on to give enhanced functionality to the engineered Nissle. Ultimately, all the design alterations will be made in response to the strength of the sensory components and output gene expression levels. The ideal probiotic treatment will halt disease progression and ameliorate symptoms/complications for the diabetic and obese population; in addition to preventing the health status of overweight patients from deteriorating to obese or diabetic.

Last but not least, the two probiotic strains should be tested out in subsequent mice studies to supplement the fructose mice model developed in this thesis; probiotics can be administered to the mice via oral gavage in fixed volumes, while control groups can be given the same gavage volumes of sterile water as placebo. The first would have to be a repeat fructose water mice study similar to Chapter 3 but with an additional natural *L. kunkeei* gavage group that is provided fructose drinking water. This murine study can help to identify any mitigating effects that *L. kunkeei* may have over the detrimental health impacts of chronic fructose consumption. Special attention should be paid to the liver histology comparisons to observe if *L. kunkeei* treatment does ameliorate the conditions of fructose-induced hepatic steatosis and early stage liver fibrosis.

The second mice study would be for the purpose of investigating the impact of engineered NAPEs-producing Nissle on the clinical characteristics of high-fat induced metabolic syndrome. There would be an identical experimental setup with high-fat diet instead of fructose drinking water, and engineered Nissle replacing *L. kunkeei* as the probiotic for oral gavage administration. Once the health benefits of the individual probiotic treatments have been validated *in vivo*, a third mice study can be conducted with the combination of both high-fat diet and high-fructose drinking water, along with co-culture administration of natural *L. kunkeei* and engineered Nissle; objective of this last murine experiment is to study the outcome and overall impact of a combined co-culture treatment.

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APPENDIX A: SEQUENCES OF PROMOTERS AND RBS USED FOR FATTY ACID BIOSENSORS

Promoter names are in bold, FabR/FadR operator sites are underlined.

Description	Sequence
pLac	AATTGTGAGCGGATAACAATTGACATTGTGAGCGGATAACAAG ATACTGAGCACATCAGCAGGACGCACTGACC
BBa_J23100	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGC
BBa_J23101	TTTACAGCTAGCTCAGTCCTAGGTATTATGCTAGC
P26	ATTAAAAGAATAAAATTCTTGACATATTAAGCACGATATGATA TAATATCTGAGGA
P_{FabR} (A10)	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGC <u>AGCGTACA</u> <u>CGTGTTAGCT</u>
P_{FabR} (B10)	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGC <u>GGCGTACA</u> <u>AGTGTACGCT</u>
P_{FabR} (A35)	TTGACG <u>AGCGTACACG</u> TGTTAGCTTACAGTGCTAGC
P_{FabR} (B35)	TTGACG <u>GGCGTACAAG</u> TGTACGCTTACAGTGCTAGC
P_{FadR} (FadH)	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGC <u>AACTCATC</u> <u>CGACCACAT</u>
P_{FadR} (FadBA)	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGC <u>ATCTGGTA</u> <u>CGACCAGAT</u>
Default RBS	TTTAAGAAGGAGATATACAT
BBa_B0034	AAAGAGGAGAAA

APPENDIX B: ADAPTATION PROTOCOL FOR HIGH-FRUCTOSE DIET (C1068)

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Dear Sir/Mdm,

RE: ADAPTATION PROTOCOL FOR FEEDING RODENTS FOR HIGH FRUCTOSE DIET (C1068)

We would like to inform you of our recommended feeding instructions as per below for the C1068 high fructose diet for the mice/rat. These steps should be followed for mice to be adapted to the high fructose diet

Week 1 -	20% C1068 ; 80% 1324 standard maintenance diet
Week 2 -	50% C1068 ; 50% 1324 standard maintenance diet
Week 3 -	80% C1068 ; 20% 1324 standard maintenance diet
Week 4 -	100% C1068

In addition - the mixture of the above-mentioned feed should not be on the cage feeder for more than 3 days. Based on our knowledge, the average daily feed intake for mice is around 5g daily.

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