Assessment of the Effect of Artificial Sweeteners on Glucose metabolism and Gut Microbiota

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Non-nutritive sweeteners (NNSs) have been popular since their introduction to the market. Currently, the NNSs approved as "table-top sweeteners" by Health Canada include acesulfame potassium, aspartame, saccharine, neotame, steviol glycosides, advantame and sucralose. The NNSs under review here are sucralose and aspartame. Recent research has focused on illuminating the negative health effects of NNSs. For example, consumption of the NNS saccharin reportedly induces glucose intolerance by altering the microbial composition of the gut (referred to as "gut microbiota dysbiosis") in mouse models. The gut microbiota may have the ability to metabolize NNSs into short-chain fatty acids (SCFAs), and this process has a wide range of consequences, including the potential to shift the normal bacterial balance and potentially lead to alterations in glucose metabolism. Therefore, the aim of this study was to investigate the effect of pure form of sucralose and aspartame consumption on the gut microbiota community and to determine whether changes in glucose metabolism will be associated with gut microbiota dysbiosis induced through daily sucralose and/or aspartame consumption. Seventeen participants (young, healthy, non-pregnant, and non-diabetic) between 18 and 45 years old, with a BMI of 20-25 and a fasting blood glucose (FBG) < 5.7 mmol/L, took part in this double-blind crossover study. They were randomly assigned to receive a beverage containing either aspartame (0.425 g) or sucralose (0.136 g) once a day during 2 week-long intervention periods separated by a minimum of a 4-week washout period.

Faecal and blood samples were collected at baseline and at the end of each phase, faecal samples were analysed for SCFA levels and microbiome composition and blood samples were analysed for glucose, insulin, incretin, and leptin levels, respectively. Seventeen participants (10 females and 7 males; age 24 ± 6.5 y; BMI 22.9±2 kg/m^2) participated in the study. There were no significant differences observed between the aspartame and sucralose periods or baseline fasting glucose, insulin, active GLP-1 and leptin concentrations. The total

area under the curve (AUC) values of glucose, insulin, active GLP-1 and leptin were similar for the aspartame or sucralose treatment compared to baseline in healthy participants. There were no significant differences in total AUC for glucose ($p=0.54$), insulin ($p=0.38$), active GLP-1 ($p=0.67$) or leptin ($p=0.80$) between sucralose and baseline in healthy participants. There were no significant differences in total AUC for glucose ($p=0.65$), insulin ($p=0.16$), active GLP-1 ($p=0.63$) and leptin ($p=0.32$) between aspartame and baseline in healthy participants. There was no significant change in HOMA-IR ($p=0.35$, $p=0.46$), HOMA-%B ($p=0.16$, $p=0.60$) and HOMA-%S ($p=0.59$, $p=0.61$) after sucralose or aspartame treatment, respectively, compared to baseline in healthy participants. The results of this research need further confirmation and assessment in obese and diseased populations.

This research will provide insights into the health consequences of NNS use and could potentially change the recommended ADI values for NNSs.

ACKNOWLEDGEMENT

I would like to acknowledge and express my deepest appreciation to my supervisor, Professor James Friel, for his continuous support, encouragement and supervision through my PhD journey. I was lucky to have you as my supervisor. I would also like to acknowledge my co-advisor, Dr. Dylan Mackay, for his invaluable assistance, guidance, mentoring, time and comprehensive advice throughout my PhD journey. Additionally, I would like to extend my appreciation and gratitude to my committee member Dr. Meghan Azad for her continuous support, feedback and kind, endless help. Also my appreciation to the rest of the committee members for their help and support. A special thanks and acknowledgement to my funding agencies, Kuwait Foundation for the Advancement of Sciences (KFAS) and University Collaborative Research Program (UCRP); the RCFFN laboratory group; and my sponsor, Kuwait Civil Service and Institute for Medical Specialization- Ministry of Health of Kuwait.

DEDICATION

This thesis is dedicated to my family.

To my husband Dr. Haitham Ali, my daughter Moudhi and my sons Tameem and Shaheen.

To my brothers and sister.

To my parents, may they rest in peace.

To my best friends Alaa & Asmaa.

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ABBREVIATIONS

NNSs	Non-nutritive sweeteners
NASs	Non-caloric artificial sweeteners
CFIA	Canadian food inspection agency
FDA	US Food and Drug Administration
ADI	Acceptable daily intake
GIT	Gastrointestinal tract
T2DM	Type 2 diabetes mellitus
GPCR	G protein-coupled receptor
SGLT1	Sodium-dependent glucose cotransporter 1
GLP-1	Glucagon-like peptide 1
GIP	Glucose-dependent insulinotropic peptide
SCFAs	Short-chain fatty acids
CVDs	Cardiovascular diseases
NHANES	National Health and Nutrition Evaluation Survey
BW	Body weight
RCT	Randomized clinical trial
Ace-k	Acesulfame-potassium
P-gp	P-glycoprotein
CYP	Cytochrome
5TRPM5	Transient receptor potential subfamily member
GLUT 2	Glucose transporter-2
CPIR	Cephalic phase insulin response
AUC	Area under curve

ADA	American diabetic association’s
OGTT	Oral glucose tolerance test
MSF	Modified sham-feeding
3-OMG	3-O-methylglucose
T1DM	Type 1 diabetes mellitus
Yr.	Year
N	Number of participants
HbA1c	Hemoglobin a1c
PABA	Para-amino benzoic acid
SSBs	Sugar-sweetened beverages
IVGTT	Intravenous glucose tolerance test
RCFFN	Richardson Centre for Functional Foods and Nutraceuticals
DHQ II	Diet history questionnaire ii
VAS	Visual analog scale
MSD	Meso scale discovery
HOMA-IR	Homeostasis Model Assessment-insulin resistance
BREB	Bannatyne campus biomedical research ethics board
SEM	Standard error of the mean
CONSORT	Consolidated Standards of Reporting Trials

CHAPTER I:

GENERAL INTRODUCTION

1.1 Introduction

Non-nutritive sweeteners (NNSs), also called non-caloric artificial sweeteners (NASs), have grown increasingly popular since their introduction to the market. This favour is a result of their affordability, advertisement as low- or zero-calorie products, and perceived health benefits for weight loss and normalization of blood glucose levels [1, 2]. According to the Canadian Food Inspection Agency (CFIA), NNSs do not provide calories or influence blood sugar levels, and their energy contribution is negligible.

NNSs are found in a wide variety of foods and beverages, including soft drinks, candy, chewing gum and yogurt. Currently, the NNSs that have been approved by the US Food and Drug Administration (FDA), including acesulfame potassium, neotame, saccharin, sucralose, aspartame, and plant-derived sweeteners stevia and monk fruit extract [2-4]. The NNSs that have been approved as “table-top sweeteners” by Health Canada are fairly similar. These include acesulfame potassium, neotame, sucralose, aspartame, steviol glycosides and erythritol [5].

The artificial sweeteners under review in this project are sucralose and aspartame, which were chosen because they are used very frequently in Canada. Sucralose is a disaccharide produced from sucrose in which three chlorine molecules replace three of the hydroxyl groups on the sucrose molecule. On the market, sucralose is sold as Splenda brand sweetener, which is 600 times sweeter than sugar, meaning that a very small amount can be used to replace sugar for sweetening beverages and foods [6].

Sucralose was discovered in 1976 and is now globally approved by international regulatory agencies as a safe food additive for sweetening food and beverages [7]. Most sucralose is not metabolized for energy or absorbed by the body; therefore, sucralose yields no calories and does not affect blood glucose concentrations. Thus, these properties make it very popular for use in edible products for diabetic people. Sucralose is excreted unchanged in the faeces in all species, including humans. The sucralose that does get absorbed is excreted unchanged in the urine [8, 9].

Aspartame, on the other hand, is a methyl ester of two amino acids, aspartic acid and phenylalanine dipeptide. This odourless, white crystalline powder, is commonly sold as either Equal or NutraSweet brand sweeteners on the market. The aspartame calorie content per gram is similar to sucrose (approximately 4 calories per gram), but since it is 200 times sweeter than sugar, only a small amount of this NNS is needed to achieve sweetness, leading to almost no calories from aspartame in sweetened food and drink products [1].

Aspartame was discovered in 1965 and is now approved by different regulatory agencies to be used as a food additive in foods and beverages [5]. In the intestinal tract, digestive enzymes such as esterases and peptidases cleave aspartame into methanol, phenylalanine, and aspartic acid, which are all absorbed into the blood circulation in the same form but in lower amounts than the human body absorbs from natural food sources such as vegetables, fruits and proteins [10, 11]. Then, the three digested products, methanol, phenylalanine, and aspartic acid, follow their natural metabolic pathways, being utilized further and absorbed by different tissues in the body or excreted; because this process occurs quite rapidly in the gastrointestinal tract (GIT) before reaching the bloodstream, aspartame is never found in circulation or internal tissues [12, 13].

According to Health Canada, the acceptable daily intake (ADI) for sucralose is 9 mg/kg body weight, while that for aspartame is 40 mg/kg body weight [14]. The ADI is the maximum amount of a food additive that can be safely consumed on a daily basis over a person's lifetime without any adverse effects and includes a 100-fold safety factor. A reference 150 pound individual would need to consume approximately 76 packets of Equal and 51 packets of Splenda to reach the respective ADIs.

There is a considerable amount of controversy in the literature on whether the use of NNSs exerts an effect on human health and physiology. A review article including large cohort studies reported that NNSs used in beverages can be a useful aid in decreasing body weight, risk of type 2 diabetes and cardiovascular diseases [15]. However, in another recent review article, Fowler et al. 2016 reported that the use of daily NNSs, specifically in beverages, is associated with negative health outcomes such as increased weight gain, obesity, cardiovascular and metabolic risk and early mortality [16]. Another review was in agreement with Fowler et al. and concluded that frequent NNS consumption was associated with an increased risk of weight gain, metabolic syndrome, cardiovascular disease and type 2 diabetes [17]. In a recent systematic review and meta-analysis, the glycaemic impact of NNSs was assessed, and the author concluded that the consumption of different types of NNS did not affect blood glucose levels [18].

There are several proposed mechanisms of action for how artificial sweeteners could alter glucose metabolism and glycaemic control. In humans, the primary sweet taste receptors, namely, T1R2 and T1R3, are found in both the lingual epithelium and the endocrine cells of the gut [19, 20, 3]. The T1R2-T1R3 heterodimer is composed of G protein-coupled receptor (GPCR) subunits, and it can detect the presence of both sugars and different types of NNSs [21, 20]. Stimulation of the taste receptor activates an intracellular signalling pathway that leads to

upregulation of the intestinal glucose transporter sodium-dependent glucose cotransporter 1 (SGLT1), as well as an increase in the capacity of the gut to absorb glucose [19]. Stimulation of the taste receptors leads to the release of incretin hormones, including glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP).

Incretins influence glucose transport, metabolism, and homeostasis by increasing the cellular uptake of glucose, thereby upregulating SGLT1 [20]. If NNSs cause this cascade of events to occur when there no glucose is present, it could lead to a change in glucose metabolism.

Moreover, there are other proposed mechanism of action that include cephalic phase insulin responses (CPIR) [1], it has been shown that CPIR is important after meal ingestion in humans for post-meal glucose tolerance [22] and it was hypothesized that lack of CPIR activation may increase obesity risk [23]. It was shown also that the presence of some NNSs such as saccharin [24] can stimulate the CPIR, while the presence of other NNSs such as sucralose or aspartame do not [25, 26]. However, further studies are needed to confirm these findings. So if NNSs stimulate sweet taste receptors in the mouth and trigger the CIPR when there is no sugar present [24], this response might cause changes in glucose metabolism and decreased hormonal responses. However, to date, sweet taste receptors role in glucose control is still not clear [29].

More recently, NNS research has focused on the potential link between NNS consumption and gut microbiota and its effect on human health. For example, consumption of the NNS saccharin reportedly induces glucose intolerance by altering the microbial composition of the gut (referred to as “gut microbiota dysbiosis”) in mouse models [4]. Some gut microbiota may have the ability to break down NNSs into SCFAs, which has a wide range of consequences, including the potential to change the normal bacterial balance and to be processed into absorbable by-products that provide calories, also SCFA play a role in regulating gene expression, stimulate gut

hormones and peptides synthesis, and in peripheral tissues they stimulate the signalling transductions pathways [27]. It is also possible that NNS consumption exerts a bacteriostatic effect on the gut microbiota, leading to a more direct alteration of the microbiome composition [8, 28]. Abou-Donia et al. (2008) demonstrated that ingestion of sucralose for 12 weeks caused a decrease in the overall abundance of the microbiome (bifidobacteria, lactobacilli, Bacteroides, and Clostridium) in rats [8].

Moreover, Suez et al. (2014) reported that ingestion of the FDA's maximal ADI of saccharin for 5 days induced glucose intolerance in healthy human participants (n=7) [4]. Furthermore, microbiome analysis of the participants showed pronounced compositional changes in the gut microbiota [4]. Evidently, this research had limitations in that it was a small study lacking a control group, used a very high dose of saccharin and evaluated only one sweetener that is not relevant in Canada. In the current study, our objective is to determine whether this effect is replicated with NNSs that are routinely used in Canada. To our knowledge, there are no data on the possible influence of aspartame or sucralose on the human gut microbiome.

Therefore, the aim of the present study was to assess the effect of the artificial sweeteners aspartame and sucralose on glucose metabolism and the gut microbiome by measuring the effect of repeated daily oral doses of aspartame or sucralose consumption on glucose, insulin, leptin and GLP-1 concentrations and to determine whether any changes in glucose metabolism are associated with changes in the gut microbiome after NNS consumption. The output of this trial is important for optimizing our knowledge regarding the effects of NNSs on glucose metabolism and the gut microbiome and increasing our awareness with regard to NNS use.

This thesis has three main objectives, we met the first objective, the second and third objectives will be met post-defence, we will have the gut microbiome data in a usable format and

write it up in the near future, but now and for the PhD thesis this is not possible because a key collaborator went on leave and left the university.

1.2 Objectives

The present research objectives are as follows:

1. To determine the effects of daily, orally administered sucralose or aspartame consumption on glucose metabolism.
2. To determine the effects of daily, orally administered sucralose or aspartame consumption on gut microbiota composition, diversity, and community structure.

1.3 Hypothesis

The hypothesis to be tested in this research is as follows:

1. Aspartame or sucralose consumption will be associated with changes in glucose, insulin, GLP-1 and leptin concentrations.
2. Aspartame or sucralose consumption will cause changes in the gut microbiota composition, diversity, and community structure.

1.4 Thesis outline

A manuscript style was used to write this thesis, which includes 4 manuscripts that follow the General Introduction (Chapter I). Chapter II (manuscript 1) shows a summary of the available data in the literature on the effect of NNSs on glucose metabolism and the gut microbiome, mainly from randomized clinical trials, and the possible mechanisms connecting NNSs use to glucose metabolism. Chapter III (manuscript 2) will present recent evidence for the effects of

NNSs on glycaemic control in the past 18 months. Chapter IV (manuscript 3) will present a detailed study protocol of our randomized double-blinded crossover controlled clinical trial to measure the effect of artificial sweeteners on glucose metabolism and gut microbiome. Moreover, Chapter V (manuscript 4) will provide a detailed investigation of the effect of artificial sweeteners on glucose metabolism in healthy adults.

Manuscripts 1 was accepted for publications in Nutrition reviews. Manuscript 2 was published in Current Opinion in Clinical Nutrition and Metabolic Care journal. Manuscript 3 was submitted to Nutrition journal and under review. Manuscript 4 was accepted for publication in the Applied Physiology, Nutrition, and Metabolism journal. Chapter VI will be the final chapter and provide an overall conclusion of the research findings, strength, limitations, future directions and final conclusion.

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BRIDGE TO CHAPTER II

Chapter II includes a manuscript that provides an overview of the effect of sucralose and aspartame on glucose metabolism and the gut microbiome, mainly from human clinical trials. Additionally, this review will shed light on the possible mechanism connecting NNSs to glucose metabolism. Samar Y. Ahmad was the principal manuscript author.

CHAPTER II

MANUSCRIPT 1: LITERATURE REVIEW

The effect of sucralose and aspartame-on glucose metabolism and gut hormones

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2.1 Abstract

Purpose of review*:* Non-nutritive sweeteners (NNSs) are thought to be good replacements for caloric sweeteners in sweet food and beverages due to the reduction in energy and carbohydrate intake having health benefits for weight management and glycaemic control. However, the potential effects of NNSs on glucose metabolism and gut hormones remains controversial. Here, the available evidence on the effects of aspartame and sucralose consumption on glucose metabolism and gut hormones was reviewed.

Recent findings*:* The majority of studies have found that aspartame and sucralose consumption have no effect on blood glucose, insulin, or gut hormone concentrations; however, two trials have shown that aspartame has effects on glucose, insulin, and glucagon-like peptide 1 (GLP-1) concentrations, while few trials have shown that sucralose has effects on glucose, insulin, and glucagon-like peptide 1 (GLP-1) concentrations.

Conclusion: Based on the available literature, one study found higher glucose concentrations after sucralose consumption, while another three studies found lower concentrations and thirty-three additional studies found no effect on glucose. Moreover, only four studies reported increased GLP-1 concentration. Also, three studies found that sucralose consumption decreased insulin sensitivity, while another trial reported an increase.

Key words: Non-nutritive sweeteners, low-calorie sweeteners, aspartame, sucralose, glucose metabolism

2.2 Introduction

The consumption of sugar- and energy-dense food and beverages has been associated with many negative health outcomes, such as weight gain, obesity, type 2 diabetes mellitus (T2DM), metabolic syndrome, and cardiovascular diseases (CVDs).[1-3] Thus, non-nutritive sweeteners (NNSs) have become popular sugar substitutes in the food and drink market globally. This is a result of their advertisement of zero- or low-calorie beverages, their affordability, and their integration into weight loss diets.[4,5] In a recent National Health and Nutrition Evaluation Survey (NHANES), the reported consumption of NNSs by US adults from 2007 to 2012 was approximately 48%[6] from multiple dietary sources, which is higher than previous reports.[7,8]

The effect of NNS consumption on health is unclear due to the mixed results reported in the literature. NNSs consumption, mainly in the form of diet soda has been associated with weight gain, as opposed to weight loss, and may increase appetite and food intake.[5,9] In contrast, it was shown that NNSs used in beverages can be beneficial for maintaining body weight (BW)[10] and decreasing CVDs and T2DM risk compared to sugar.[11]

Evidence regarding the effect of NNS on glucose metabolism and gut hormones is still inconclusive. For example, Romo-Romo et al.[12] and colleagues conducted a systematic review of fourteen observational prospective studies, twenty-eight clinical trials (1985 to 2015) and two meta-analyses to assess the effect of NNS consumption on glucose metabolism in addition to other gut hormones in adults. The authors concluded that the effect of NNS consumption on glucose metabolism was unclear.[13-15] Uncertainty remains regarding the effects of NNSs on weight, metabolic health, glucose homeostasis and glycaemic responses.

In this narrative review, our aim is to contribute to the available evidence regarding the effect of aspartame and sucralose on glucose metabolism. First, an overview of aspartame and sucralose absorption, metabolism, and excretion is provided, and then the possible mechanisms through which NNSs consumption could influence glucose metabolism. This overview is followed by a summary of the clinical trials on the effects of aspartame and sucralose on glucose metabolism. The objectives of this paper were achieved by searching the PubMed database and PMC using different keywords and all possible combinations of keywords from the following two groups: (1) "artificial sweeteners", "non-nutritive sweeteners", "nonnutritive sweetener ", "non nutritive sweetener", "low calorie sweetener", "low-calorie sweeteners", "zero-calorie sweeteners", "sucralose", "aspartame", "splenda" "nutrasweet", "equal", "sugar twin", "saccharin", "stevia", "neotame", "acesulfame", "saccharin", "diet soda", "diet drink", "diet beverage"; and (2) " glucose tolerance", "glycemic load", "glycaemic load", "glycemic responses", " glucose load", "blood glucose", "oral glucose", "oral ingestion", "glucose metabolism", "glucose homeostasis", "glucose profiles", "insulin", "insulin sensitivity", "GLP-1", "glucagon-like peptide-1", "GIP", "incretin hormone", "hormonal responses", "clinical trials" and "randomized clinical trial" . Titles and abstracts of the articles recognized through the keyword search were retrieved for evaluation of the full text and were included in the review if they met all of the following criteria: study design: randomized clinical trial (RCT); Study subject: human; intervention: oral, intragastric and intraduodenal NNS consumption, administration of NNS alone or in combination with other beverage or food; outcome: effect on glucose metabolism; Article type: peer-reviewed publication; and Language: English.

2.3 Types of non-nutritive sweeteners

NNSs, also called a zero- or low-calorie sweeteners, can be extracted from natural sources or synthesized. NNSs are chemosensory compounds that have the ability to produce an intense sweet taste with no or few calories depending on the type of NNS used, NNS are often used at very low concentrations compared to caloric sweeteners. This characteristic has made NNSs very popular in the food and beverage market due to their potential for controlling BW and glycemia.[4,5]

To date, several NNSs have been approved by the US Food and Drug Administration (FDA), including seven artificial NNSs (aspartame, sucralose, ~~neotame,~~ saccharin, acesulfame-potassium (Ace-k), neotame, and advantame) and two natural NNSs (steviol glycosides and Luo Han Guo fruit extracts) (Table 1).[16] In Canada, approved NNSs include aspartame, sucralose, neotame, Ace-k, steviol glycosides, monk fruit extract.[17] The NNSs that was reviewed in this paper are aspartame and sucralose because they are the most frequently consumed in artificial sweeteners.[18]

2.3.1 Aspartame

Aspartame (L-alpha-aspartyl-L-phenylalanine methyl ester) was discovered in 1965. Aspartame is a low-calorie, artificial, non-saccharide sweetener used to sweeten food and drink products. This white crystalline powder has a sweet taste very similar to sucrose, which is why it is very commonly used in food and beverages. Aspartame is approximately 200 times as sweet as sucrose; therefore, very small amounts are needed to achieve a similar sweet taste intensity as sucrose.[4,16] Aspartame is commonly sold as Equal®, NutraSweet® or Sugar Twin®.

In the small intestine, digestive enzymes break aspartame down into methanol, phenylalanine, and aspartic acid. These metabolites are further broken down into formaldehyde, formic acid,[19] each of which follow a natural metabolic pathway just as they would be metabolized from other dietary sources. Metabolism and absorption of aspartame occur quickly, which is why it is never found circulating in the blood.[20,21] Unlike other NNSs, aspartame has some nutritive value when metabolized in the body: one gram of aspartame provides approximately 4 kcal.[22] The FDA and Health Canada have established acceptable daily intakes (ADIs) of 50 and 40 mg/kg bw/day, respectively.[16,17] The ADI is an estimate of the maximum amount of a food additive in food or beverages (expressed on a BW basis) that can be safely consumed on a daily basis over a person's lifetime without any health risk to the consumer, including a 100-fold safety factor.[23]

2.3.2 Sucralose

Sucralose was discovered in 1976. Sucralose is a disaccharide made from sucrose by a chemical process that replaces the hydroxyl groups on sucrose molecules with three chloride atoms.[24] Sucralose is approximately 600 times sweeter than sucrose, with a pleasant sweet taste very similar to sucrose, which is why it is used in variety of food and beverages.[4] On the market, sucralose is sold under the brand name Splenda®.

It is generally thought that 11-27% of sucralose is absorbed in the intestines and is removed from the blood circulation by the kidneys and excreted in urine, while the remaining sucralose is directly excreted unchanged in faeces.[25] However, a recent animal study did not support the findings of early metabolic studies; it was reported that acetylated sucralose metabolites accumulate in the urine and faeces of rats after a repeated dosing for 40 days at an

average dosage of 80.4 mg/kg/day.[26] This dose was within the normal range of the toxicological studies submitted in North America and other countries. Therefore, this recent finding showed that ingested sucralose may not be excreted unchanged in faeces and urine and the regulatory agencies might need to revisit the safety status of sucralose which was based on early metabolism studies.

Furthermore, another animal study found that sucralose ingestion for 12 weeks in male rats increased the expression of the intestinal efflux transporter P-glycoprotein (P-gp) and cytochrome P-450 (CYP).[27] These two transporters are involved in drug detoxification, suggesting the body might be treating sucralose as a toxin that needs to be removed from the body.[27] Every food additives is assigned to and ADI, this ADI is usually based on toxicological evaluations. The FDA and Health Canada established ADI of 5 and 9 mg/kg bw/day, respectively for sucralose.[16,17]

2.4 Possible mechanisms connecting non-nutritive sweeteners to glucose metabolism

2.4.1 Activation of oral, gut sweet taste receptors and gut hormones

To understand the physiological role of NNSs, it is important to understand the physiology of taste receptors. The focus here will be on the sweet taste, which allows the recognition of energy-rich nutrients. In brief, the tongue has various types of taste papillae including the foliate, the fungiform, and circumvallate. Each taste papillae have hundreds of taste buds. The human tongue has different regions that are able to sense different tastes (sweet, bitter, sour, salty and savoury) in addition to carrying out different functions. They all work in a harmonized fashion to carry out their anticipated function.[28] Taste receptor cells are anatomical

substrates in units that are grouped into taste buds that are present across the papillae of the palate epithelium and the tongue.

Sweet and umami taste processes are mediated by a family of three G protein-coupled receptors (GPCRs): T1R1, T1R2 and T1R3. These GPCRs are grouped into heterodimeric receptor complexes.[29] When T1R3 combines with T1R2 they form a sweet taste receptor that responds to all different classes of sweet tastes, including NNSs (e.g., aspartame and sucralose), natural caloric sugars (e.g., glucose, fructose and sucrose), sweet proteins (monellin and thaumatin), and D-amino acids, as well as various other types of organic compounds.[28]

Activation of the oral sweet taste receptors in the mouth occurs when a sweet substance binds to T1R2-T1R3 receptors, leading to the release of alpha-gustducin, which activates the release of different signalling effectors, including phospholipase C-β2, inositol, and diacylglycerol. Then, the transient receptor potential subfamily member (5TRPM5) channel is activated, which depolarizes the plasma membrane to allow for calcium entry. Upon effector activation and taste cell depolarization, neuronal signals are sent to the brain to transmit a sweetness sensation.[30]

Interestingly, sweet taste receptors have also been found in the gastrointestinal tract, especially in the small intestine and colon, as well as on pancreatic β cells. Stimulation of the sweet taste receptors in the gastrointestinal tract activates an intracellular signalling pathway similar to that in the mouth,[31] which leads to the upregulation of the intestinal glucose transporter, sodium-dependent glucose co-transporter-1 (SGLT1), which is a major route for dietary glucose transportation from the intestine into enterocytes. Afterward, glucose transporter-2 (GLUT2) is activated on the basolateral membrane to facilitate the movement of glucose into

blood circulation.[32,33] Accordingly, sweet taste receptors, whether they are in the mouth or in the gut, are important for recognizing and metabolizing different energy sources in the human body.

Interestingly, the SGLT1 transporter likely plays a more important role in glucose absorption in the human intestine than GLUT2. This was shown in a previous study by Kim et al.[34] where the intestinal expression of SGLT1 transporters was much higher in human participants than in mice and rats, while rats had a higher expression of GLUT2.[34]

Stimulation of the gut taste receptors T1R2 and T1R3 also leads to the release of incretin hormones including GLP-1 and glucose-dependent insulinotrophic peptide (GIP). These GLP-1 and GIP are gut derived peptides that stimulate insulin secretion when there is an increase in blood glucose concentrations.[35-37]

The in vivo data regarding the effects of NNSs on glucose metabolism and gut hormone secretions are inconsistent. For example, Margolskee et al.[37] showed that mice supplied with regular sugar and NNS solutions for two weeks had increased SGLT-1 expression and glucose absorptive capacity in the gastrointestinal tract.[37] Moreover, Mace et al.[38] reported that artificial sweeteners could increase glucose absorption rate in rats by enhancing apical GLUT2 insertion into enterocytes.[38] By contrast, Saada et al.[39] reported a decrease in serum glucose in normal and diabetic rats and that there was no effect on insulin in the diabetic rats compared to other groups examined after the administration of 11 mg/kg of sucralose (Splenda) by oral gavage for six weeks.[39] Also, Fujita et al.[40] reported that 1 g/kg of different types of sweeteners, including sucralose, Ace-k, Stevia, and d-tryptophan, given to male rats by gastric gavage failed to cause any effect on glucose absorption or incretin hormonal release.[40]

The conflicting data continues in human trials, as few studies have reported that NNS consumption can increase GLP-1 in healthy participants without any effect on blood glucose,[41,42]

while another study showed that NNS consumption decreased GLP-1 concentration.[43] However, other human trials found that NNSs causes changes in blood glucose concentrations without any changes in GLP-1[44-46] (Table 2[41-75]). These contradictory findings from human trials may be due to the many variations in the protocols used including the NNS type used, the route of administration, and the durations of the previous and current exposures to NNSs.

2.4.2 Cephalic phase insulin release

When humans ingest a sweet meal, the activation of oral sweet receptors will send sensory information to the brain, which in turn will cause the release of insulin[76] to prepare the body for energy metabolism.[77,78] These physiological responses, which last for approximately 10 minutes, are known as the cephalic phase insulin response (CPIR) and have been demonstrated in human and animal studies.[79-82] It was shown that the CPIR to meal ingestion in humans is important for post-meal glucose tolerance and that blocking of the CPIR causes a prolonged increase in blood glucose concentrations.[83] It was shown that the presence of some NNSs can stimulate the CPIR, while others do not. For example, oral taste receptor activation via mouthwash solutions sweetened with saccharin activates a CPIR and causes a significant increase in the plasma insulin concentration,[84] while the presence of sucralose, aspartame and saccharin did not activate the CPIR in other studies[56,68]; however, further studies are needed to confirm these findings. In humans, it was reported that oral sensory stimulation with food significantly lowered the plasma glucose area under the curve (AUC) ($p<0.03$) in healthy humans. This suggests that oral sensory stimulation is important for glucose metabolism and homeostasis.[80] If NNSs stimulate oral sweet taste receptors and cause the CPIR to occur when there is no sugar present,[84] this response could lead to changes in glucose metabolism and

depressed hormonal responses with regular use of NNSs. Generally, NNSs are usually consumed in foods or drinks, which could stimulate the CPIR. When conducting a clinical trial looking at NNS, it is important to consider the route of delivery, for example, giving a NNS treatment in a capsule form may not trigger CPIR and therefore may not mimic real NNS consumption.

2.4.3 Gut microbiota

The human gut microbiota is a collection of microorganisms that live symbiotically with the digestive tract. It is estimated that the human gut microbiota could contains as many as one hundred trillion bacteria (10^{14}) in the large intestine.[85] Microbiota colonization is present in the proximal gastrointestinal tract (GIT) starting from the stomach and ending with a huge diversity and number of microorganisms in the distal GIT, or the colon. Every host has a specific microbial community that evolves rapidly throughout their life and can be affected by external and internal modifications,[86] mainly exposure to host diet and xenobiotics.

The human-associated microbes evolution might involve changes at different scales, such as changes that occur within the microbial community, evolution of microbial genomes within individual, and exchange between microbiomes and the physical environment outside the host.[87]

Up to 90% of the adult human gut microbiota is dominated by two bacterial phyla: the Bacteroidetes and the Firmicutes, whereas Actinobacteria, Proteobacteria, Verrucomicrobia, Fusobacteria and Cyanobacteria represent the bacterial minority.[88] Gut microbiota have many different physiological roles, such as maintaining the immune system, GIT motility, drug metabolism, defence against pathogen attack, the production of vitamins, carbohydrate metabolism, and metabolizing indigestible polysaccharides into short-chain fatty acids (SCFAs), such as propionate, acetate and butyrate.[89,90] These roles can be interfered with when there is a

shift in the quality, quantity or composition of the gut microbiome (referred to as "gut microbiota dysbiosis").[91] Westernized diets that are rich in fat and sugar, in addition to a sedentary lifestyle, have been linked to dysbiosis, which can lead to impaired glycaemic control.[92] Exposure to NNSs has also been shown to cause dysbiosis. For example, repeated exposure to the NNS saccharin in mouse models induces glucose intolerance by altering the microbial composition.[93] The gut microbiota has the ability to metabolize NNS saccharine into SCFAs, which may have a wide range of consequences, including the potential to shift the normal bacterial balance and the capability of being processed into absorbable by-products that may provide calories.[94] Some NNS may be able also to exert a bacteriostatic effects on the gut microbiota when consumed, leading to dysbiosis at least in rodent models.[27,95] For example, Abou-Donia et al.[27] reported that when Splenda (which contains sucralose) was given by oral gavage at different concentrations of up to 1000 mg/kg to rats for 12 weeks, there was a significant decrease in the beneficial gut microbiome. Moreover, this decrease remained during the 12 week recovery period.[27] Furthermore, Palmnas et al.[95] examined the effect of low-dose aspartame consumption on the gut microbiota of rats. Faecal analysis of the gut microbiota showed that daily consumption of aspartame (5-7 mg/kg/d) for 8 weeks resulted in an increase in the total bacterial content, especially the abundances of Enterobacteriaceae and *Clostridium* leptum.[21]

NNS consumption has also been linked to an increased risk of weight gain and obesity, type 2 diabetes, metabolic syndrome, and cardiovascular events in humans.[96] It is possible that these negative consequences are partially related to alterations in glucose metabolism. A study by Suez et al.[93], they demonstrated that four out of seven individuals who consumed the FDA's maximal ADI of the sweetener saccharin during a 5 day period displayed significantly worse glycaemic responses after this intervention compared to before. Furthermore, microbiome

analysis of all seven healthy participants showed that "NNS responders" (those who developed poor glycaemic responses) experienced pronounced compositional changes, whereas "NNS non-responders" experienced little change in microbiota composition.[93] In the same study, Suez et al.[93] also showed how NNS consumption can be associated with changes in glucose metabolism that are mediated by gut microbiota dysbiosis by transplanting faecal microbiota from mice on saccharin into germ-free mice. Mice receiving a microbiota transplant from saccharin-treated mice developed glucose intolerance compared to the control group.[93]

However, there are few clinical trials investigating the effect of NNSs on the human gut microbiota. It is clear that more studies are needed because of the established association between dietary habits, gut microbiota, and human health,[97] and with the advancements in molecular and microbial DNA sequencing techniques that enable us to better study microbial communities.[98]

2.5 Randomized clinical trials (RCTs) measuring the effects of aspartame and sucralose on glucose metabolism and gut hormones

There has been an increased interest on the impact of NNS consumption on glucose metabolism based on many reports investigating the effects of NNS on incretin hormones GLP-1 and GIP (both of which influence glucose absorption and metabolism) in in vitro and in vivo animal studies.[36,37] The number of human studies evaluating the effects of repeated daily exposure to sucralose and/or aspartame on glucose metabolism is far fewer than the number of studies evaluating the effects of an acute single dose of NNS. Human trials are summarized in Table 2.[41-75]

2.5.1 Studies evaluating the effect of repeated doses of sucralose on glucose metabolism

The effect of repeated doses of sucralose has been assessed in healthy, obese and diabetic participants. For example, Baird et al.[48] evaluated the effect of different doses of sucralose in two single-blinded, randomized trials in healthy human participants. In the first study, ascending doses of sucralose (1, 2.5, 5 and 10 mg/kg) were given to healthy volunteers (n=8) every other day for 10 days, followed by a daily dose at 2 mg/kg for 3 days and 5 mg/kg for 4 days (total duration 17 days). In the second study, participants (n=77) consumed up to 500 mg/day of sucralose or 50 g/day of fructose (n=31) two times daily for 13 weeks. The results demonstrated that sucralose had no effect on fasting insulin or blood glucose levels over the course of both studies.[48] While the dose of sucralose used in the second study was high compared to the ADI, there was no effect on glucose or insulin after prolonged exposure. Also, the participants were not screened for past NNS usage.

Consistent with Baird et al.[48] and Grotz et al.[49] found no effect of sucralose on glucose metabolism. Obese participants with T2DM (n=136) were randomly assigned to receive either encapsulated sucralose (333.5 mg) twice daily for 13 weeks or placebo (cellulose capsules for the same period). All participants continued to receive blind placebo capsules during the last 4 weeks and the entire follow-up period phase. There were no significant differences in the main outcome measures between the sucralose and placebo groups, including in fasting plasma glucose, haemoglobin A1c (HbA1c), C-peptides, from baseline or throughout the experiment.[49] The sucralose dose used in this study was 3x the estimated maximum intake (2.4 mg/kg/day), however there was no effect of repeated daily consumption of sucralose in these participants with T2DM. In this trial participants were not screened for past NNS usage, and sucralose was given in a capsule.

Additionally, Reyna et al.[50] conducted a study of male patients with well-controlled T2DM (n=16) to compare metabolic and anthropometric changes induced by the American Diabetic Association's (ADA) nutritional recommendations with a modified, low-calorie diet containing fat replacers and NNS (sucralose). Both groups received their diets for 4 weeks in addition to a daily aerobic exercise (60 minutes walking). After 4 weeks both diets showed a significant improvements in HbA1c, weight, body mass index and lipid profile, but not in fasting blood glucose. However, greater improvement was observed in the group who received the low-calorie diet and sucralose.[50] Despite the reported results, the sucralose amount used was not reported in this trial. Additionally, it cannot be confirmed whether the improvements observed in group receiving the diet containing sucralose were related to the sucralose because the composition of the two diets were different.

Grotz et al.[62] examined the effect of repeated doses of sucralose on glucose and insulin HbA1c in healthy male volunteers (n=47) in a randomized, double-blinded, placebo-controlled study. Participants consumed encapsulated sucralose (333.3 mg) or placebo 3x/day at mealtimes for 12 weeks. The results showed no differences in fasting glucose, insulin and HbA1c concentrations between the sucralose and placebo (cellulose) groups.[62] However, giving NNSs in capsule form will bypass the activation of oral sweet taste receptors, which might have affected the measured outcomes. The sucralose concentration used in this trial was higher than the ADI for sucralose.

The effect of repeated doses of sucralose has been assessed in healthy and diabetic participants (Table 2[41-75]). For example Romo-Romo et al.[63] conducted a randomized, open label, parallel arm study to measure the effects of sucralose ingestion on blood glucose, insulin and insulin sensitivity. Healthy female participants (n=66) received a commercial sucralose sachet

(958 mg dextrose, 30 mg maltodextrin, and 12 mg sucralose) 3 times/day for 14 days. The results showed that insulin sensitivity was lower in the sucralose group compared to the control group ($p=0.04$). Moreover, the acute insulin response increased in the sucralose group ($p=0.04$) compared to the control group.[63] However, the amount and contents of the sachets given to the control group were not mentioned; therefore, these results should be interpreted with caution.

Lertrit et al.[42] conducted a study to measure the effects of repeated consumption of sucralose on the glycaemic response, insulin secretion and sensitivity, and GLP-1 secretion in a randomized, crossover, double-blinded trial. Healthy participants (n=15) were included in this study and received sucralose capsules (200 mg) or empty placebo capsules for 4 weeks. They reported in their findings that AUCs for GLP-1 were higher in the sucralose group compared to control group ($p<0.001$) and that the acute insulin response and sensitivity were decreased in the sucralose group compared to the control group ($p<0.005$) after performing an OGTT with 75 g glucose the next morning.[42] The delivery of the NNS in capsule form bypasses the oral sweet taste receptors and this may play a role in the outcomes measured.

In summary, majority of the clinical trials to date with repeated daily consumption of sucralose by healthy and diabetic patients showed no significant effects on blood glucose, insulin or C-peptides.[48-50] However, these studies had limitations including: 1) the participants were not screened for past NNS usage; 2) sucralose was given in a capsule; 3) unreported NNS amount used in one of the trials and whether there was other ingredients present with NNS.

2.5.2 Studies evaluating the effect of a single dose of sucralose on glucose metabolism

There are many human studies that have evaluated single dose effects of sucralose on blood glucose, insulin and gut hormones in healthy and diabetic patients. For example,

Mezitis et al.[47] reported in a randomized, crossover trial that sucralose ingestion has no effect on glucose or C-peptide concentrations compared to placebo. In this study, participants with T1DM and T2DM (n=26) were recruited to receive a single dose of sucralose (1000 mg capsule) or placebo (cellulose capsules) followed by a meal. The results showed that sucralose consumption had no effect on glucose or C-peptide levels compared to the placebo.[47] However, the sucralose this study was given in a capsule.

Moreover, Ma et al.[51] conducted a randomized, single-blinded, crossover study to measure the effect of sucralose on blood glucose levels, incretin hormone release of GLP-1 and GIP, and insulin in healthy male and female participants (n=7). All participants were fasted and received an intragastric infusion of saline (500 ml), sucrose (50 g), or sucralose (80 mg or 800 mg), all of which were labelled with 150 mg ^{13}C-acetate. The results showed that the sucralose and saline solutions had no effect on blood glucose, insulin, GLP-1, or GIP levels.[51] Bypassing oral activation of sweet taste receptors might affect the result observed in this study.

Another randomized crossover trial was conducted by Brown et al.[52] in healthy male and female participants (n=22) to determine the effects of a single doses of sucralose on glucose metabolism. The fasted volunteers received either 240 ml of diet soda containing sucralose of an unknown concentration with Ace-k or 240 ml carbonated water. An oral glucose tolerance test (OGTT) with 75 g of glucose was carried out 10 minutes after receiving the drinks. There was no effect of sucralose and Ace-K consumption on glucose, insulin, GLP-1, or C-peptides observed. However, after the oral glucose load, the GLP-1 peak ($p=0.003$) and the AUC ($p=0.003$) were significantly higher with diet soda than with carbonated water.[52,54] The other ingredients in diet soda (including colour, caramel, natural flavours, citric acid, potassium benzoate, phosphoric acid, potassium citrate, and gum acacia) might interfere with the effect observed and should be

controlled for in studies looking to replicate this effect. Additionally, the sucralose concentration was not reported in this trial and participants were not screened for past NNS usage.

Ma et al.[53] conducted a randomized, single-blinded, crossover study to measure the acute effects of sucralose on glucose absorption in healthy male and female participants (n=10). Each subject was infused intraduodenally with either sucralose (960 mg) or control (0.9% saline) at 4 ml/min for 150 min. The results showed no significant difference in the blood glucose or GLP-1 between the NNS and saline infusions.[53] However, the delivery of sucralose intraduodenally bypasses the activation of oral sweet receptors which may not replicate normal route of consumption of sucralose.

In addition, Brown et al.[54] conducted a randomized crossover trial in healthy females (n=8) to determine the acute effect of sucralose on blood glucose, insulin and glucagon in fasting and post-prandial states. Participants consumed sucrose and/or sucralose dissolved in water in a factorial design consisting of water only (355 ml), sucrose (50 g) in 355 ml water, Splenda (6 g) in 355 ml water, or sucrose (50 g) and Splenda (6 g) in 355 ml water. The results showed that there was no significant effect of sucralose on insulin, glucose, or glucagon compared to water alone.[54] However, in this study there was no past NNS usage measured.

Steinert et al.[55] conducted a double-blinded, placebo-controlled, crossover study in healthy male and female participants (n=12) to measure the acute effect of NNSs on blood glucose, insulin, GLP-1 and appetite hormones. After an overnight fast, each subject received one of the following treatments dissolved in 250 ml of water by intragastric infusion: glucose (50 g in water), fructose (25 g in water), sucralose (62 mg in water), aspartame (169 mg in water), Ace-K (220 mg in water), or water alone as control. There was no glucose, insulin, GLP-1, or

appetite hormone response observed when NNSs were administered.[55] Again, there was no screening for past NNS usage in this study.

Ford et al.[56] conducted a randomized, crossover, single-blinded and placebo-controlled trial in healthy male and female participants (n=8) investigating the acute effect of oral sucralose consumption on plasma glucose, insulin and GLP-1. After an overnight fast, participants received 50 ml of water, 50 ml of sucralose (0.083% w/v), or 50 ml of sucralose (0.083% w/v) and maltodextrin (50% w/v), followed by a modified sham-feeding protocol (MSF) of the same solution, which was consumed to stimulate oral sweet receptors, or 50 ml of water followed by MSF combined with sucralose (0.083% w/v). Consumption of sucralose or water followed by MSF combined with sucralose had no effect on plasma glucose, insulin or GLP-1. However, consumption of sucralose with maltodextrin caused an increase in plasma glucose and insulin AUCs without affecting the GLP-1 hormone.[56] Furthermore, past use of NNSs may have affected the results of this study, however there was no screening for past NNS usage.

Wu et al.[57] assessed the acute effects of different types of preload (small load of macronutrients that are given at fixed interval before meal) sweet drinks on blood glucose, insulin and incretin hormone secretion in a randomized, single-blinded trial. Healthy male and female participants (n=10) were fasted, then they ingested 4 preloaded drinks containing glucose (40 g), sucralose (60 mg), 3-O-methylglucose (3-OMG) (40 g) or a tagatose/isomalt mixture (TIM) (40 g) 15 min before a labelled mashed potato meal containing ^{13}C-octanoic acid. Sucralose had no effect on blood glucose, GLP-1, GIP, or insulin.[57] In this trial, adding a control preloaded drink, such as water, would have been helpful in interpreting the findings, as well as screening for the past NNS usage.

Wu et al.[58] examined the acute effect of NNSs on blood glucose, plasma insulin and GLP-1 levels in healthy males (n=10) in a randomized, single-blinded, crossover trial. Participants consumed either water alone (240 ml or a similar amount of water sweetened with sucralose (52 mg), Ace-K (200 mg), or sucralose (46 mg) and Ace-K (26 mg) after an overnight fast. An OGTT with 75 g glucose and containing 13 C-acetate (150 mg) was administered 10 minutes after each treatment. Prior to glucose ingestion, blood glucose, plasma insulin and GLP-1 levels did not change with any of the sweetened drinks or water, but all increased after the OGTT ($P<0.001$ for each) and were similar among all the treatment groups.[58]

Moreover, Stellingwerff et al.[59] investigated the acute effect of sucralose ingestion on blood glucose and plasma insulin during exercise in a randomized, double-blinded, crossover study. Healthy male cyclists (n=23) participated in this study and consumed 8 x 50 ml doses of either a 1 mM (20 mg) sucralose solution (SUCRA) or a placebo (sucralose mouthwash followed by drinking 50 ml of water) every 15 minutes for 2 hours, starting 120 min before the beginning of exercise and followed by ingestion of a maltodextrin drink (34 g) over a 2 hour cycling period. Ingestion of sucralose had no effect on blood glucose or insulin levels.[59]

Sylvetsky et al.[61] investigated the acute metabolic effects of NNSs in diet sodas in a randomized, crossover study. In study arm 1, healthy adults (n=30) consumed 355 ml of water alone or with different doses of preloaded sucralose (68 mg, 170 mg, and 250 mg).

In study arm 2, adults (n=31) consumed 355 ml of diet soda containing 18 mg sucralose, 18 mg Ace-K and 57 mg aspartame, 355 ml diet soda containing 68 mg sucralose and 41 mg Ace-k, or 355 ml carbonated water with 68 mg sucralose and 41 mg Ace-k in a randomized design. The results of study arm 1 showed that the different doses of preloaded sucralose had no effect on glucose, insulin, C-peptide, glucose absorption, or GLP-1 or GIP gut hormones.

Carbonated water with NNSs also had no effect on the parameters measured compared to carbonated water alone. However, study arm 2 results showed that the GLP-1 hormone was augmented after diet soda intake, but not after carbonated water intake.[61] These reported results are similar to the results reported by Brown et al.[52] and may be because the additional ingredients present in the diet soda cause an augmentation of GLP-1 hormones.

Few trials have demonstrated effects of a single doses of NNS consumption on either glucose, insulin, insulin sensitivity, acute insulin response or gut hormones. For example, Brown et al.[41] examined the acute effects of diet soda on blood glucose, insulin and gut hormones in young participants with type 1 diabetes mellitus (T1DM) (n=9), T2DM (n=10), or healthy volunteers (n=25) in a randomized, crossover study. Participants received 240 ml of diet soda containing sucralose (190 ± 38 mg/ml) and Ace-k (108 ± 0.6 mg/ml) or 240 ml of carbonated water as a placebo. After 10 minutes, an OGTT with a 75 g glucose load was carried out. The results showed no difference in blood glucose, C-peptides, or GIP between the two conditions in all groups. However, GLP-1 AUC was 43% higher after diet soda ingestion (p=0.02) vs. carbonated water in the T1DM, but not the T2DM patients,[41] and GLP-1 AUC was 34% higher after diet soda consumption in healthy participants. These reported increases in GLP-1 AUC are similar to the results reported by Brown et al.[52] and Sylvetsky et al.,[61] which may be due to the presence of other ingredients in diet soda. Past use of NNSs by the participants might have also affected the outcomes measured here, but this was not reported in this study.

Pepino et al.[44] examined the acute effect of sucralose consumption on glucose metabolism in participants who were morbidly obese (n=17). In this randomized, crossover design trial, participants received either 60 ml of distilled water (control condition) or 60 ml of a sucralose solution (2 mmol/L; 48 mg sucralose) after an overnight fast. Following the treatments,

an OGTT with a 75 g glucose load was performed. The results showed that peak values for glucose, C-peptides, insulin, insulin AUC and insulin secretion rates were higher following the OGTT in the sucralose group compared to water group. GLP-1, GIP and glucagon concentrations and AUCs in the water and sucralose-treated groups were similar.[44] Additionally, there were decreases in insulin sensitivity and insulin clearance of 23 ± 20% (p=0.01) and 7 ± 4% (p=0.04), respectively.

Temizkan et al.[60] assessed the acute metabolic effects of NNSs in healthy participants (n=8) and newly diagnosed T2DM patients (n=8) not on medication. In this randomized, single-blinded, crossover study, participants received sucralose (24 mg; table top formulation) or aspartame (72 mg; table top formulation) in 200 ml of water or water alone 15 minutes before an OGTT (75 g glucose). The results showed that consumption of sucralose was associated with lower blood glucose and increased GLP-1 levels (AUC), however, there was no effect on insulin or C-peptide concentrations in the healthy participants. In the T2DM patients there was no effect on the same outcome measured and the AUCs were similar for blood glucose, insulin, C-peptides and GLP-1 after treatment with sucralose and water.[60] The table top sweeteners used here were commercial sachets and other ingredients and fillers in the sweetener brands could have mediated the GLP-1 effect because they were not controlled for.

2.5.3 Studies evaluating the effect of repeated doses of aspartame on glucose metabolism

The effect of repeated doses of aspartame has been assessed in healthy and diabetic participants (Table 2[41-75]). For example, Nehrling et al.[64] conducted a randomized, double-blinded and placebo-controlled trial in participants with T1DM and T2DM (n=62) to measure the effect of repeated daily consumption of aspartame on glucose and HbA1c. They received either aspartame capsules (2.7 g) or placebo capsules with corn starch (20 mg) every day with meals for 18 weeks. Blood glucose concentrations and HbA1c were similar in both groups.[64] Receiving NNS in capsule form might bypass the activation of oral sweet taste receptors and the presence of diabetes might have affected the measured outcomes.

Additionally, Colagiuri et al.[66] tested the effect of adding aspartame to meals on the blood glucose and insulin levels of participants with controlled T2DM in a double-blinded, crossover design trial for 6 weeks. Participants (n=9) received aspartame (162 mg) during the aspartame period and sucrose (45 g) during the sucrose period received, added to meals and beverages. The results showed that aspartame did not cause changes in glucose and HbA1c.[66] However, T2DM might have affected the development of these outcomes.

Higgins et al.[74] assessed the repeated daily effect of aspartame on glucose metabolism in a randomized, parallel arm trial. Healthy participants (n=93) consumed different doses of aspartame (350 mg or 1050 mg) dissolved in 500 ml of beverage with or without capsules once a day for 12 weeks. The reported results of this trial showed similar concentrations of glucose, insulin, GIP and GLP-1 hormones in all groups.[74]

Bonnet et al.[75] reported similar findings when they investigated the effects of aspartame and Ace-k on insulin sensitivity and secretion in a randomized, crossover, double-blinded study. Healthy men (n=50) received 2 cans of carbonated beverage (330ml) containing aspartame (129

mg) and Ace-k (13 mg) or unsweetened carbonated beverage as a control, daily for 12 weeks. The results showed no significant differences in insulin sensitivity or secretion between the two groups.[75] However, the generalizability of this study is limited since only male participants were included.

2.5.4 Studies evaluating the effect of a single dose of aspartame on glucose metabolism

Similar to sucralose, the acute effects of single doses of aspartame have also been assessed in healthy, obese and diabetic participants (Table 2[41-75]). For example, Rodin[67] conducted a randomized, crossover trial to evaluate the acute effect of aspartame in overweight and normal weight healthy participants (n=24). Participants consumed aspartame (250 mg), fructose (50 g) or glucose (50 g) and 500 ml of water or water alone as a control. The results showed no effects of aspartame on glucose, insulin or glucagon.[67]

Additionally, findings from Hartel et al.[68] were consistent with the results from Rodin.[67] They examined the acute effect of a single dose of aspartame on glucose and insulin in healthy participants (n=14). In this RCT each male received either 330 ml of water with aspartame (165 mg), sucrose (33 g), Ace-k (165 mg), cyclamate (800 mg) or saccharin (75 mg). The results showed no effects on insulin or glucose concentration after NNS consumption compared to sucrose.[68]

Anton et al.[69] tested the acute effect of preloaded NNSs on post-prandial glucose and insulin concentrations in a crossover study. Healthy lean (n=19) and obese (n=12) participants received preloads containing tea sweetened with aspartame, sucrose, or stevia before meals. The results showed that plasma glucose concentrations were lower with stevia consumption compared to aspartame and sucrose ($p<0.01$). Additionally, insulin concentrations were lower

with stevia compared to aspartame and sucrose preloads ($p<0.05$).[69] However, the exact concentration of NNSs used in this trial were not reported and additionally, the tea could have had other ingredients that may have affected the measured outcomes.

Maersk et al.[70] assessed the acute effect of NNSs on glucose concentration and incretin hormones in a crossover trial. Healthy participants with obesity (n=24) received 500 ml of regular soft drink, skim milk, diet soft drink sweetened with aspartame, or water. The results showed no effect of diet cola drink on glucose, insulin, GLP-1, GIP, or ghrelin.[70] The concentrations of the NNSs in the diet cola drink were not reported.

Olalde-Mendoza et al.[71] measured the acute effect of diet soda on capillary blood glucose in patients with T2DM (n=80). After an overnight fast, patients received either 200 ml of diet soda (contains 40 mg aspartame and 100 g Ace-k) or 200 ml of regular soda. There was no effect of diet soda consumption on capillary blood glucose.[71] However, diabetes may have played a role in the development of these outcomes.

Bryant et al.[72] examined the acute effect of aspartame on glycaemic responses in a randomized, crossover trial. Healthy participants (n=10) were studied on 4 separate days. After an overnight fast, participants received glucose only (45 g), glucose (45 g) and aspartame (150 mg), glucose (45 g) and Ace-k (85 mg), or glucose (45 g) and saccharin (20 mg) in 250 ml of water. The results showed that fasting blood glucose concentrations between the conditions were similar.[72]

Moreover, Tey et al.[73] examined the acute effect of NNSs on 24 hour glucose profiles in a randomized, crossover, double-blinded study where healthy men (n=30) consumed one of the following treatments as beverages: aspartame (0.44 g) in 500 ml of water, monk-fruit (0.63 g) in 500 ml of water, stevia (0.33 g) in 500 ml of water, or sucrose (65 g) in 500 ml of water.

Treatments were masked with strawberry flavour and pink colour. Glucose and insulin AUCs were similar in all groups.[73] However, the generalizability is limited to men as only male participants were included.

In contrast, few trials have shown the acute effects after a single dose of aspartame consumption. For example, Horwitz et al.[65] carried out an open, crossover, randomized study to measure the acute effect of a single dose of NNSs in healthy females (n=12) and T2DM patients (n=10) taking oral hypoglycaemic medication. Participants were asked to drink 300 ml of cherry-flavoured Kool-Aid sweetened with aspartame (400 mg) or saccharin (135 mg) after an overnight fast and to continue fasting throughout the 3 hour study period. The results showed no significant changes in plasma glucose levels or insulin in any of the treatment groups following the treatments. However, the mean insulin AUC was higher following aspartame consumption compared to saccharin or control beverages in the normal participants.[65]

Moreover, Moller et al.[45] determined the acute effects of single doses of aspartame on glucose and insulin levels in healthy participants (n=10). During their visits, participants consumed aspartame (1 g) or bovine albumin (12.2 g) in 200 ml water or water alone as a control. There was a decrease in glucose concentrations after aspartame compared to water ($p<0.05$), but insulin levels were not affected.[45]

The findings of Melanson et al.[46] agreed with Moller et al.[45] regarding the effect of aspartame on glucose concentrations. Melanson et al.[46] examined the acute effect of aspartame on blood glucose concentrations in healthy participants (n=10) in a crossover study. Participants consumed beverages with aspartame or high-fat or simple carbohydrate followed by ad libitum meals. There was a decrease in blood glucose concentration ($p=0.014$) in 40% of the participants after aspartame consumption.[46]

However, Hall et al.[43] did not report any increase in blood glucose levels when they investigated the acute effects of aspartame on glucose, insulin and GLP-1 hormones in a crossover study. In their study, healthy participants (n=6) consumed aspartame capsules (400 mg), aspartic acid (176 mg) with phenylalanine (224 mg), or corn flour (400 mg) as control. The results showed that aspartame and amino acid consumption decreased plasma GLP-1, while aspartame ingestion did not affect glucose, insulin, or GIP concentrations.[43]

2.6 Discussion and conclusion

Overall, based on the totality of clinical trials to date (1969 - 2019), there is insufficient evidence to support adverse or beneficial effects of sucralose or aspartame consumption on glucose metabolism and gut hormones. The aim of this review was to provide a summary of RCTs that measured the effects of aspartame and sucralose on glucose metabolism and gut hormones. Unfortunately, it is still unclear if repeated daily or single exposures to aspartame and/or sucralose are associated with changes in glucose metabolism or gut hormones because of the many variations in and between and limitations of the existing RCTs.

In particular, screening for exposure to past use of NNSs needs to be considered when conducting future RCTs. Because there may be variation in the outcomes measured, The participants needs to be screened to determine if they are non-consumers or light NNS users, as well as if they have prolonged use or abstinence from NNSs because repeated NNS intake might affect glucose metabolism,[37] and might cause human gut microbiome adaptation.

Another limitation that needs to be considered is the unjustified, unreported, and/or unmeasured concentrations of NNSs used in trials that make it difficult to determine if the dosage in a trial is applicable to real life situations, and to replicate the results in future trials.

Moreover, there are significant variations in the routes of administration used, as some studies use the intragastric or intraduodenally route of administration, while others use capsules. These unusual routes of NNS administration that bypass the activation of oral sweet receptors might cause variations in the outcomes measured and do not reflect normal intake of NNS. Additionally, the presence of other ingredients in the diet drinks or sachets used might interact with the NNSs. The other ingredients could also inhibit or boost the potential adverse effects of the NNSs; therefore, controlling for these ingredients is important. Furthermore, some trials selected one sex, which limits their generalizability. Also, the variation in the microbiome composition of the human gut might affect every individual response to NNS consumption differently which result in variable results. Finally, some studies used NNSs at intake higher than would be consumed during a normal pattern of use. While it is difficult to design a perfect study, it is always important to consider the limitations of RCTs when interpreting the results.

In conclusion, few clinical trials have found any effects of aspartame or sucralose on glucose metabolism (Table 3[41-75]). However, the results are conflicting and variability exists between the available studies, which makes it difficult to compare them. For example, one study found higher glucose concentrations in morbidly obese participants after sucralose consumption, while another three studies found lower concentrations and thirty-three additional studies found no effect on glucose. Moreover, only four of the studies available in Table 3[41-75] reported increased GLP-1 concentrations in healthy and T1DM participants compared to water. Three studies found that sucralose consumption decreased insulin sensitivity in healthy and morbidly obese participants, and one study reported a decrease in acute insulin response, while another trial reported an increase. However, studies that involved the intragastric or intraduodenal administration of NNSs have been consistent in their findings, which show no effect of sucralose

on glucose, insulin or gut hormone secretion. For aspartame, two of the 16 studies reported a decrease in glucose concentration and one study showed that aspartame decreased the GLP-1 concentration compared to a placebo.

Finally, based on the available literature, it is clear from the limitations that exist in the available RCTs that more clinical trials are required. In particular, future trials should consider: 1) the measurement of previous to, or incidental during, NNS exposure, as contamination of control groups or treatment groups is likely with the increased use of NNSs; 2) the route of NNS administration, avoiding delivery routes such as capsules which avoid oral taste receptor activation and CPIR and does not replicate the usual route of NNS consumption, 3) the concentration of NNS used, doses which are not relevant to real life intakes should be reconsidered; 4) the study population, including healthy and non-healthy participants, 5) appropriate study design, especially with regards to controls and statistical power; and 6) study duration, with longer intervention periods because there may be consequences of consumption of NNSs that occur only after adaptations to long-term NNS exposure that should be investigated.

2.7 Acknowledgments

SYA work was supported by Kuwait Civil Service and Institute for Medical Specialization-Ministry of Health of Kuwait. The funding agencies had no role in the writing of the manuscript; or in the decision to submit the manuscript for publication. This manuscript did not undergo a peer-review by the external funding agency.

Authors' contributions. SYA wrote the manuscript, created and executed the literature search and provided input on the literature tables. JKF and DSM contributed significantly to the

improvement of this manuscript. All authors read, made critical revisions and approved the final manuscript.

Funding. The authors have not received any funding or benefits from industry or elsewhere to write this manuscript.

Declaration of interest. DSM was an invited speaker at a seminar entitled "Conflicting Outcomes from Systematic Reviews: Is the Consumption of Low-Calorie Sweeteners a Benefit or a Risk for Weight Management?" at Nutrition 2018 in Boston, DSM which sponsored by PepsiCo. PepsiCo paid for his accommodation, conference fee and honorarium. PepsiCo is a company which sells products that contain non-nutritive sweeteners. The authors have no potential conflicts of interest to declare.

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Table legands

Table 1. Common current approved non-nutritive sweeteners and their uses.

Table 2. Summary of studies evaluating the effect of non-nutritive sweeteners consumption on glucose metabolism.

Table 3. Summary of effects of sucralose and aspartame consumption on glucose metabolism and gut hormone.

Tables

Table 1 Common current approved non-nutritive sweeteners and their uses.

Sweetener generic chemical name	Trade name	Discovery year	Known metabolites	Multiplier of sweetness intensity (compared to table sugar sucrose)	Acceptable daily intake (US) (ADI) milligrams per kilogram body weight per day (mg/kg bw/d)	Acceptable daily intake (Canada) (ADI) milligrams per kilogram body weight per day (mg/kg bw/d)	Common uses
Acesulfame potassium	Sunett®, Sweet One®	1967	Not metabolized	200 x	15	15	Frozen desserts, candies, beverages, and baked goods
Aspartame	Equal®, NutraSweet®, Sugar Twin®	1965	Methanol, aspartic acid, phenylalanine	200 x	50	40	Table top sweetener, chewing gum, cold breakfast cereals, and dry bases for certain foods
Advantame	-	2014 (FDA approved)	-	20,000 x	32.8	N/A	General purpose sweetener, flavour enhancer, baked goods
Neotame	Newtame®	2002 (FDA approved)	De-esterified derivative, methanol	7000-13,000 x	0.3	N/A	Baked goods
Saccharin	Sweet'N Low®, Sweet Twin®, Necta Sweet®	1879	O-sulfamoylbenzoic acid	200-700 x	5	5 (Banned in 1970s, then approved in 2014)	Beverages, fruit juice drinks, bases or mixes, cooking and table top use
Sucralose	Splenda®	1976	Not metabolized	~600 x	5	9	Baked goods, beverages, chewing gum, gelatines, and frozen dairy desserts
Steviol glycosides	Truvia®, Enliten®, PureVia®	1955	-	200-400 x	4	NA	Baked goods, soft drinks
Luo Han Guo fruit extracts	Nectresse®, PureLo®, Monk Fruit in the Raw®	13 centuries	-	100-250 x	Not specified	NA	

NA: not associated.

Table 2 Summary of studies evaluating the effect of non-nutritive sweeteners consumption on glucose metabolism.

Study	Study design	Population, Gender, Sample size , Age (yrs.), BMI (kg/m2)	Duration	Type of non-nutritive sweetener used	Dose of sweetener used and method of administration	Main outcome reported
Mezitis et al.[47]	Ranomized, cross-over trial	T1DM and T2DM, M, F, n=26, Age <65 yrs.	Single dose	Sucralose	Subjectes received sucralose (1000 mg capsule) or placebo (cellulose capsules) followed by a meal.	Sucralose consumption had no effects on glucose or c-peptides levels compared to placebo
Baird et al.[48]	Randomized, single blinded trial	Healthy adults, M,F, study 1: n= 8, Mean. Age : 32 yr., Mean. wt: 70 kg	Repated daily dose, 17 days	Sucralose	Subjects fasted before the doses. Sucralose supplied in aqueous solution (5mg/ml), Phase 1: day 1= 0 mg/kg, day 3= 1mg/kg, day 5= 2.5 mg/kg, day 7= 5 mg/kg, day 9= 10 mg/kg, Phase 2: day 11-13= 2mg/kg/day, day 14-17= 5mg/kg/day, day 18- 25= 0 mg/kg.	No effect on fasting glucose or insulin.
Baird et al.[48]	Randomized, single blinded trial	Healthy adults, M,F, study 2: sucralose group: n=77, Mean. Age: 34.6 yr. Mean. wt: 71.5 34.6 kg, control group: n=31, Mean. Age: 33.9 yr., Mean.wt:69.3 kg	Repated daily dose, 13 weeks	Sucralose	Sucralose supplied in aqueous solution (5mg/ml) consumed 2x day for a total daily dose: Sucralose group: Weeks 1-3: 125 mg/day, weeks 4-7: 250 mg/day, weeks 5- 12: 500 mg/day, Control group: Weeks 1-13: 50 g/day of fructose.	No effect on fasting glucose or insulin
Grotz et al.[49]	Randomized, double-blind, placebo controlled trial	T2DM obese adults, M, F N=136, age 31-70 yrs, Mean. BMI: 31.6 kg/m2, Sucralose group: n= 67, Placebo group: n=69	Repated daily dose, 13 weeks Follow-up: 4 weeks	Sucralose	Sucralose group: sucralose (333.5 mg) supplied in capsules 2x day. Total daily intake was 667 mg. Placebo group: celloulose capsules supplies 2x day containing cellulose. Follow-up phase: 2 cellulose capsules/day .	No significant differences in plasma glucose , insulin, or serum-c-peptides or HbA1c levels between sucralose and placebo groups for the entire study duration.
Reyna et al.[50]	Randomized, controlled clinical trial	T2DM adults, M, American Diabetic Association (ADA) group: Mean. Age: 45±55 yr., Control group: n=8, Mean. BMI: 28.9 ± 2.0 kg/m2, Modified diet group: n=8, Mean. BMI: 28.5 ± 1.7 kg/m2	Repated daily dose, 4 weeks	Sucralose	ADA diet group: they did not mention components of the diet. Modified diet group: fructose and sucralose was used in a ratio of 30:70 as a sweeteners. Low calorie diet included fat free bread with 8% fat replacer with beta-glucans and oats ,and cookies prepared with 50% fat replacer with beta-glucans, Bread was consumed 2x day (60 g each bread and 3 cookies (20 g/cookie).	There was significant improvement in HbA1c, lipid profile and BMI in both groups, but not fasting blood glucose. Improvement was greater with the diet containg sucralose compared to ADA diet with a greater decrease in HbA1c
Ma et al.[51]	Randomized, cross-over, single blinded trial	Healthy subjects, M,F, n=7, Mean. BMI: 21.6 ± 1.2 kg/m2, Mean. Age: 24 ± 2 yr.	Single dose , 4 visits	Sucralose	All subjects received intragastric infusion labeled with 150 mg 13C-acetate: 500 ml saline, 50 g sucrose (positive control), 80 mg sucralose, and 800 mg sucralose.	Sucralose showed no effect on blood glucose, insulin, plasma GLP-1 or GIP levels when consumed alone

Brown et al.[52]	Randomized, cross-over trial	Healthy subjects, M,F, n=22 , Mean. BMI: 25.6 ± 4.6 kg/m2 , Age: 12-25 yrs.	Single dose, Post-prandial (OGTT)	Sucralose	Subject fasted before treatment, then they ingested either 240 ml carbonated water or 240 ml diet drink containing sucralose (unknown concentration) + Ace-K. 10 minutes following treatment an OGTT with 75 g glucose was done.	There was no effect seen on glucose, insulin, and GLP-1or c-peptides following OGTT. The GLP-1 peak (P= 0.003) and AUC (P= 0.003) were significantly higher with diet soda than carbonated water
Ma et al.[53]	Randomized, cross-over, single-blinded trial	Healthy subjects, n=10, Mean. BMI: 23.4 ± 0.8 kg/m2, Mean. Age: 27 ± 2yrs.	Single dose, 2 visits	Sucralose	Subjects received intraduodenaly infusion of sucralose (960 mg) in saline or saline only for 150 minutes.	Sucralose infusion did not affect glucose or GLP-1
Brown et al.[54]	Randomized, cross-over trial	Healthy subjects, n=8, F, Mean. BMI 22.1 ± 1.7 kg/m2, Mean. Age: 21.8 ± 2.5 yrs.	Single dose, 4 visits, fasting state and post-prandial	Sucralose	Subjects fasted before treatment of 355 ml water, 50 g sucrose in 355 ml water, 6 g Splenda in 355 ml water, 50 g sucrose and 6 g Splenda in 355 ml water. One hour after treatment breakfast was given.	There was no significant effect of sucralose on insulin, glucose, and glucagon compared to water.
Steinert et al.[55]	Randomized, placebo-controlled, double blinded, cross-over trial	Healthy subjects, n=12, M,F, Mean. BMI: 23 ± 0.5 kg/m2, Mean. Age:23.3 ± 0.7 yrs.	Single dose, 6 visits	Sucralose Aspartame Ace-k	Subjects fasted before intragastric infusion of treatments: glucose (50 g in water), fructose (25 g in water), sucralose (62 mg in water), aspartame (169 mg in water), and ace-k (220 mg in water), or water (250 ml) only as control.	No significant glucose, insulin, GLP-1, and appetite hormones response observed compared to water,
Ford et al.[56]	Randomized, cross-over, placebo-controlled, single-blinded trial	Healthy subjects, M,F, n=8, BMI: 18.8- 23.9 kg/m2, Age: 22- 27 yrs.	Single dose, 3 visits	Sucralose	Subjects fasted before treatment of 50 ml of water, 50 ml of sucralose (0.083% w/v), 50 ml of sucralose (0.083% w/v) + maltodextrin (50% w/v) followed by modified sham-feeding protocol (MSF) of same solution that was consumed to stimulate oral sweet receptors.	Consumption of sucralose or water followed by MSF combined with sucralose had no effect on plasma glucose, insulin and GLP-1 concentrations. Consumption of sucralose with maltodextrin caused an increase in plasma glucose and insulin AUCs without affecting GLP-1 hormone.
Wu et al.[57]	Randomized, cross-over, single-blinded trial	Helthy subjects, M,F, n=10, Mean. BMI 25.5 ± 1.5 kg/m2 , Mean. Age: 28.8 ± 4.0 yrs.	Single dose, 4 visits	Sucralose	Subjects fasted before preload drinks containing 40 g glucose, 60 mg sucralose, 40 g 3-OMG or 40 g tagatose/isomalt mixture (TIM) 15 min before a labeled mashed potato meal containing 13C-octanoic acid.	Sucralose had no effect on plasma blood glucose, GLP-1, GIP, insulin.
Brown et al.[41]	Randomized, cross-over trial	3 groups of subjects:M, Age: 13-24 yrs., Healthy: n=25, T1DM: n=9, Mean. BMI: 21.7 ± 2.4 kg/m2, Obese T2DM :n=10, Mean. BMI: 35.0 ± 6.8 kg/m2	Single dose, 2 visits, Post-prandial (OGTT)	Sucralose	Subjects fasted before drink, minus 10 minutes subjcets consumed 240 ml of diet soda containing sucralose (190 ± 38 mg/ml) and ace-K (108 ± 0.6 mg/ml) or 240 ml of carbonated water as a placebo. 3-hour OGTT with 75 g glucose load was done.	T1DM subjects: GLP-1 AUC 43 % higher after diet soda consumption (p=0.02). Healthy subjects: GLP-1 AUC 34 % higher after diet soda consumption (p=0.029). Diet soda had no effect on glucose , c-peptides, and GIP in all subjects.

Pepino et al.[44]	Randomized, cross-over trial trial	Morbidly Obese subjects, M,F, n=17, Mean. Age: 35.1 ± 1.0, Mean. BMI: 41.0 ± 1.5 kg/m2, Low consumption of NNS(< one can of diet soda or 1 spoonful of NNS /week)	Single dose, 2 visits, Post-prandial (OGTT)	Sucralose	Subjects fasted before drinks then consumed 60 ml of distilled water (control condition), 60 ml sucralose solution (2 mmol/L; 48 mg sucralose). OGTT with 75 g glucose load was performed after treatments.	Glucose, insulin, c-peptides were higher in sucralose group compared to water group (p<0.004). There was a decrease in Insulin sensitivity and insulin clearance 23 ± 20 % (p=0.01) and 7 ± 4 % (p=0.04) respectively. No differences in GLP-1, GIP, glucagon concentrations
Wu et al.[58]	Randomized, single-blinded, cross-over trial	Helthy subjects, n=10, M, Mean. BMI :25.5 ± 1.0 kg/m2, 33.6 ± 5.9 yrs.	Single dose, 240 minutes, fasting and post-prandial (OGTT)	Sucralose Ace-K	Subjects consumed either 240 mL water alone or similar amount sweetened with 52 mg sucralose, 200 mg, Ace-K, or 46 mg sucralose + 26 mg Ace-K after an overnight fast. An OGTT with 75 g glucose and containing 150 mg 13 C-acetate was administered 10 minutes after each treatment.	Prior to glucose ingestion, blood glucose, plasma insulin and GLP-1 levels did not change with any of the sweetened drinks or water. Blood glucose, plasma insulin and GLP-1 all increased after OGTT (P<0.001 for each) and were similar among all the treatment group.
Stellingwerff et al.[59]	Randomized, double-blinded, crossover study trial	Helthy subjects,M, n=23, Mean. BMI 23.1 ± 1.9 kg/m2, Mean. Age: 29 ± 7 yrs.	Single dose, Before , during and after 2 hours of exercise (cycling).	Sucralose	8 x 50 ml doses of either 1mM (20 mg) sucralose solution (SUCRA) or placebo (sucralose mouthwash followed by drinking 50 ml of water) every 15 minutes for 2 hours starting 120 min before the beginning of exercise.	Ingestion of sucralose had no effect on blood glucose and insulin levels during the study.
Temizkan et al.[60]	Randomized, single-blinded , cross over trial	Healthy subjects: M,F, n=8, Mean. Age: 45.0 ± 4.1 yrs., Mean. BMI : 30.3 ± 4.5 kg/m2, Newly diagnosed T2DM subjects: M,F, n=8, Mean. Age: 51.5 ± 9.2 yrs., Mean. BMI: 33.7 ± 5.4 kg/m2.	Single dose, 3 visits, Post-prandial (OGTT)	Sucralose, Aspartame	Subjects consumed:24 mg of sucralose (tabletop formulation) in 200 ml of water, or 72 mg aspartame (tabletop formulation in 200 ml of water or, or 200 ml water alone. 15 minutes after treatments, OGTT (75g glucose) was performed.	Consumption of sucralose was associated with lower blood glucose AUC (p=0.002) and increase GLP-1 AUC (p=0.04) compared to water in healthy subjects. No effect on insulin or c-peptides levels between the sucralose and water groups. No effect on glucose, insulin, c-peptides or Glp-1 after treatment with sucralose and water in T2DM subjects.
Sylvetsky et al.[61]	Randomized, crossover trial	Study arm 1: healthy subjects n=30, M,F, Mean. BMI: 25.8 ± 4.2 kg/m2, Mean. Age: 29.7 ± 7.6 yrs., Study arm 2: healthy subjects, n=31, M,F Mean. BMI: 26.3 ± 7.5 kg/m2, Mean. Age: 27.4 ± 6.7 yr.	Single dose, Post-prandial (OGTT)	Sucralose, Aspartame, Ace-K	Subjects fasted before drinks: Study arm 1 consumed 355 mL water or 355 mL water + sucralose (68 mg) or 355 mL water + sucralose (170 mg) or 355 mL water + sucralose (250 mg). Study arm 2 consumed 355 ml carbonated water or 355 mL diet soda (18 mg sucralose +18 mg ace-K + 57 mg aspartame + other ingredients) or 355 mL diet soda (68 mg sucralose +41 mg ace-K+ other ingredients) or 355 ml carbonated water (68 mg sucralose + 41 mg Ace-K).	Study arm 1: Sucralose preload of different doses had no effect on glucose, insulin, C-peptide, glucose absorption, gut hormones GLP-1 and GIP. Also carbonated water with NNSs had no effect on the parameters measured in comparison to carbonated water alone Study arm 2: sucralose preload with Ace-K has no effect on metabolic outcomes. GLP-1 hormone was augmented after diet soda intake .
Grotz et al.[62]	Randomized, double-blinded, Parallel trial	Healthy adults, n= 47, M, Age: 18-45 yrs., BMI: 19.4 - 27.0 kg/m2.	Repated daily dose, 12 weeks	Sucralose	Subjects consumed ~333.3 mg sucralose capsules 3x/day or placebo (cellulose) 3x/day at mealtimes.	No differences between groups in change from baseline for fasting glucose, insulin and HbA1c.
Romo-Romo et al.[63]	Randomized, open label, parallel arm design	Healthy adults, n= 66, F , Age: 18- 55 yrs., BMI: 18.5- 24.9 kg/m2, low NNS consumers (< 5 portion/ week regardless of product type).	Repated daily dose, 14 days	Sucralose	Subjects consumed 1 commercial sachet 3x/ day (12 mg sucralose, 958 mg dextrose, and 30 mg maltodextrin) added to beverage or meals, control group followed similar procedure without sucralose.	Sucralose group showed significant decrease in insulin sensitivity compared to control group (p = 0.04). Acute insulin response increased in sucralose group (p = 0.04).

Lertrit et al.[42]	Randomized, cross-over design, double-blinded trial	Healthy adults, n=15, M, F, Age: 18-59 yrs., BMI: 18.5 – 27 kg/m2.	Repated daily dose, 4 weeks, Post-prandial (OGTT)	Sucralose	Subjects consumed 200 mg sucralose or placebo capsules.	Acute insulin response and sensitivity decreased in sucralose group ($p < 0.005$). AUC of active GLP-1 increased in sucralose group ($p< 0.001$).
Nehrling et al.[64]	Randomized, double-blinded, and placebo controlled trial	IDDM & NIDD subjects: n =62, Age: 18–65 yrs.	Repated daily dose, 18 weeks	Aspartame	Subjects consumed aspartame capsules (2.7 g) per day with meals or placebo capsules (20 mg of corn starch) with meals.	During treatment blood glucose or HbA1c levels were similar in both groups.
Horwitz et al.[65]	Randomized, cross-over trial	Healthy subjects: F, n=12, Mean. age: 28 ± 8 yrs., Mean. BMI: 62.1± 6.9 kg/m2, NIDDM on oral hypglycemic agents:F, n=10, Mean. age: 57 ±8 yrs., Mean. BMI: 94.1± 15.7 kg/m2.	Single dose, 3 visits	Aspartame, Saccharin	After an overnight fast subject consumed 400 mg aspartame +300 ml of cherry-flavored Kool-Aid sweetened or 135 mg saccharin +300 ml of cherry-flavored Kool-Aid sweetened.	No significant changes in plasma glucose, insulin or glucagon levels in all treatment groups. In normal subjects, mean AUC insulin concentrations was higher following aspartame consumption compared to saccharin or control beverage ($p<0.05$).
Colagiuri et al.[66]	Controlled, cross-over double-blind trial	NIDDM, M,F, n=9, Mean. age: 66 ± 5 yrs., Mean. BMI: 26.4 ± 2.1 kg/m2.	Repated daily dose, 6 weeks	Aspartame, Sucrose	Subjects consumed 45 g sucrose and 162 mg aspartame, added to meals and beverages daily	Aspartame consumption did not cause changes in glucose and HbA1c levels.
Rodin[67]	Randomized, cross over trial	Overweight and normal weight healthy subjects (n=24), Age: 22-50 yrs.	Single dose	Aspartame	Subjects consumed aspartame (250 mg) or fructose (50 g) or glucose (50 g) + 500 ml water or water alone as a control.	No significant effects of aspartame on glucose, insulin or glucagon.
Moller et al.[45]	Randomized trial	Healthy men: n=6, M, Age: 22–37 yrs., BMI: 63-83 kg/m2.	Single dose	Aspartame	Subjects consumed aspartame (1g) or bovine albumin (12.2 g) in 200 ml water or water alone as a control.	Glucose concentration were decreased compared to control after aspartame consumption ($p<0.05$). Insulin levels were not affected in all three groups
Hartel et al.[68]	Randomized clinical trial	Healthy subjects,M, n=14, Age: 19- 52 yrs.	Single dose	Aspartame Ace-k Saccharin	Subjects consumed 330 ml water with aspartame (165 mg) or sucrose (33g) or Ace-k (165mg) or cyclamate (800 mg) or saccharin (75 mg).	No effect on insulin and glucose concentration after NNS consumption compared to sucrose
Melanson et al.[46]	Randomized, cross-over trial	Healthy subjects, n=10, Age: 19-31 yrs., Mean. BMI: 23.4 ± 1.9 kg/m2.	Single dose	Aspartame	Subjects consumed drinks with aspartame or high-fat or carbohydrate followed by ad libitum meals.	There was a decrease in blood glucose concentration ($p=0.014$) in 40 % of the participants after aspartame consumption
Hall et al.[43]	Cross-over trial	Healthy subjects, n=6, age 24-31yrs., BMI < 25 kg/m2.	Single dose, 3 visits	Aspartame	Subjects consumedaAspartame capsules (400 mg) or aspartic acid (176 mg) + phenylalanine (224 mg) or corn flour (400 mg) as control.	Aspartame and amino acids consumption decreased plasma GLP-1 ($p<0.05$). Aspartame ingestion did not affect glucose, insulin, or GIP concentrations
Anton et al.[69].	Cross-over trial	Healthy lean subjects: M,F, n=19, age 18 - 50 yrs., BMI: 20.0 – 24.9 kg/m2,	Single dose, 3 visits	Aspartame, Stevia, Sucrose	Subjects consumed tea sweetened with aspartame or stevia or sucrose before they consume buffet ad libitum. No quantity specified in mg only kcal.	Plasma glucose and insulin were lowered with stevia consumption compared to sucrose ($p<0.01$, $P<0.05$ respectively).

		Obese subjects: M,F, n=12, age:18 - 50 yrs., BMI: 30.0 – 39.9 kg/m2.				
Maersk et al.[70]	Open, cross-over trial	Obese healthy subjects: n=24 Mean. age: 33.5 ± 9.2 yrs., Mean. BMI: 31.4 ± 3.11 kg/m2.	Single dose, 4 visits	Aspartame	Subjects consumed 500 ml of regular cola drink (sweetened with sucrose) or 500 ml skimmed milk or 500 ml diet cola drink (sweetened with aspartame) or 500 ml water.	Diet cola drink (sweetened with aspartame) did not show an effect on glucose, insulin, GLP-1, GIP or ghrelin.
Olalde-Mendoza et al.[71]	Randomized trial	T2DM subjects on medication: n=80, Mean. age: 49.3± 9.0 yrs., Mean. BMI: 30.5 ± 4.3 kg/m2.	Single dose	Aspartame, Ace-K, Regular soda	Subjects consumed after an overnight fast: group 1 : 200 ml of diet soda (contains 40 mg aspartame + 100 g acesulfame potassium) , Group2: 200 ml regular soda	There was no effect of diet soda on capillary blood glucose levels
Bryant et al.[72]	Randomized, cross-over trial	Healthy subjects, M, F, n=10, age 18-24 yrs., Mean. BMI: 21.8 ± 1.8 kg/m2.	Single dose, 4 visits	Aspartame, Saccharine, Ace-K	Subjects consumed after an overnight fast 45 g glucose or, 45 g glucose + 150 mg aspartame or, 45 g glucose + 85 mg ace-K or, 45 g glucose + 20 mg saccharin in 250 ml water.	There was no effect after NNS consumption on glucose concentrations.
Tey et al.[73]	Randomized, cross-over, double-blinded trial	Healthy subjects, n= 30, M, Age: 21-50 yrs., BMI: 18.5 - 25.0 kg/m2.	Single dose, 1 visit	Aspartame, monk fruit extract, stevia, sucrose	Subjects consumed beverages given as pre-load. containing 0.44 g aspartame + 500 ml water or 0.63 g monk fruit extract (50 % mogroside + 500 ml water or 0.33 g stevia (steviol glycoside, rebaudioside A) + 500 ml water or 65 g sucrose + 500 ml water. Consumed 1 hour before ad libitum lunch.	There was no effect of any treatment on glucose and insulin AUC
Higgins et al.[74]	Randomized, parallel arm trial	Healthy Subjects, n=93, M, F, Age: 18-60 yrs., BMI: 18-25 kg/m2, non NNS consumers.	Repated daily dose, 12 weeks	Aspartame	Subjects consumed aspartame 1x/ day. 0-mg/day aspartame group: took 2 capsules collectively containing 680 mg dextrose + 80 mg para-amino benzoic acid (PABA) + 2 empty capsules, 350-mg/day aspartame group: beverage with 350 mg aspartame + 80 mg PABA+ 2 capsules collectively containing 680 mg dextrose + 2 empty capsules, 1050 mg/day aspartame group: took beverage with 350 mg aspartame + 80 mg PABA+ 4 capsules collectively containing 700 mg aspartame and 680 mg dextrose.	No significant difference in glucose, insulin, GLP-1 or GIP at baseline or week 12 between groups.
Bonnet et al.[75]	Randomized, cross-over design, double-blinded trial	Healthy subjects, n= 50, M, Mean. Age: 31.1 ± 10.3 yr., BMI: 19-29 kg/m2, not regular users of NNS (consuming < can of beverage with high intensity sweeteners/week).	Repated daily dose, 12 weeks	Aspartame, Ace-K	Subjects consumed 330 ml beverage 2x/ day containing 129 mg of aspartame + 13 mg of Ace-K in carbonated water, control group received 330 ml carbonated water 2x/ day.	No significant difference in insulin sensitivity or secretion between groups.

Yr.= year, N= number of participants, M= male, F= female, Kg/m^2= kilograms per square meter, HbA1c= hemoglobin A1c, h= hour, NNS= non-nutritive sweetener, AUC= area under curve, GLP-1= glucagon-like peptide 1, GIP= gastric inhibitory polypeptide, PABA: para-amino benzoic acid, Ace-K= acesulfame- potassium, OGTT= oral glucose tolerance test, T1DM= Type 1 diabetes mellitus, T2DM= Type 2 diabetes mellitus.

Table 3 Summary of effects of sucralose and aspartame consumption on glucose metabolism and gut hormones.

NNS used	Dose	Route	Reference	Subjects	Summary of effects on glucose metabolism									
					Blood glucose	Insulin	GLP-1	GIP	Insulin sensitivity	Acute insulin response	Insulin clearance	HbA1c	C-peptides	Glucagon
Sucralose	Single dose													
		Oral	Mezitis et al.[47]	T1DM	No effect								No effect	
		Oral		T2DM	No effect								No effect	
		Intragastric	Ma et al.[51]	Healthy	No effect	No effect	No effect	No effect						
		Oral	Brown et al.[52]	Healthy	No effect	No effect	No effect						No effect	
		Intraduodenal	Ma et al.[53]	Healthy	No effect		No effect							
		Oral	Brown et al.[54]	Healthy	No effect	No effect								No effect
		Intragastric	Steinert et al.[55]	Healthy	No effect	No effect	No effect							
		Oral	Ford et al.[56]	Healthy	No effect	No effect	No effect							
		Oral	Wu et al.[57]	Healthy	No effect	No effect	No effect	No effect						
		Oral	Brown et al.[41]	Healthy	No effect		Increased (p= 0.02)	No effect						
		Oral		T1DM	No effect		Increased (p= 0.02)	No effect						
		Oral		T2DM, obese	No effect		No effect	No effect						
		Oral	Wu et al.[58]	Healthy	No effect	No effect	No effect							
		Oral	Stellingwerff et al.[59]	Healthy	No effect	No effect								
		Oral	Pepino et al.[44]	Morbidly obese	Increased (p< 0.004)	Increased	No effect	No effect	Decreased (p= 0.01)		Decreased (p= 0.04)		No effect	
		Oral	Temizkan et al.[60]*	Healthy	Decreased (p=0.002)	No effect	Increased (p= 0.04)						No effect	
		Oral		T2DM	No effect	No effect	No effect						No effect	
		Oral	Sylvetsky et al.[61]	Healthy	No effect	No effect	No effect	No effect					No effect	
	Repeated daily dose													
		Oral	Grotz et al.[62]	Healthy	No effect	No effect						No effect		
		Oral	Romo-Romo et al.[63]	Healthy	No effect				Decreased (p= 0.04)	Increased (p= 0.04)				
		Oral	Lertrit et al.[42]	Healthy	No effect		Increased AUC (p< 0.001)		Decreased (p< 0.005)	Decreased (p< 0.005)				
		Oral	Baird et al.[48]	Healthy	No effect	No effect								
		Oral	Baird et al.[48]	Healthy	No effect	No effect								
		Oral	Grotz et al.[49]	T2DM, obese	No effect	No effect						No effect	No effect	
		Oral	Reyna et al.[50]	T2DM, obese	No effect							Decreased		
Aspartame	Single dose													

		Oral	Horwitz et al.[65]	Healthy	No effect	Mean AUC increased (p< 0.05)								No effect
		Oral		T2DM	No effect	No effect								No effect
		Oral	Rodin[67]	Healthy	No effect	No effect								No effect
		Oral		Obese	No effect	No effect								No effect
		Oral	Moller et al.[45]	Healthy	Decreased (p< 0.05)	No effect								
		Oral	Härtel et al.[68]	Healthy	No effect	No effect								
		Oral	Melanson et al.[46]	Healthy	40% decreased									
		Oral	Hall et al.[43]	Healthy	No effect	No effect	Decreased (p< 0.05)	No effect						
		Oral	Anton et al.[69]	Healthy	No effect	No effect								
		Oral		Obese	No effect	No effect								
		Oral	Maersk et al.[70]	Healthy, obese	No effect	No effect	No effect	No effect						
		Oral	Olaldemendoza et al.[71]	T2DM	No effect									
		Oral	Bryant et al.[72]	Healthy	No effect									
		Oral	Temizkan et al.[60]*.	Healthy	No effect	No effect	No effect						No effect	
		Oral		T2DM	No effect	No effect	No effect						No effect	
		Oral	Tey et al.[73]	Healthy	No effect	No effect								
	Repeated daily dose													
		Oral	Nehrling et al.[64]	T1DM	No effect							No effect		
		Oral		T2DM	No effect							No effect		
		Oral	Colagiuri et al.[66]	T2DM	No effect							No effect		
		Oral	Higgins et al.[74]	Healthy	No effect	No effect	No effect	No effect						
		Oral	Bonnet et al.[75]	Healthy	No effect	No effect								

*This study tested both sweeteners aspartame and sucralose,

AUC= area under curve**,** T1DM= Type 1 diabetes mellitus, T2DM= Type 2 diabetes mellitus.

BRIDGE TO CHAPTER III

The following chapter consists of a manuscript that provides recent evidence (between 2017 and 2018) for the effects of NNSs on glycemic control. Samar Y. Ahmad was the principal manuscript author.

CHAPTER III

MANUSCRIPT 2

This manuscript has been published in the Current Opinion in Clinical Nutrition and Metabolic Care journal 2019,

RECENT EVIDENCE FOR THE EFFECTS OF NON-NUTRITIVE SWEETENERS ON GLYCAEMIC CONTROL

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3.1 Abstract

Purpose of review: By replacing sugar, non-nutritive sweeteners (NNSs) are thought to aid in weight management and decrease insulin resistance. We reviewed the latest randomized clinical trials (RCTs) investigating the effects NNSs on glycaemic control.

Recent findings: Six RCTs addressed this topic between 2017 and 2018; the majority tested artificial NNS (sucralose or aspartame), with only one testing natural NNS (stevia and monk fruit extract). Most found no effect of NNS on blood glucose, insulin, gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) levels; however, two trials showed an effect of sucralose on the acute insulin response.

Summary: We are still incapable of reaching a definite judgement on which types of NNS, if any, impact glycaemic control. There is a need for more research to overcome the limitations of recent RCTs, related to sample size, intervention duration, dose, form of NNSs used, and inclusion of males or females only. Future studies should also compare different NNS types with each other, and include the increasingly popular "natural" NNS.

Keywords: Non-nutritive sweeteners, low-calorie sweeteners, aspartame, sucralose, acesulfame potassium, monk fruit extract, stevia, glucose metabolism, glycaemic control, blood glucose, insulin, randomized clinical trial.

3.2 Introduction

Awareness of the potential harmful effects of sugars is increasing due to the rising consumption of food and beverages high in sugar, particularly sucrose or high-fructose corn syrup (1). Examples of sugar-sweetened beverages (SSBs) include sodas, fruit drinks and sport drinks; these sources account for 50% of the total added sugar in the Western diet. Moreover, added sugar consumption accounts for approximately 11% to 13% of the total energy intake for Canadian adults (2) and higher than 13% in the United States (3), substantially exceeding the limit of 10% recommended by the World Health Organization (4).

Current evidence suggests that excess sugar intake is associated with the development of insulin resistance, which can lead to an increased risk of cardiovascular diseases and type 2 diabetes mellitus (T2DM) (5). In addition, overconsumption of food and beverages saturated with sugar can be a major risk factor for developing obesity in adults and children (6). Thus, non-nutritive sweeteners (NNSs) have become a popular alternative and have attracted increasing attention worldwide.

NNSs are zero- or low-calorie sweeteners that can be artificially synthesized or derived from natural sources. They that are added in small quantities when replacing caloric sweeteners because they are 30 to 37,000 times sweeter than sucrose (7). NNSs are popular worldwide as a substitute for regular sugar providing a desired sweet taste with few or no calories. Currently, nine types of NNSs are approved by the Food and Drug Administration (FDA) for use in the United States as food additives. The approved artificial NNS include aspartame (NutraSweet and Equal), advantame, saccharin (Sweet'N Low), sucralose (Splenda), acesulfame-potassium (Ace-K and Sweet One), neotame (Newtame). Approved natural NNS are stevioside, rebaudioside A and monk fruit extract (8). Marked increases in regular NNS consumption were reported in both children

and adults in the United States between 1999-2000 and 2009-2012, with the consumption prevalence increasing from 8.7% to 25% (200% increase) in children and from 27% to 41.5% (54% increase) in adults (9). This increase may have multiple explanations, including 1) increasing awareness of recommendations to reduce added sugar intake, 2) increasing prevalence of T2DM and obesity, and 3) increasing availability of NNS-containing food and drink products (9).

Replacing caloric sweeteners with NNS is expected to be beneficial for people with obesity or metabolic diseases, such as T2DM, which is characterized by high plasma glucose levels and insulin resistance. However, limited evidence in the literature supports this benefit (10, 11). A recent systematic review and meta-analysis focused on long-term (minimum 6 months) studies (11). The authors complied evidence (1997 to 2016) from seven RCTs with 1,003 participants and 30 observational prospective studies with 405,907 participants and found that regular consumption of NNSs did not fulfil their proposed purpose of weight management. In fact, in observational studies, the NNSs were associated with a moderate increase in body mass index (BMI) and cardio-metabolic risk (e.g., overweight, obesity, metabolic syndrome, T2DM, hypertension), while evidence from RCTs suggest that NNSs consumption is not associated with decrease in BMI, weight, and waist circumference due to the inherent limitations of observational studies and the paucity of long-term RCTs, the authors suggested that further studies are needed to substantiate the risk and benefits of long term NNS use.

Another recent meta-analysis was conducted to assess the glycaemic impact of NNSs (12*) . The reported findings were from 29 RCTs with 741 subjects (1985 to 2018) comparing the acute effect of four types of NNSs (aspartame, saccharin, sucralose and steviosides) on blood glucose concentrations over time after NNS consumption. The authors concluded that NNS consumption did not increase blood glucose concentrations compared to baseline, and that blood glucose

concentrations gradually declined following NNS consumption. The glycaemic impact of NNS consumption did not differ by the type of NNS used; however, the impact was reduced in older participants and those with high BMI.

The present review aims to provide an updated summary of recent RCTs (published between July 2017 and January 2019) measuring the effect of NNSs on glycaemic control in humans of any gender, age or health status. Outcomes considered for glycaemic control are glucose, insulin, haemoglobin A1c (HbA1c), GLP-1, GIP, and insulin response and sensitivity.

3.3 Recent evidence from RCTS examining NNSs and glycaemic control

Between 2017 and 2019, six RCTs were published addressing this topic (Table 1). Grotz et al. investigated whether sucralose affected glucose homeostasis in 47 healthy male volunteers who consumed 333.3 mg of encapsulated sucralose or placebo 3x/day at mealtimes (total dose of 999.9 mg/day) for 12 weeks. In this randomized, double-blinded, placebo controlled study, weekly measures of fasting glucose, haemoglobin A1c (HbA1c), insulin and C-peptide revealed no significant differences between the sucralose and placebo (cellulose) groups, leading the authors to conclude that sucralose had no effect on glycaemic control (13). However, this study design had limitations including: 1) only male participants, limiting generalizability; 2) sucralose given by capsules, thereby bypassing the oral activation of sweet taste receptors; 3) a very high dose of sucralose (999.9 mg/day, roughly equivalent to sucralose from thirteen 12-ounce cans of diet-flavoured soda). Study strengths included the relatively long intervention duration, compliance measured through a pill count (weekly), electronic records of capsules bottle opening, and urine tests to detect sucralose presence in the urine.

Consistent with Grtoz et al., Tey et al. found no effect of NNS on glycaemic control. In a randomized, double-blind crossover trial, they examined the immediate effects of consuming three types of NNSs (aspartame, monk fruit, stevia) or sucrose in sweetened beverages on blood glucose and insulin responses in 30 healthy male volunteers. The results did not show a significant difference in the total area under the curve (AUC) for either glucose or insulin for any of the NNS types (14). This study was the first to investigate the effects of monk fruit consumption on glycaemic control in humans. Limitations included: 1) the treatment exposure was very short (single acute challenge), a longer exposure period may be important for measurement of NNS effects on glycaemic control; 2) only healthy males were included in this trial, limiting generalizability.

A RCT with a parallel arm design by Higgins et al. examined the effect of aspartame consumption in 93 healthy lean adults. During the 12-week intervention, 3 groups received differing aspartame doses once per day: 1. Two capsules collectively containing 680 mg dextrose + 80 mg para-amino benzoic acid (PABA) + 2 empty capsules, 2. Beverage with 350 mg aspartame + 80 mg PABA+ 2 capsules collectively containing 680 mg dextrose + 2 empty capsules, 3. Beverage with 350 mg aspartame + 80 mg PABA+ 4 capsules collectively containing 700 mg aspartame and 680 mg dextrose. The beverages contained PABA, which was measured in participant urine to assess compliance. The trial found no significant differences between the groups in the glycaemic response, insulin, GIP or GLP-1 hormones (15). This study had a reasonably large sample size for glycaemic response, included males and females, and measured compliance using PABA.

Bonnet et al. compared the effects of carbonated beverages sweetened with NNSs on insulin sensitivity and secretion in 50 healthy men using a randomized, double-blinded cross-over

design consisting of two 12-week intervention periods. Participants were randomized to drink a carbonated beverage (2 cans 330 mL each day) containing aspartame (129 mg) and acesulfame K (13 mg), or an unsweetened carbonated beverage. The NNS-sweetened beverage had no significant effect on insulin sensitivity, assessed by the Matsuda Insulin Sensitivity Index (MISI) after an oral glucose challenge, or insulin secretion assessed by Stumvoll indexes (16). NNSs intake did not influence body weight, food intake, or physical activity. Strengths of this trial are the sample size. Limitations included: 1) compliance was not assessed; 2) different flavor between the 2 drinks tested in this study can be distinguished.

An RCT conducted by Romo-Romo et al. examined the effects of sucralose consumption on glucose metabolism, including insulin sensitivity, acute insulin response to glucose, and glucose effectiveness. In this trial, 66 healthy female subjects with a history of low NNS consumption received a commercial sucralose sachet (contains 958 mg dextrose, 30 mg maltodextrin, and 12 mg sucralose) 3 times/day for 2 weeks. The results showed a significant decrease in insulin sensitivity compared to that of the control group (P= 0.04) who received similar sachets but without sucralose. Additionally, a significant increase in the acute insulin response to glucose (P = 0.04) was observed in the sucralose group for the participants who were adherent to the protocol (17**). However, there was not enough details regarding the amount and contents of the sachets received by the control group.

Lertrit et al. also examined the effects of sucralose on glucose metabolism. In this randomized, double-blinded, placebo controlled study, 15 healthy non-NNS consumers received capsules containing either 200 mg of sucralose or placebo (empty capsules) for 4 weeks (18**). After a 75-g oral glucose tolerance test (OGTT) on two separate occasions, the AUC for active GLP-1 was significantly higher in the sucralose vs. control group ($p<0.001$). Interestingly, insulin

secretion (measured by Insulinogenic index) was significantly higher, and insulin sensitivity (measured by Matsuda index) was lower after exposure to sucralose than to placebo ($p<0.001$ and $p<0.005$, respectively). Conflictingly, acute phase insulin response was lower in the sucralose than in the placebo group measured during an intravenous glucose tolerance test (IVGTT) (18). These results suggest that the route of glucose delivery may be important in how sucralose impacts insulin secretion, and the increase in GLP-1 via oral glucose exposure under sucralose presence may be necessary for the increase in insulin secretion. Moreover, continuous exposure to sucralose may decrease the insulin sensitivity and increase GLP-1 and insulin release in response to oral glucose in healthy volunteers, but longer follow-up studies are needed to support these results. Limitations of this trial are the small sample size with majority of participants being female. Also, the authors did not mention how many sucralose and placebo capsules were consumed per day. Similarly, to Grotz et al. (13), the activation of taste receptors in the oral cavity was bypassed in this study because sucralose was administered in capsules but this was intentional, because the authors aim was to determine the effect of chronic sucralose intestinal exposure by avoiding the activation of oral sweet receptors which can be mediated by cephalic phase (gastric hormones secretion before food reach stomach induced by oral receptors stimulation) responses.

3.4 Discussion

Despite these recent contributions to the literature, the uncertainty continues regarding the potential effects of NNS on impairment of glycaemic control. For example, the study by Romo-Romo et al demonstrated a significant increase in the insulin response in women after sucralose consumption (19**), which was also seen by Lertrit et al. in both men and women during OGTT, but not in IVGTT (18). This discrepancy may be suggestive of a mechanism by which sucralose

exposure increases insulin secretion. In an IVGTT glucose bypasses the oral cavity, stomach and intestines, leading to lower insulin responses (20) compared to OGTT, likely due to the action of GLP-1 (21). However, the RCT conducted in men by Grotz et al. (13) did not replicate this association between sucralose consumption and insulin secretion in response to glucose. All three of these studies were relatively small and none were powered specifically for insulin response in an OGTT. Therefore, the potential effect of sucralose on insulin secretion in response to glucose requires further investigation in clinical trial powered specifically for that purpose, that uses a real-world sucralose delivery method and recruits both men and women. Moreover, bypassing the oral sweet taste receptors in Grotz et al. (13) and Lertrit et al. (18) trials by using sucralose capsules might have an effect on the outcome measured, because stimulating the oral sweet receptors causes an increase in glucose absorption through the release of incretins hormones GLP_1 and GIP which influence glucose metabolism (22).

In agreement with the majority of previous research (reviewed in (23)), recent RCTs by Tey et al. (14), Higgins et al. (15), and Bonnet et al. (16) did not show any effect of aspartame consumption on measures of glycaemic control. This may be due to aspartame's unique composition and metabolism relative to other NNS such as sucralose. Aspartame is quickly broken down to aspartic acid, phenylalanine and methanol, so there is almost no systematic absorption or exposure of the gut microbiome to intact aspartame (24). Moreover, very few studies have tested natural NNS (e.g. only 1 of these 6 trials mentioned above), this paucity of RCTs must be addressed in future trials.

Overall, the findings from the above RCTs suggest that more research is needed to determine if and how NNS impact glycaemic control. Comparative trials of different NNS types

will be especially useful, as different NNS may have different mechanisms of action which may or may not influence glycaemic control.

3.5 Conclusion

In conclusion, the consumption of different types of NNSs has mixed effects on blood glucose, insulin levels, and gut hormones. Based on the current evidence, we are still incapable of establishing a definite judgement on whether NNS use truly affects glycaemic control. To address the limitations in the previous trials, future studies should strive to use an appropriate forms and doses of NNSs similar to those used in beverages and foods, include men and women, and compare various NNS to each other and/or appropriate controls. Meanwhile, NNSs should be used with caution until further research fully elucidates their impact on human health.

An interesting factor to consider when interpreting results of RCTs using NNSs, participants can often be exposed to sources of NNSs in the treatment and control groups, potentially confounding the results. This was shown in as 2-week trial (25**) where the authors found that more than one-third of the participants (N=18) were exposed to sucralose prior to randomization and at baseline even after receiving detailed verbal and written instructions to stay away from products containing NNSs. Sucralose exposure was measured using a spot urine sample analysis.

3.6 Key points

- Conclusion cannot be reached on which types of NNS, if any, impact glycaemic control.
- There is a need for more research to overcome the limitations of recent RCTs, related to sample size, intervention duration, dose, form of NNSs used, and inclusion of males or females only.

- Future studies should compare different NNS types with each other, and include the "natural" NNS.

3.7 Acknowledgments

None

3.8 Final support and sponsorship

SYA work was supported by Kuwait Civil Service and Institute for Medical Specialization-Ministry of Health of Kuwait. No funding was specifically obtained for this review. MBA holds a Tier 2 Canada Research Chair in the Developmental Origins of Chronic Disease.

3.9 Conflicts of interest

DM was an invited speaker at a seminar entitled "Conflicting Outcomes from Systematic Reviews: Is the Consumption of Low-Calorie Sweeteners a Benefit or a Risk for Weight Management?" at Nutrition 2018 in Boston, MA which sponsored by PepsiCo. PepsiCo paid for his accommodation, conference fee and honorarium. PepsiCo is a company which sells products that contain non-nutritive sweeteners. The other authors have no conflict of interest to declare.

3.10 References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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** of outstanding interest

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- Intresting new finding.

Table 4 Summary of RCTs evaluating the effect of NNS on glycaemic control included in this review.

Author	Study design	Population, Number of subjects	Gender	Age (yr.)	Body mass index (kg/m2)	Duration	Type of sweetener	Dose of sweetener	Intervention	Main outcome to NNS consumption
Grotz et al., 2017	RCT-double-blinded, parallel.	Healthy adults, n= 47	M	18-45 yrs.	19.4 - 27.0 kg/m^2	12 weeks	Sucralose	~333.3 mg sucralose 3x/day	Encapsulated sucralose or placebo (cellulose) 3x/day at mealtimes	No differences between groups in change from baseline for fasting glucose, insulin and HbA1c.
Tey et al., 2017	RCT, cross-over design, double-blinded.	Healthy adults, n= 30	M	21-50 yrs.	18.5 – 25.0 kg/m^2	1 day	Aspartame, monk fruit extract, stevia, sucrose	0.44 g aspartame, 0.63 g monk fruit extract (50 % mogroside, 0.33 g stevia (steviol glycoside, rebaudioside A), 65 g sucrose	Beverages given as pre-load containing sweeteners + 500 ml water consumed 1 h before ad libitum lunch	There were no significant differences in total AUC for glucose and insulin between the 4 treatments.
Higgins et al., 2018	RCT, parallel arm design.	Healthy adults, n=93, non NNS consumers	M, F	18-60 yrs.	18- 25 kg/m^2	12 weeks	Aspartame	350 or 1050 mg aspartame 1x/ day.	0-mg/day aspartame group: took 2 capsules collectively containing 680 mg dextrose + 80 mg para-amino benzoic acid (PABA) + 2 empty capsules, 350-mg/day aspartame group: beverage with 350 mg aspartame + 80 mg PABA+ 2 capsules collectively containing 680 mg dextrose + 2 empty capsules, 1050 mg/day aspartame group: took beverage with 350 mg aspartame + 80 mg PABA+ 4 capsules collectively containing 700 mg aspartame and 680 mg dextrose.	No significant difference in glucose, insulin, GLP-1 or GIP at baseline or week 12 between groups.

Bonnet et al., 2018	RCT, cross-over design, double-blinded.	Healthy adults, n= 50, not regular users of NNS (consuming < can of beverage with high intensity sweeteners/week)	M	Mean age: 31.1 ± 10.3 yrs.	19-29 kg/m^2	12 weeks	Aspartame, acesulfame potassium	129 mg of aspartame and 13 mg of Acesulfame potassium 2x/day	330 mL beverage 2x/day containing 129 mg of aspartame and 13 mg of Acesulfame potassium in carbonated water, control group received 330 ml carbonated water 2x/day.	No significant difference in insulin sensitivity or secretion between groups
Romo-Romo et al., 2018	RCT, open label, parallel arm design.	Healthy adults, n= 66, low NNS consumers (< 5 portion/ week regardless of product type)	F	18- 55 yrs.	18.5- 24.9 kg/m^2	14 days	Sucralose	12 mg sucralose 3x/day	1 commercial sachet 3x/ day (12 mg sucralose, 958 mg dextrose, and 30 mg maltodextrin) added to beverage or meals, control group followed similar procedure without sucralose,	Sucralose group showed significant decrease in insulin sensitivity compared to control group ($p = 0.04$) Acute insulin response increased in sucralose group ($p = 0.04$)
Lertrit et al., 2018	RCT, cross-over design, double-blinded.	Healthy adults n=15	M, F	18-59 yrs.	18.5 – 27 kg/m^2	4 weeks	Sucralose	200 mg sucralose	200 mg sucralose vs. placebo capsules	Acute insulin response and sensitivity decreased in sucralose group ($p < 0.005$) AUC of active GLP-1 increased in sucralose group ($p< 0.001$)

Yr.= year, N= number of participants, M= male, Kg/m^2= kilograms per square meter, HbA1c= hemoglobin A1c, h= hour, NNS= non-nutritive sweetener, AUC= area under curve, F= female, GLP-1= glucagon-like peptide 1, GIP= gastric inhibitory polypeptide, PABA: para-amino benzoic acid.

BRIDGE TO CHAPTER IV

The following chapter consists of a manuscript that provides a detailed study protocol of the randomized clinical trial presented in Chapter V. Samar Y. Ahmad was the principal manuscript author.

CHAPTER IV

MANUSCRIPT 3

The effect of artificial sweeteners on glucose metabolism and gut microbiome in healthy adults: a study protocol for a randomized double-blinded crossover controlled clinical trial

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4.1 Abstract

Background: Non-nutritive artificial sweeteners (NNSs) have been widely used since their introduction to the food market. Sucralose and aspartame are the NNSs which are most frequently used in Canada. Recent research has shown potential negative health effects of NNSs. NNSs may have the ability to change the bacterial balance of the gut microbiota which could potentially lead to alterations in glucose metabolism, and this was shown for example in mouse models consuming very high doses of NNS saccharin. This study aims to determine the effect of sucralose and aspartame consumption on glucose metabolism, gut microbiota composition using a realistic dose of NNSs.

Methods: Nineteen Healthy participants will be selected, between the ages of 18-45 years, with a BMI of 20-25, and a fasting blood glucose (FBG) < 5.7 mmol/L. They will undertake two two-week treatments periods, separated by four weeks washout periods in a randomized, double-blind crossover design. The sweeteners each participant consumes will be a standardized dose of 14% (0.425 g) of the acceptable daily intake (ADI) for aspartame and 20% (0.136 g) of the ADI for sucralose. Blood samples will be analyzed for glucose, insulin, glucagon, incretins, and leptin. Fecal samples will be collected and analyzed for (SCFAs) and microbiome.

Discussion: This research will provide insight into the potential impact of daily NNSs use reflective of high habitual diet soda consumers on glucose tolerance and gut microbiota. This will contribute to an under-researched area in NNSs safety and has the potential to inform future NNSs recommendations.

Trial registration: The study was registered at ClinicalTrials.gov (Identifier: NCT02569762) in October 2015.

Keywords: Non-nutritive sweetener, aspartame, sucralose, gut microbiome, randomized clinical trial, protocol.

4.2 Background

Non-nutritive sweeteners (NNSs) are chemicals that produce an intense sweet taste at a very low concentration compared to caloric sweeteners such as sucrose, dextrose and high-fructose corn syrup. Also known as non-caloric or artificial sweeteners, NNSs have grown increasingly popular since their introduction to the food and beverage market. This is a result of their low cost, their low- or zero-calories, and their perceived health benefits for weight loss/management and the normalization of blood glucose levels [1, 2]. For these reasons, NNSs are found in a wide variety of foods and beverages marketed as "sugar-free" or "diet " including baked goods, soft drinks, powdered drink mixes, candy, puddings, canned foods, jams and jellies, dairy products, gum, and yogurt.

Currently, there are seven NNSs that have been approved by the US Food and Drug Administration (FDA), including acesulfame potassium, neotame, saccharin, sucralose, aspartame, monk fruit extract and plant-derived stevia [2-5]. In Canada, the approved NNS are acesulfame potassium, neotame, sucralose, aspartame, saccharine and steviol glycosides [6]. The NNSs that will be used in this study are sucralose and aspartame, because they are used more often than others especially in diet soft drinks [7-9].

Sucralose is a disaccharide in which three chlorine molecules replace three hydroxyl groups on the sucrose molecule. Sucralose is 600 times sweeter than table sugar [10]. Most sucralose is not absorbed by the human body and is excreted unchanged in the feces, while sucralose that is absorbed is excreted unchanged in the urine [11, 12]. Aspartame, is a methyl

ester of aspartic acid and phenylalanine dipeptide. It is it is 200 times sweeter than sugar [1]. Aspartame is digested into methanol, phenylalanine, and aspartic acid in the upper intestinal tract of the digestive system. This process occurs rapidly, such that aspartame is never found in the blood circulation. The 3-digestion product of aspartame will follow their normal metabolic pathway and excreted in urine and feces [11, 13, 14].

Health Canada defined the acceptable daily intake (ADI) of sucralose as 9 mg/kg body weight, while that of aspartame is 40 mg/kg body weight [15]. This ADI is the maximum amount of a food additive that can be ingested safely on a daily basis over a person's lifetime without any adverse effects and it has a 100-fold safety factor. A 150-pound individual would need to consume approximately twenty 355 mL of diet coke containing 131 mg aspartame or fourteen 341 mL of diet ice teas containing 41 mg sucralose in order to reach the respective ADIs mentioned above.

There are mixed results on whether NNSs use is associated with positive or negative health outcomes [16]. On the one hand, NNSs have the potential to be effective as a weight loss tool, but only if their use truly leads to a lower overall energy intake [1]. On the other hand, NNS consumption has been associated with increased risk of overweight and obesity, type 2 diabetes, metabolic syndrome, and cardiovascular diseases in humans in observational cohorts [16, 17].

There are several mechanisms by which NNS could adversely affect glucose metabolism. Recently, the lingual taste receptor type 1 member 2 (T1R2) and taste receptor type 1 member 3 (T1R3) were found in the endocrine cells of the gut [4, 18, 19]. Stimulation of the sweet taste receptors activates an intracellular signaling pathway causing an up-regulation of the intestinal glucose transporter, sodium-dependent glucose cotransporter 1 (SGLT1), in addition to an increase in the capacity of the gut to absorb glucose [18]. Incretin hormones such as glucagon-

like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are released as a result of taste receptors stimulation. These incretins improve insulin secretion and affect glucose transport, metabolism, and homeostasis by increasing the cellular uptake of glucose, thereby up-regulating SGLT1 [19]. It is hypothesized if NNSs cause this cascade of events to happen when there is not any glucose present, it could potentially lead to a change in glucose metabolism, especially overtime with chronic exposure [20, 21]. There are other suggested mechanisms of action for how artificial sweeteners could change glucose metabolism and glycemic control include cephalic phase insulin response [1, 2], altered composition of short-chain fatty acids (SCFAs) [9], and failure to respond to real sugars appropriately [17, 22].

More recently, NNS research has focused on the gut microbiota. Saccharin consumption in mouse models has been shown to induce marked glucose intolerance via gut microbiota dysbiosis (alteration of microbial composition of the gut) [3]. Some microbes in gut microbiota may have the capability to metabolize NNSs, which can cause a shift in the normal bacterial balance [9]. It is also possible that NNS intake may have a bacteriostatic effect on certain gut microbes, causing changes to the microbiome composition [12, 13]. However, it is important to highlight the paucity of human studies on gut microbiome in relations to NNS consumption [3], so more research is needed in this area.

In Suez et al. (2014) it was reported that four out of seven healthy subjects who consumed the FDA's maximal ADI of the saccharin during a 5-day period presented with a poorer glycemic response after this intervention compared to before [3]. Moreover, there was a pronounced change in the microbiome composition of the four participants with the poor glycemic response compared to other participants [3].

A limited number of clinical trials have addressed the effect of NNSs such as aspartame and sucralose on glucose metabolism and gut hormones [17], Most of which used different doses and method of ingestion. Importantly, none of these studies were designed to focus on the effect of NNSs use that reflect the habitual intake of diet soft drinks such as diet soda in healthy subjects. For example, Hall WL, et al. (2003) investigated the effect of aspartame on glucose metabolism, insulin and GLP-1 hormone in 6 subjects who consumed 400 mg encapsulated aspartame. They found no effect on glucose concentration or gut hormones. However, this study had very small samples size and the form of sweetener used is capsule, which might bypass the mouth taste receptors [23].

A 2012 study examined the acute effect of diet soda (240 ml) on gut hormones secretion in three groups, healthy control (n=25), type 1 diabetes (n=9) and type 2 diabetes (n=10) patients using a cross over design, they found that diet soda increased GLP-1 hormone secretion by 34% in healthy subjects, and by 43 % in subjects with type 1 diabetes but not type 2 diabetes group. Some limitation to this study might affect the outcome measured, for example, diet soda used in this trial have other ingredients which might affect the gut hormones secretions, so maybe it would be more beneficial to examine the effect of pure NNSs first, then look at the overall effect of diet soda. Additionally, it was unclear whether the enhanced glucose-stimulated GLP-1 response was caused by acesulfame potassium or sucralose or both [24].

Moreover, Pepino et al. (2013) evaluated the acute effect of sucralose (48 mg) in 17 obese subjects who were insulin sensitive, they consumed either sucralose or water 10 min before the glucose load in a randomized crossover design. The results showed a significant increase in plasma glucose concentration and an increase in insulin levels as well in the sucralose group. There could be some limiting factors to this study such as the absence of food intake record the

days before the self-reported overnight fast [25]. Temizkan et al. (2015) found that 24 mg of sucralose enhances GLP-1 release and decrease blood glucose in the presence of carbohydrate in healthy subjects (n=8) but not in patients with type 2 diabetes (n=8). The 72 mg of aspartame given to the subjects had no effect on glucose or gut hormones [26].

Regarding NNSs use and gut microbiome dysbiosis, there are mostly just animal studies which have examined their effect in mice and rat models [12, 13].

To the best of our knowledge, there is no clinical trial that has investigated the effects of aspartame and sucralose, the most commonly used sweeteners in Canada, at intakes reflecting high habitual diet soda intakes, in healthy participants or addressed their possible effect on the gut microbiome. Therefore, this study aims to determine the effect of sucralose and aspartame consumption on glucose metabolism and gut microbiota composition, diversity, and community structure. Moreover, the selection of healthy individuals in this study help us to explore the effect of NNSs in normal healthy population before moving forward to observe such an effect in people who are obese or have type 2 diabetes mellitus.

4.3 Study outcomes

The primary outcome of this clinical trial are changes in blood glucose. The secondary outcomes of this clinical trial are the changes in gut hormones such as insulin, leptin and GLP-1 and in fecal microbiome composition, diversity, community structure and SCFA.

4.5 Methods

4.5.1 Study design

This study is a randomized, double-blind crossover and controlled clinical trial that will take place in the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) at the University of Manitoba in Winnipeg, Canada. The study protocol flow chart is shown in Figure 1.

Participants will consent to follow a 12-week diet regimen, in a crossover design. For the first four weeks, all participants will go through a baseline period, where no artificial sweeteners will be consumed. During weeks 5 and 6, half of the participants will consume aspartame and half of the participants will consume sucralose. For weeks 7 through 10, all participants will undergo a washout period, where no artificial sweeteners will be consumed. Lastly, during weeks 11 and 12, the two groups of participants will consume the sweetener, which they did not previously consume. The study schedule of enrollment, interventions, and assessments is summarized in Table 5

Table 5:The schedule of enrollment, interventions, and assessments.

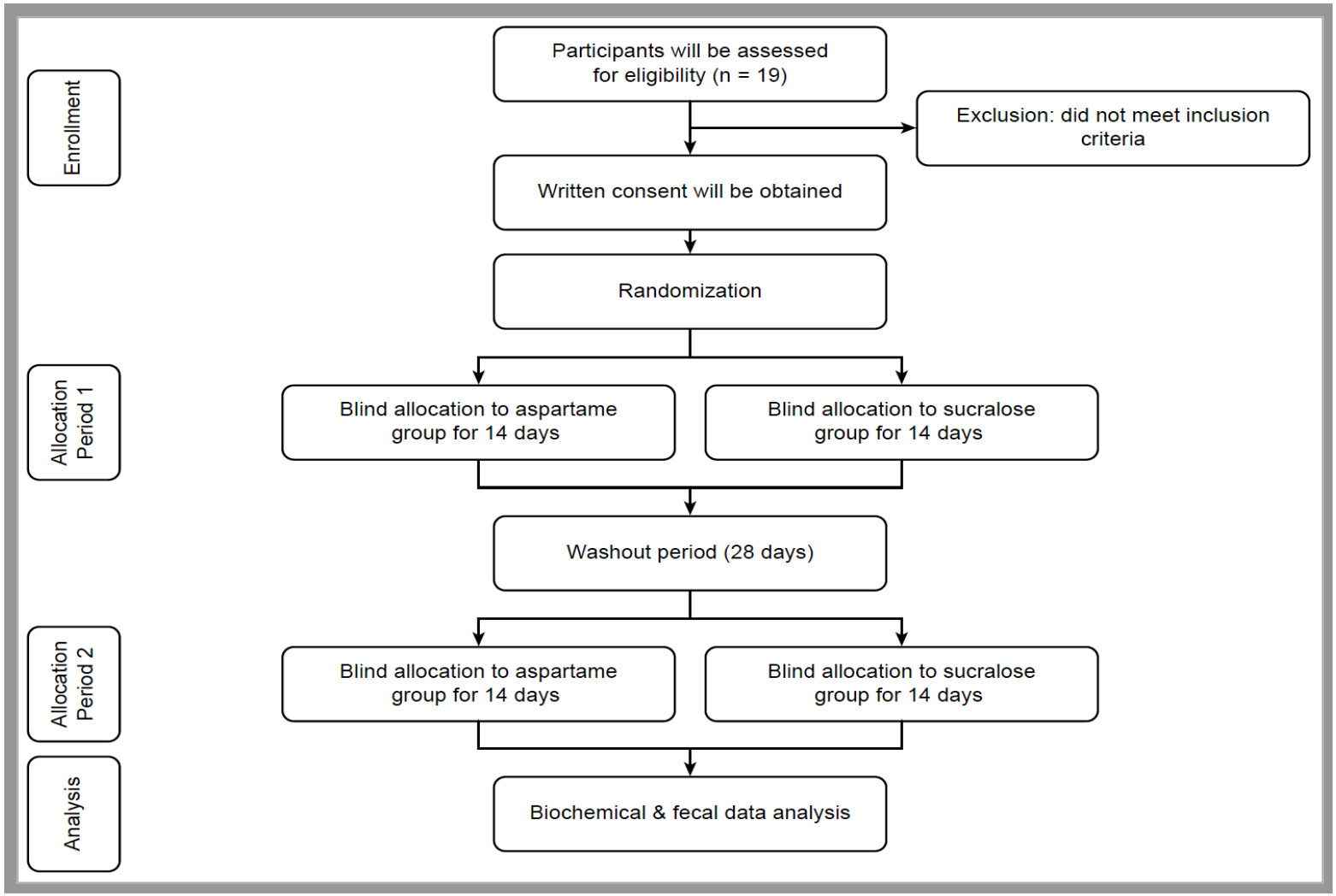

Figure 1 Study protocol flow chart: effect of artificial sweeteners on gut microbiome and glucose metabolism.

Assessments	Screening & enrollment	Visit 1	Visit 2	Visit 3	Visit 4
Day in study		1	28	42	84
Week in study	-4	1- 4	5 & 6	7-10	11 &12
General information form	✓				
Informed consent	✓	✓			
Medical history	✓	✓			
Weight	✓	✓	✓	✓	✓
Non-nutritive sweetener supplementation[1]			✓		✓
Blood draw[2] (1.5 ml) session		✓	✓	✓	✓
Stool sample collection		✓	✓	✓	✓
Laboratory measurements in plasma: glucose, insulin, GLP-1, leptin		✓	✓	✓	✓

Laboratory measurements in stool: fecal microbiome, fecal short-chain fatty acids		✓	✓	✓	✓
Oral glucose tolerance test[3]		✓	✓		✓
Fasting blood glucose test[4]	✓	✓	✓	✓	✓
Visual analogue scales (taste panel)[5]			✓		✓
Diet history questionnaire	✓				
Food diary[6]			✓		✓
Adverse event log		✓	✓	✓	✓

[1]This will be either aspartame or sucralose; visits to collect the supply will vary.

[2]There will be five blood draws after the visits, and they will be conducted at the RCFFN.

[3]OGTT will involve drinking a sweet liquid containing 75 g of glucose.

[4]FBG will be conducted after a 10-12 hour fast.

[5]This test is to measure the participants' tolerance to the sweetener mixed into beverages.

[6]Food diaries documenting 2 weekdays and 1 weekend day.

4.6 Study participants

Adult's males and females aged 18-45 years will be selected for inclusion in this study from the Winnipeg Region. Posters and flyers will be posted and distributed in Winnipeg. An advertisement will be placed in the local newspapers and via the internet (i.e., kijiji) notifying them of the study.

The determination of the eligibility of those who express an interest will be via questionnaire. They will come to the research center for screening and to be provided with necessary information. So, determination for eligibility will be through the inclusion criteria. Participants will be remunerated for participation in the study.

4.7 Inclusion and exclusion criteria

Participants who are healthy, non-diabetic will be included in the study. Participants must be 18-45 years old, with a BMI of 20-25 (i.e., normal weight), and a fasting blood glucose < 5.7 mmol/L (i.e., normal fasting glucose). Participant who do not consume NNS regularly will be included in the trial, we define regular consumers as any person who consume ≥ one can of diet beverage or one spoonful of NNS/week or it is equivalent in food product. Women with regular cycles who are not taking oral contraceptive pills will be included in the study because taking oral contraceptive pills will affect their blood glucose levels, and every female participants will begin the study at approximately the same phase (follicular) of their respective menstrual cycles. This is because it has been shown that healthy women experience diminished insulin sensitivity in certain phases of menstruation [27].

Participants will be excluded if they have a history of alcohol or drug abuse, are taking any antibiotic medications or probiotics within the 6-month period prior to the study because it will have an impact on the gut microbiome composition and function; have any medical conditions that could potentially affect outcomes. These include metabolic or gastrointestinal

disorders (e.g., diabetes, malabsorption syndrome, inflammatory bowel disease, irritable bowel syndrome, celiac disease, phenylketonuria); are taking medications which impact glucose metabolism (e.g., metformin), change gastric pH (e.g., proton pump inhibitors) or gastric emptying (e.g., metoclopramide); or have known allergy, sensitivity or another contraindication to aspartame or sucralose.

Pregnant and lactating women will also be excluded because there is not enough evidence about the negative health effect of NNS during pregnancy or lactation as it may affect the fetus or can be excreted in the breast milk during lactation. Investigators may decide to take participants off this study if participants decided to stop drinking the artificial sweeteners drink as described by the protocol, or if they will be using any steroids or beta agonists (orally, intranasal or inhaled) within a week of any oral glucose tolerance test. Female participants may also be taken off this study if they become pregnant.

4.7.1 Randomization

Eligible participants will go through assessment at baseline and will be randomly assigned by trial coordinator to two groups aspartame then sucralose group or sucralose group then aspartame group by simple randomization (coin flip) after enrollment into the trial. The assignment of this intervention will be blinded for both the investigators and the participants. The randomization codes will be concealed in opaque sealed envelopes, and will be released to participants after all baseline measurements have been completed

4.7.2 Dietary recommendation during the time of the study

Participants will be advised to avoid consuming any NNSs during the study period and will be taught about the hidden sources of any NNSs in different foods, beverages products and medication. Examples of other NNSs that should be avoided are aspartame, acesulfame

potassium, neotame or E961, saccharin, sucralose, stevia and monk fruit extract. Additionally, they will receive a recommendation regarding their caffeinated beverage intake because caffeine ingestion has an effect on glucose uptake rates [28] to 2 cups (250 ml) per day of drinks such as tea, coffee, energy drinks and soft drinks. In addition, they should limit their alcohol drink to no more than 2 units of alcohol (unit = 10 mL pure alcohol) due to their effect on the blood glucose and insulin levels [29]. During the time of the study participants will refrain from consuming any probiotic supplements or food contains probiotics such as kefir, coconut kefir, yogurt, natto, miso soup, raw cheese, kombucha tea, tempeh, fermented soy bean and fermented cabbage. Participants will be restricted from using ibuprofen (Advil and Motrin) during the study; only acetaminophen (Tylenol) may be taken if needed, and in case of any other medication being used, the study coordinator must be notified. Probiotics and ibuprofen have an effect on gut microbiome function and composition [30, 31].

4.8 Interventions

Half of the participants will be receiving aspartame during week 5 and 6, while the other half of the participants will consume sucralose. During weeks 11 and 12, the two groups of participants will consume the sweetener, which they did not previously consume. The amount each participant consumes will be determined based on average body weight in adults in order to meet 14% of the ADI for aspartame, and 20% of the ADI for sucralose. These dosages are based on the patterns of regular soft drink intakes in Canadian men and women [8]. This dosage level is high, but reasonable and realistic reflecting intakes of consumer who drink about 3 cans of diet soda a day. Fourteen percent of the ADI for aspartame is approximately equivalent to 0.425 g of aspartame (10 packets of aspartame), while 20% of the ADI for sucralose is approximately equivalent to 0.136 g of sucralose (approximately

10.5 packets of sucralose). Participants will be given the sweeteners in a blinded fashion with beverages in identical bottles labelled “A” or “B”.

4.9 Treatments

Beverages will be designed as 1000-mL drinks. Beverages will be created as follows. The Aspartame beverage: 1000 mL of water, 0.08 g of citric acid, 0.037 g of pure lemon extract (club house brand, McCORMICK CANADA LONDON, CANADA N6A 4Z2) and 0.425 g of pure aspartame powder (Walnut, CA 91788, USA). Aspartame will be dissolved in the water using a high frequency ultrasonic bath for 10 minutes. The sucralose beverage: 1000 mL of water, 0.08 g of citric acid, 0.037 g of pure lemon extract and 0.136 g of sucralose pure powder (Walnut, CA 91788, USA). During the washout period participants will receive 1000 mL of water mixed with 0.08 g of citric acid and 0.037 g of pure lemon extract only.

4.10 Assessment and evaluation

4.10.1 Self-reported

Participants will be asked to fill out a web-based version of a Canadian Diet History Questionnaire II (C-DHQ II) at baseline prior to the start of the study [32], followed by a weekly 3-day food diary (2 weekdays and 1 weekend day) of food and drink consumption [33]. The C-DHQ II records food intake patterns over the past year, while the food diary measures compliance.

Palatability, motivation to eat, energy fatigue, and physical comfort will be measured by visual analog scale (VAS, fixed length 100 mm) in participants when they receive treatments A and B. Participants will be asked to rate themselves by placing a small” x” across the horizontal line at the point that best reflects their present findings, where the extreme limits of the measured parameter will be oriented from the left (worst) to the right

(best). Once the VAS score is received from the participant, the determination of the score will be done by measuring in millimeters from left hand end of the horizontal line to the point marked by the participant [34]. VAS score will allow us to look at changes within individuals receiving each beverage.

4.11 Compliance

During each period, participants will complete a weekly 3-day food diary (2 weekdays and 1 weekend day), which will be used to monitor their compliance with the study requirements. Additionally, the beverage will be distributed in two 500 mL bottles, and they will be instructed to return all empty bottles for counting purposes.

4.12 Study measures

4.12.1 Anthropometric

Anthropometric

All anthropometric measurements will be performed on first day after a 10- to 12-hour overnight fast. Standing height (cm) without footwear will be measured to the nearest 0.5 cm on the first day using a stadiometer. For the calculation of BMI ($kg \cdot m^{-2}$), weight (kg) will be recorded on days 1, 14, 28, and 84. A calibrated digital scale with increments of 0.1 kg will be used to measure the weight of each participant.

4.12.2 Biochemical measurements

The participants will be asked to abstain from consuming alcohol (48 hours) and caffeinated beverages (12 hours) before their blood draws. Participant will undergo an overnight (10- to 12-hour) fasting blood draw performed by a registered nurse (RN). The RN will check participants fasting blood glucose with a glucometer, then insert an intravenous catheter into the participant arm and a fasting blood sample will be drawn at time 0. Subjects

will be given a 75g glucose tolerance test beverage (Trutol) followed by additional blood samples taken at time 15, 30, 45, 60, 90 and 120 minutes. During the intervention periods, a baseline sample will be drawn at day 1, then at the beginning and the end of each treatment period, i.e., days 28, 42 and 84. For female participants, the fasted blood draws will be scheduled after the end of the monthly menstrual cycle.

All blood samples will be collected in BD Vacutainer EDTA tubes (BD; Franklin Lakes, NJ, USA), placed on ice, then plasma will be separated by centrifuging at 3000*g* for 15 minutes at 4˚C. Plasma aliquots will be treated with 20 µl (10 µl/1 mL blood) dipeptidyl peptidase-4 inhibitor (DPP-IV) inhibitor before they are kept at -80°C in a microfuge tube for active GLP-1 analysis in multiplex with, insulin, glucagon and leptin. Untreated aliquots of plasma will be stored at -80°C for glucose analysis.

Measurement of glucose content in plasma will be done using The Cobas 311 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Measurement of insulin, glucagon-like peptide-1 (GLP-1) and leptin contents in plasma will be done using the Meso Scale Discovery (MSD) multiplex assay (Meso Quickplex SQ 120, Rockville, Maryland, USA). All analysis will be performed according to manufacturer's protocol.

Homeostasis Model Assessment-insulin resistance (HOMA-IR) will be used to assess insulin resistance [35].

Fecal samples will be collected also on days1, 28, 42 and 84 using sterile cups after defecation and separated into two aliquots for storage. The first aliquot will be stored as is at -80°C for later microbiota analysis, and the second will be stored in RNAlater storage reagent at -80°C for possible future metagenomic analysis. The fecal microbiota will be analyzed by 16S rRNA Illumina-based gene sequencing, and quantitative PCR (for selected taxa of interest), using select methods [36]. This will allow to detect any alterations in the composition of the gut microbiota. The concentration of SCFAs in feces will be measured,

100 mg of frozen stool will be homogenized in 1 mL 0.15 mmol/L H_2SO_4, and centrifuged [37]. The fatty acid composition will be determined following appropriate protocol.

4.13 Safety

All blood draws will be conducted by a registered trained nurse. The trial will follow the guidelines for the collection of blood samples in research involving humans released by the University of Manitoba to assure safe practice [38].

4.14 Statistical analysis

All data will be entered in duplicate into a password-protected computer after data collection is complete. All statistical procedures will be done using SPSS 22.0 for Macintosh. The normality of the data distribution will be tested using Shapiro Wilk test and the non-normal variables will be normalized using a log transformation. Changes in the data from baseline within treatment groups will be assessed using linear mixed model with REML estimation. Repeat measures ANOVA will be used to measure any biochemical or anthropometric changes. Demographic data will be reported as the average ± standard deviation. The results will be reported as least-squared means ± standard error of the mean (SEM) unless otherwise specified. Statistical significance will be set at $P < 0.05$ for all analyses.

QIIME version 2 2018.11 (qiime2.org) and SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) will be used for microbiome analyses. The Shannon Diversity Index of species richness will be calculated to determine microbiota diversity within samples; permutational multivariate analysis of variance (PERMANOVA) and principal coordinates analysis (PCoA) of UniFrac distance matrices will be used to determine between sample differences in community structures [39].

G-power was used for sample size calculation. A sample size of 12 was sufficient to detect a difference in treatment effect on glucose AUC of 139 mmol/l at 120 minutes with 85% power and a 5% level of significance. Considering a dropout rate of 35%, the sample size required was 19 [3].

4.15 Confidentiality

All study-related information will be kept in a locked secure area (Locked cabinets in a locked room, password protected computers and no identifying information on flash drives) and only those persons identified will have access to these records. If any of medical/research records need to be copied for study staff, participant name and all identifying information will be removed and they will sign agreements to preserve the confidentiality of all participants. No information revealing any personal information such as participant name, address or telephone number will leave the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN). The samples will be given non-identifiable numbers to protect confidentiality, and a researcher at RCFFN who is not involved in the study will maintain the link to identifying information.

4.16 Discussion

Aspartame and sucralose are the most common NNSs used in Canada [8]. Since the extensive introduction of artificial sweeteners to our diet, their consumption has been associated with an increased risk of overweight, obesity and diabetes in some populations [17]. However, the majority of this research has been undertaken in observational studies and/or metabolically unhealthy individuals. The lack of randomized clinical trials in healthy individuals involving NNS and gut microbiome studies makes this study an important

addition to the literature, and inform further research on the relationship between NNSs, glucose metabolism and the gut microbiota in the context of human health and disease.

4.17 List of abbreviations

NNSs: Non-Nutritive Sweeteners; SCFA: Short-Chain Fatty Acids; BMI: Body Mass Index; FBG: Fasting Blood Glucose; mmol/L: Millimoles Per Liter; g: Gram; ADI: Allowed Daily Intake; FDA: the US Food and Drug Administration; mg/kg: Milligram Per Kilogram; T1R2: Taste Receptor Type 1 Member 2; T1R3: Taste Receptor Type 1 Member 3; GPCR: G Protein-Coupled Receptor; SGLT1: Sodium-Dependent Glucose Cotransporter 1; GLP-1: Glucagon-Like Peptide 1; GIP: Glucose-Dependent Insulinotropic Peptide; RCFFN: Richardson Centre For Functional Foods And Nutraceuticals; VAS: Visual Analogue Scale; C-DHQ II: Diet History Questionnaire II; CM: Centimeter; OGTT: Oral Glucose Tolerance Test; EDTA: Ethylenediamine Tetraacetic Acid; °C: Celsius; MSD: Meso Scale Discovery; HOMA-IR: Homeostasis Model Assessment-Insulin Resistance; PCR: Polymerase Chain Reaction; SAE: Severe Adverse Event; REB: Research Ethics Board; PHIA: Personal Health Information Act; SEM: Standard Error Of The Mean; AUC: Area Under Curve; KFAS: Kuwait Foundation For The Advancement Of Sciences; UCRP: University Collaborative Research Program.

4.18 Declarations

4.18.1 Ethics approval and consent to participate

The Bannatyne Campus Biomedical Research Ethics Board (BREB) in Winnipeg, Manitoba, Canada has approved this study protocol (HS18698 (B2015:0690). This trial is registered at clinicaltrials.gov (Identifier: NCT02569762). All participants will provide a

written informed consent before they are enrolled in this trial, this consent form will be dated and signed by participants and the principal investigators at the beginning of the study.

4.19.2 Consent for publication

Not applicable

4.18.3 Availability of data and materials

A de-identified dataset that will be used and/or analyzed during the current study will be available from the corresponding author on reasonable request.

4.18.4 Competing interests

The authors declare that they have no competing interests.

4.18.5 Funding

This study is funded by Kuwait Foundation for the Advancement of Sciences (KFAS) and University Collaborative Research Program (UCRP) and supported, in part, by the Canada Research Chairs Program. SYA work was supported by Kuwait Civil Service and Institute for Medical Specialization- Ministry of Health of Kuwait. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

4.18.6 Authors' contributions

SYA conceived the study, sought funding and ethical approval, contributed to development of the study protocol and wrote the manuscript. DM designed the study protocol, sought funding and ethical approval. JF, DM contributed to development of the

study protocol. SYA, DM and JF designed the selection criteria of patients, contributed to the sample size estimation and statistical analysis, advised on protocol. All authors contributed to the research design and read, made critical revisions, wrote and approved the final manuscript.

4.18.7 Acknowledgements

Not applicable.

4.18.8 Authors' information

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BRIDGE TO CHAPTER V

The following chapter comprises a manuscript that presents the results from the clinical trial conducted to assess the effects of the artificial sweeteners sucralose and aspartame on glucose metabolism in a randomized double-blinded crossover design. The findings in this chapter showed that daily repeated consumption of 1 L of a beverage sweetened with aspartame or sucralose for 2 weeks had no effect on glucose metabolism in healthy adults.

Samar Y. Ahmad was the principal manuscript author and participated in study coordination, data collection, analysis and interpretation of the human data.

CHAPTER V

MANUSCRIPT 4

This manuscript is submitted to Applied Physiology, Nutrition, and Metabolism journal of Nutrition and Disease for publication 2019,

The effect of the artificial sweeteners on glucose metabolism in healthy adults: a randomized double-blinded crossover clinical trial

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5.1 Abstract

Background: This study aims to determine the effect of pure forms of sucralose and aspartame, in doses reflective of common consumption, on glucose metabolism.

Methods: Healthy participants consumed pure forms of a non-nutritive sweetener (NNS) mixed with water that were standardized to doses of 14% (0.425 g) of the acceptable daily intake (ADI) for aspartame and 20% (0.136 g) of the ADI for sucralose every day for two weeks. Blood samples were collected and analysed for glucose, insulin, active glucagon-like peptide-1 (GLP-1), and leptin.

Results: Seventeen participants (10 females and 7 males; age 24 ± 6.8 years; BMI 22.9 ± 2.5 kg/m^2) participated in the study. The total area under the curve (AUC) values of glucose, insulin, active GLP-1 and leptin were similar for the aspartame and sucralose treatment groups compared to the baseline values in healthy participants. There was no change in insulin sensitivity after NNS treatment compared to the baseline values.

Conclusions: These findings suggest that daily repeated consumption of pure sucralose or aspartame for 2 weeks had no effect on glucose metabolism among normoglycaemic adults. However, these results need to be tested in studies with longer durations.

Novelty:

- Daily consumption of pure aspartame or sucralose for 2 weeks had no effect on glucose metabolism.
- Daily consumption of pure aspartame or sucralose for 2 weeks had no effect on insulin sensitivity among healthy adults.

Keywords: non-nutritive sweetener, aspartame, sucralose, protocol, glucose metabolism, insulin, glucose, active GLP-1, leptin

5.2 Introduction

Non-nutritive sweeteners (NNSs) are novel chemosensory compounds in the food additive class that have been commonly used in different foods and beverages to provide an intense sweet taste and decrease caloric content (Gardner et al. 2012). NNSs are also used and recommended for managing weight and controlling blood glucose levels in individuals with obesity and diabetes (Gardner et al. 2012; Mattes and Popkin 2009). The US Food and Drug Administration (FDA) has approved some artificial sweeteners, including acesulfame potassium, neotame, saccharin, sucralose, aspartame and some natural sweeteners, including monk fruit extract and plant-derived stevia (U.S. Food & Drug Administration 2018). In Canada, acesulfame potassium, neotame, sucralose, aspartame, monk fruit extract, steviol glycosides and erythritol have been approved by Health Canada (Government of Canada 2019).

Evidence shows conflicting results regarding the effects of NNSs on health. For example, some studies have reported that NNSs can be associated with weight loss (Benton 2005; Mattes and Popkin 2009). Other observational and cohort studies have found that repeated consumption may be associated with an increased risk of overweight and obesity, diabetes mellitus, cardiovascular diseases and metabolic syndrome (Azad et al. 2017; Swithers 2013). In particular, repeated consumption of diet soda sweetened with NNSs may be associated with an increased risk of type 2 diabetes mellitus (T2DM) and metabolic syndrome, including abdominal obesity, impaired glucose tolerance, insulin resistance, high blood pressure (BP) and dyslipidaemia (Nettleton et al. 2009).

There are many proposed mechanisms for how NNSs may alter glucose metabolism and glycaemic control, such via cephalic phase insulin response, which might be weakened by repeated NNS use, causing eventual failure of the body to respond to actual sugar appropriately (Swithers et al. 2009, 2010). Another interesting possible mechanism is that

changes in glucose metabolism could be mediated by alterations in the gut microbiota (dysbiosis) caused by NNS consumption. Suez et al. demonstrated that daily intake of saccharine for 7 days caused glucose intolerance in four out of seven individuals. Furthermore, the change in glucose metabolism was shown to be mediated by gut microbiota dysbiosis, which was demonstrated when the faecal microbiota of humans were transplanted into germ-free mice. The recipient mice developed glucose intolerance as well (Suez et al. 2014). It is possible that NNSs might influence the growth of certain gut bacteria, leading to a microbial imbalance (Abou-Donia et al. 2008; Palmnas et al. 2014).

Recent studies that have investigated the effect of aspartame and sucralose on glucose metabolism and gut hormones are limited and have conflicting results (Ahmad et al. 2019). Randomized clinical trials have reported an effect of repeated daily doses of sucralose on insulin sensitivity and acute insulin response (Lertrit et al. 2018; Romo-Romo et al. 2018) and active glucagon-like peptide 1 (GLP-1) concentrations (Lertrit et al. 2018). Other randomized clinical trials have reported an acute effect of a single doses of sucralose on glucose (Pepino et al. 2013; Temizkan et al. 2015), insulin and insulin sensitivity (Pepino et al. 2013), and active GLP-1 concentrations (Brown et al. 2012; Temizkan et al. 2015). These results have not always been consistently replicated in other studies (Baird et al. 2000; Brown et al. 2011; Grotz et al. 2003, 2017; Sylvetsky et al. 2016; Wu et al. 2012, 2013).

Additionally, a few clinical trials have reported an acute effect of a single doses of aspartame on glucose (Melanson et al. 1999; Moller 1991), insulin (Horwitz et al. 1988), and active GLP-1 concentrations (Hall et al. 2003), while other studies investigating either repeated daily doses of aspartame (Bonnet et al. 2018; Higgins et al. 2018) or a single dose of aspartame (Anton et al. 2010; Bryant et al. 2014; Temizkan et al. 2015; Tey et al. 2017) could not confirm these results.

A recent systematic review analysed twenty-eight clinical trials evaluating the effects of NNSs on glucose metabolism. Most of the evaluated studies measured the acute effect of a single dose of an NNS (n=20), and the remaining studies (n=8) evaluated the effect of repeated doses of NNSs. In a systematic review, it was concluded that the effects of NNS consumption on glucose metabolism are still unclear and incomparable due to major protocol differences that exist between studies (Romo-Romo et al. 2016). Another systematic review and meta-analysis of randomized clinical trials (RCTs) (n=21) assessed the effect of aspartame consumption on fasting blood glucose and insulin concentrations. They reported that aspartame consumption had no association with changes in blood glucose or insulin concentration compared to the concentrations observed in the control group (Santos et al. 2018).

To date, few studies have assessed the effect of repeated daily consumption of aspartame and sucralose in beverages on glucose metabolism, insulin and GLP-1 hormone (Bonnet et al. 2018; Colagiuri et al. 1989; Lertrit et al. 2018; Romo-Romo et al. 2018). Aspartame and sucralose are the most commonly used sweeteners in diet beverages in Canada; therefore, these two artificial NNSs were investigated in this study (Garriguet 2008; Nikpartow et al. 2012). The acceptable daily intake (ADI) in Canada is 9 mg/kg body weight for sucralose, while that for aspartame is 40 mg/kg body weight (Pepsico Canada 2011).

Therefore, we decided to investigate the effect of pure aspartame and sucralose, without contamination from other ingredients present in diet sodas and at intakes reflecting normal daily consumption, on glucose metabolism in healthy adults.

5.3 Materials and methods

5.3.1 Recruitment and population

Participants were recruited in Winnipeg, Canada by using posters, flyers and advertisements around the University of Manitoba campus. Participants aged 18-45 years old were included if they had a body mass index (BMI) of 20-25 kg/m^2 and a fasting blood glucose (FBG) < 5.7 mmol/L (i.e., normal FBG) and were not regular users of NNSs. We defined regular users of NNSs as those consuming ≥ 1 can of diet beverages, one spoonful of NNSs or the equivalent per week in food products. We used the web-based version of the Canadian Diet History Questionnaire II (C-DHQ II) (Lo Siou et al. 2017) to screen for NNS intake over the last 12 months and to assess nutrient intake. The DHQ II includes questions that inquire about the type, quantity and frequency of consumption of artificial sweeteners used for tea, coffee, and other drinks and the intake of diet beverages (including fruit drinks, diet soda, iced tea and flavoured water).

Adherence to dietary recommendations was evaluated once a week during the treatment periods, and a 3-day food record was completed (Yang et al. 2010). Women who were taking oral contraceptive pills and/or who had irregular menstrual cycles were excluded from the study. Individuals were excluded from the trial if they were pregnant or lactating, had a history of alcohol or drug abuse, were on antibiotic medication or took probiotics within the 6-month period before the study, had any past or present medical conditions including metabolic or gastrointestinal disorders, or used medications known to impact glucose metabolism, gastric pH or gastric emptying. The protocol was reviewed and approved by the University of Manitoba Bannatyne Campus Biomedical Research Ethics Board (BREB) in Winnipeg, Manitoba, Canada. This trial was registered at clinicaltrials.gov under the number NCT02569762.

5.3.2 Study design

This study used a randomized, controlled, double-blinded, crossover design to investigate the effect of NNS consumption on glucose metabolism. This trial was conducted from 2016-2018 at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) at the University of Manitoba in Winnipeg. The trial consisted of two 2-week periods separated by a minimum of a 4-week washout period during which the participants were instructed to maintain their regular water intake and habitual diet. Participants were randomly assigned to 1 of 2 treatment orders by simple randomization after enrolment in the trial. Treatments were beverages containing either 14% of the ADI for aspartame or 20% of the ADI for sucralose to be consumed by participants daily. The doses of NNSs used in this study were similar to the amount of aspartame or sucralose present in ~ 3 cans of diet soda (355 ml can) (Franz 2010; Garriguet 2008).

For the first 2-week intervention period, participants consumed either an aspartame beverage, which contained 1000 ml water, 0.425 g (425 mg) of pure aspartame powder (HerbStoreUSA, Walnut, CA 91788, USA), 0.08 g of citric acid and 0.037 g of pure lemon extract (Club House brand, McCormick London On, Canada N6A 4Z2), or a sucralose beverage, which contained 1000 ml water, 0.136 g (136 mg) of sucralose pure powder (HerbStoreUSA, Walnut, CA 91788, USA), 0.08 g of citric acid and 0.037 g of pure lemon extract, depending on the group to which they were randomized. During the second 2-week intervention period, participants received the treatment they did not receive in the first 2-week intervention period. Beverages were given to the participants in a blinded fashion in identical bottles labelled “A” or “B”. Participants were instructed to drink their beverages throughout the day. Participants were instructed to consume their habitual diets and maintain their physical activity levels throughout the entire study duration. Additionally, they were advised to avoid consuming food or drink products that contained NNSs during the entire

duration of the study. Participants were asked to complete a 3-day food diary for 2 weekdays and 1 weekend day over the 14-day intervention period.

To increase compliance, participants were also asked to complete a daily checklist to verify beverage consumption and to return all empty beverage containers each week for counting purposes.

Palatability, motivation to eat, energy, fatigue, and physical comfort were measured by visual analogue scale (VAS) in participants when they received the aspartame and sucralose drinks. Participants were asked to rate themselves after having their first drinks during the treatment period.

5.3.4 Blood sampling and analysis

Participants were instructed to abstain from consuming caffeinated beverages for 12 hours and alcoholic beverages for 48 hours prior to blood draws.

On the first and last day of each period, 12-hour fasting blood samples were collected. A registered nurse collected blood samples immediately before and 15, 30, 45, 60, 90 and 120 minutes after the administration of a 75 g glucose challenge. For female participants, glucose challenges were scheduled after the cessation of menses. Plasma was separated from whole blood samples within 1 hour of collection. Blood was centrifuged at 3000 × *g* for 15 minutes at 4˚C. Plasma aliquots were treated with 20 µl (10 µl/1 mL blood) of dipeptidyl peptidase-4 (DPP-IV) inhibitor before they were stored at -80°C. Untreated aliquots of plasma were stored immediately at -80°C for further analysis.

Glucose and fructosamine concentrations were measured in plasma by a Cobas 311 analyser (Roche Diagnostic, Germany) at baseline and after each treatment period. Insulin, active GLP-1 and leptin contents in plasma were measured at baseline and after each treatment phase by a Meso Scale Discovery (MSD) multiplex assay (Meso Quickplex SQ

120, Rockville, Maryland, USA) according to the manufacturer's protocol. The incremental area under the curve (AUC) values were calculated for glucose, insulin, active GLP-1 and leptin by the trapezoidal method (Allison et al. 1995). The homeostasis model assessment-insulin resistance (HOMA-IR) was calculated using the formula ([insulin, μIU/L]*[glucose, mg/L])/405. Additionally, HOMA-%beta was calculated as 20 × fasting insulin (μIU/ml)/fasting glucose (mmol/ml) − 3.5 (Matthews et al. 1985).

5.3.5 Statistical analysis

A sample size of 12 was sufficient to detect a difference in treatment effect on glucose AUC of 139 mmol/l at 120 minutes with 85% power and a 5% level of significance. Considering a dropout rate of 35%, the sample size required was 19 (Suez et al. 2014). Missing data points were imputed in the calculation of AUC values. The missing data were assumed to be missing at random. The average value for all participants at the same time-point and treatment, was used as the parameter estimates for the missing value. AUC values were then calculated as above.

Statistical analyses were performed using SPSS 22.0 for Macintosh. The normality of the data was assessed using the Shapiro Wilk test, and non-normal variables were normalized using log transformation. The results are expressed as estimated least-squares means ± standard errors of the means (SEMs) for all values unless otherwise stated, and statistical significance was set at $p<0.05$ for all analyses. Changes in the data from baseline within treatment groups were assessed using a linear mixed model with REML estimation. Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality. P-values were obtained by likelihood ratio tests of the full model with the effect in question against the model without the effect in question. Missing values at random were calculated using the mean values of variables.

5.4 Results

5.4.1 Participants

A total of ten female and seven male participants completed the 2-stage trial and were included in all analyses. Two participants dropped out without providing a reason.

Fig. 1 is a flow diagram of the trial according to the CONSORT (Consolidated Standards of Reporting Trials) guidelines. The participants were young healthy adults with a mean age of 24±6.8 years, a BMI of 22.9±2.5 kg/m^2 and biochemical variables within the normal range. The participants' BP and FBG levels were within the normal range. Table 1 shows the baseline characteristics of the participants who completed the trial.

Based on returned empty bottle counts, there were no significant violations of the protocol. The mean bottle count adherence for sucralose drinks was 100% compared to 99.47±1.49% for the aspartame treatment.

5.4.2 Fasting glucose, insulin, active GLP-1 and leptin concentrations following OGTT

There were no differences in mean fasting glucose, insulin, active GLP-1 or leptin concentrations between the baseline visit and the visit at the start of period 2 after the washout period (Table 2).

There were no significant differences in glucose, insulin, active GLP-1 or leptin concentrations between the aspartame or sucralose treatments and baseline. The curves of mean fasting glucose, insulin, active GLP-1 and leptin concentration during the 75 g OGTT in the 17 healthy participants are given in Fig. 2.

The total AUC values for glucose, insulin, GLP-1 and leptin were not different between baseline and the aspartame or sucralose treatments (Table 3). There were no differences in the total AUC for glucose (p=0.54), insulin (p=0.38), active GLP-1 (p=0.67) or

leptin (p=0.80) between the end of the sucralose treatment and baseline. There were no differences in the total AUC for glucose (p=0.65), insulin (p=0.16), active GLP-1 (p=0.63) or leptin (p=0.32) between the end of the aspartame treatment and baseline (Table 3). The % change in AUC from baseline to after sucralose treatment was -4.2% for glucose, +19.8% for insulin, +8.2% for active GLP-1 and +7.8% for leptin. The % change in AUC from baseline to after the aspartame treatment was +3.1% for glucose, +31.9% for insulin, +9.2% for active GLP-1 and +31% for leptin. There were no differences in the percentage change between the treatment groups and baseline (Table 3)**.**

5.4.3 Insulin secretion and sensitivity derived from OGTTs

The linear mixed model showed no difference in HOMA-IR, HOMA-%B, or HOMA-%S in healthy participants after sucralose or aspartame consumption compared to the baseline values (Table 4). There were no differences in HOMA-IR (p=0.35), HOMA-%B (p=0.16) or HOMA-%S (p=0.59) after sucralose treatment compared to the values at baseline. There was no difference in HOMA-IR (p=0.46), HOMA-%B (p=0.60) or HOMA-%S (p=0.61) after aspartame treatment compared to the values at baseline.

5.6 Discussion

To the best of our knowledge, this study is the first to evaluate repeated oral daily consumption of beverages sweetened with pure aspartame or sucralose powder in healthy adults in a randomized, double-blind, crossover trial. The primary outcome of this study was the effect of repeated daily consumption of NNSs on glucose metabolism. Daily oral ingestion of flavoured beverages containing pure aspartame or sucralose for 2 weeks did not affect plasma glucose, insulin, active GLP-1 or leptin concentrations in healthy participants. HOMA-IR, HOMA-%B and HOMA-%S were also unaffected by aspartame and sucralose

ingestion, suggesting that daily consumption of aspartame or sucralose does not impact the outcomes measured here, at least at the doses of 425 mg/day of aspartame and 136 mg/day of sucralose, which corresponded to an intake of ~3 cans (355 ml) of NNS sweetened beverages/day (Garriguet 2008).

Our results confirm and add to the present understanding of the effects of aspartame and sucralose on glucose metabolism. Many recent clinical trials have shown that NNSs, especially repeated daily doses of aspartame or sucralose, do not alter glucose metabolism in healthy or unhealthy individuals (Ahmad et al. 2019; Bonnet et al. 2018; Grotz et al. 2017; Higgins et al. 2018; Lertrit et al. 2018; Romo-Romo et al. 2018).

The effect of repeated daily doses of aspartame has been examined in recent studies. A study in 8 patients with newly diagnosed type 2 diabetes and 8 healthy participants found that consuming a daily table top formulation containing aspartame did not alter glucose, insulin or GLP-1 concentrations (Temizkan et al. 2015). Another study of 93 healthy participants found that consuming different doses of aspartame lower (350 mg) and higher (1050 mg) than the aspartame dose (425 mg) we used daily for 12 weeks had no effect on glucose, insulin or GLP-1 concentrations in all groups (Higgins et al. 2018). Similarly, a study in 50 healthy men found that daily oral ingestion of 2 cans of carbonated beverages containing aspartame and acesulfame potassium for 12 weeks did not change glucose or insulin concentrations (Bonnet et al. 2018).

The effect of repeated daily doses of sucralose has been examined previously in a few studies, which have shown mixed results. Two studies examined the effect of daily sucralose consumption; the first study was conducted with 77 participants and the second with 8 healthy participants, and both found that daily consumption of a beverage containing different doses of sucralose for >17 days did not alter blood glucose or insulin levels (Baird et al. 2000). Furthermore, a study of 67 patients with obesity and T2DM showed that

consuming a daily sucralose dose 3x the estimated maximum intake for 13 weeks had no effect on blood glucose or insulin concentrations (Grotz et al. 2003). A recent study in 47 healthy males demonstrated that consuming a high dose of sucralose in capsule form daily for 12 weeks did not affect glucose metabolism (Grotz et al. 2017).

Another recent trial of 66 healthy female participants reported that the daily consumption of commercial sucralose sachets of unknown concentration did not have an effect on glucose but did decrease insulin sensitivity and increase the acute insulin response (Romo-Romo et al. 2018). Another study in 15 healthy participants showed that oral ingestion of sucralose capsules for 4 weeks enhanced GLP-1 secretion and decreased insulin sensitivity without any effect on glucose levels (Lertrit et al. 2018).

These differing results could be due to the longer period of exposure to sucralose or the high dose of NNSs used in this trial, which is 1.47-times higher than the dose we used in our trial.

Previous studies have used a wide range of designs, and only two studies have assessed pure forms of sucralose in beverages in healthy individuals and individuals with obesity (Pepino et al. 2013; Sylvetsky et al. 2016). Most previous human trials were carried out with oral ingestion of NNS to measure the acute single dosing effects (Anton et al. 2010; Brown et al. 2009, 2012; Bryant et al. 2014; Pepino et al. 2013; Sylvetsky et al. 2016; Temizkan et al. 2015; Tey et al. 2017; Wu et al. 2012), but studies assessing the repeated daily consumption of aspartame or sucralose are far less common than studies of a single dosing (Bonnet et al. 2018; Grotz et al. 2017; Higgins et al. 2018; Lertrit et al. 2018; Romo-Romo et al. 2018).

The strengths of our current trial include its double-blind, crossover design and the selection of a healthy population with good adherence to the protocol and the inclusion of male and female participants, extending the generalizability of the results of this trial to the

general population. Additionally, females were assessed after the cessation of their menses to avoid diminished insulin sensitivity during some phases of the menstrual cycle (Diamond et al. 1989). Moreover, the NNSs used in this study were in a pure form to avoid any contamination by any other ingredients present, for example, in varying types of diet soda. However, this study has some potential limitations. We did not measure aspartame or sucralose consumption compliance through urinary biomarkers, and our study intervention period was only 2 weeks. This intervention period length may have been too short to observe a change, especially in healthy participants. Additionally, we did not have equal numbers of men and women in our RCT.

Future studies should recruit participants with higher BMI values, including individuals with obesity and people with prediabetes or type 2 diabetes. Additionally, more studies are needed with longer exposure durations to assess the effect of the chronic use of NNSs on metabolism.

In our future research, we plan to examine the impact of the consumption of the pure form of sucralose and aspartame on the gut microbiome using samples from this trial. We will explore the potential changes in the gut microbiota that might be induced by regular oral consumption of NNSs in humans. These changes in the gut microbiome could have occurred prior to the development of glucose metabolism dysregulation, so they may be captured in the timescale of this study even though no changes in glucose metabolism were observed.

In conclusion, our study showed that sucralose or aspartame consumption, reflecting high but realistic daily intakes, for 2 weeks had no effect on glucose, insulin, active GLP-1 or leptin concentrations in healthy participants. Further research is needed to confirm the findings of this trial.

5.8 Acknowledgements

The authors would like to thank all the participants for their effort and time to take part in this study. We would like to thank the clinical coordinators, laboratory staff, and nurses for their help in this trial.

5.10 Funding

This study was funded externally by Kuwait Foundation for the Advancement of Sciences (KFAS) and internally by University Collaborative Research Program (UCRP) and was supported, in part, by the Canada Research Chairs Program. SYA was a recipient of a scholarship from Kuwait Civil Service and Institute for Medical Specialization- Ministry of Health of Kuwait. The funding agencies had no role in defining the study design, in the collection and analysis of data, in the writing of the manuscript, or in the decision to submit the manuscript for publication.

5.7 Conflict of interest

D.M. was an invited speaker at a seminar entitled ‘Conflicting Outcomes from Systematic Reviews: Is the Consumption of Low-Calorie Sweeteners a Benefit or a Risk for Weight Management?’ at Nutrition 2018 in Boston, Massachusetts, USA, which was sponsored by PepsiCo. PepsiCo paid for his accommodation, conference fee and honorarium. PepsiCo is a company that sells products that contain non-nutritive sweeteners. The other authors declare no conflicts of interest.

5.9 Authors contributions

The authors' responsibilities were as follows: SYA and DM conceived the study, sought funding and ethical approval, contributed to development of the study protocol. SYA performed the laboratory work, interpreted the data, and wrote the manuscript. JF contributed to the design and development of the study protocol. DM, and JF designed the project and supervised the study. All authors read, made critical revisions to, and approved the final version.

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Tables

Table 6 Characteristics of participants at baseline1.

Variables	Value
Total participants (F/M)	17 (10 /7)
Age (years)	24 ± 6.8*
Body weight (kg)	68.9 ± 10.5*
BMI (kg/m^2)	22.9 ± 2.5*
Race (n)	
White	4
Asian	10
Middle Eastern	2
Not reported	1
Systolic blood pressure (mmHg)	119.9 ± 2.5
Diastolic blood pressure (mmHg)	78 ± 1.8
FBG	4.8 ± 0.1
Fasting plasma glucose (mmol/L)	5.3 ± 0.1
Fasting plasma insulin (pmol/L)	67.7 ± 8.5
Fasting plasma GLP-1 (pmol/L)	3.2 ± 0.5
Fasting plasma leptin (ng/ml)	7.6 ± 1.2
Fasting plasma fructosamine (μmol/L)	248.6 ± 4.9
HOMA-IR	1.3 ± 0.2
HOMA-%B	98.5 ± 6.6
HOMA-%S	90.6 ± 6.8

[1]Values are means ± SEMs unless otherwise indicated; * standard deviation; Concentrations were determined from plasma; M, males; F, Females; FBG, fasting blood glucose; GLP-1, glucagon-like peptide-1; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-%B, homeostasis model assessment of beta cell function; HOMA-%S, homeostasis model assessment of insulin sensitivity.

Table 7 Mean fasting glucose, insulin, active GLP-1 and leptin concentrations between the baseline visit and the baseline visit after the washout period measured in healthy participants, n=17.

Variables n=17	Values at baseline	Values after washout period	P-value
Mean fasting plasma glucose (mmol/L)	5.3 ± 0.1	5.9 ± 0.6	0.30
Mean fasting plasma insulin (pmol/l)	67.7 ± 8.5	81.9 ± 8.0	0.51
Mean fasting plasma GLP-1 (pmol/L)	3.2 ± 0.5	3.6 ± 0.4	0.99
Mean fasting plasma leptin (ng/ml)	7.6 ± 1.2	14.3 ± 4.4	0.20

Values are means ± SEMs unless otherwise indicated; concentrations were determined from plasma; GLP-1, glucagon-like peptide-1.

T-test was used to compare values between phases.

*Significant value if $p < 0.05$

Table 8 Changes in the AUCs of glucose, insulin, GLP-1 and leptin in healthy participants.

	Baseline	Sucralose	Aspartame	% change [a]	% change [b]	P-value [c*]	P-value [d*]
Glucose (mmol/l 120 min)	833 ± 143	798 ± 145	860 ± 205	- 4.2	+3.1	0.54	0.65
Insulin (nmol/l 120 min)	68 ± 39	81 ± 50	89 ± 42	+ 19.8	+ 31.9	0.38	0.16
GLP-1 (pmol/l 120 min)	695 ± 386	752 ± 373	759 ± 404	+ 8.2	+ 9.2	0.67	0.63
Leptin (ng/ml 120 min)	898 ± 512	968 ± 960	1177 ± 915	+ 7.8	+ 31.0	0.80	0.32

Abbreviations: AUC, area under the curve during 75 g OGTT; GLP-1, glucagon-like peptide-1; OGTT, oral glucose tolerance test; [a] % change in AUC from baseline to after sucralose treatment; [b] % change in AUC from baseline to after aspartame treatment; [c] differences between the end of the sucralose-sweetened beverage treatment and baseline; [d] differences between the end of the aspartame-sweetened beverage treatment and baseline; Values are means ± SDs, * Linear mixed model with REML estimation.

Table 9 Summary of insulin sensitivity and insulin secretion derived from OGTT results in healthy participants (n=17) who had consumed aspartame or sucralose for 14 days.

	Baseline	Sucralose	Aspartame	% change [a]	% change [b]	P-value [c*]	P-value [d*]
HOMA-IR	1.2 ± 0.6	1.5 ± 0.7	1.4 ± 0.5	+17.3	+13.3	0.35	0.46
HOMA-%B	98.4 ± 27.9	115.4 ± 40.1	104.6 ± 34.4	+17.24	+6.30	0.16	0.60
HOMA-%S	90.5 ± 28.9	83.5 ± 40.9	83.8 ± 43.3	-7.7	-7.4	0.59	0.61

OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-%B, homeostasis model assessment of beta cell function; HOMA-%S, homeostasis model assessment of insulin sensitivity; [a] % change from baseline to after sucralose treatment; [b] % change from baseline to after aspartame treatment; [c] differences between the end of the sucralose-sweetened beverage treatment and baseline; [d] differences between the end of the aspartame-sweetened beverage treatment and baseline. ($p<0.05$); Values are means ± SDs; *Linear mixed model with REML estimation.

CHAPTER VI

OVERALL CONCLUSIONS

6.1 Summary and implications

The findings of the present research contribute to the existing knowledge regarding the effect of NNSs on glucose metabolism and are in agreement with the majority of findings. With the increased awareness that consumption of food and beverages high in sugars can have harmful effects [1], and can be associated with many health risks such as increased risk of cardiovascular diseases, type 2 diabetes [2] and development of obesity in children and adults [3], there has been a demand to find alternative solutions. Thus, NNS have been introduced into the market as a popular alternative in many drinks such as diet sodas.

Regardless of the beneficial health effects of NNS use, such as weight management and control of blood glucose levels in different populations [4], NNS use has been associated with an increased risk of obesity, overweight, type 2 diabetes, cardiovascular diseases and metabolic syndrome, as shown in human studies [5, 6]. Taking into account the debate regarding the effect of NNSs on glucose metabolism [7-9], we evaluated whether NNSs have an effect on glucose, insulin, GLP-1 levels and leptin. Although our results showed that aspartame and sucralose have no effect on glucose metabolism, these results are also open to debate due to mixed results in the literature. Our findings are an important addition to the literature evaluating the effect of NNSs, specifically aspartame and sucralose, on glucose metabolism and our findings increase our knowledge of whether NNS consumption is associated with metabolic changes. Herein, we tried our best to design a study protocol that takes into consideration many of the limitations that exist in previous clinical trials. Therefore, we examined the effect of repeated daily consumption of aspartame and sucralose, the most popular artificial sweeteners used in Canada [8], in healthy males and females. We

used a pure form of NNS to avoid contamination by other ingredients that are present in diet drinks and might have independent metabolic effects and/or that may interact with NNS [10]. The route of NNS administration by oral consumption reflects NNS intake in real life versus administration by capsule form or intra-duodenal route, which is the case in many previous clinical trials. Our design of the study was carefully considered in the context of participant characteristics and inclusion criteria with more attention to current and previous NNS exposure. However, our results are consistent with the growing evidence that suggests no favourable effects of aspartame and sucralose consumption on glucose, insulin, and active GLP-1 [12-14], although comparing our results to those of other trials is should be done with caution due to many variations in the protocols used.

Our findings that sucralose or aspartame have no effect on glucose metabolism and insulin index may suggest that these artificial compounds might be metabolically inert and exert no effect when used in doses similar to the one we used in our clinical trial. However, these findings does not support the uncontrolled use of products containing NNSs, so I suggest that NNS should be used in caution until further research is done and provides conclusive answers. Our study, being of an exploratory nature, gives a number of opportunities for future research in terms of protocol and methods development. More research is needed to confirm our findings.

6.2 Strengths, limitations and future directions

The strength of this study was the double blind, crossover and randomized design. The use of the pure form of aspartame and sucralose dissolved in water in doses approximately similar to those in diet drinks replicating a real life situation is one of the strengths in our trial. Moreover, screening for previous use of NNSs and the recommendation for the participant to stay away from any products containing NNSs add strength to our trial. The

selection of a healthy population with a good adherence to the protocol and the generalizability of our study result can be extended to general population because we included males and females volunteers. Females participants were assessed after the cessation of their menses to avoid the possible effect of menstruation-related hormone changes on glucose homeostasis and diminished insulin sensitivity in some phases of the menstrual cycle [19].

However, a few limitations apply to this study. The study participants may not represent the target population, especially if we need to extrapolate our results to individuals with obesity or T2DM. Additionally, our findings cannot be applied to other NNSs, as each artificial or natural sweetener has a different chemical structure with a different metabolic fate and might exert different effects. A good compliance measure for sucralose consumption would be through measuring urinary biomarkers twice weekly during treatment phase from participants in the sucralose treatment group, this measurement was not included in our protocol. Also, detection of sucralose in urine samples during washout period would be a good tool to monitor adherence to the study recommendations during washout period. The exposure period of (2 weeks) might not be enough duration to observe changes in glucose metabolism in healthy participants and we cannot declare that there will no effect with aspartame or sucralose consumption for a longer exposure durations.

Although we included measures to support compliance during treatment phases such as bottle count, the intake of the beverages was outside our supervision. Our study design allowed the participants to consume sucralose or aspartame beverages during the day while maintaining their normal daily eating habits which was monitored through the 3-day food record during treatment phases; studies have shown that instructing the participants to record their diet in clinical trials might have an impact on the study outcomes [18],

Future studies should also focus on enrolling obese and diabetic adults to study the effect of NNSs, because these individuals are the most frequent users of NNSs [16] and already have metabolic complications.

Studies are also warranted to assess different blood glucose measurement techniques and how the efficiency and sensitivity of the instrument may affect the outcomes measured. The health implications of frequent and chronic NNS consumption need to be evaluated in future studies and if there is any observed metabolic effect of NNS consumption, their mechanisms can be explored as well.

The effect of sucralose and aspartame consumption on gut microbiota diversity and SCFA was measured in healthy adults. The data of the gut microbiome and SCFA analysis will be published later.

6.3 Final conclusion

The consumption of food and beverages rich in sugar, particularly sucrose or high-fructose corn syrup, is increasing globally [1]. In particular, sugar-sweetened beverages (SSBs), which include sodas, sport drinks and fruit drinks, account for more than fifty % of the Western diet, which exceeds the 10 % limit recommended by the World Health Organization(4). This statistic is important because overconsumption of SSBs has been associated with an increased risk of cardiovascular diseases and type 2 diabetes (5). Thus, beverages sweetened with NNSs were promoted as a healthier alternatives to SSBs to control weight and blood glucose levels worldwide.

However, whether NNSs are beneficial to human health has been a topic of debate over the past few decades, and the majority of data do not support this claim [6, 17]. Given the importance of many limitations in previous studies and our study, future studies with improved design are needed to determine the metabolic health consequences of NNS

consumption under real-world conditions. Moreover, even though the safety of NNSs has already been established and confirmed by health authorities, concerns regarding their metabolism and metabolic effect in the long run continue. Thus, until these findings are confirmed, diet drinks should not be marketed as a healthier option. I recommend using products sweetened with NNSs in moderation, keeping in mind that the best alternative to SSBs is water or carbonated water. The overall conclusion, aspartame and sucralose consumption is not associated with changes in glucose, insulin, GLP-1 and leptin concentrations. Also daily consumption of pure aspartame or sucralose for 2 weeks had no detectable effect on insulin sensitivity among healthy adults.

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APPENDICES

Appendix I

Ethics approval for study corresponding to chapter V

Ethics approval for study corresponding to chapter V

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PRINCIPAL INVESTIGATOR:	INSTITUTION/DEPARTMENT:	ETHICS #:
Dr. James Friel	J of M and RCFFN/Human Nutritional Sciences	HS18698 (B2015:069)
BREB MEETING DATE (if applicable):	**APPROVAL DATE:** March 13, 2017	
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (if applicable):		

PROTOCOL NUMBER:	PROJECT OR PROTOCOL TITLE:
NA	Assessment of the Effect of Artificial Sweeteners on Gut Microbiota and Glucose Metabolism
SPONSORING AGENCIES AND/OR COORDINATING GROUPS: CIHR	

REMINDER: THE CURRENT BREB APPROVAL FOR THIS STUDY EXPIRES: June 22, 2017

REVIEW CATEGORY OF AMENDMENT:	Full Board Review ☐	Delegated Review ☒
Submission Date of Investigator Documents: February 15, 2017	BREB receipt date of Documents: March 6, 2017	

THE FOLLOWING AMENDMENT(S) and DOCUMENTS ARE APPROVED FOR USE:

Document Name	Version(if applicable)	Date
Protocol: Protocol		2017-02-15
Consent and Assent Form(s): Research Participant Information and Consent Form		03/10/2017
Other: Poster		submitted February 15, 2017

CERTIFICATION
The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the amendment to the research study/project named on this ***Certificate of Approval*** as per the category of review listed above and was found to be acceptable on ethical grounds for research involving human participants. The amendment and documents listed above were granted final approval by the Chair or Acting Chair, UM BREB.

BREB ATTESTATION
The University of Manitoba (UM) Biomedical Research Board (BREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulation of Manitoba.

Ethics approval for study corresponding to chapter V

In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

QUALITY ASSURANCE
The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

CONDITIONS OF APPROVAL:

1. This amendment is acceptable on scientific and ethical grounds for the ethics of human use only. ***For logistics of performing the study, approval must be sought from the relevant institution(s).***
2. This research study/project is to be conducted by the local principal investigator listed on this certificate of approval.
3. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to the research study/project, and for ensuring that the authorized research is carried out according to governing law.
4. **This approval is valid until the expiry date noted on this certificate of approval. A Bannatyne Campus Annual Study Status Report** must be submitted to the REB within 15-30 days of this expiry date.
5. Any changes of the protocol (including recruitment procedures, etc.), informed consent form(s) or documents must be reported to the BREB for consideration in advance of implementation of such changes on the **Bannatyne Campus Research Amendment Form.**
6. Adverse events and unanticipated problems must be reported to the REB as per Bannatyne Campus Research Boards Standard Operating procedures.
7. The UM BREB must be notified regarding discontinuation or study/project closure on the **Bannatyne Campus Final Study Status Report**.

Sincerely,

Lindsay Nicolle, MD, FRCPC
Chair, Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above Human Ethics Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone**: (204) 789-3255/ **Fax:** (204) 789-3414

Clinical trial protocol registration corresponding to chapters IV, V

ClinicalTrials.gov PRS
Protocol Registration and Results System

ClinicalTrials.gov PRS DRAFT Receipt (Working Version)
Last Update: 03/10/2019 03:13

ClinicalTrials.gov ID: NCT02569762

Study Identification

Unique Protocol ID:	B2015:069
Brief Title:	Effects of Artificial Sweeteners on Gut Microbiota and Glucose Metabolism
Official Title:	Assessment of the Effect of Artificial Sweeteners on Gut Microbiota and Glucose Metabolism
Secondary IDs:	

Study Status

Record Verification:	May 2018
Overall Status:	Completed
Study Start:	July 2016 [Actual]
Primary Completion:	January 2018 [Actual]
Study Completion:	January 2018 [Actual]

Sponsor/Collaborators

Sponsor:	University of Manitoba
Responsible Party:	Sponsor
Collaborators:	

Oversight

U.S. FDA-regulated Drug:	
U.S. FDA-regulated Device:	
U.S. FDA IND/IDE:	No
Human Subjects Review:	Board Status: Approved Approval Number: HS18698(B2015:069) Board Name: University of Manitoba Bannatyne Campus Biomedical Research Ethics Board Board Affiliation: Biomedical Research Ethics Board (BREB) Phone: Email: Address: Research Ethics Board P126-770 Bannatyne Avenue Winnipeg, MB, Canada

Appendix II

Forms corresponding to study described in chapter V

Study advertisement - poster

VOLUNTEERS NEEDED FOR A NUTRITION STUDY!

Are you between 18-45 years old?

If so, you may be eligible to participate in a study to examine the effects of zero-caloric sweeteners on:
Blood sugar
Gut microbiome

Contact Us:
Richardson Centre for Functional Foods and Nautracitucals
196 Innovation Drive
University of Manitoba
Phone
Email:
james.friel@umanitoba.ca
Investigator: Dr.James Friel .

You will be compensated for your participation
This study has research ethical board approval

Participants consent form, page1

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

UNIVERSITY OF MANITOBA | Richardson Centre for Functional Foods and Nutraceuticals

Title of Study: "Assessment of the Effect of Artificial Sweeteners on Gut Microbiota and Glucose Metabolism."

Principal Investigator: Dr. James Friel
Richardson Centre, 196 Innovation Drive, Winnipeg, Manitoba R3T6C5

Co-Investigator: Dr. Dylan Mackay,
Richardson Centre, 196 Innovation Drive, Winnipeg, Manitoba R3T6C5

You are being asked to participate in a Clinical Trial (a human research study). Please take your time to review this consent form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family before you make your decision. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand. The study doctor and institution are receiving funds from the sponsor to conduct this study.

Purpose of Study

This Clinical Trial is being conducted to study the effect of non-caloric artificial sweeteners (NASs) on the composition of the human gut microbiome, and the consequent effect on glucose metabolism. You are being asked to take part in this study because you are between ~~20-30~~ 18 and 45 years old, with a BMI of 20-25 (i.e. normal weight), and a fasting blood glucose < 5.7 mmol/L (i.e. normal fasting glucose). You are not currently taking, and have not taken any antibiotic medications or probiotics in the last six months. You have not been diagnosed with any metabolic or gastrointestinal disorders, including diabetes, malabsorption syndrome inflammatory bowel disease, irritable bowel syndrome, phenylketonuria and/or celiac disease. For female participants: you are not pregnant or lactating, **and you have regular menstrual cycle**

The purpose of this study is to determine the effect of daily sucralose or aspartame (NAS) consumption on gut microbiota composition, diversity, and community structure, and to find out whether changes in glucose metabolism are mediated by deviations in gut microbiota composition, diversity, and community structure induced through NAS consumption.

Participants consent form, page 2

This research is being done because NASs are among the most commonly used food additives worldwide, and there is currently a lack of scientific data to support their perceived benefits for weight loss and control of blood sugar concentrations. The safety and potential health risks of NASs will be evaluated.

Study procedures

In this study, you will complete both treatments described below, in a randomly chosen order. You will agree to follow a 12-week diet regimen as follows. For the first four weeks, you will go through a run-in period, where no artificial sweeteners will be consumed. During weeks 5 and 6, you will consume either aspartame or sucralose. For weeks 7 through 10, you will undergo a washout period, where again, no artificial sweeteners will be consumed but you will be asked to consume at least one liter a day of water. Lastly, during weeks 11 and 12, you will consume the sweetener, which you did not previously consume. The amount of each sweetener that you will consume will be a **standard dose**, in order to meet the 14% of the Canadian acceptable daily intake (ADI) for aspartame and 20% of the Canadian ADI for sucralose. You will be given the appropriately calculated dose of sweetener in a double-blind procedure. This will involve having the sweeteners made into mixed, flavored beverages by a third party technician. Neither you nor the study investigators will know which order of treatment you are receiving. In an emergency, this information will be made available.

If you take part in this study, you will have the following tests and procedures:

1) You will fill out a food frequency questionnaire (FFQ) prior to the start of the study, followed by weekly food diaries (documenting two weekdays and one weekend day) of food and drink consumption. The FFQ will be used to determine your average consumption of NAS-containing foods and drinks on a regular basis. The weekly food diaries will be used to monitor your compliance with the study requirements.
2) You will be weighed prior to the start of the study, as well as in between each treatment condition, for a total of four weight measurements (on days 1, 28, 42, and 84).
3) You will provide a stool sample in between each treatment condition, for a total of ~~nine~~ **four stool** collections (on days 1~~,~~, 28, 42~~,~~ and 84). Stool samples will be collected using sterile cups after defecation and will be separated into two aliquots for storage in the RCFFN. The first aliquot will be stored as is at -80°C for later microbiota analysis, and the second will be stored in RNA*later* storage reagent. This second aliquot will be kept in order to keep open the possibility of future analysis of the genetic material from this community of microbiota (metagenomics analysis). These samples will be kept for up to 5 years after the end of the study.
4) You will have blood drawn at-**your first visit**, as well as in between each treatment condition, for a total of ~~f~~our blood sample collections (on days 1, 28, 42, ~~70,~~ and 84). Volume of the blood samples that will be drawn will be 1.5 tsps. At each visit. Blood samples will be taken by a registered nurse at the RCFFN, following both an overnight fast of at least 8 hours (fasting blood glucose test), and throughout a 2-hour oral glucose tolerance test (OGTT). This will involve drinking a sweet liquid containing a measured amount of glucose, in order to measure your body's ability to use glucose, the body's main source of energy. On days 1, 28, and 84 a fasting blood

Participants consent form, page 3

2-hour oral glucose tolerance test (OGTT). This will involve drinking a sweet liquid containing a measured amount of glucose, in order to measure your body's ability to use glucose, the body's main source of energy. On days 1, 28, 42**,** ~~70,~~ and 84 a fasting blood glucose test will be done as well. These samples will be kept for up to 5 years after the end of the study.

Participation in the study will be for 12 weeks, as shown below. The run-in and washout periods will each be 4 weeks long, while the treatment phases will each be 2 weeks long. At screening, a weight measurement and a blood sample will be taken in order to determine BMI and fasting blood glucose, respectively**.** Those who meet all screening criteria will participate in a total of **five** visits to the RCFFN, including the initial screening visit. During each treatment phase, you will be asked to come to the RCFFN every week once, and you will be given drinks for the rest of the week. At each "sample collection" period, you will be asked to come to the RCFFN for a blood draw, a weight measurement and to provide a stool sample. You will bring your food diaries to these visits, which should take about 2 hours.

The researcher may decide to take you off this study if funding is stopped. You may also be taken off this study if you either fail to ingest, or refrain from ingesting the artificial sweeteners as described. Female participants: you may also be taken off this study if you become pregnant or stop taking oral contraceptive pills.

You can stop participating at any time. However, if you decide to stop participating in the study, we encourage you to talk to the study staff first. There are no foreseeable health-related consequences of sudden withdrawal from the study.

The results will be given to you when the analyses are completed. You will receive a sealed and confidential letter, which will state your individual results of the parameters measured for the study purposes, along with the mean values obtained from the entire study population. The letter will be sent by the principal investigator at the RCFFN to the mailing address on the personal information form that you filled out prior to enrollment in the study.

Risks and Discomforts

While on the study, you are at risk for certain minor side effects. Blood draws have the potential to cause you discomfort or anxiety. When the needle is inserted to draw blood, some people feel moderate pain, while others feel only a prick or stinging. Afterward, there may be some throbbing or slight bruising, which soon goes away. Other risks associated with having blood

Participants consent form, page 4

human gut microbiome, and the possible resultant alterations in glucose metabolism. There is no expected, direct medical benefit to you from participating in this study. We hope the information learned from this study will benefit other participants with diabetes or uncontrolled glucose metabolism in the future.

Costs

All clinic and professional fees, diagnostic and laboratory tests, which will be performed as part of this study, are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Payment for participation

You will re ach "sample collection" period involving an oral glucose gift card at the end of the 12 weeks period of the study, t

Alternatives

You do not have to participate in this study to receive recommendations about safe NAS consumption. Please talk to your regular doctor about all your dietary concerns.

Confidentiality

Information gathered in this research study may be published or presented in public forums, however your name and other identifying information will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The records will be coded with identifying information and code maintained on a master list and all documents of participant's data only bearing the unique code generated. If information must be transported or is to leave the site it will be only the coded information.

Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. All study documents related to you will bear only your assigned participant code.

The University of Manitoba Biomedical Research Ethics Board may review research-related records for quality assurance purposes.

All records will be kept in a locked secure area (Locked cabinets in a locked room, password protected computers and no identifying information on flash drives) and only those persons identified will have access to these records. If any of your medical/research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN). The samples will be given non-identifiable numbers to protect confidentiality, and a researcher at RCFFN who is not involved in the study will maintain the link to identifying information.

Participants consent form, page 5

With your permission your Family Physician (GP) will be notified about your participation in this study.

The study is registered on a publicly available Registry Databank and the selected registry is Clinical Trials.gov. *ClinicalTrials.gov* is a website that provides information about federally and privately supported clinical trials. A description of this clinical trial will be available on *http://ClinicalTrials.gov.* This website will not include information that can identify you. At most, the website will include a summary of the results. You can search this website at any time.

Voluntary Participation/Withdrawal From the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision not to participate or to withdraw from the study will not affect your other medical care at this site. If your study doctor feels that it is in your best interest to withdraw you from the study, your study doctor will remove you without your consent.

We will tell you about any new information that may affect your health, welfare, or willingness to stay in this study.

As a student or employee of the University of Manitoba, any and all relevant evaluations of your performance will not be affected by your decision not to participate.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research participant. If any questions come up during or after the study or if you have

For questions about your rights as a research participant, you may contact The University of Manitoba Biomedical Research Ethics Board at

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent

For Study Subject: I have read this consent form and I agree to my family physician being notified of my participation in this study.

Yes ☐ No ☐

Participants consent form, page 6

1. I have had the opportunity to discuss this research study with Dr. James Friel and/or his study staff.
2. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me.
3. I believe that I have not been unduly influenced by any study team member to participate in the research study by any statement or implied statements. Any relationship (such as employee, student or family member) I may have with the study team has not affected my decision to participate.
4. I understand that I will be given a copy of this consent form after signing it.
5. I understand that my participation in this clinical trial is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.
6. I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of my medical records by The University of Manitoba Biomedical Research Ethics Board.
7. By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

I agree to being contacted in relation to this study. Yes ☐ No ☐

Participant signature_____ Date_____
(day/month/year)

Participant printed name: _____

For study Coordinator: I confirm that I have fully explained the details of this research study to the volunteer whose name and signature appears above. I confirm that I believe that the volunteer has understood and knowingly given their consent to participate by his/her personally dated signature.

Signature_____ Date _____
(day/month/year)

Printed name of above: _____

Dietary recommendation form, page 1

UNIVERSITY OF MANITOBA

NNS project-Recommendations

Dietary recommendations during the study

- Eat healthy balanced diet (Eatwell plate), which consist of
  - Vegetable (half the size of your plate)
  - Grains (your fist size)
  - Quarter portion for meat, fish and alternatives (size of the palm of the hand).
- Consume fruits for your dessert.

SPECIFICATION DURING THE TIME OF THE STUDY:

During the run in period (4 weeks before the start of the study) and the following weeks of the trial you **should not consume any non-nutritive sweeteners** and be ware of **hidden sources** of non-nutritive sweeteners in different food and beverages products and medication.

- Aspartame (**Equal**® or **NutraSweet**®) or **E951** found in a wide variety of foods and beverages, including **cereals**, **yogurt, frozen and gelatin desserts**, **candy, sugar-free gum, juices, diet sodas, ice cream** and many other products. It is also used in drugs such as **vitamin supplements and laxatives**.
- Acesulfame potassium (**Sunett**® and **Sweet One**®) or **E950** is generally used in combination with other non-nutritive sweeteners and is frequently found in sugar-free sodas (eg. **Pepsi Max**® **and Fruche**®).
- Neotame or **E961** is also used in low-calorie foods and beverages,
- Saccharin (Sweet 'n Low®, Sweet Twin®, and Sugar Twin®)
- Sucralose (**Splenda**® and **Equa**l "yellow packet") or **E955** It is found in many

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Dietary recommendation form, page 2

low-calorie foods and beverages, such as baked goods and other **desserts, canned fruits, dairy products** and **syrups**.

- Stevia (**Truvia®, A Sweet Leaf®, Sun Crystals®, and PureVia®**) Stevia is used in a wide range of foods and beverages, including **teas and juices**.
- Lo han guo (Monk fruit extract) (**Nectresse®**) is a natural sweetener made from crushed monk fruit. It is the newest nonnutritive sweetener on the market.

BEVERAGES

- Make water your beverage of choice.
- Milk, 100% juice are also healthy options.
- Make them part of your recommended number of Food Guide Servings each day.
- Limit soft drinks, sports drinks, energy drinks, fruit drinks, sweetened hot and cold beverages.

CAFFEINATED BEVERAGES

- **Limit your intake to 2 cups (250 ml) per day** of caffeinated beverages like tea, coffee, energy drinks and soft drinks.

ALCOHOL

- Limit **to 1 drink per day** of alcohol intake for a maximum of 5 drinks per week.
- What a one serving of alcohol is?
  - ✓ A glass of wine (150ml)
  - ✓ A normal size bottle of beer (340 ml)
  - ✓ A glass of liquor (45 ml)

***keep in mind that these servings are not cumulative.

PROBIOTICS AND ANTIBIOTICS/MEDICATION

- During the time of the study **don't take any probiotics supplements or food containg probiotics** such as:

× Kefir	× Coconut kefir	× Sauerkraut (fermented cabbage)
× Yougurt	× Natto (fermented soybean)	× Miso soup
× Raw cheese	× Kombucha tea	× Tempeh (fermented soy bean)

General information form

General information

STUDY NAME:

Site Numbe: **Visit Date**:

Pt_ID:

Visit Type (check one): ☐ **Screening** ☐ **Baseline**

Family name:_____ Name:_____

Gender: ☐Male ☐Female

Date of Birth (DD/MM/YY)_____ Age:_____

Address:_____

Postal code:_____

Telephone number:_____

E-mail:_____

Interest and motivation:

What is your interest in participating in this study?

_____

How did you get information regarding this study?

_____

Availability for meetings at the research center:

Availability in the morning: ☐Yes ☐No time:_____

Availability in the afternoon: ☐Yes ☐No time:_____

Availability in the evening: ☐Yes ☐No time:_____

Are you planning a trip or travel outside Manitoba over the next few weeks or months? ☐Yes ☐No

If yes, precise:_____

Comments: _____

General information Version 1.0

Medical questionnaire form , page 1

Medical Questionnaire/Life Habits

STUDY NAME	
DOB: _____ **Age:** **Pt_ID:** _____	**Visit Date:** __ __ / __ __ __ / 2 0 __ __ d d m m m y y y y

Samplings: ☐ left arm ☐ right arm

ANTHROPOMETRIC MEASUREMENTS

Height (m)_____

Body weight (kg)_____

BMI (kg/m2)_____

Completed by: _____

Comments:

_____

_____

Medical questionnaire

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Version 1.0

form

Medical questionnaire form , page 2

Medical Questionnaire/Life Habits

- **Did you see a doctor for any health concern in the past/present or future?** ☐ Yes ☐ No
- **Does the participant have a medical or surgical history, current or resolved, of any of the following?**

MEDICAL HISTORY	Yes / No	Unknown	If Yes, Explain	Current / Resolved
1. Head, Eye, Ear, Nose, Throat	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
3. Respiratory	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
4. Cardiovascular	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
5. Gastrointestinal	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
6. Genitourinary	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
7. Musculoskeletal	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
8. Neurological	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
9. Endocrine-Metabolic	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
10. Blood/Lymphatic	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
11. Dermatologic	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
12. Psychiatric	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
13. Allergy	☐ Yes ☐ No	☐		☐ Current ☐ Resolved
14. Other, specify: _____	☐ Yes ☐ No	☐		☐ Current ☐ Resolved

Medical questionnaire
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Version 1.0

Medical questionnaire form, page 3

Medical Questionnaire/Life Habits

MEDICATION: ☐ Yes ☐ No

Name of drug	Dose	Freq.	Indication	Start	Stop	*

* *Check box if medication is stopped.*

Drug allergies: ☐ Yes ☐ No
If yes, please specify: _____

NATURAL HEALTH PRODUCTS: ☐ Yes ☐ No

Do you take any dietary supplements, vitamins and or/minerals, homeopathic remedies and other natural health products (medicinal plants, omega3-fatty acids…etc.)

Name of drug	Dose	Freq.	Indication	Start	Stop	*

Check box if medication is stopped.

Are willing to stop taking these supplements at least 6 weeks before the begging of the study?
☐ Yes ☐ No

Medical questionnaire
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Version 1.0

Medical questionnaire form, page 4

Medical Questionnaire/Life Habits

FEMALE ONLY

Do you have a regular menstrual cycle? ☐ Yes ☐ No

What is the average length of your menstrual cycle?

When was the last day of you menstrual cycle? _____

Follicular Phase: _____

Are you currently taking oral contraceptives pills or other forms of hormonal contraception?

☐ Yes ☐ No Type:_____

Are you planning a pregnancy soon? ☐ Yes ☐ No

SOCIAL HISTORY/ LIFE HABITS:

- Participant's marital status:_____
- The nature of the current job _____
- Hobbies: _____
- Interests: _____
- Physical activity:

  _____
- Do you smoke? ☐ Yes ☐ No
- If yes: -How many cigarettes per day do you smoke? _____

  - Over what period of time? _____

Medical questionnaire form, page 5

Medical Questionnaire/Life Habits

- Have you ever taken any street drugs? ☐ Yes ☐ No
- Do you drink alcohol ☐ Yes ☐ No
- If yes: how much alcohol do you drink? _____Units/week –refer to table below.

Measures of Alcohol
1 unit (8g) = ½ pint beer or 1 glass wine (15 cl) or single measure of spirit (1/6 gill).

NUTRITION:

Allergies:

- Foods ☐ Yes ☐ No specify_____
- Others ☐ Yes ☐ No specify_____
- Special dietary habits ☐ Yes ☐ No specify_____

Consumption of:

- Soft drinks ☐ Yes ☐ No specify_____
- Diet drinks ☐ Yes ☐ No specify_____
- Coffee/ ☐ Yes ☐ No specify_____
- Tea ☐ Yes ☐ No specify_____
- Non caloric artificial sweeteners ☐ Yes ☐ No specify_____
- Number of meals /day: _____

FUNCTIONAL HISTORY:

If you participate in this study, what type of transportation would you use- e.g. walking, driving, cycling, bus?

_____

Medical questionnaire
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Version 1.0

Screening form, page 1

Inclusion/Exclusion Criteria

STUDY NAME:	
Pt. ID	Visit Date.
Visit Type (check one): ☐ Screening	☐ Baseline

Inclusion Criteria

Participant must:

1.	Age between 1	☐Yes	☐No
2.	Normal weight (BMI 20-25)	☐Yes	☐No
3.	Fasting blood glucose < 5.7 mmol/l	☐Yes	☐No
4.	Women: regular menstrual cycle	☐Yes	☐No
5.	Women: not taking oral contraceptive pills	☐Yes	☐No
6.	No Known active medical condition	☐Yes	☐No
7.	Low consumption of non-nutritive sweetener	☐Yes	☐No

Screening form, page 2

Inclusion/Exclusion Criteria

Exclusion Criteria

Participant must not:

1.	Have you smoked in the last 6 months?	☐Yes	☐No
2.	Have you taken any antibiotics within the last 6 months?	☐Yes	☐No
3.	Have you taken any probiotics within the last 6 months?	☐Yes	☐No
4.	Do you have any metabolic or gastrointestinal disorder (e.g. Diabetes, malabsorption syndrome, inflammatory bowl disease, irritable bowl syndrome, celiac disease, phenylketonuria,	☐Yes	☐No
5.	Women: are you pregnant?	☐Yes	☐No
6.	Women: are you breastfeeding?	☐Yes	☐No
7.	Have you used in the Recent past 2 months any drugs that change glucose metabolism (e.g. metformin), change gastric pH (e.g. proton pump inhibitors) or gastric emptying (e.g. metoclopramide)	☐Yes	☐No
8.	Positive urine pregnancy test for females	☐Yes	☐No
9.	Is there any Known allergy, sensitivity or other contraindication to any study food or drug or its vehicle	☐Yes	☐No

Inclusion/Exclusion Criteria
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Version 1.0

Screening form, page 3

Inclusion/Exclusion Criteria

Did the participant meet the eligibility requirements for this study?	☐Yes	☐No

Investigator Signature:_____ Date: _____

- Regular use of non nutritive sweetener (consuming > one can of diet beverage or one spoonful of NNS (e.g. sucralose, aspartame or saccharine a week).
- Active illegal drug user (self-reported).
- Use of steroids or beta agonists (orally, intranasal or inhaled) within a week of any OGGT.

Anthropometric measurements form

ANTHROPOMETRIC MEASURMENTS

STUDY NAME	
Site Number: _____	**Visit Date:** ___ ___ / ___ ___ ___ / 2 0 ___ ___ (d d m m m y y y y)
Pt_ID: _____	

Visit Type (circle one): **Screening** **Completion Visit**

Baseline

Visit _____

1. Time ____:____ ☐ am ☐ pm

2. Heart Rate _____bpm ☐ Not Done

3. Blood Pressure_____/_____ (systolic/diastolic) ☐ Not Done

3.a BP Position

☐ Sitting
☐ Supine
☐ Standing

5. BMI (kg/m²): _____

6. Weight (kg): _____ ☐ kilograms ☐ Estimated? ☐ Not Done

7. Height (cm): _____ ☐ centimeters ☐ Estimated? ☐ Not Done

Completed by : _____

Vital Signs

Version 1.0

Adverse event form

Adverse event log

Participant ID	Description of adverse event	Start Date End date	Severity*: Mild, moderate, severe, life threating, death	Relation to study	Expected	Outcome

*mild: Can be tolerated by the volunteer, causing minimal discomfort and not affecting everyday activities.

Moderate: the event that is sufficiently discomforting and interfering with daily normal activities

Severe: an event which is preventing normal everyday activites

Food journal form, page 1

UNIVERSITY OF MANITOBA

FOOD JOURNAL

Subject number: _____

Reception date: _____

Food journal form , page 2

FOOD JOURNAL *SUBJECT#:*

PLEASE *REMEMBER* THE FOLLOWING:

- List everything you eat and drink during 3 days:
  - **2 days** in the **week**
  - **1 day** in the **week-end**
- This food journal will represent your normal diet.
- Please indicate the day, time, and place of the meal.
- List **only one food item per line**, and you can use new sheets for each day.
- Always note progressively what you eat.
- Provide more details as much as possible for every food, the more the better.
- Always indicate the **quantity by volume** for everything you eat or drink, for example
  - Cup, ml, teaspoon and tablespoon
  - Or in weight (grams or ounce)
  - And always indicate whether the measurement was taken before or after cooking and if it includes bone, fat or skin.
- Make sure to **list condiments** and other **additives** (ketchup, sauce, salt, oil, butter, etc)
- Indicating the **trademark** would be great when ever it is possible, even cutting off the nutrition fact label if applicable.
- Include everything you eat such as gums, candy, snacks, beverage and medications.
- If you consume a mixed meal, please **write down the recipe** with all ingredients and their quantity used to prepare the meal.
- If you eat at a **restaurant**, please provide the name of the restaurant, type of food you ordered (name of the dish would help) and the serving size consumed.

Thank for taking part in this study and we appreciate your collaboration

DAY: [redacted] *DATE:* [redacted] *SUBJECT#:* _____

Food journal form , page 3

FOOD JOURNAL _____ *SUBJECT#:*

EXAMPLE

Time	Place	Quantity (Grams/vol.)	Description of food or drink consumed (One item per line)	Trademark	Don't write in this column
		2	*Bread and butter*		
		1 bowl	*Noodle soup*		
		A little	*Salad with dressing*		
		1 glass	*Juice*		
		3	*Cookies*		
14:20	*Home*	*2 slice=46 g*	*Whole wheat bread*	*Silver hill*	
		1 teaspoon	*Butter*	*Organic meadow*	
		300 ml	*Canned noodle soup with vegetables*	*campbell*	
		60 g	*Iceberg lettuce*		
		10 g	*Cubes carrot*		
		30 ml	*Regular Italian dressing*	*kraft*	
16:00	*Home*	*200 ml*	*Orange juice from concentrate*	*ocean*	
		3 cookies= 60g	*Chocolate sandwich cookies*	*oreo*	

BAD EXAMPLE

GOOD EXAMPLE to follow

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Food journal form , page 4

FOOD JOURNAL *SUBJECT#:*

DAY: *DATE:*

Time	Place	Quantity (Grams/vol.)	Description of food or drink consumed (One item per line)	Trademark	Please make sure to mention these?
					-juice? Sweet beverages, powder, fresh. -Bread? White, wheat, cereals -Muffin? Size, homemade -Egg? Fried scrambled or boiled, poached, fat added? Amount? -Sugar, syrup or cream added to coffee? -Soup? Homemade, canned, bagged -Salad? Additives: amount of veggies, cheese, croutons, seeds…etc. -Sandwich? Bread, additives? Mayonnaise, mustard..etc. -Meat/fish? Method of cooking. -Poultry? With/without skin -Fat added during cooking? Amount please -Dessert? Size, toppings -Snacks? Type, amount.

DAY: *DATE:*

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Food journal form, page 5

FOOD JOURNAL _____ *SUBJECT#:*

Recipe: **EXAMPLE** *Vegetable pie* Recipe's yield: 5 servings		Recipe: Recipe's yield_____	
2 large	*Minced onion*		
2ml	*Salt and pepper*		
14 oz	*Canned mushroom*		
4 sheets	*Buff pastry sheets*		
2 large	*Zucchini*		
1 cup	*Carrots*		
1 cup	*Sour cream*		

EXAMPLE

Recipe: Recipe's yield: _____		Recipe: Recipe's yield_____	

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Visual analogue scales, page 1

ID: _____ DATE (DD/MMM/YYYY):_____

Part: _____ Study: _____

SESSION: _____ TREATMENT (Code): _____

Visual Analogue Scales
Palatability: Treatment

This question relates to the palatability of the beverage/food you just consumed. Please rate yourself by placing a small "**x**" across the horizontal line at the point which best reflects your present findings.

1. How pleasant have you found the beverage?

NOT at all pleasant _____ VERY pleasant

2. How tasty have you found the treatment?

NOT at all tasty _____ VERY tasty

3. How did you like the texture of the treatment?

NOT at all _____ VERY much

4. How sweet have you found the beverage?

NOT at all sweet _____ VERY sweet

Visual analogue scales, page 2

ID: _____ DATE (DD/MMM/YYYY):_____

Part: _____ Study: _____

SESSION: _____ TREATMENT (Code): _____

Visual Analogue Scales
Motivation to Eat

Time: _____

These questions relate to your "motivation to eat" at this time. Please rate yourself by placing a small "x" across the horizontal line at the point which best reflects your present feelings.

1. **How strong is your desire to eat?**

   VERY weak _____ VERY strong

2. **How hungry do you feel?**

   NOT hungry at all _____ As hungry as I have ever felt

3. **How full do you feel?**

   NOT full at all _____ VERY full

4. **How much food do you think you could eat?**

   NOTHING at all _____ A LARGE amount

5. **How thirsty do you feel?**

   NOT thirsty at all _____ As thirsty as I have ever felt

Visual analogue scales, page 3

ID: _____ DATE (DD/MMM/YYYY):_____

Part: _____ Study: _____

SESSION: _____ TREATMENT (Code): _____

Visual Analogue Scales
Energy and Fatigue

Time: _____

These questions relate to your energy level and fatigue at this time. Please rate yourself by placing a small "**x**" across the horizontal line at the point which best reflects your present feelings.

1. How energetic do you feel right now?

NOT at all _____ VERY energetic

2. How tired do you feel right now?

NOT at all _____ VERY tired

Visual analogue scales, page 4

ID: _____ DATE (DD/MMM/YYYY):_____

Part: _____ Study: _____

SESSION: _____ TREATMENT (Code): _____

Visual Analogue Scales
Physical Comfort

Time:_____

These questions relate to your "motivation to eat" at this time. Please rate yourself by placing a small "x" across the horizontal line at the point which best reflects your present feelings.

1. **Do you feel nauseous?**

   NOT at all _____ VERY much

2. **Does your stomach hurt?**

   NOT at all _____ VERY much

3. **How well do you feel?**

   NOT well at all _____ VERY well

4. **Do you feel like you have gas?**

   NOT at all _____ VERY much

5. **Do you feel like you have diarrhea?**

   NOT at all _____ VERY much

Appendix III

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Chapter II: Manuscript 1

The effect of sucralose and aspartame on glucose metabolism and gut hormones

Chapter III: Manuscript 2

Recent evidence for the effect of non-nutritive sweeteners on glycemic control

Chapter V: Manuscript 4

The effect of artificial sweeteners on glucose metabolism in healthy adults: a randomized double-blinded crossover clinical trial

Appendix IV

Supplementary figures and tables

Chapter II: Manuscript 1

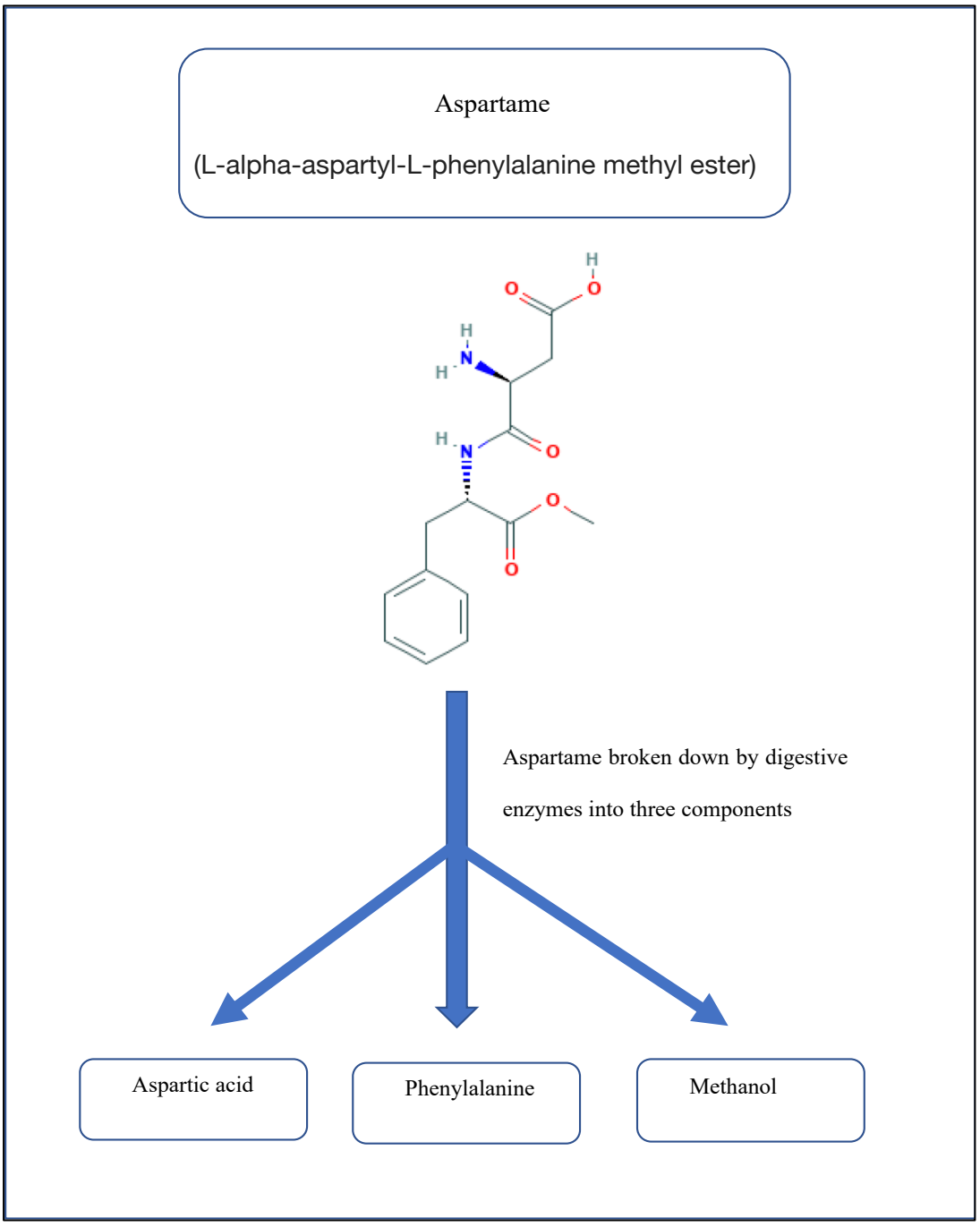


Figure 1 :Aspartame metabolism. Digestive enzymes break aspartame down into methanol, phenylalanine, and aspartic acid.

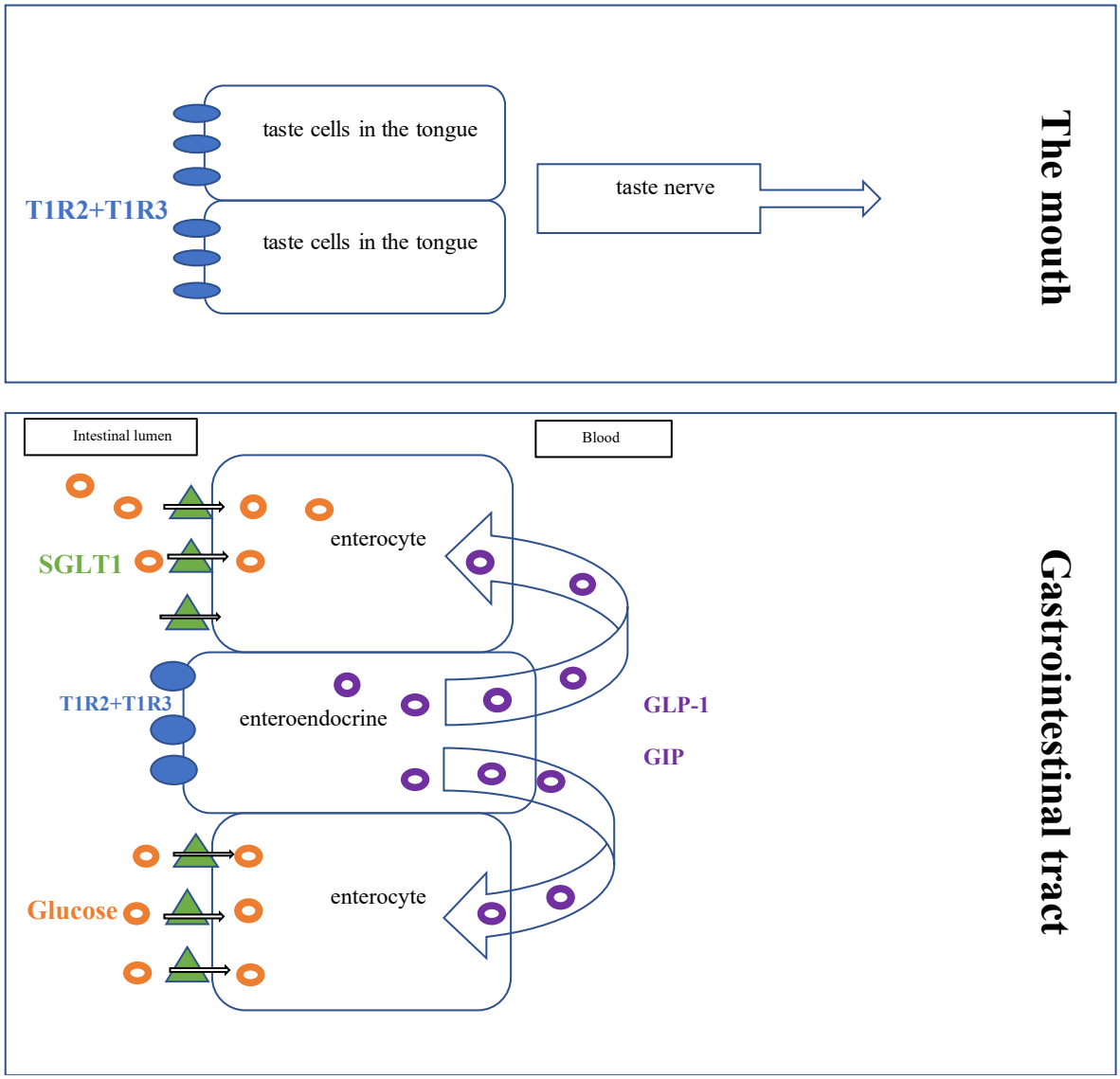


Figure 2 NNS and intestinal glucose absorption. Stimulation of the gut taste receptors T1R2 and T1R3 (blue circles) will leads to the release of incretin hormones including GLP-1 (purple circles) and glucose-dependent insulinotrophic peptide (GIP) (purple circle. These GLP-1 and GIP are gut derived peptides that stimulate insulin secretion when there is an increase in blood glucose (orange circles) concentrations. (green triangles= SGLT1 receptors)

Chapter V: Manuscript 4

Table 10 Visual analogue scale collected from participants (n=17) receiving aspartame or sucralose treatment.

Visual analogue scale (VAS)	Aspartame drinks n=17	Sucralose drinks n=17	P-value [c*]
Palatability:			
Pleasantness of drink	53.47 ± 23.88	52.00 ± 25.73	0.86
Taste	48.41 ± 21.96	53.76 ± 25.69	0.49
Texture	55.65 ± 23.62	59.76 ± 23.06	0.47
Sweetness	63.94 ± 23.56	65.53 ± 20.95	0.82
Motivation to eat:			
Desire	45.41 ± 21.41	46.47 ± 20.68	0.89
Hunger	41.06 ± 18.36	51.71 ± 21.35	0.17
Fullness	46.06 ± 22.30	49.24 ± 24.00	0.59
Amount	49.59 ± 22.36	54.76 ± 22.97	0.46
Thirst	43.82 ± 26.30	35.24 ± 21.82	0.20
Energy and fatigue:			
Energy	51.53 ± 19.31	54.47 ± 22.81	0.60
Tiredness	41.06 ±23.37	39.12 ± 19.60	0.83
Physical comfort:			
Nausea	16.65 ± 18.03	26.82 ± 23.73	0.21
Stomach pain	16.24 ± 19.99	15.88 ± 13.99	0.95
Wellness	49.59 ± 31.09	55.12 ± 28.63	0.46
Gas	23.41 ± 26.98	25.59 ± 25.86	0.80
Diarrhea	15.53 ± 22.86	8.18 ± 11.59	0.19

Data presented as mean ± standard deviation (SD).

T-test was used to compare scores between two drinks.

Table 11 Self-reported 3-day food record (n=17) during treatment phases.

Dietary method					
Nutrients intake n=16	3 days food record Sucralose treatment		3 days food record Aspartame treatment		
	Mean	SD	Mean	SD	P-value
Energy (kcal)	1733.69	628.15	1520.89	612.97	0.08
Proteins (g)	90.26	53.73	81.64	47.94	0.09
Total fat (g)	63.95	29.84	56.71	26.93	0.13
Carbohydrates (g)	202.34	78.47	173.28	81.28	0.01 *
Fiber (g)	14.76	5.92	13.05	7.35	0.04 *
Caffeine (mg)	69.05	63.82	59.48	65.80	0.31

Data presented as mean ± standard deviation (SD).

T-test was used to compare nutrient intake between phases.

*Significant value if $p<0.05$

Table 12 Nutrient intake and NNS use over the past year reported from the Diet History Questionnaire II which was collected from participants at baseline.

Dietary method		
Diet history questionnaire over the past year		
	Mean	SD
Energy (kcal) (n=17)	1825.84	693.40
Total fat (g) (n=17)	72.04	31.13
Carbohydrates (g) (n=17)	228.60	98.29
Proteins (g) (n=17)	70.87	29.32
Fiber (g) (n=17)	17.43	9.21
Caffeine (mg) (n=17)	176.87	186.72
Aspartame (mg) (n=15)	11.81	11.61
Saccharin (mg) (n=13)	2.54	7.45
Acesulfame potassium (mg) (n=9)	0.02	0.03
Sucralose (mg) (n=12)	65.83	168.55

Data presented as mean ± standard deviation (SD).

Table 13 Mean fasting glucose, insulin, active GLP-1 and leptin concentration between baseline visit and the baseline visit after the washout period measured in healthy participants, n=17.

Variables n=17	Values at baseline	Values after washout period	P-value
Mean fasting plasma glucose (mmol/L)	6.00 ± 0.48	5.99 ± 0.63	0.98
Mean fasting plasma insulin (pmol/l)	67.66 ± 8.73	81.86 ± 8.30	0.14
Mean fasting plasma GLP-1 (pmol/L)	3.24 ± 0.49	3.19 ± 0.38	0.94
Mean fasting plasma leptin (ng/ml)	7.55 ± 1.23	6.62 ± 1.42	0.98
Values are mean ± SEMs unless otherwise indicated; concentrations are determined from plasma; GLP-1 , glucagon like peptide-1. T-test was used to compare values between phases. *Significant value if $p<0.05$			

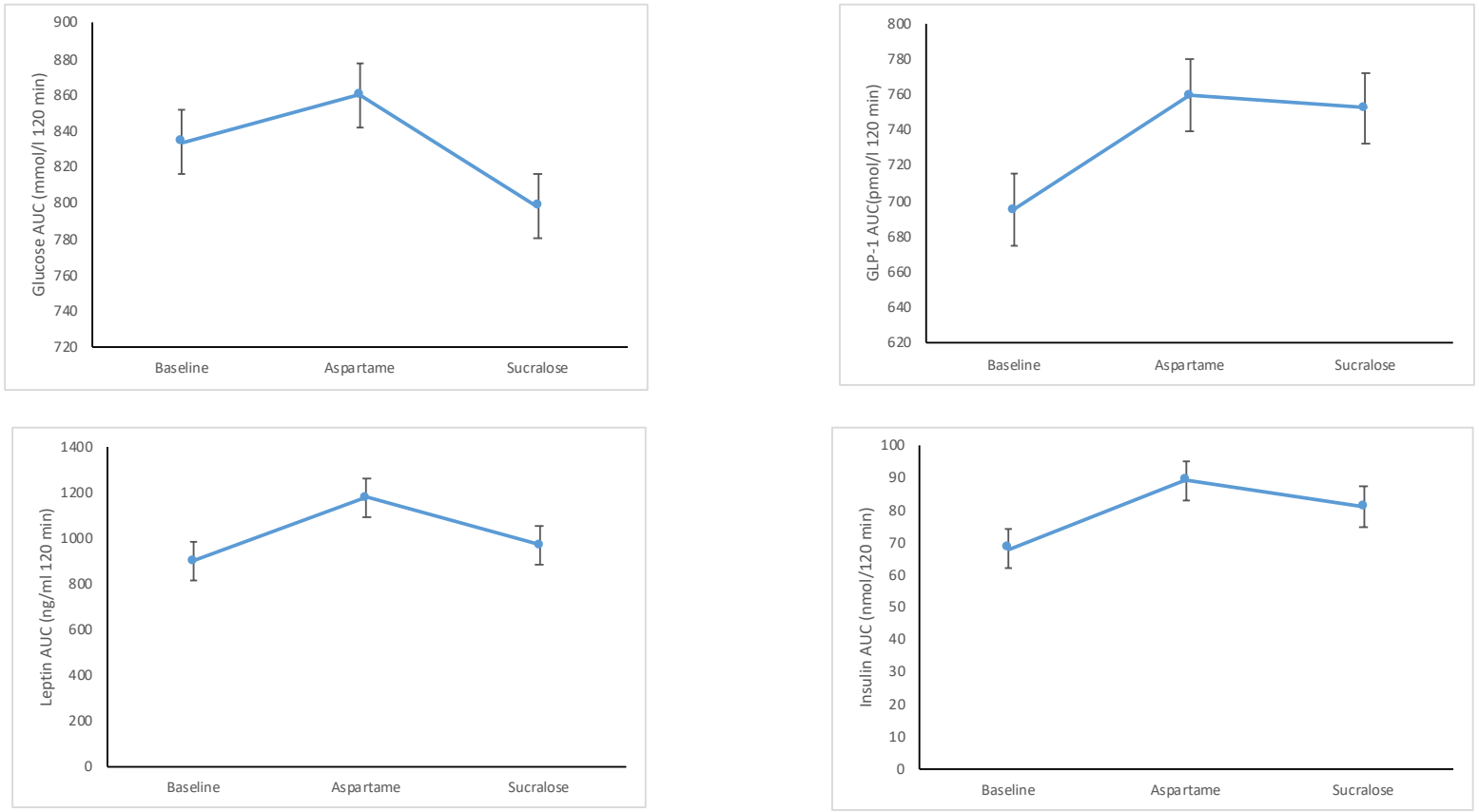
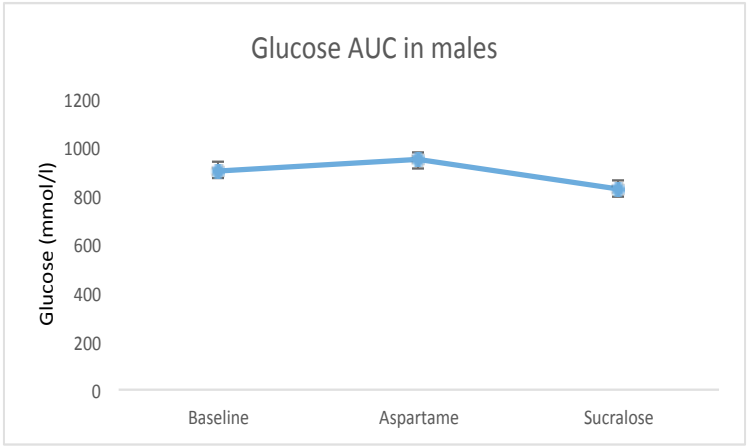


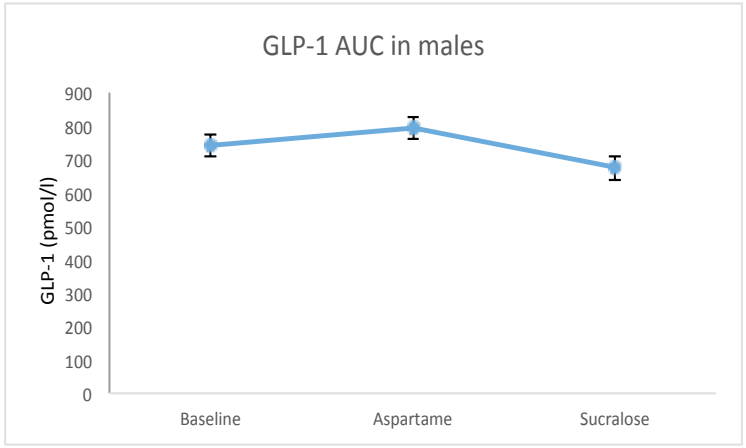
Figure 3 Change in AUC of glucose, insulin, GLP-1 and leptin in healthy participants (n=17) from baseline to after receiving aspartame or sucralose treatment for 14 days.

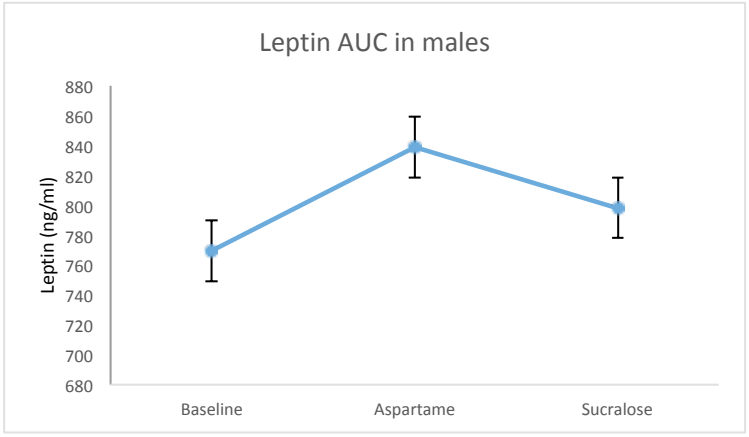
Table 14 Changes in AUC of glucose, insulin, GLP-1 and leptin in healthy males participants (n=10) receiving aspartame or sucralose treatment for 14 days.

	Baseline	Sucralose	Aspartame	% change [a]	% change [b]	P-value [c*]	P-value [d*]
Glucose (mmol/l 120 min)	904±125	826±149	945±180	-8.65	4.54	0.24	0.54
Insulin (nmol/l 120 min)	75±46	103±46	100±38	37.86	33.43	0.49	0.54
GLP-1 (pmol/l 120 min)	673±401	793±319	741±401	17.85	10.07	0.73	0.85
Leptin (ng/ml 120 min)	769±354	798±586	838±342	3.78	9.01	0.96	0.90

AUC, area under the curve during 75 g OGTT; GLP-1, glucagon like peptide-1; OGTT, oral glucose tolerance test; [a] % change in AUC from baseline to after sucralose treatment; [b] % change in AUC from baseline to after aspartame treatment; [c] differences between sucralose-sweetened beverage and baseline; [d] differences between aspartame-sweetened beverage and baseline; Values are means ± SD, * Linear mixed model with REML estimation.







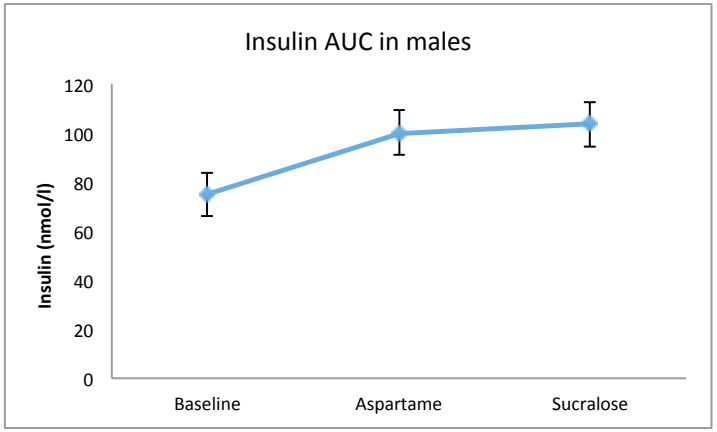


Figure 4 Change in AUC of glucose, insulin, GLP-1 and leptin in healthy males participants (n=10) from baseline to after receiving aspartame or sucralose treatment for 14 days.

Table 15 Changes in AUC of glucose, insulin, GLP-1 and leptin in healthy females participants (n=7)receiving aspartame or sucralose treatment for 14 days.

	Baseline	Sucralose	Aspartame	% change [a]	% change [b]	P-value [c*]	P-value [d*]
Glucose (mmol/l 120 min)	718±58	754±122	740±156	3.78	3.11	0.65	0.78
Insulin (nmol/l 120 min)	50±22	49±31	74±39	-0.71	48.56	0.40	0.10
GLP-1 (pmol/l 120 min)	658±426	750±501	742±416	13.94	12.64	0.13	0.13
Leptin (ng/ml 120 min)	974±1352	1121±1013	1660±669	15.90	70.43	0.09	0.30

AUC, area under the curve during 75 g OGTT; GLP-1, glucagon like peptide-1; OGTT, oral glucose tolerance test; [a] % change in AUC from baseline to after sucralose treatment; [b] % change in AUC from baseline to after aspartame treatment; [c] differences between sucralose-sweetened beverage and baseline; [d] differences between aspartame-sweetened beverage and baseline; Values are means ± SD, * Linear mixed model with REML estimation.

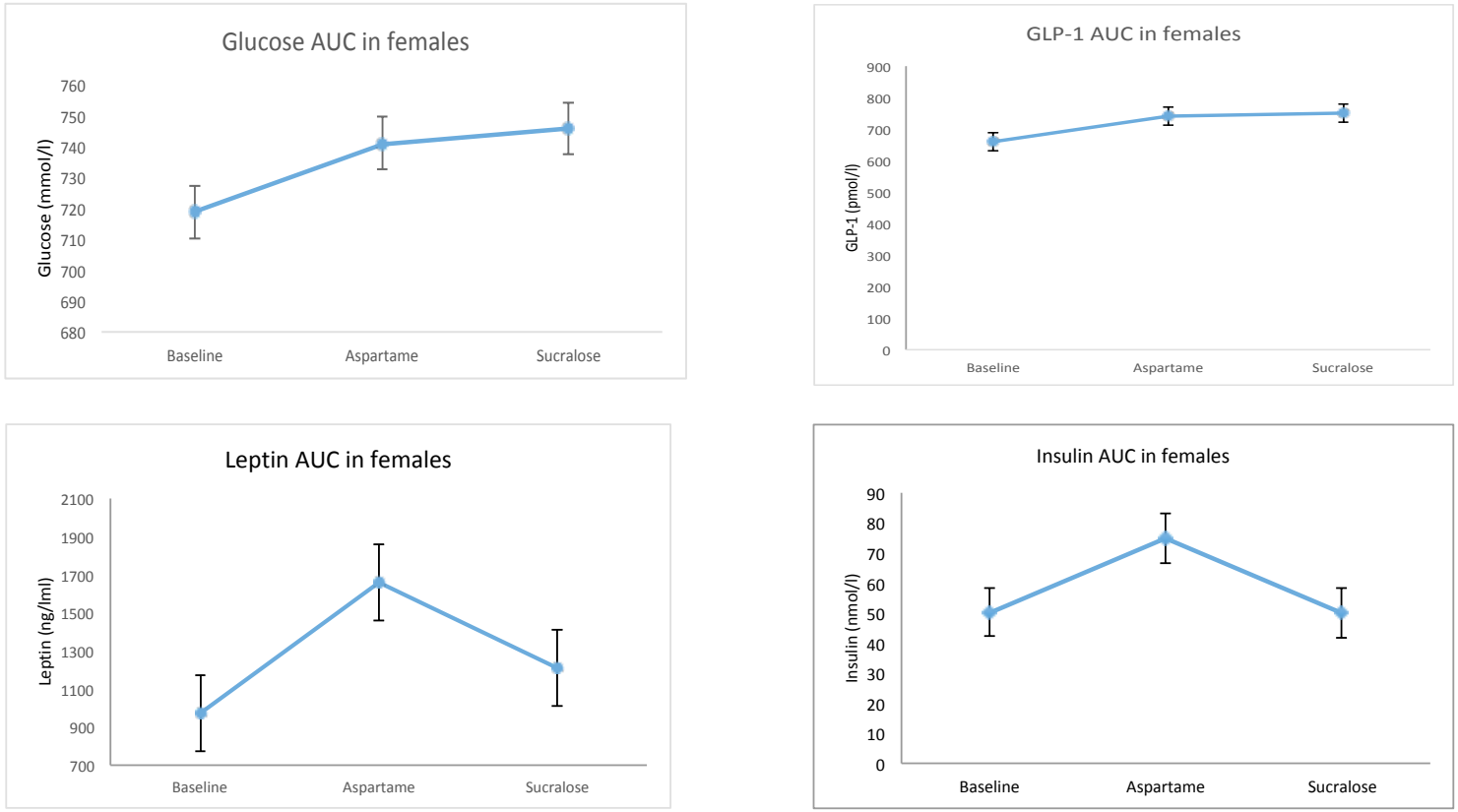
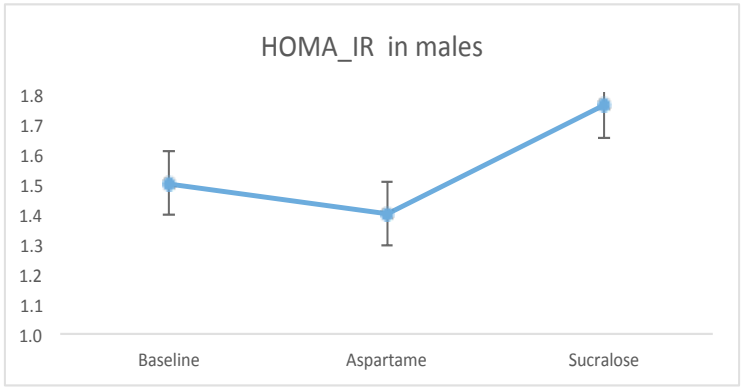


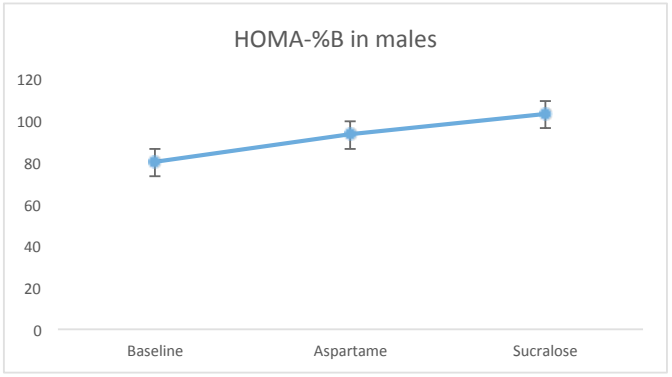
Figure 5 Change in AUC of glucose, insulin, GLP-1 and leptin in healthy females participants (n=7)from baseline to after receiving aspartame or sucralose treatment for 14 days.

Table Summary of insulin sensitivity and insulin secretion derived from OGTT in healthy males participants (n=10) who had consumed aspartame or sucralose for 14 days.

	Baseline	Sucralose	Aspartame	% change [a]	% change [b]	P-value [c*]	P-value [d*]
HOMA-IR	1.45 ± 0.76	1.46 ± 0.82	1.76 ± 0.61	17.22	6.75	0.32	0.88
HOMA-%B	79 ± 34	103 ±32	93 ± 32	28.84	16.33	0.14	0.40
HOMA-%S	79 ± 21	68 ± 26	90 ± 48	-14.35	14.11	0.51	0.50

OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-%B, homeostasis model assessment of beta cell function; HOMA-%S, homeostasis model assessment of insulin sensitivity; [a] % change from baseline to after sucralose treatment; [b] % change from baseline to after aspartame treatment; [c] differences between sucralose-sweetened beverage and baseline; [d] differences between aspartame-sweetened beverage and baseline. ($p<0.05$); Values are means ± SD; *Linear mixed model with REML estimation.





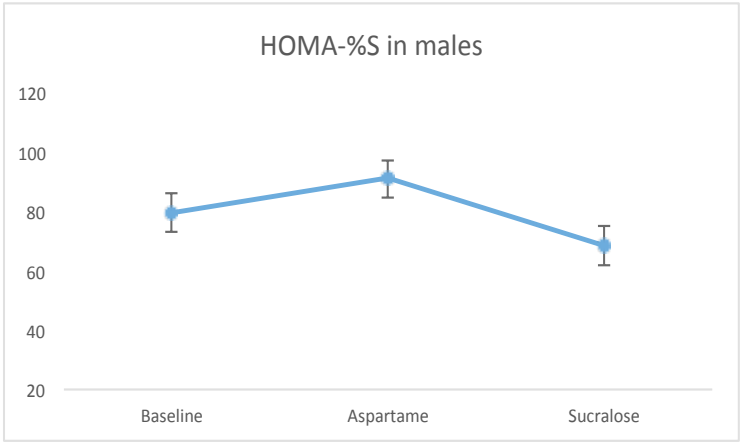
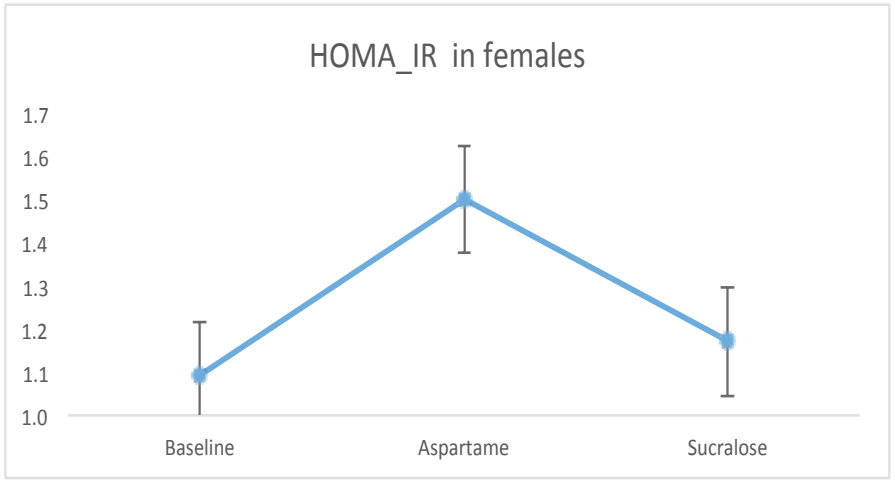


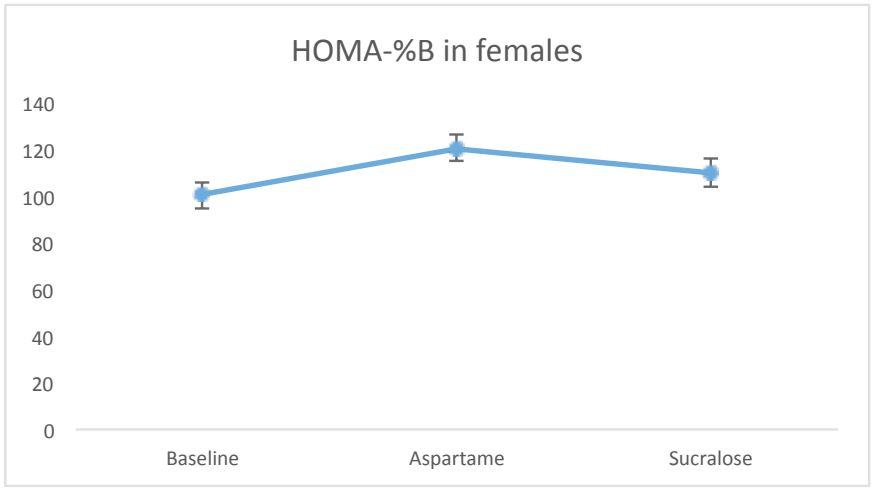
Figure 6 Change in insulin sensitivity and insulin secretion derived from OGTT in healthy males participants (n=10) from baseline to after treatment with aspartame or sucralose for 14 days.

Table 16 Summary of insulin sensitivity and insulin secretion derived from OGTT in healthy females participants (n=7) who had consumed aspartame or sucralose for 14 days.

	Baseline	Sucralose	Aspartame	% change [a]	% change [b]	P-value [c*]	P-value [d*]
HOMA-IR	1.09 ± 0.40	1.17 ± 0.51	1.50 ± 0.40	7.33	37.61	0.82	0.26
HOMA-%B	100 ± 32	109 ± 35	120± 27	9.25	19.99	0.62	0.28
HOMA-%S	102± 31	73 ± 27	104 ± 45	-2.23	27.96	0.90	0.15

OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-%B, homeostasis model assessment of beta cell function; HOMA-%S, homeostasis model assessment of insulin sensitivity; [a] % change from baseline to after sucralose treatment; [b] % change from baseline to after aspartame treatment; [c] differences between sucralose-sweetened beverage and baseline; [d] differences between aspartame-sweetened beverage and baseline. ($p<0.05$); Values are means ± SD; *Linear mixed model with REML estimation.





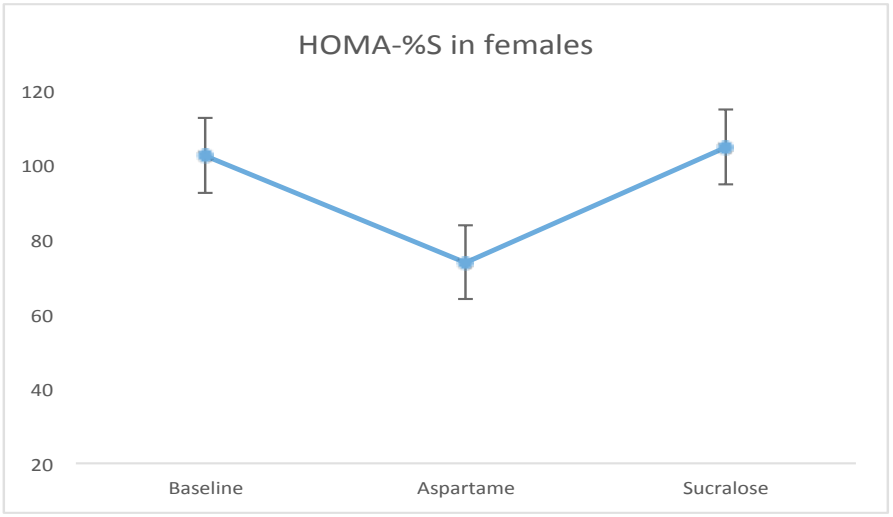


Figure 7 Change in insulin sensitivity and insulin secretion derived from OGTT in healthy females participants (n=7) from baseline to after treatment with aspartame or sucralose for 14 days.

Table 17 Changes in plasma glucose at different time points in healthy participants receiving aspartame or sucralose treatment for 14 days.

	Baseline	Sucralose	Aspartame	% change [a]	% change [b]	P-value [c*]	P-value [d*]
Glucose_0 (mmol/l)	5.28±0.47	5.15±0.37	5.41±0.60	-2.46	2.46	0.44	0.53
Glucose_15 (mmol/l)	6.37±0.79	6.41±0.79	7.15±1.24	0.62	4.75	0.90	0.02*
Glucose_30 (mmol/l)	7.57±1.15	7.21±1.76	8.49±1.77	-4.76	12.15	0.50	0.09
Glucose_45 (mmol/l)	8.29±1.84	7.95±2.54	8.25±2.52	-4.10	-0.48	0.66	0.95
Glucose_60 (mmol/l)	7.88±2.41	7.51±2.18	7.77±2.51	-4.70	-1.39	0.65	0.89
Glucose_90 (mmol/l)	6.52±1.62	6.01±1.68	6.65±1.80	-7.82	2.00	0.38	0.82
Glucose_120 (mmol/l)	5.83±1.25	5.76±1.25	6.25±1.53	-1.20	7.20	0.87	0.37

[a] % change in plasma glucose from baseline to after sucralose treatment; [b] % change in plasma glucose from baseline to after aspartame treatment; [c] differences between sucralose-sweetened beverage and baseline; [d] differences between aspartame-sweetened beverage and baseline; Values are means ± SD, * Linear mixed model with REML estimation.