EFFECTS OF PROBIOTIC SUPPLEMENTATION ON INDUCING REMISSION TO DRUG-FREE NORMOGLYCEMIA IN ADULTS WITH PREDIABETES – STUDY DESIGN.

by FARAH SULTAN, H.B.Sc., R.D

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CONTENTS

ABBREVIATIONS

7DD	7-day diary
2hrPG	2-hour post-prandial glucose
AE	Adverse events
BMI	Body mass index
CBT	Cognitive behaviour therapy
CRF	Case-report form
CVD	Cardiovascular disease
DPP	Diabetes Prevention Program
eCRF	Electronic case-report form
eGFR	Estimated glomerular filtration rate
FFQ	Food frequency questionnaire
FPG	Fasting plasma glucose
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide1
GOS	Galacto-oligosaccharides
GPAQ	Global physical activity questionnaire
HbA1c	Hemoglobin A1c
HDL	High density lipoprotein
HFD	High fat diet
HOMA-B	Homeostatic model of beta-cell function
HOMA-IR	Homeostatic model of insulin resistance
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IP	Intestinal permeability
IQR	Interquartile range
IS	Insulin secretion
IR	Insulin resistance

ITF	Inulin-type fructans
ITT	Intention-to-treat analysis
IVGTT	Intravenous glucose tolerance test
KG	Kilograms
LPS	Lipopolysaccharide
ME	Metabolic Endotoxemia
MVPA	Moderate and vigorous physical activity
NGT	Normal glucose tolerance
OGTT	Oral glucose tolerance test
O.R	Odds ratio
OTU	Operational taxonomic unit
PA	Physical activity
PHIPA	Personal Health Information Protection Act
PID	Participant identification
PYY	Peptide YY
RD	Registered dietitian
RCT	Randomized-controlled trial
SAE	Serious adverse event
SCFA	Short-chain fatty acids
TCPS	Tri-Council Policy Statement
TG	Triglycerides
T2D	Type 2 diabetes
WC	Waist circumference

ABSTRACT

BACKGROUND: Patients with prediabetes are at a high risk of developing type 2 diabetes (T2D) and the current strategies to prevent the progression of prediabetes to T2D are difficult to implement at the population level. Recently, the role of gut microbiota has emerged as a possible link to metabolic disease. The modulation of the gut microbiota in individuals with prediabetes through probiotic supplementation may improve metabolic dysfunction and induce remission of prediabetes to normoglycemia.

OBJECTIVES: The primary objective of this trial is to determine the effect of 900 billion CFU/day of VSL#3®, a multi-strain probiotic supplement for 20 weeks, on induction of drug-free remission to normoglycemia (HbA1c<6.0%) in adults with prediabetes compared to placebo 20 weeks post-randomization.

METHODS: In a randomized, triple-blind, controlled multi-centre trial, 568 adults with prediabetes will undergo a 2-week run-in after which they will be randomly allocated to 20 weeks of either 900 billion CFUs of VSL#3® per day or placebo. Prediabetes remission will be measured using HbA1C at week 20 and 32. Change in insulin resistance (HOMA-IR), beta-cell function (HOMA-B), weight, BMI, waist circumference and fecal relative abundance of bacteria will be measured from baseline at week 20 and 32. Exploratory regression analyses will involve a multiple logistic regression model to assess whether the change in relative abundance of the *Rosburia* genus from baseline at week 20 is an independent predictor of drug-free prediabetes remission at week 20.

DISCUSSION: Individuals with prediabetes are at high risk of developing T2D and the induction of prediabetes remission would be important to patients and clinicians. The role of microbiota in metabolic processes presents the potential for therapeutic applications of probiotics. If successful, probiotics would offer a therapeutic option for reversing prediabetes to normoglycemia that is simple, cheap and easy to incorporate into standard clinical care.

CHAPTER 1: BACKGROUND AND THEORETICAL FRAMEWORK CONTENT

1.1. TYPE 2 DIABETES

The International Diabetes Federation estimates that 8.8% of the global adult population has diabetes and this number is projected to increase to 10.4% in the next 20 years[1]. In 2015, 8.9% (3.3 million) of the Canadian adult population was affected by diabetes and this is expected to increase by 40% in the next 10 years[2].

Type 2 diabetes (T2D) is a complex metabolic disorder that is characterized by abnormal glucose control resulting from defective insulin secretion, defective insulin action or a combination of the two[3]. Chronic hyperglycemia is associated with long-term microvascular complications affecting the eyes, kidneys and nerves, as well as an increased risk for cardiovascular disease (CVD)[4]. The diagnostic criteria for diabetes are based on thresholds of glycemia that are associated with retinopathy[5]. Rates of microvascular complications are reduced in individuals who are treated with pharmacological interventions that lower blood glucose levels[4]. Canadian diabetes practice guidelines classify the diagnostic criteria of T2D as either a fasting plasma glucose (FPG) of $\geq$7.0 mmol/L, a 2-hour postprandial glucose (2hrPG) of $\geq$11.1 mmol/L (from a 75g oral glucose tolerance test),a random plasma glucose of $\geq$11.1 mmol/L or a glycated hemoglobin A1c (HbA1c) of $\geq$6.5%[6].

There are a variety of pharmacological therapies dedicated to diabetes, however glycemic control remains a challenging hurdle for many patients. Patients endure personal and financial burdens associated with polypharmacy, daily blood glucose testing, and complications of diabetes. Enormous burdens are also placed on health care systems. In 2010, diabetes-related costs in Canada were $11.7 billion and these costs are projected to exceed $16 billion by 2020 which would threaten the sustainability of the healthcare system[5]. Although this is daunting, a 2% decrease in prevalence rates would reduce direct healthcare costs by 9%[5]. Due to the non-relenting increase in T2D prevalence, the World Health Organization's Report on

Diabetes (2016) urges that research and treatment efforts focus on diabetes prevention[7]. It is especially imperative to focus on people at highest risk of developing diabetes, notably individuals with prediabetes.

Prediabetes is a term referring to impaired fasting glucose (IFG) defined as a FPG of 6.1-6.9 mmol/L, impaired glucose tolerance (IGT) defined as a 2hrPG of 7.8-11.0 mmol/L, or both[6]. A HbA1c of 6.0% to 6.4% is also indicative of prediabetes[6]. Prediabetes places individuals at high risk of developing diabetes and its complications especially if both IFG and IGT are present[3]. A recent meta-analysis revealed individuals with various presentations of dysglycemia have a 5-10 time greater likelihood of developing T2D within one year when compared to individuals with normal glucose tolerance (NGT), and that individuals with both IFG and IGT are approximately 12 times more likely to develop T2D within that time frame compared to those with NGT[8].

The annual incidence of progression of IGT to diabetes is approximately 4-6%, 6-9% for IFG and 15-19% for both IGT and IFG[8]. In 2015, the global prevalence of IGT was estimated to be approximately 7% of the population, ranging up to 14% in North America, while prediabetes prevalence was estimated to be as high as 15% in Canada[9]. Prediabetes is not simply a warning-system signaling that T2D is imminent, rather it has been associated with microvascular complications such as chronic kidney disease[10]. A recent meta-analysis also highlighted the positive association of prediabetes with coronary heart disease[11].

Metabolic syndrome is another common risk factor for T2D[6,12]. It is thought to develop from a pro-inflammatory state and indicates insulin resistance as a major underlying component[12]. Metabolic syndrome is characterized by the presence of at least three of the following metabolic abnormalities: i) abdominal obesity as indicated by waist circumference measurements ii) elevated triglycerides (TG) or drug treatment to reduce TG iii) reduced high-density lipoprotein (HDL) or drug treatment to reduce HDL iv) hypertension and v) IFG or drug treatment for hyperglycemia[12]. Individuals with metabolic syndrome are at a 5 time higher risk of

developing T2D[6,12]. Weight reduction and increased physical activity are the primary recommended interventions that address all 5 criteria of metabolic syndrome and may ultimately prevent the development of T2D[12].

1.1.1. Type 2 Diabetes Prevention

Substantial evidence exists that demonstrates the possible prevention or delay of the development of T2D. Strategies that have been proven effective mainly pertain to lifestyle modification and pharmacological interventions[1,13–28]. Results from the American Diabetes Prevention Program (DPP) trial showed that an intensive lifestyle intervention which involved weight loss and exercise reduced the incidence of T2D in those with IFG and IGT by 58% when compared to placebo, more than with metformin when compared to placebo (31%)[23]. Follow-up studies revealed that 10 years after the trial, diabetes incidence continued to be lower in participants from the lifestyle intervention group when compared to the metformin group and the placebo group[29]. The majority of successful lifestyle interventions require that participants either reduce caloric intake, achieve $\geq$150 minutes of moderate intensity physical activity per week or both. Most interventions had the goal of a 5 -7% reduction of baseline weight[23–28]. Current diabetes prevention recommendations are centered on findings of such trials and include two options. The first focuses on lifestyle modification and encourages a structured program that consists of regular physical activity and moderate weight loss (5-7% of body weight)[30]. The other recommendation is the initiation of metformin in individuals with IGT[30]. Although these recommendations have produced significant results within the confines of randomized trials, their implementation in the real world, specifically those involving lifestyle modifications, is challenging. To begin with, the trials that implemented these lifestyle changes did so using a comprehensive, structured program that involved guidance from a healthcare team. It is recognized that the best results are achieved when a multi-disciplinary team of physicians and allied health professionals such as dietitians, kinesiologists and behavioral therapists are

involved in helping individuals meet their weight loss and physical activity goals[31]. It is challenging for individuals to achieve weight loss and physical activity goals without some guidance or assistance from a professional. Indeed, the role of standard behavioral approaches and cognitive behaviour therapy (CBT) in weight loss and physical activity has become increasingly important. Studies show that participants who receive CBT along with dietary advice lose more weight and keep it off longer than those who received only dietary guidance[32,33]. Furthermore, the application of behavior therapy significantly increases the amount of physical activity of participants compared with usual care[34,35]. Implementing this type of program on a population level is difficult. What is required is a simple, cost-effective and accessible intervention that can target the underlying pathophysiology that contributes to the development of T2D.

1.1.2. Pathophysiology of Type 2 Diabetes

Type 2 diabetes is preceded by changes in metabolic processes such as insulin secretion and sensitivity, resulting in elevated fasting and post-prandial glucose levels[36]. These changes can begin up to 6 years or more prior to the development of T2D[36]. Abnormal beta-cell function and beta-cell loss is a key feature of T2D[37]. The pancreatic beta-cell is a vital component of glucose control as it is responsible for releasing regulatory hormones such as insulin in response to rising plasma glucose levels[38]. The secretion of insulin is stimulated by a group of hormones known as incretins[39]. Incretins such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are secreted by the small intestine and colon in response to the meal ingested[39]. In healthy individuals, incretins are estimated to account for as much as 70% of the insulin secreted in response to a meal[40]. Recent evidence determined the presence of incretin system abnormalities within the realm of diabetes. Individuals with T2D have reduced sensitivity to the insulinotropic effects of GIP for unknown reasons[41]. Apart from stimulating glucose-dependent insulin secretion, GLP-1 helps to preserve insulin stores by

activating glucose-stimulated insulin gene transcription and production as well as decreasing the secretion of glucagon by the pancreas[40]. Incretins, particularly GLP-1, have additional beneficial effects including delaying gastric emptying which slows the digestion of carbohydrates, slowing the rate of glucose absorption into the blood stream after meals[39]. In addition, GLP-1 suppresses appetite, energy intake and contributes to weight control in patients with T2D[42]. Evidence from animal studies has shown that GLP-1 may contribute to beta-cell regeneration and survival[43,44]. Moreover some evidence also suggests that GLP-1 suppresses hepatic glucose production and increases glycogen stores and glucose uptake by muscle as well as regulates fat metabolism in adipocytes[40]. The ensuing reduction in hyperglycemia results in diminished burden on the beta-cell which in turn relieves beta-cell stress and dysfunction[38]. The gut is believed to play a central role in the development of T2D, through mechanisms related to inflammation, hormone production, and intestinal permeability[45]. A bidirectional relationship exists between the gastrointestinal tract and diabetes. Diabetes affects gastrointestinal morphology and function and emerging data highlights that intestinal permeability (IP) may precede the development of T2D[45]. Increased IP may induce inflammatory responses which may contribute to the development of insulin resistance, impacting the uptake of glucose into target tissues[46]. Low-grade inflammation is a common feature of T2D and is characterized by a two to threefold increase in systemic plasma concentrations of cytokines[47]. An increase in cytokines such as interleukins and TNF-α is observed in T2D, both of which are known to induce insulin resistance[46]. Interestingly, it has been demonstrated that with insulin-resistance, there is translocation of intestinal bacteria to the adipose tissue and bloodstream, where the bacteria can then induce further inflammation[48]. This translocation can be reversed by treatment with probiotic bacterial strains, thereby improving overall inflammatory status[48]**.** The role of the gut bacteria in the development of metabolic disease has become increasingly evident over the past decade[49,50].

1.2. GUT MICROBIOTA

It is estimated that the human adult gut contains anywhere from 10 to 100 trillion microorganisms spanning more than 1000 different species[51]. The genetic material of the microbiota exceeds the magnitude of the human genome by over 100 times[52]. The combined genetic material of the microbiota is collectively known as the microbiome[52]. The microbiota and its microbiome provide the human host with additional gene products that are lacking in humans and these products serve a multitude of functions which contribute to overall homeostasis including gut-level defense against pathogens, immunity-related metabolites and signals, the development of intestinal microvilli and the synthesis of certain vitamins[52–54]. The vastness, complexity, and multiple functions of the microbiome has resulted in it often being regarded as an organ[52,53]. The microbiome is a diverse entity that begins to develop at the time of birth through colonization by the mother and environment[55]. Within a number of years, the microbiome establishes itself and remains relatively stable in healthy adults[51,55]. Healthy individuals have a microbiome unique to themselves, shaped by their genotype, diet, age and sex[52]. External stressors such as dietary changes, antibiotics and bacterial infections can temporarily influence the composition of the microbiome[51,55]. Diet-induced changes in gut microbiota have been found to occur within a relatively short time frame of three to four days, however these changes are easily reversed if the diet is not maintained, depending on the host's initial microbiome composition[56]. Although there is marked inter-individual diversity in microbial composition in humans, most gut bacteria belong to one of three dominant microbial phyla: *Firmicutes*, *Bacteroidetes* or *Proteobacteria*[57–59]. One of the essential functions of each of these phyla include the production of short-chain fatty acids (SCFAs)[52]. The production of SCFAs is one of the many ways microbiota contribute to human health. Other known functions include digestive and metabolic processes, maintenance of epithelial integrity and anti-oxidant production[53]. There are a number of proposed pathways through which microbiota are

implicated in the development of metabolic disorders including T2D which will be described below.

1.2.1. Microbial Metabolites and Host Metabolism

Intestinal microbes ferment dietary carbohydrates that humans are not capable of digesting such as oligo- or polysaccarhides[6,7]. The fermentation process results in the production of SCFAs, most dominantly acetate, butyrate and propionate[60,61]. Propionate can be used as a source of energy for the host and is involved in *de novo* glucose and lipid synthesis[62]. SCFAs are involved in energy homeostasis and are able to modify the levels of several gut peptides involved in glucose metabolism and gut barrier function[63]. Studies have suggested that SCFAs may influence metabolism via G protein-coupled receptors[62,64]. The binding of SCFAs to these receptors increases plasma levels of GLP-1 and of a hormone related to satiety called peptide YY (PYY)[62,64]. PYY also reinforces insulin action in muscle and adipose tissue, leading to improved glucose homeostasis and decreased appetite[62,64]. Moreover, the production of indole, a microbiota metabolite produced from tryptophan may also contribute to the secretion of GLP-1 by intestinal enteroendocrine cells[65]. Gut microbes also produce secondary bile acids such as lithocholic and deoxycholic acids which activate a G protein-coupled receptor that has been associated with improved glucose tolerance by regulating GLP-1 production in mice[66]. The effects of some SCFAs are also mediated via gene expression. For instance in animals, butyrate and propionate activate the expression of genes that are involved in intestinal gluconeogenesis[67]. The resulting release of glucose into the portal vein contributes to a reduction in hepatic gluconeogenesis and overall regulation of glycemia[67].

1.2.2. Gut Permeability and Metabolic Endotoxemia

Lipopolysaccharide (LPS), a known as a bacterial endotoxin, is a glycolipid molecule that is found in the walls of gram negative bacteria and is released into the gut during bacterial lysis[68]. **‘**Leaky gut’ or increased gut permeability results in transportation of LPS through the intestinal epithelium which in turn results in high levels of circulating LPS, a state termed

metabolic endotoxemia (ME). LPS then reaches target tissues where it binds to receptors on immune cells, triggering an inflammatory response and potentially leading to chronic inflammation and ultimately insulin resistance[48, 68]. High levels of LPS have been associated with high-fat diets (HFDs) and HFDs have been shown to increase intestinal permeability, promoting intestinal translocation of LPS through the intestinal wall[69,70]. It was demonstrated that changes in the gut microbiota are responsible for endotoxemia and the resulting inflammatory response in HFD-fed mice[69]. Studies have found that HFDs induce changes in the gut microbiome, favoring the colonization of gram negative bacteria, and that obese individuals present with increased variety of LPS-producing bacteria[71]. Furthermore LPS levels have been found to be strongly correlated with metabolic syndrome features and a significant increase in LPS was found in individuals with T2D and in those with impaired glucose tolerance[68,72,73]. LPS levels in non-diabetic individuals have been found to be significantly associated with fasting insulin even when age, sex and BMI were controlled for[72]. This further suggests that LPS may act as a gut microbiota-related factor involved in the development of T2D and obesity in humans.

Colonization of the intestine with *Bifidobacteria* spp, a gram positive microbe, has been shown to reduce gut endotoxemia in mice[74]. A number of studies have demonstrated that an increase in *Bifidobacteria* spp colonization was associated with an increase in GLP-1 and GLP-2 which are involved in modulation of IP[75,76]. Higher endogenous GLP-2 was found to improve gut barrier function, leading to reduced LPS translocation and a reduced inflammatory response[77]. SCFAs also play a role in gut barrier function. For instance, butyrate has trophic effects on the mucosa as well as anti-inflammatory properties[46,78]. These findings strongly suggest that microbiota-derived products play a role in metabolic functions and homeostasis.

1.3. MICROBIOTA AND METABOLIC DISEASE

Recent evidence revealed that alterations in the microbiome (dysbiosis) are associated with diseases such as metabolic syndrome, obesity and diabetes[50,62,79,80]. Metagenomics data revealed that people with T2D exhibit a moderate degree of microbial dysbiosis. It has also been reported that adults with T2D have significantly lower counts of *Firmicutes* and a higher proportion of gram-negative bacteria belonging to the phyla *Bacteroidetes* and *Proteobacteria*[80]. The same study also found that the ratio of *Bacteroidetes* to *Firmicutes* was significantly and positively correlated with decreasing glucose tolerance[80]. These findings are in agreement with evidence of microbiome alterations in overweight and obese individuals[62]. The *Lactobacillus* group, specifically *L. gasseri*, was also significantly higher in the diabetes population and was positively correlated with fasting plasma glucose and HbA1c levels[79, 80]. *L. gasseri* has been proven to have pro-inflammatory properties which may be contributing to the underlying chronic inflammation in diabetes[81]. *Clostridium clostridioforme* (representing three opportunistic pathogens) was significantly higher in T2D whereas *Roseburia*, a butyrate-producing bacteria, was significantly diminished[79,82]. Interestingly, fecal transplants from lean donors to recipients with metabolic syndrome resulted in an increase in *Roseburia* and butyrate levels, as well as improved insulin sensitivity[83]. A summary of the changes in microbial profiles with the development of T2D is provided in Appendix 1.

Metagenomics studies such as the ones mentioned above spurred observational studies that aimed to assess whether microbial genetic markers from blood samples can be used to predict or assess risk of metabolic disease development. The blood of non-obese, non-diabetic participants was tested for 16S rDNA (a bacterial gene and marker of the overall microbiota) at baseline and again after 9 years[84]. The 16S rDNA concentration was higher in individuals who developed diabetes and was a significant predictor of the onset of diabetes after adjustments were made for sex, baseline age, family history of T2D, hypertension, waist circumference, BMI, smoking status and FPG[84]. The 16S rDNA was sequenced to identify the phyla of bacteria

present and the results revealed that 80-90% of the phyla was that of *Proteobacteria*, a major group of bacteria that includes a wide variety of pathogens[84,85]. These findings further support the association between compositional and functional alterations in the gut microbiome and the development of T2D.

1.4. DIETARY MODULATION OF GUT MICROBIOTA AND METABOLIC CONSEQUENCES

Modification of the gut microbiome composition via dietary or pharmacological interventions may confer beneficial changes to the host's metabolism. However, there is uncertainty in whether these changes depend on the host's pre-existing gut microbiome composition, the host's phenotype, or the mode and combination of bacterial strains administered[52]. Furthermore, the mechanisms that are implicated in providing metabolic benefits remain unclear[52]. Although animal studies may lend to this knowledge, extending observations from such trials to humans may not be entirely appropriate[52]. Some small randomized control trials using dietary interventions to manipulate gut microbiota composition have been able to link the intervention to beneficial phenotypic changes, however, most studies did not assess simultaneous quantitative and qualitative changes in gut microbiota[59].

One of the ways that the gut microbiota may be altered is through probiotics. The joint report by the Food and Agriculture Organization and the World Health Organization (2001) accepts the definition of probiotics as "*live micro-organisms, which when consumed in adequate amounts, confer a health effect on the host"* [86]*.* More recent definitions have been expanded to specify that the health benefits are a result of changes in the gut microbiota that are both transient and that diminish gradually with time after cessation of consumption[87]. There are certain characteristics which must be met when classifying a bacterial strain as a probiotic: i) the strain must reach the target site of action alive and must survive the physiological stressors encountered from the point of ingestion, stomach acid and biliary salts; ii) its beneficial effect in the host must be evident iii) it must not pose any risk for the host and iv) it must remain viable

during manufacturing and incorporation into an ingestible form[86]. Once these conditions have been met, the probiotic strain is classified by the genus, species and an alphanumeric designation[54].

Other methods of altering the microbiome that have been trialed include prebiotics. Prebiotics are fermentable dietary components that result in selective changes in the composition and/or activity of the gut microbiota which confer benefits to the host[88]. The majority of experimental and clinical trials on prebiotic effects have been conducted using ingredients from two chemical groups: inulin-type fructans (ITF) and the galacto-oligosaccharides (GOS)[88]. These have been demonstrated to have the capacity to *selectively* stimulate the growth of *bifidobacteria* and in some cases *lactobacilli*, which leads to a significant change in gut microbiota composition and a reduction of the metabolic activity of potentially harmful bacteria[88]. The main substrates for bacterial growth are dietary non-digestible carbohydrates that elude upper intestinal hydrolysis and absorption[89]. Non-digestible carbohydrates are comprised of resistant starch and resistant dextrins, non-starch polysaccarhides (such as pectins, arabinogalactans, gum Arabic, guar gum and hemicellulose), non-digestible oligosaccharides (such as raffinose, stachyose, ITF, galactans and mannans) as well as undigested portions of disaccharides (e.g. lactose) and sugar alcohols (such as lactitol and isomalt)[89].

The amalgamation of probiotics with prebiotics is known as synbiotics and is administered so that the selectivity of the prebiotic favors the combined probiotic[90]. The synergistic combination of the pre and probiotic together is thought to enhance the efficacy of the individual components[90]. However, the selection of the appropriate prebiotic to combine with the probiotic is a critical process in the formation of the synbiotic product[90]. The prebiotic choice should improve the probiotic's survival and implantation in the gut by selectively stimulating and/or activating metabolic functions of one or more bacterial strains[91]. Prebiotics, whether used alone or in combination with probiotics have been associated with abdominal discomfort, specifically bloating, distention and significant amounts of flatulence[92].

1.5. PROBIOTICS AND GLYCEMIC OUTCOMES

A number of clinical trials have tested the impact of probiotics on various glycemic measures such as plasma glucose, HbA1c, plasma insulin and insulin resistance in healthy individuals, those with metabolic syndrome, pre-diabetes and T2D[93–98]. These trials yielded inconsistent results. A trial that administered *L. acidophilus* La-5 and *B. animalis subsp. lactis* BB-12 to 45 patients with T2D resulted in a statistically significant difference in HbA1c between groups, with the probiotic group having an average HbA1c that was lower by 0.78% compared to the placebo group ($p=0.02$)[99]. Another trial using the same strains in individuals with T2D observed a significant decrease in FPG by an average of 0.70 mmol/L in the probiotic group compared to the control group ($p<0.05$)[94]. HbA1c levels post-treatment were also significantly different between the groups with the placebo group having an HbA1c that was on average higher by 0.42% ($p<0.05$)[94]. In a study that had males with NGT, IGT and T2D, a 4 week treatment of *L .acidophilus* NCFM preserved insulin sensitivity compared to placebo[100].

A meta-analysis of 11 trials that included populations with T2D, prediabetes and NGT concluded that probiotics are effective in reducing FPG, HbA1c and insulin resistance in those with T2D[101]. However, only one of the 11 trials included participants with prediabetes. Very few trials exist that have studied the efficacy of probiotics in participants with prediabetes or metabolic syndrome. A trial that tested the effect of *Lactobacillus gasseri* BNR17 capsules on glycemic outcomes in overweight and obese adults with FPG$\geq$5.5 mmol/L did not find significant differences between the probiotic and placebo group in FPG, HbA1c, or plasma insulin after 12 weeks, however, the study was powered to detect changes in body weight, not glycemic outcomes[102]. Another trial tested the effect of kefir containing lactic acid bacteria in participants with prediabetes and T2D and compared glycemic outcomes to a control group on a 'standard diet'[98]. The analysis was not stratified based on diabetes status but the results demonstrated a significant reduction in FPG in the probiotic group compared to the control group (-1.89 mmol/L vs.-0.16 mmol/L respectively, $p=0.015$) [98]. The probiotic group also showed a significant

reduction in HbA1c compared to the control group (-0.13% vs. 0.001% respectively, p=0.001). These results are to be interpreted with caution as there are evident methodological limitations that were not addressed by the authors. These included lack of randomization, blinding and a description of the control group's 'standard diet'. Furthermore, statistical analysis methods are not presented in the report[98]. A pilot trial testing the impact of *Lactobacillus casei Shirota* supplements on inflammatory markers in participants with metabolic syndrome found no significant changes in insulin resistance[103]. However, the authors highlight a number of limitations of the trial including the fact that the study is a pilot and is thus likely underpowered. They also questioned whether the probiotic concentration (10^8 CFU/ml) and the intervention duration (12 weeks) were adequate, and address the lack of placebo for the control group[103]. Additional trials have been summarized in Appendix 2.

Trials investigating the effect of prebiotics on glucose homeostasis have also generated inconsistent results. The administration of 20g of short-chain fructans for 4 weeks in a healthy population resulted in a decrease in hepatic glucose production but had no effect on insulin-stimulated glucose metabolism[104]. When the same intervention was tested in individuals with T2D, no effect was observed on glucose homeostasis[105]. However, another study in healthy subjects found that prebiotic administration with the evening meal improved glucose response after the breakfast meal the following day, increased GLP-1 production and reduced inflammatory cytokines[106]. Similarly, supplementation with inulin-type fructans in hypercholesterolemic participants increased GLP-1 production and decreased 2hrPG response after the breakfast meal while another trial in a similar population observed a decrease in 2-hour post-prandial insulin response[64,107]. A systematic review and meta-analysis of the effects of prebiotics on glycemic indices found that prebiotics did not reduce fasting glucose or insulin, however, only 2 of the 8 trials included participants with diabetes. The remaining trials had a heterogeneous population of individuals with excess weight, obesity, or obesity with other comorbidities[108]. The same review found that synbiotics significantly reduced fasting insulin in

obese individuals with non-alcoholic fatty liver disease based on results from 2 trials, however, no significant reduction in FPG was seen in those trials as well as 2 others that involved individuals with diabetes[108].

The inconsistencies in findings of all these trials are likely due to the heterogeneity of the probiotic strains, prebiotic choice, intervention form, duration and study population. Most trials were relatively small in size (less than 50 participants), short in duration (30 days to 12 weeks), and were not powered to detect changes in glycemic outcomes. Probiotic viability was seldom assessed in most trials and only a select few analyzed fecal samples for changes in bacterial gene markers.

Large, well designed, long- term trials are needed to accurately assess the impact of probiotics on glycemic control and their potential role in preventing the progression of prediabetes to T2D. An ideal design would be a multi-centre randomized placebo-controlled trial that implements blinding of participants, research staff and trial investigators. This proposed trial aims to investigate whether the administration of probiotics improves metabolic homeostasis in individuals with prediabetes, resulting in remission to normoglycemia. Careful consideration of the literature was undertaken when selecting our trial intervention. The intervention strain, form and duration are described in detail below.

1.6. TRIAL INTERVENTION

1.6.1. Probiotic Strain Selection

One of the biggest challenges with probiotic research and use is the selection of an appropriate strain(s). The reason being is the fact that there are many different strains of microorganisms with diverse and overlapping functions. Although some strains have been associated with certain health benefits, the mechanisms involved remain uncertain. Furthermore, the use of one versus a combination of multiple strains is another important point

for consideration. A subgroup analysis of a systematic review of the effect of probiotics on glycemic indices revealed that interventions with multiple species of probiotics resulted in a more pronounced reduction in FPG compared to single species[109]. The analysis of another systematic review also demonstrated that mixed strains resulted in significant reductions in HbA1c and insulin in comparison to single strains[101]. VSL#3® is a multi-species probiotic product that has been mostly used and tested in gastrointestinal disorders, however its promising results are more recently being investigated in other conditions including metabolic diseases. In a study with HFD-fed mice, 8 weeks of VSL#3® resulted in a significant reduction in FPG, 2hrPG and suppressed hyperinsulinemia[110]. Furthermore, VSL#3® improved metabolic function by reducing plasma insulin, triglycerides, free fatty acids, and increased levels of adiponectin (a protein involved in glucose regulation)[110]. It also improved the inflammatory response and hepatic steatosis compared to placebo[110]. Further findings indicated that VSL#3® protected against HFD-induced diabetes and obesity, reduced food intake and reversed obesity and diabetes in diet-induced obese mice and leptin-deficient mice[110]. These results are believed to have been elicited by the significant increase in butyrate and GLP-1 which were positively correlated in the VSL#3® group[110]. Another study in a mouse model of dyslipidemia and intestinal inflammation demonstrated significant improvements in plasma insulin, insulin signaling and insulin resistance[111]. VSL#3® also resulted in improved epithelial integrity and a reduction in mucosal and adipose levels of inflammatory markers such as TNFα[111].

A clinical trial testing the effect of 6 weeks of VSL#3® supplementation on inflammatory and glycemic indices in obese adults found significant reductions in FPG, insulin and insulin resistance when compared to placebo[112]. Interestingly, fecal analysis of insulin resistant individuals at baseline revealed lower *Lactobacilli, Bifidobacteria, Streptococcus* and higher *E. coli and Bacteroides*[10]. Following probiotic treatment, fecal microbial profiles demonstrated a significant increase in concentrations of *Lactobacillus*, *Bifidobacteria*, and *Streptococcus*[10].

VSL#3® was also tested in women with gestational diabetes and after 8 weeks of supplementation, plasma insulin, insulin resistance and inflammatory cytokines (IL-6, hs-CRP and TNF) were signficiantly lower in the probiotic group compared to the control group[113]. These results along with others make VSL#3® a promising agent that may induce microbial and metabolic changes and that can ultimately alter glucose homeostasis. Its standardized production ensures quality control and its ease of use make it an optimal choice for our study intervention.

1.6.2. Probiotic Form and Concentration

The most common form of probiotics are dairy products, fermented foods and capsule supplements. The fermented foods include fermented milk (e.g. yogurt, buttermilk, kefir), fermented (pickled) vegetables (e.g. sauerkraut, cabbage kimchee, pickled ginger), fermented bean paste (e.g. miso, tempeh) and other fermented foods and beverages[86]. Other forms include capsules and sachets containing freeze-dried probiotics[54]. The role of the vehicle/filler substances used to deliver the probiotic must be considered since some effects may not be reproduced using different vehicles or fillers due to reduced viability of the strain[54]. Subgroup analysis from two systematic reviews of the effect of probiotics on glycemic measures showed that probiotics in the form of supplements resulted in a greater reduction in FPG and insulin compared to fermented milk and yogurt[109,101]. This is likely due to the fact that not all organisms survive in milk products or withstand a long shelf-life[114].

The concentration or dose of probiotic varies greatly depending on the strain and product[54]. Results of a systematic review of trials testing different probiotics in adults with diabetes showed that a daily dose of probiotic supplementation of ≥10 billion CFU/day resulted in a greater reduction in FPG compared to doses that were <10 billion CFU/day; however, the reduction observed was not statistically significant[109]. A small 4-week clinical trial testing 900 billion CFU/day of VSL#3® in adults on a high-fat diet found no statistically significant change in

FPG, fasting insulin, or insulin sensitivity compared to the control group[115].

VSL#3® is available in sachets, each of which contains 450 billion CFU's[116]. Although the maximum daily number of sachets is not specified, 1 to 2 sachets per day is suggested by the manufacturers[116]. We chose 2 sachets (900 billion CFU/day) for our intervention to minimize the risk of missing an effect due to inadequate probiotic dose.

This trial will investigate the effects of a commercially available multi-strain, high-concentration probiotic on induction of normoglycemia in adults diagnosed with prediabetes. The subsequent chapters will present in detail the trial protocol, methodological considerations, and the associated strengths and limitations of the proposed study. Implications of potential study findings and future research directions are also discussed in the final chapter.

CHAPTER 2: STUDY PROTOCOL

2.1. RESEARCH QUESTION, HYPOTHESIS AND STUDY OBJECTIVES

2.1.1. Primary Research Question

In adults with prediabetes, will 900 billion CFU/day of VSL#3® probiotic supplementation for 20 weeks result in a significantly higher proportion of remission to drug-free normoglycemia as defined by a HbA1c<6.0% 20 weeks after randomization in comparison to those taking placebo?

2.1.2. Hypothesis

We hypothesize that 900 billion CFU/day of VSL#3® probiotic supplementation for 20 weeks will result in a higher proportion of patients undergoing prediabetes remission to normoglycemia compared to placebo 20 weeks post-randomization.

2.1.3. Primary Objective

The primary objective of this trial is to determine the effect of 900 billion CFU/day of VSL#3® supplementation for 20 weeks on induction of drug-free remission to normoglycemia (HbA1c<6.0%) in adults with prediabetes compared to placebo.

2.1.4. Secondary Objectives

The secondary objectives are to:

i) assess the sustainability of drug-free prediabetes remission in all participants12 weeks after discontinuation of the study intervention, at week 32;

ii) assess the effects of VSL#3® on the proportion of individuals who progress to T2D at any point in time throughout the trial, at week 20 and 32;

iii) determine the effects of VSL#3® on insulin resistance and beta-cell function as denoted by HOMA-IR and HOMA-B respectively in all participants at week 20 and 32;

iv) assess the effects of VSL#3® on anthropometric indices such as weight, waist circumference and BMI in all participants at week 20 and 32;

v) assess the effects of VSL#3® on gut microbiota via fecal analysis and to investigate whether any changes from baseline at week 20 in relative abundance of bacterial and archaeal phyla, classes and genera may be associated with changes in glucose homeostasis. Fecal analysis will be exploratory in nature and hypothesis-generating;

vi) assess the safety and tolerability of this particular probiotic supplement.

2.2. RECRUITMENT, RANDOMIZATION AND BLINDING

This study will be a multi-centre, parallel-group, two-arm, triple-blinded randomized placebo-controlled trial with a 1:1 allocation ratio and a 2-week run-in period, testing the effect of probiotic supplementation on inducing drug-free normoglycemia in adults with prediabetes.

2.2.1. Recruitment

A total of 568 participants will be recruited from 19 sites consisting of outpatient endocrinology clinics, diabetes education centres, and primary care clinics across Canada. We anticipate a recruitment rate of 1 participant per month from each site over a period of 30 months. Additional study sites may be added if recruitment targets are not being met. The trial will be advertised through posters in waiting rooms as well as through various outlets such as *Diabetes Canada* (formally Canadian Diabetes Association). Potentially eligible subjects will be approached and introduced to the study by the research nurse or coordinator after the patient's permission is obtained from their diabetes care provider. Patients can also self-refer by contacting the study coordinator directly. If a patient is interested in participating they must provide voluntary written consent prior to being screened for eligibility.

2.2.2. Randomization and Allocation Concealment

Eligible participants who have provided voluntary and informed consent will be randomized to either the treatment group or the control group in a fixed 1:1 ratio. The randomization schedule will be prepared by an independent statistician and will be stratified by study centre. To avoid an imbalance in the number of participants assigned to each group, random block sizes will be used when creating a randomization schedule for each study centre. Group allocation will be done using an online computer program that utilizes a randomization algorithm and provides a treatment assignment number which will be linked to the pre-packaged sachets. The assignment numbers will be four random digits that will prevent identification of the contents. The probiotic supplier will number the sachet packages according to the randomization schedule prepared by the statistician. The package numbering will be performed distant from any enrolling site.

Randomization will take place during the last study visit of the 2-week run-in period or within a 1-week window after the run-in period is completed to avoid subject withdrawals or change in underlying disease status. Participants incorrectly enrolled and randomized in the study will be followed until the end of the study and they will remain in the group they were randomized to.

2.2.3. Blinding

Study staff who will be enrolling participants and collecting the data (coordinators and assistants), the investigators, the study statistician, and the participants will be blinded to treatment group allocation. Code breaking will be done if necessary for participants who may present, for example, with an acute gastrointestinal illness and require management in hospital.

2.3. ELIGIBILITY CRITERIA

Eligible participants will include adults between the ages of 18-70 diagnosed with prediabetes within the previous 5 years as defined by the *Diabetes Canada guidelines*[3] (Table

1). The diagnosis will be confirmed at screening using an HbA1C blood test.

Individuals on any pharmacological treatment for prediabetes will not be eligible to participate. Patients who have type 1 or type 2 diabetes and those with a history of anti-hyperglycemic agent use within 3 months prior to screening will not be eligible for participation. Individuals with conditions that may influence glucose metabolism such as: hepatic disease, pancreatic disease such as current acute or chronic pancreatitis, and pancreatic cancer are excluded from participation in the study. The use of medications that may cause glucose intolerance such as chronic steroids will deem an individual as ineligible. Individuals with significant and chronic gastrointestinal disease (e.g., irritable bowel syndrome, inflammatory bowel disease, ulcerative colitis and celiac disease) will be excluded[117].

Additional exclusion criteria include acute thyroid disease, a history of or planned bariatric surgery within the next 8 months and use of antibiotics or immunosuppressive medication within 30 days prior to randomization.

Frequent antibiotic use, defined as the completion of more than 3 courses of antibiotics within the past 12 months, will make an individual ineligible for participation.

Individuals who are had been using probiotics, prebiotics or synbiotics within 3 months prior to screening will be asked to discontinue their use for 3 months then return for screening.

Disorders that may significantly influence HbA1c levels such as severe renal disease (as defined by a an estimated glomerular filtration rate (eGFR) of <30 mL/min/1.73m^2 (measured using the "Modification of Diet in Renal Disease" equation[118]), or history of iron deficiency anemia and hemoglobinopathies will make individuals ineligible to participate.

Probiotics pose safety risks in immunocompromised individuals as well as in women who are pregnant or breast feeding[119]. Therefore such individuals including women who are planning to become pregnant within the next 8 months will not be eligible to participate.

Individuals with current or a history of alcohol abuse (>14 standard drinks/week for men, >7 standard drinks/week for women[120]) or drug abuse will not be eligible to participate. Anyone

with a disease that is expected to reduce life expectancy to less than 3 years will be excluded from participation in the study.

All potentially eligible individuals must be willing and able to give voluntary informed consent. Inclusion and exclusion criteria are summarized in Table 2.

Table 1: Diabetes Canada Prediabetes Diagnostic Criteria[6]

Test	Result	Prediabetes category
FPG (mmol/L)	6.1–6.9	IFG
2hPG in a 75 g OGTT (mmol/L)	7.8–11.0	IGT
HbA1C (%)	6.0–6.4	Prediabetes

2hPG, 2-hour plasma glucose; *A1C*, glycated hemoglobin; *FPG*, fasting plasma glucose; *IFG*, impaired fasting glucose; *IGT*, impaired glucose tolerance; *OGTT*, oral glucose tolerance test.

Table 2: Inclusion and Exclusion Criteria

Inclusion Criteria:
- Prediabetes as defined by a HbA1c of 6.0-6.4% and diagnosed within the previous 5 years - 18-70 years of age - Males/Females - All ethnicities - Willing and able to give voluntary informed consent
Exclusion Criteria
- Use of anti-hyperglycemic medication within the past 3 months - Currently have type 1 or type 2 diabetes - Use of any medication that may influence glucose tolerance (e.g. chronic steroids) - Chronic hepatic disease - Pancreatic disease (e.g. acute or chronic pancreatitis and current or history of pancreatic cancer) - Significant and chronic gastrointestinal disease (e.g. irritable bowel syndrome, inflammatory bowel disease, ulcerative colitis and celiac disease) - History of or planned bariatric surgery within the next 8 months - Acute thyroid disease - Use of probiotics, prebiotics or synbiotics within 3 months prior to screening - Antibiotic use within 30 days prior to randomization - Completion of > 3 courses of antibiotics within the past 12 months - Immunosuppressive medication use within the 30 days prior to randomization - Immunocompromised individuals - Severe renal disease as determined by an eGFR<30 mL/min/1.73m^2 [118] - History of iron deficiency anemia or hemoglobinopathy

- Women who are pregnant, breastfeeding or planning to become pregnant within the next 8 months
- Current or history of alcohol abuse (> 14 standard drinks/week for men, >7 standard drinks/week for women[120]) or drug abuse
- Any disease expected to reduce life expectancy to < 3 years

2.4. INTERVENTION

2.4.1. Probiotic Intervention

VSL#3® is a commonly used commercial mixture of 8 live freeze-dried bacterial strains in high concentrations of 300 billion CFU/gram or 450 billion CFU/dose (*Lactobacillus plantarum, Lactobacillus delbrueckii* subsp. *Bulgaricus, Lactobacillus casei, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis* and *Streptococcus salivarius* subsp. *thermophilus)*[116]. It provides the highest commercially available concentration of bacteria per dose[116]. VSL#3® is manufactured by Ferring Pharmaceuticals and is produced in a standardized manner, ensuring consistency of product quality.

2.4.2. Probiotic Form and Concentration

The probiotic intervention in this trial will consist of 2 VSL#3® sachets/day which is equivalent to 900 billion CFU/day. The sachets are available in a lemon flavour or unflavoured. For the purpose of this trial, the unflavoured option would be most appropriate in order to be indistinguishable from the placebo intervention. The VSL#3® sachets will be delivered to research sites by the manufacturer. All sites are to store the study sachets (VSL#3® and placebo) in their research pharmacy or clinic refrigerator. Refrigerator temperature maintenance and calibration logs must be completed weekly by all sites. Participants will be instructed to store the product at a temperature between 2 to 8ºC to maintain product viability, even though the probiotic can be stored at room temperature (25ºC or less) for a maximum of 7 days[1]. The VSL#3® is to be mixed in cold liquids such as water or milk, and not hot liquids as that can

impact bacterial viability[1]. Participants will be instructed to mix the probiotic powder daily in water prior to consumption.

2.4.3. Intervention Duration

We considered a number of factors when deciding upon the trial duration. One such factor is the amount of time that may be required for changes in the gut microbiota population to take place. Previous trials indicate a *minimum* of 20 days may be required to elicit changes in fecal microflora[7,8]. Another factor we considered is our primary outcome which is measured using HbA1c, a measure of glucose control over the previous 2-3 months[6]. The probiotics may also modulate gut permeability and consequently reduce metabolic endotoxemia, reducing insulin resistance. There may be other mechanisms through which the probiotics might exert their effects. We wanted to allow for adequate time for these potential alterations to take place and to be accurately captured. Therefore we selected an intervention period of 20 weeks (5 months). Participants' glycemic status will be reassessed 12 weeks after completing the intervention. The 12-week follow-up period will allow for any lasting changes in glycemic homeostasis to be adequately reflected in an HbA1c test. Assessing glycemic status 12 weeks after discontinuation of the probiotics will shed light on whether or not their glycemic effects last beyond their consumption.

2.5. CONTROL GROUP

The VSL#3® probiotic intervention group will be compared against a control group which will be administered placebo sachets provided by the probiotic manufacturer. The probiotic and placebo sachets will look and taste identical to each other in order to ensure blinding of participants and research staff. All participants will be given the same ingestion and storage instructions.

2.6. ADHERENCE

Participant adherence to the study sachets will initially be assessed during a 2-week run-in period when all participants will be requested to consume the placebo sachets. The participants will not be told that the sachets are placebo. The run-in will help identify participants who may demonstrate low adherence and highlight which individuals may need some adherence support and aids during the post-randomization period. Participants who exhibit an average of <80% adherence to the placebo sachets during run-in will be excluded from participating in the remainder of the trial[123]. During run-in and post-randomization, all participants will be instructed to take the study sachets once daily with water at approximately the same time each day, with or without food. If the once-daily dose is missed, participants will be instructed to take the next dose as scheduled. Once randomized to a treatment group, participants will remain on the same intervention and placebo regimen for 20 weeks. The probiotics and placebo sachets will be dispensed to the participants at the follow-up visits that are scheduled at 4, 8, 12 and 16 weeks after randomization (see Appendix 3 for visit schedule) and participants will be asked to return their used and unused sachets at their next follow-up visit during which the importance of adherence will be reinforced. Returned sachets will be counted at each visit, and adherence will be calculated as the percentage of used doses compared to the expected number. Research staff will also contact participants by phone between study visits to assess adherence and reinforce its importance as well as to inquire about adverse events. Participants are defined as non-adherent if they have taken less than 80% of the study product on average throughout the trial[124]. A record of the date, the visit and the amount of study product dispensed and returned will be documented for each participant.

2.7. STUDY TIMELINE

2.7.1. Screening, run-in and randomization

Potential participants will be recruited through local advertising and from diabetes clinics and education centres. Patients within each study site will be pre-screened by the site's research nurse or coordinator. The research coordinator will explain the study as well as the randomization and concealed allocation to the patients. If patients are interested in participating they must provide signed consent.

A CRF will be completed by the research coordinator to ensure potential participants meet all inclusion criteria prior to randomization. The CRF will collect information regarding participants' medical history, medication use and lifestyle, including alcohol intake and smoking habits. Screening will involve an HbA1c blood test to confirm prediabetes status. We will also collect serum creatinine in order to measure eGFR[118]. Women of child-bearing potential will be requested to provide a urine sample to rule out pregnancy.

Participants will be given instructions during screening on completion of a 3-day food diary to complete during the period of time (approximately a week) while their eligibility is confirmed. The 3-day food diary is to cover 2 week-days and 1 day of the weekend. If they are eligible to participate in the trial they will return to complete a two-week run-in period. They will be asked to provide the completed food diary at their initial run-in visit which will include a session with a registered dietitian (RD) who will use the food diary to assess their intake and will provide teaching on healthy eating. This will be done to standardize participants' dietary intake as diet may interact with the gut microbiota[56]. They will also be provided with a list of probiotic and prebiotic-containing and fermented foods to avoid during the run-in period and throughout the remainder of the trial. The participants will meet with the RD midway through the run-in period to receive additional dietary teaching and guidance. They will also meet with the RD during the final visit of the run-in period for which they will complete a 3-day food diary. Participants will be encouraged to continue with healthy eating habits throughout the remainder

of the trial. They will also be asked to maintain their current physical activity levels throughout the trial as increases in moderate and vigorous-intensity physical activity may influence the microbiome[125–127].

As outlined earlier, the run-in will also involve administration of placebo sachets and adherence to the study sachets will be assessed. Participants demonstrating an average adherence of $\geq$80% will move on to randomization. The final run-in visit will also serve as the randomization visit to minimize participant burden however a one-week window after the last run-in visit will be allowed for randomization if needed.

Participants will be randomized into the probiotic group or the placebo group and their assigned treatment will be dispensed by the research pharmacy. Participants will be provided with instructions on how to take and store the sachets and will be reminded to maintain a healthy diet intake and consistent physical activity levels from the run-in period throughout the entire study period. The Global Physical Activity Questionnaire (GPAQ) will also be administered to participants during the randomization visit to capture baseline physical activity levels. Although it is unlikely that physical activity levels will differ dramatically between study groups, we are capturing average minutes of moderate and vigorous-intensity physical activity per day over the course of the trial as this will be required for our regression analysis.

During the randomization visit anthropometric measurements such as weight, height, and waist circumference (WC) will be measured. A fasting plasma glucose and insulin sample will be collected for the assessment of insulin resistance and beta-cell function using the homeostatic model assessment of insulin resistance (HOMA-IR) index and homeostatic model of beta-cell function (HOMA-B) index respectively. A stool sample will also be collected, however, if participants are unable to provide a stool sample during their visit, they will be given a stool sample collection kit to take home. The kit will allow participants to safely store their sample in the freezer for a maximum of three days after which the sample can either be dropped off at the study site or to a LifeLabs for shipment to the study site. All samples will be

frozen in a -80°C freezer in the study site until they are analyzed. A breakdown of study visits and assessments can be found in Appendix 3.

2.7.2. Follow-up Visits

Participants will return to the clinic for a study visit at week 4, 8, 12, 16, 20 and 32 after randomization. During these visits anthropometric measurements (weight and WC) and a medical events CRF will be completed. Participants will also meet with the RD to receive ongoing dietary guidance. They will be asked to complete a 3-day food diary for the 8, 20 and 32-week visit. The RD will review the food diaries for consistency in overall intake and to help direct the dietary counselling. The physical activity questionnaire (GPAQ) will be administered to participants during the 8, 20 and 32 week visit as well. A stool sample will be collected during the week 20 and 32 study visit.

Participants will be asked about change in any medications, especially the initiation of antibiotics since they can impact the underlying microbiota and potentially blunt the effect of the probiotic treatment[55]. Despite this, participants who start on antibiotics will continue with their assigned trial treatment.

If a participant reports that they have been diagnosed with T2D and has started on anti-hyperglycemic medication then they will be treated as a treatment failure. Those that have been diagnosed with T2D during the trial but have not been prescribed any medication will have a confirmatory HbA1c test done and if the results are positive for T2D then the participant will be labeled as treatment failure.

Participants will be asked about their adherence to the study sachets and if they have experienced any adverse events. They will also be given a supply of study sachets to last them until their next visit. According to the VSL#3® manufacturer, the probiotic viability will be preserved between visits as long as the product is stored between 2 to 8 °C [116]. Participants will be asked to bring any remaining sachets from their last supply to each follow-up visit. The

sachets will be counted and retained by study staff.

Losses to follow-up are expected to be minimal considering the short duration of the intervention, which is only 20 weeks. Frequent contact will be maintained between research staff and participants via follow-up telephone calls. In order to reduce loss to follow-up, participants will be offered convenient and flexible times for their clinic visits. A 7 day window before or after the scheduled follow-up appointment will be offered to participants. Participants will also be compensated for their travel and parking costs upon each visit. If participants are unable to attend a study visit then research staff will administer the study visit CRFs and questionnaires over the phone. If participants miss their final visit they will be contacted by research staff and offered the option of remote blood collection via a test kit.

Participants who are lost-to-follow up may be fundamentally different than those that remain in the study. It is essential to identify whether the reason for loss to follow-up or participant withdrawal is related to the study intervention. Staff will call participants, then attempt a second call, and finally send an email and/or letter by mail if the participant does not respond to earlier attempts. Study staff will attempt to obtain an HbA1c sample from the participant at week 20 and will inquire about any anti-hyperglycemic medication that the participant may have been started on. If the participant is unwilling or unable to come to the study site to provide a blood sample, a remote collection kit will be sent to the participant to take into a LifeLabs with a bloodwork requisition form. If the participant is still unable or unwilling to provide a blood sample, then the last option would be to request the participant's latest HbA1c result from their physician, with the participant's signed consent which will be obtained at the start of the study.

2.8. OUTCOMES

2.8.1. Primary Outcome

The primary outcome of this study is prediabetes remission to normoglycemia at week 20 of the trial, while being off of any anti-hyperglycemic medications for 20 weeks post-

randomization. There is no set definition of prediabetes remission, however, the *Diabetes Canada* cutoff for a prediabetes diagnosis is an HbA1c of 6.0%-6.4%, thus we can define normoglycemia as a HbA1c of <6.0% without the use of any anti-hyperglycemic medications[6].

2.8.2. Secondary outcomes

Prediabetes remission will also be assessed in all participants during week 32 which is 12 weeks after the discontinuation of the study sachets. The proportion of participants in drug-free remission at week 32 will be compared between groups.

In addition to assessing the proportion of individuals that revert to normoglycemia, we will also capture the proportion of participants that develop T2D at any point during the first 20 weeks of the trial within each group. The proportion of individuals in each group that develop T2D during the 32 weeks of the trial will also be reported. The proportions will be compared between-groups at both time points.

Insulin resistance and beta-cell function will be measured using the HOMA-IR and HOMA-B index respectively in all participants using plasma glucose and insulin samples collected from participants after an overnight fast[37]. Change in HOMA-IR and HOMA-B values from baseline will be calculated and compared between study groups at 20 weeks and 32 weeks. Both HOMA models are appropriate for measuring longitudinal changes in insulin resistance and beta-cell function, and are based on fasting plasma glucose and insulin[37,128,129]. Estimates from these models have been found to be independently associated with diabetes risk after adjustment for other diabetes risk factors[37,128]. The HOMA-IR reference value is 1 which reflects a healthy individual without insulin resistance and a HOMA-B of 100% denotes perfect beta-cell function[130].

Changes in anthropometric measures will also be assessed. A reduction in weight, WC and BMI from baseline have been associated with probiotic administration and are predictors of prediabetes remission[100,131–134]. Thus, the % change in weight, BMI and WC from baseline at

week 20 and at week 32 between groups will be compared. Capturing changes in these anthropometric measurements may help shed some light on possible mechanisms through which the intervention may be eliciting the outcome of prediabetes remission.

Another secondary outcome is the assessment of change in relative abundance of bacterial and archaeal phyla, classes and genera from baseline at week 20 and at week 32 in all participants. Results for changes between study groups will be reported at both time points. Change in fecal flora abundance will be calculated using stool samples that will be collected at baseline, week 20 and week 32. Bacterial abundance will be determined using 16S rRNA sequencing.

Finally, we will be assessing the safety and tolerability of the study probiotic. Some probiotic strains have been associated with infections, albeit rare and mostly in immunocompromised individuals[119]. VSL#3® was found to safe and did not result in serious adverse events (AE's) in previous trials[121,135]. A meta-analysis found that out of 355 participants receiving VSL#3®, only 33 reported mild side effects, predominantly temporary bloating and there were no incidents where the treatment had to be stopped[135]. However it is still recommended that all trials employing a probiotic intervention monitor participants for cases of infection as well as other adverse events[119]. As such, participants will be asked during follow-up visits as well as during phone calls whether they have experienced any AE's. AE's are undesirable signs or symptoms that occur during the study and may or may not be causally related to the treatment. All AE's considered to be possibly, probably or definitely related to the tested product will be recorded in a case report form (CRF). Serious adverse events (SAE's) are defined as events that are fatal, life-threatening, disabling, incapacitating or result in hospitalization or prolonged hospital stay. All SAE's, whether they are related to the study intervention or not, will be recorded in a CRF and will be reported to the research ethics board. Participants will be discontinued from the probiotic if it is decided that the SAE was related to probiotic consumption.

2.9. DATA COLLECTION

The research coordinator and research assistant(s) will be responsible for the data collection and entry. All research staff will be certified in *Good Clinical Practice* and will receive thorough training in appropriate data collection and entry methods.

Data will be collected using CRFs and will be manually entered into electronic case report forms (eCRFs) in an electronic data management system. Research staff will be trained on operation of the data management system and data entry procedures. All CRFs will be reviewed by the research coordinator(s) shortly after data collection to ensure data are accurate and complete. Whenever possible any missing data will be obtained before the participant leaves their study visit. A random sample of data will be double-entered to ensure accurate data entry. The data management system will be programmed to flag data that may be inconsistent or out of range minimizing the chance of erroneous data entry.

2.9.1. Anthropometric Measurements and Blood Specimens

Participants' height and weight will be measured with light clothing on and without shoes. Waist circumference will be measured in the horizontal plane midway between the lowest rib and the iliac crest[136]. Body mass index (BMI) will be calculated as weight in kilograms (KG) divided by the square of height in metres.

Insulin resistance and beta-cell function will be estimated using the HOMA-IR and HOMA-B index respectively, based on the formula: (HOMA- IR) = ((insulin mU/L)(glucose mmol/L))/22.5, and (HOMA-B)= (20 x insulin mU/L)/(glucose mmol/L- 3.5)[37]. Fasting plasma glucose and insulin will be collected from participants after an overnight fast at randomization visit, week 20 and 32. HbA1c will be measured using a validated assay standardized to the National Glycohemoglobin Standardization Program-Diabetes Control and Complications Trial reference[6]. The proper collection and processing of blood samples will be detailed in a laboratory manual that will be provided to all the study sites.

2.9.2. Fecal sample collection

Stool samples will be collected at randomization as well as at week 20 and 32. Ideally, samples are to be collected during the study visits but if the participants are unable to provide a stool sample at that time, they will be given a stool sample collection kit to take home. Participants will be instructed to store the sample (using the provided kit) in their freezer for a maximum of three days after which they must either drop it off to the study site or to LifeLabs using icepacks and a Styrofoam cooler provided as part of the kit. LifeLabs will ship the samples on dry ice to our research centre[137]. Fecal samples will be frozen in a -80°C freezer immediately upon collection during a study visit or upon arrival to the study site. All samples will be labeled using participants' study identification number, date of collection and date of storage in the study site. Fecal sample analysis will occur through a centralized lab specializing in 16S rRNA sequencing.

2.10. MEASURING POTENTIAL CO-INTERVENTIONS

Shifts in dietary intake may influence gut microbiota composition as can regular physical activity[138,139]. Therefore diet and physical activity will be monitored in all participants throughout the trial.

2.10.1. Diet Intake Measurement

All participants will meet with a study research dietitian throughout the run-in period and their final 3-day food diary will be used to denote their intake at 'baseline' or 'randomization'. Additional 3-day food diaries will be measured and submitted to the dietitian during week 8, 20 and 32 of the trial for the purpose of monitoring and reinforcing healthy eating habits. Diet diaries will also be assessed for intake of probiotic and prebiotic-containing foods and participants will be reminded to refrain from consuming these foods for the remainder of the trial.

2.10.2. Physical Activity Measurement

The *Global Physical Activity Questionnaire* (GPAQ) will be administered by a trained research staff member. It incorporates the use of images as well as lists that depict moderate and vigorous-intensity activities which will aid the participants in recalling their activity during a typical week[15]. The GPAQ has been validated against accelerometer data for measurement of moderate and vigorous-intensity physical activity (MVPA) over 7 days and for assessing change in MVPA over a period spanning 1 to 6 months and has shown moderate agreement for MVPA minutes/day (r=0.48) and for change in MVPA (r=0.52).[140]

The GPAQ will be administered at randomization, approximately midway through at week 8, 20 and 32 to assess average total minutes of MVPA per week.

2.11. SAMPLE SIZE

Several trials have demonstrated remission of prediabetes with proportions of individuals reverting to normoglycemia ranging from 16% to 58% over time frames reaching up to 10 years[14,133,141–144]. However, to the best of our knowledge, no studies have attempted induction of normoglycemia in individuals with prediabetes using probiotics. As such, to help us determine an estimation of our required sample size we resorted to remission proportions from observational studies and remission proportions of control groups from trials aiming to prevent T2D in adults with prediabetes and trials aiming to reverse prediabetes. Considering our proposed intervention duration is 20 weeks (5 months), we focused on remission proportions from trials with durations of 1 year or less to select an anticipated remission proportion for our control group. An Iranian observational study of prediabetic first-degree relatives of individuals with T2D found that 15% of their participants reverted to normal glucose tolerance after one year of dietary and weight-management education[132]. A non-randomized study aiming to prevent T2D compared a group on a combination of oral anti-hyperglycemic medications with a group that was given printed information about healthy eating and encouraged to walk 30

minutes per day[145]. The pharmacological intervention group was also given the same print-outs and told to walk 30 minutes per day, so in essence, the lifestyle group functioned as the control group[145]. After a median follow-up of 8.9 months, the control group demonstrated a remission proportion of 12.5%[145]. The Diabetes Prevention Program trial studied the prevention of T2D using metformin or lifestyle modification compared to a control group[146]. The remission proportion in the control group was approximately 25% after a one year follow-up but this figure decreased to 10% after adjusting for baseline age, sex, ethnicity, weight, FPG, 2hrPG, insulin sensitivity and secretion[146, 21].

We also found an observational study in Japanese population that reported a remission proportion of 43% after a one year follow-up period[147].

We leaned on the conservative side by selecting a remission proportion of 8% in the control group given our trial duration is 20 weeks (5 months). We would like to detect a 2-fold increase in the proportion achieving remission in the probiotic group. Using a power-based approach, a prediabetes remission proportion of 8% in the control group and a proportion of 16% in the probiotic group, an alpha error probability of 5% in a two-sided test, and a power of 80%, our estimated sample size is 516, or 258 per group (see sample size calculator output in Appendix 4). Accounting for a hypothesized attrition rate of 10%, our sample size increases to a total of 568 or 284 participants per group. Alternative sample size calculations are provided in Appendix 5.

2.12. STATISTICAL CONSIDERATIONS

All participants will be followed until they withdraw, develop T2D or until the completion of the study. Using intention-to-treat analyses, participants who withdraw or are lost-to-follow up will still be included in the analysis for the treatment group that they were randomly allocated to and be used in the final analyses. Missing data will be dealt with using multiple imputation for all variables with the exception of the primary endpoint. Study staff will obtain the participant's

most recent HbA1c from their family physician or diabetes care provider in order to ascertain if the study outcome has been achieved. If for any reason we are unable to obtain an HbA1c we will adopt a conservative approach and assume that remission was not achieved. Participants that develop T2D will be considered treatment failures.

An exploratory per-protocol analysis will be conducted using data from participants who demonstrated $\geq$80% mean adherence throughout the trial. Results of the per-protocol analysis will be compared with the ITT analysis results. Inconsistencies between the ITT and per-protocol results will help explore the impact of poor adherence (<80%) on the treatment effect of probiotics[148,149].

Descriptive statistics will be used to summarize participants' baseline characteristics and to assess the distribution of the data. Continuous variables will graphed using box plots to identify outliers beyond the interquartile range (IQR). The IQR provides an approximation of the variability near the center of the data and is calculated as the 75th percentile (denoted as Q3) minus the 25th percentile (denoted as Q1). Any data points falling above the upper and lower limits as calculated by $[Q_3+1.5(IQR)]$ and $[Q_1-(1.5IQR)]$ respectively will be considered extreme outliers. We will investigate the nature of extreme outliers before deciding whether or not to drop the observation. If it is clear that the outlier is due to incorrectly entered or measured data, then it will be corrected if possible. Remaining outliers will be kept in the analysis and the data will be winsorized to reduce the influence of the outliers on the results. The winsorization cut-off will be determined after the data has been collected and examined. Continuous variables will be presented as means and their standard deviations or medians and interquartile ranges depending on the distribution. Categorical variables will be presented as counts (percentages). Differences in participants' baseline characteristics between the two groups will be analyzed using a chi-square test for categorical variables and the t-test for normally distributed continuous data. Non-normally distributed continuous data will be tested using the Mann-Whitney test. Changes in all of our outcome measures pre and post-intervention will be

reported within groups and compared between groups. Between-group differences will be tested for significance using the same methods as the baseline characteristics.

Our primary analysis will serve the purpose of hypothesis-testing while the secondary analyses will be hypothesis-generating. A p-value of <0.05 will indicate statistical significance for all analyses.

The proportion of participants that achieve prediabetes remission at week 20 will be calculated in the probiotic and placebo groups. The chi-square test will be used to identify if the proportion of individuals who achieve drug-free prediabetes remission is statistically different between the probiotic and placebo group. A Fisher's exact test will be used if counts within the contingency table's cells are <5. Prediabetes remission will also be assessed at week 32 and the proportion of individuals who achieve remission to normoglycemia will be compared between the two groups. The proportion of individuals that develop T2D at any point during the trial will be compared between groups in the same way as prediabetes remission. The odds ratios (O.R) and their 95% confidence intervals will be reported for these analyses.

The other secondary outcomes (HOMA indices, weight, BMI, WC and relative abundance of microbiota) will be analyzed using an independent sample's t-test to compare changes from baseline between groups. HOMA-B and HOMA-IR estimates are usually not normally distributed and so a Mann-Whitney test will be used to assess differences between the groups. Comparisons between groups will be reported as an estimate of effect along with the corresponding 95% confidence interval and associated p-values.

Mean relative abundance and standard deviation will be reported for phyla, class, and genera. To account for the multiple testing within the microbial composition analysis, a false discovery rate-adjusted $p<0.05$ will be used to denote statistical significance[150].

Our exploratory regression analyses will involve a multiple logistic regression model to assess whether the change in fecal microbiota, specifically the change in relative abundance of the *Rosburia* genus from baseline at week 20 is an independent predictor of drug-free

prediabetes remission at week 20 (categorized as yes/no). The purpose of this analysis is to assess whether the change in this microbial population is associated with prediabetes remission. To assess whether the change in fecal microbiota is an independent predictor of remission, we will adjust for variables that have been found to be significant predictors of prediabetes remission. Previous studies investigating predictors of prediabetes remission have found that BMI reduction from baseline, but not baseline BMI, was a significant predictor of prediabetes remission to normoglycemia after adjusting for age, sex and baseline fasting glucose and lifestyle factors. They also found that change in WC from baseline but not initial WC was significantly associated with prediabetes remission[17]. On the other hand some trials found baseline weight, WC and BMI and not necessarily changes in these variables to be important predictors of regression to NGT[21,132,141]. Furthermore, age, sex, ethnicity, family history of type 2 diabetes; physical exercise (minutes of total MVPA per week); FPG; as well as insulin resistance (HOMA-IR) and beta-cell function (HOMA-B) have been identified as important predictors [20,21,132,133]. We will adjust for age, sex, ethnicity, mean total minutes of MVPA per week (average of baseline, week 8 and week 20) and baseline HbA1c. All these variables will initially be added in our model at once and backward elimination will be performed to find the best model that predicts our outcome. Variables will be retained in the model if they change the main effect estimate by $\geq$10%. However we will first test our independent variables for multi-collinearity and if variables are found to be significantly correlated ($r>0.7$) then only one of the two collinear variables will be included in the model. The O.Rs and 95% confidence intervals will be reported for the final adjusted multiple logistic regression model.

All analyses will be conducted using the SPSS V.23. Statistical Software package and be reported according to CONSORT guidelines.

CHAPTER 3: ADDITIONAL METHODOLIGICAL CONSIDERATIONS

3.1. ETHICS AND RISK TO PARTICIPANTS

The trial will be conducted in accordance with the *Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans* (TCPS 2). TCPS 2 training will be mandatory for all research staff involved in the trial.

Voluntary written consent will be mandatory prior to participation in the trial. Subjects may withdraw from the trial at any time at their own request, without requiring to disclose the reason unless it is due to discomfort or adverse events.

Identifying information of patients who become participants will be protected under the Personal Health Information Protection Act (PHIPA). Each participant will be assigned a unique participant identifier (PID) by the data management system. The PID will not contain any information that would link back to the participant's name, initials, date of birth or medical record number. The database is maintained on a secure server and is accessible only to authorized members of the research team who will each have their own user I.D and password. The database tracks the activity of each user, thus ensuring that all data entry and editing can be traced back to the correct staff member. Participant charts will be securely stored in a locked cabinet within a locked room. Identifying information will be stored separately from the de-identified data.

Participant safety will be carefully monitored and protected throughout the trial. We have selected exclusion criteria that prevents certain vulnerable individuals from participating. For instance, immunocompromised people as well as women who are pregnant are restricted from participating due to risk of infection with probiotics, even though the risk is minimal[119]. Furthermore, women of child-bearing potential will be asked to complete a urine pregnancy test at the start of the trial and will be asked to use reliable contraception methods to avoid pregnancy during the trial. All participants will be clearly informed of potential risks and adverse

events associated with the intervention and any new information that emerges which impacts their welfare or continuation in the trial will also be shared.

3.2. POTENTIAL CO-INTERVENTIONS

3.2.1. Dietary Intake

Dietary components and short-term or acute changes in diet can influence gut microbiota in different ways[56,151]. Animal studies suggest HFDs result in shifts in certain microbiota, primarily reductions in *Bifidobacterium spp*. and *Bacteroides* and an increase in *Protebacteria*[69,152,153]. These observed changes in microbiota may lead to increased levels of LPS and intestinal permeability, resulting in a cascading inflammatory response that may ultimately lead to insulin resistance[152]. High LPS levels have been associated with metabolic syndrome features and a significant increase in LPS was found in individuals with T2D as well as in those with impaired glucose tolerance[68,72,73]. In an animal study, changes in gut microbiota and metabolic indices appear to result from diets high in milk and lard fat (saturated fatty acid sources), or safflower oil (polyunsaturated fatty acid), with fat totaling approximately 60% of total energy intake[154]. These HFDs induced changes in phylogenic profiles that were associated with inflammatory profiles[154]. In a small human study, the HFD was comprised of an animal-based diet with over 60% of caloric intake from fat and this diet arm was compared to individuals on a plant-based diet with less than 30% fat intake[155]. Participants on the HFD had increases in bile-tolerant microbiota such as *Bacteroides* and decreased levels of *Firmicutes* that metabolize dietary plant polysaccharides compared to the plant-based diet arm[155].

We don't anticipate that there will be drastic differences in intake between our two study groups. However, there is a concern that perturbations in fat intake by individuals in either group may blur the treatment effect of the probiotics. As such, we plan to standardize participants' intake as much as possible during the run-in period when participants will meet with a RD. The RD will provide teaching on healthy eating and will provide instructions on thorough and

accurate completion of 3-day food diaries. The RD will use the collected data to assess whether participants require further teaching reinforcement to achieve a healthy diet as well as to monitor intake to ensure consistency throughout the trial.

3.2.2. Physical Activity

The impact of physical activity on the gut microbiota has been mainly studied in animal models. Studies using mice have demonstrated that moderate-intensity exercise can alter the gut microbiota and increase SCFA production[125,126]. Data from humans is limited and available data is from trials comparing athletes to sedentary controls[127,156]. A trial done in a small group of women did not find any significant differences in the phylum level between the active and sedentary group, however there was a trend towards higher presence of *Firmicutes* and a lower presence of *Bacteroidetes* in the active group compared to the inactive group[139].

Although the relationship between physical activity and the gut microbiota is uncertain, the glucose-lowering effects of physical activity as well as its role in weight loss have been established[23,24]. Vigorous as well as non-vigorous physical activity can reduce insulin resistance regardless of weight loss and is an important predictor of diabetes prevention[3,156,157]. It is unlikely that the groups will differ in physical activity levels following randomization. However, we plan to test whether change in the microbiome is an important predictor of prediabetes remission using a regression model and will need to adjust for physical activity levels. Therefore we will capture the average total minutes of MVPA per week in both groups at baseline, week 8 and week 20.

3.3. DATA COLLECTION CONSIDERATIONS

3.3.1. Dietary Intake Measurement

There are a number of ways to collect diet intake data. One of the most commonly used tools is the *food frequency questionnaire* (FFQ). The FFQ is a validated measure of dietary intake and is a retrospective measurement of *usual intake* over the previous 12 months[158].

There is no current 'gold standard' for measuring food intake, however the FFQ has been validated against weighted food records and a series of 24-hour dietary recalls[158]. Compared with other approaches, such as 24-hour dietary recalls and food records, the FFQ generally collects less detail regarding the foods consumed, cooking methods, and portion size[158]. Overall, the questionnaire allows the estimation of the average daily consumption of foods by asking about sub-items for several food items[158]. The FFQ is usually completed independently by the respondent and is considered low-burden[159]. It is relatively inexpensive and therefore is usually the method of choice for diet measures in large-scale epidemiologic studies[159]. Its intended use is to assess ranking of intakes within a population, and so it is important to note that FFQ's may not produce reliable estimates of absolute intake. This method is also subject to errors common to tools that rely on recall and self-administration. These include the reliance on long-term memory, interpretations of the frequencies and serving sizes and a poor ability of some individuals to estimate and describe their usual food intake[160].

Another method of dietary data collection is the 7-day diary (7DD) which is a prospective record of food and beverages at the time of consumption[159]. It can include details such as portions, brand names, cooking and preparation methods[159]. Participants can take photographs of their food/portions or measure their food using household measures and record their intake by hand or electronically[159]. The immediate logging of food intake results in more accurate quantification compared to methods that rely on recall[161]. This method has been found to be in closest agreement to a 16-day record of weighed food and the second highest correlation with biomarkers compared to the FFQ and a 24-hour recall[161]. Concerns with this method include the burden it places on participants as well as the risk that it may be completed from memory after the food is consumed rather than at the time of consumption[162]. Additionally, the process of filling out any food record may influence eating behavior, however that is likely due to the nature of subjective measurement of intake rather than the tool itself[162]. Altering behavior or self-collected data to align with societal norms or expectations, also known as social desirability

bias, is a common limitation associated with collection of lifestyle data[163]. However, this will likely be a non-differential bias as it can occur in both groups.

A 3-day food diary is similar to the 7DD but with less burden placed on participants. This approach is the most suitable method for our study given that we are monitoring diet for the purpose of maintaining consistency of healthy eating habits throughout the trial. The RD can visually review the 3-day food dairies without the need to input data into software and can provide personalized feedback and teaching to participants.

3.3.2. Physical Activity Measurement

Similar to diet measurement, physical activity can be measured in various ways. Objective measurement includes the use of pedometers which are small motion sensor devices that count the number of steps taken in one day. Although pedometers are simple to use they present a number of drawbacks. To begin with, they only capture walking activity and do not account for other forms of exercise such as swimming or resistance training. Secondly, pedometer accuracy is variable as it can erroneously perceive other motions as steps, resulting in recorded steps not reflecting actual steps taken[164]. Additionally, pedometer step counts are influenced by body size and speed of movement[165]. Some pedometers now include a function that records distance walked and can be used to assess kilocalorie expenditure[165]. However a study that validated 10 different pedometer devices concluded that pedometers are most accurate for capturing number of steps and less accurate for distance and kilocalorie assessment. Accelerometers are similar to pedometers in that they are small wearable devices but the main difference, as the name suggests, is that they capture acceleration of movement in up to three planes (anteroposterior, mediolateral, and vertical)[166]. Accelerometers are an attractive option because they can be used to compute physical activity durations, rates and time spent in different intensities of activity[167]. However, accelerometers are expensive and require individual programming which necessitates technical expertise and specialized

software[166,13]. Also, some accelerometers are unable to differentiate body position (e.g., sitting, standing) and may miss upper body movements if worn on the waist[167].

Subjective methods include self-report via diaries or questionnaires and are the cheapest and most convenient way to capture physical activity data from a large number of people in a short time. Activity diaries or logs are similar to diet diaries in that they can vary in duration of recall and amount of detail provided. They also present similar drawbacks as diet diaries. Many activity questionnaires have been designed to capture physical activity levels for the purpose of either surveillance or assessment of behavior change within different populations[168]. Since physical activity is not a primary variable of interest, we do not require detailed accounts from participants but rather an estimate of their activity levels. The *Global Physical Activity Questionnaire* (GPAQ) has been designed for that purpose and is a valid measure of moderate and vigorous-intensity activity as well as of change in those activity levels[140,169]. The questionnaire covers intensity, duration, and frequency, and it assesses occupational physical activity, transport-related physical activity, and leisure physical activity of a typical week[15]. GPAQ is designed for administration by a trained interviewer which is advantageous as it reduces error associated with self-completion[15]. It also incorporates the use of images and lists that depict moderate and vigorous-intensity activities which will aid the participants with their responses[15]. The GPAQ will be used to measure average total minutes of MVPA per week in our study.

3.3.3. Fecal Sample Collection

Stool samples can be collected as a bulk fecal sample or using fecal occult blood test cards[170]. Although the test cards are inexpensive and easy to ship to and from the participant, they are designed for colon cancer screening and contain a chemical that detects blood in the stool which could potentially affect the gut microbiome[171]. Thus, bulk stool collection would be the more appropriate option for our study.

The method of collection has not been found to result in significant differences in microbiome stability and detection, however storage temperature can impact result reproducibility. There is conflicting evidence related to the stability of microbial communities in fecal samples stored at room temperature (for a short period of 3 days) in comparison to frozen samples[170,137]. Frozen samples (at -80 °C) are the gold standard for microbial storage and some evidence demonstrates changes in fecal microbial viability with delayed freezing[137,170,172]. Furthermore, evidence shows that fecal samples which were immediately frozen at −80 °C, stored on ice for 24 hours, or stored on ice for 48 hours before DNA extraction and analysis did not exhibit significant differences due to the storage method[173]. There is little concern about the sample thawing from the time it is removed from the participant's freezer to the time it is placed on dry ice as viability was preserved in a study where samples were thawed and re-frozen 4 times, totaling 28 minutes of thawing time[137].

Another important point for consideration is the variation in results that can occur from different batches of DNA extraction kit reagents used in prospective studies[174]. It is recommended that all extraction kits be purchased at the start of the study to minimize potential discrepancies among reagent batches and to record the kit and batch used to process each sample[174].

3.4. OUTCOME MEASUREMENT

3.4.1. Primary outcome

Our primary outcome is prediabetes remission to normoglycemia, as defined by an HbA1c of <6.0% at week 20 of the trial without the use of any anti-hyperglycemic medication for 20 weeks post-randomization.

Alternative methods of assessing normoglycemia include a FPG<6.1mmol/L and/or a 2hrPG<7.8mmol/L, measured using a 75g-OGTT[6]. Both methods are acceptable prediabetes diagnostic tests however each presents certain limitations[6]. For instance an OGTT is a time-

consuming test that requires an overnight fast and must be conducted between 7:00 to 9:00 a.m., making it burdensome to participants[175]. Often times patients forget to fast or consume an inadequate amount of carbohydrates over the proceeding 3 days (150 grams/day), impacting result accuracy[176]. Furthermore, when compared to FPG and HbA1c, the 2hrPG is associated with substantially more intra-individual variability, as high as 16.7%[177].

Although FPG is accepted as a diagnostic criterion of prediabetes, it has been reported to have low reproducibility[6]. There are a number of factors that contribute to its poor reproducibility, namely inter and intra-individual biological variability[6,42]. In a person with normal glucose tolerance, day to day FPG can vary by 5.7-8.3% and inter-individual variability can reach as high as 12.5%[177,178]. FPG levels can also be influenced by factors that occur prior to sample collection such as food intake, length of pre-test fasting and exercise[179]. Furthermore, illness and acute stress can increase FPG[180]. These limitations highlight a major drawback of FPG which is that it is a reflection of glucose levels at a single point in time[176].

HbA1c is advantageous in that it is a measure of the average glucose concentrations over the previous 2-3 months and thus reflects long term glucose control[181]. Another advantage is that no preparation is required by participants and the test is not time-sensitive[6]. Additionally, intra-individual variation is minimal (<1%), however variation between individuals is greater despite similarities in glucose concentrations[182,183]. This variation is believed to be due to differences in rate of hemoglobin glycation, termed as the 'glycation gap', the clinical significance of which is unclear[176,184]. Other important points to keep in mind include the influence of race and some chronic medical conditions on HbA1c levels. For instance African Americans, Indian Americans, Hispanics and Asians have HbA1C values that are up to 0.4% higher than Caucasians at similar glycemic levels[185,186]. It is unknown whether these differences are present in Canadian Africans and Canadian Aboriginals[176]. Despite the ethnic variation in HbA1c, diagnostic cut-offs based on ethnicity have not been developed[6].

HbA1c measures may be inaccurate in individuals with hemoglobin-related disorders,

iron-deficiency anemia, hemolytic anemias, and severe liver and kidney disease[187]. Although some of these conditions such as iron-deficiency anemia have been observed to increase HbA1c levels minimally, correction of the iron deficiency is recommended prior to measuring HbA1c[187]. Despite these cautions, HbA1c is a convenient, stable and accurate measure of glucose control in the overall population[187]. For these reasons, it will be used to determine the outcome of prediabetes remission in this trial.

3.4.2. Secondary Outcomes

Although our overall objective is to induce prediabetes remission, it is crucial to investigate the impact of our intervention on underlying pathophysiology. Prediabetes starts with insulin resistance, resulting in hyperinsulinemia, which is also accompanied by overproduction of endogenous glucose[130]. Evidence shows that individuals with IFG or IGT have decreased beta-cell function irrespective of HbA1c level and that insulin resistance has to be considered when assessing insulin release by the beta-cell[37,188]. Furthermore the ACT NOW study demonstrated that the risk of developing diabetes was related to diminishing b-cell function (as determined by the insulin secretion/insulin resistance (IS/IR) index)[189]. As such, it would be important to assess whether changes in beta-cell function and insulin resistance are taking place in response to probiotic therapy. It would also be of interest to assess whether changes in beta-cell function and insulin resistance continue beyond the administration of probiotics. HOMA-IR and HOMA-B are relatively simple and inexpensive surrogate measures of insulin resistance and beta-cell function respectively[129]. Both indices are calculated using the HOMA2 computer calculator which accounts for hepatic and peripheral glucose resistance[129]. Both measures have been validated against more complex tests such as the intravenous glucose tolerance test (IVGTT), the hyperglycemic clamp, and the euglycemic-hyperinsulinemic clamp[130]. However, it is important to mention that the equations the HOMA2 utilizes are based on the original HOMA1 model which was developed in 1985 and was calibrated to an insulin

assay from the 1970's which when compared to modern assays results in an underestimate of insulin resistance and an overestimate of beta-cell function. This is not problematic when assessing relative change but more so for the assessment of absolute insulin resistance or beta-cell function[129].

The mechanisms by which probiotics may be exerting their effects are not fully understood. It is unclear whether they exert their effects by altering the host's native microflora population or through indirect mechanisms or both[85]. To help elucidate some of the uncertainty, we will be measuring changes in fecal flora relative abundance using the 16s rRNA sequencing method. This method allows for analysis of the composition and diversity of microbial communities, providing information about taxonomy and function[190]. 16s rRNA is especially useful for characterizing which kinds of organisms are present in a wide range of samples[191]. To obtain a detailed structural overview of the microbiome of each subject enrolled in the study, operational taxonomic unit (OTU) analysis will be conducted[190].

We are particularly interested in evaluating whether changes in the *Roseburia* genus is associated with prediabetes remission. *Roseburia* are members of the *Firmicutes* phylum and are butyrate-producing bacteria[192]. As previously mentioned, butyrate has been found to have anti-inflammatory and trophic effects in the epithelial mucosa, maintaining gut-barrier integrity[46,78]. Butyrate has also been correlated with elevated levels of GLP-1[110]. Fecal transplants from lean donors to adults with metabolic syndrome resulted in an increase in *Roseburia* bacteria as well as butyrate[83]. The transplant recipients also demonstrated a significant improvement in peripheral insulin sensitivity[83]. *Roseburia* has been found to be significantly diminished in adults with type 2 diabetes and it would be interesting to explore whether a change in relative abundance of *Roseburia* is associated with remission from prediabetes to normoglycemia.

3.5. INTERNAL AND EXTERNAL VALIDITY

Sources of systematic error or bias can present themselves prior to randomization, during the intervention phase and during data analysis. These sources of bias are mitigated through careful consideration and implementation of a randomized controlled trial design and methodology. The randomization, concealed allocation and triple-blinded design minimize sources of bias that would otherwise impose threats to internal validity within observational study designs. For instance if participants are not blinded to their group allocation, those in the control group may use co-interventions in order to achieve similar benefits as participants in the active treatment group. Given that our participants will be blinded it is likely that any co-intervention use will be balanced between the two groups of participants. Participants will be advised to maintain their current lifestyle behaviors and will be counseled on avoiding co-interventions that may impact the study outcome, with the exception of antibiotics.

Similarly, the risk of contamination between study groups is avoided with the triple blinded design. Blinding prevents the placebo group participants from knowing what treatment group they are in and from attempting to achieve the same benefits as the probiotic-group participants by consuming probiotics outside of the study. The triple-blinded design also prevents study staff from influencing or manipulating study results during the data collection and analysis phase in order to demonstrate the desired study outcome. There is a possibility that the probiotic group participants experience symptoms associated with probiotic use such as increased bloating and flatulence which may make them privy to their group allocation.

Information bias can result from how participants recall their diet and physical activity. Diet and physical activity estimates may be less accurate to their true intake given that they are measured indirectly using subjective methods. Also, social desirability bias results when participants intentionally under or over-report intake of certain foods based on what they believe will be favorably viewed or expected of them. Any reporting bias related to physical activity would impact the results of our regression analysis since our regression model will be adjusted

for physical activity.

The use of probiotics, prebiotics or synbiotics outside of trial protocol can influence the trial outcomes. As previously discussed, prebiotics are non-digestible but fermentable food components that selectively stimulate the growth or activity of one or multiple gut microbes[114]. Bifidobacterium bacteria are known to respond to the administration of certain prebiotics[114]. For example, amylase-resistant starch increases *Lactobacilli* and *Bifidobacteria* as well as *Lactobacilli*, while also decreasing *Enterobacteria*[193]. However it is important to highlight that translation of these findings from animal studies to humans has resulted in variable results. The administration of oligofructose in humans reduced plasma endotoxin levels and improved glucose tolerance and weight loss in humans[69]. Further attempts to assess the efficacy of prebiotics in humans have demonstrated that reductions in energy intake and increase in PPY concentrations may be dose-dependent[194,195]. The amount of evidence from human trials is limited and beneficial changes of metabolic markers remains to be demonstrated in large randomized controlled trials. Similarly, probiotic and symbiotic use can impact our outcomes and so participants will be questioned about their use of probiotics, prebiotics and synbiotics during the screening process. They will also be instructed to refrain from using these items during the trial. Dietary recalls will be conducted throughout the trial to assess intake of prebiotic and probiotic food sources and will be used to assess whether intake of these foods has remained unchanged from baseline. As previously discussed, it is unlikely that there will be significant differences in intake between the groups given the randomized and blinded design.

Changes in the microbiome that occur with antibiotic use can last beyond the completion of antibiotic treatment. Broad-spectrum antibiotics have been found to reduce the abundance of approximately 30% of the taxa in the human gut, reducing microbial diversity[196]. Pre-treatment microbial diversity was mostly restored approximately 4 weeks after the completion of the antibiotic course in healthy adults, however several taxa failed to recuperate 6 months post-treatment[196]. Long-term consequences of antibiotic use have also been reported. A human trial

demonstrated that after 7 days of antibiotic exposure, a sharp decline in *Bacteriodes* was detected and pre-treatment diversity was not regained after a period of two years post-treatment[197]. It is important to note that individualized responses to the same antibiotic vary, and different antibiotics may have different effects on underlying microbiota[196,197]. Participants who have been on antibiotics within 30 days prior to randomization and those who have had more than 3 antibiotic courses within the previous 12 months will not be eligible to participate in the trial. Enrolled participants will be questioned about changes in medications and health at every study visit to monitor initiation of antibiotics.

In regards to external validity, the proposed inclusion and exclusion criteria would extend the generalizability of our results to individuals with prediabetes but may not apply to those with T2D or with current gestational diabetes.

The run-in period is useful for increasing the proportion of participants who adhere to the study intervention. Excluding those that demonstrate <80% adherence will allow us to explore the treatment effect in participants who actually take the probiotic[198]. Although the exclusion of non-compliant participants limits the external generalizability to those who take an average of 80% or more of the intervention, the number or participants who end up being excluded tends to be relatively small[198].

If probiotics demonstrate efficacy in inducing prediabetes remission, we will not be certain of the applicability of our findings to those with the comorbidities listed in the exclusion criteria. On the other hand, the trial will be recruiting from populations across Canada, capturing a wide variety of ethnicities and cultures.

CHAPTER 4: TRIAL STRENGTHS, LIMITATIONS AND IMPLICATIONS

4.1. STUDY STRENGTHS AND LIMITATIONS

To the best of our knowledge, this is the first randomized trial of its kind to investigate the effects of a high concentration multi-strain probiotic on inducing drug-free remission of prediabetes to normoglycemia. In designing this trial, several key considerations were made to address limitations of previous studies. Most trials using probiotics have relatively small sample sizes and short durations. Our duration of 20 weeks should allow for any changes in gut microbiota to take place, for the microbiota to exert their effects and for any metabolic changes to be reflected in the HbA1c. Furthermore, our follow-up 12 weeks after the discontinuation of probiotics will help reveal if the effects of the probiotic intervention last beyond its administration.

The decision to measure prediabetes remission using HbA1c rather than an OGTT is in-part due to the high OGTT intra-individual variability and problems with reproducibility. Also, if we used an OGTT to determine our outcome, given the high intra-individual variation and chance of measurement error, we might end up with a less accurate estimate of remission. Furthermore, the OGTT is more burdensome to participants, requiring patients to fast overnight whereas the HbA1c test does not require any preparation by participants.

The run-in period will help ensure that participants who demonstrated high-compliance be included in the study, thereby increasing our ability to detect a true effect. Similarly, compliance to the study intervention will be frequently assessed throughout the trial. The run-in period will also function to reduce the variability in participants' baseline dietary intake and will allow for co-interventions to be standardized and for their effects to be reduced.

Many of the existing probiotic trials had a mixed population of individuals with obesity, metabolic syndrome, gestational diabetes, prediabetes and T2D. Our carefully defined population will enable the results to be tested in a specific and comparable patient population in which probiotics may be efficacious.

A number of previously conducted trials also used inappropriate placebos such as yogurt. We selected an intervention which would ensure that the placebo is indistinguishable from the probiotic treatment in appearance and administration without conferring any potential benefits.

Although our blinded design will reduce risk of contamination and differential use of interventions in the treatment and control group, we will nonetheless be collecting dietary and physical activity data which are both potential co-interventions. This will allow us to assess whether a possible reduction in the rate of our outcome is due to the influence of co-interventions in both groups.

The selection of our secondary outcomes was done with the objective of exploring if probiotics may be conferring their effects through indirect pathways. For instance, both HOMA-IR and HOMA-B are surrogate markers of insulin resistance and beta-cell function respectively[129]. By measuring changes in these indices we are attempting to contribute data to the understanding of the pathophysiology underlying the probiotic-induced remission to normoglycemia.

The fecal analysis is also a valuable component of our investigation as it will enable us to identify whether probiotic supplementation results in shifts in the gut microbiota and whether any population shifts are associated with our primary outcome. This information will contribute to filling some of the gaps in knowledge related to probiotics.

There remains considerable uncertainty regarding effective probiotic strain combinations, concentrations and forms. Consequently, we are uncertain of whether the dose of probiotics we are administering is high enough to induce prediabetes remission. Even though our chosen concentration is one of the highest that we have seen in the literature, the number of organisms in the sachets may need to be even higher to induce remission of prediabetes.

Although one of our strengths is the minimal burden associated with the outcome determination using an HbA1c test at week 20, there is a chance that we may miss those that

change status from prediabetes to normoglycemia more than once throughout the trial (spontaneous remission and reverting back to prediabetes). While we won't be capturing cases of transient remission, its occurrence is advantageous to our participants considering that the achievement of normoglycemia at least once during an intervention has been associated with a 56% relative risk reduction in T2D in comparison to individuals who did not achieve remission[199]. Another trial observed a 75% lower incidence of T2D in 24 months compared to individuals who did not revert[20].

Another limitation of using HbA1c rather than OGTT is that we are unable to distinguish the basis of prediabetes as IGT, IFG or a combination of the two. The type of glucose impairment may result in differences in remission proportions as observed in other studies[132]. These differences are thought to be due to different mechanisms and pathophysiology underlying IFG and IGT. A reoccurring finding from various studies has been that HOMA-IR is increased in IFG but not IGT, and HOMA-B tends to be higher in IGT compared to IFG; however, this is often attributed to HOMA indices being more likely to reflect the pathophysiology of IFG than IGT[200–203]. Interestingly, in one trial, *both* HOMA-IR and HOMA-B were lower at baseline in participants with prediabetes that reverted compared to those that didn't, regardless of the type of glucose impairement[204]. The authors believe the lower HOMA-B indicates a lower demand on insulin secretion in those that reverted to normoglycemia[204].

Finally, the inter-individual variation in gut microbiota is one of the major limitations in any probiotic and microbiota-based research. It is not clearly understood how this variability may influence probiotic function, and ultimately our outcome of remission.

4.2. CLINICAL IMPLICATIONS AND KNOWLEDGE TRANSLATION

If our intervention proves to be effective, additional trials are needed to confirm our results. However, in the meantime, assuming the incidence of AE's in our trial is low, the use of VSL#3® can be initiated in individuals with prediabetes with minimal concern of harm. Trials that

assessed T2D diabetes remission found that a diabetes duration of 2 years or less since diagnosis was an important predictor of remission[205,206]. It is possible that prediabetes duration may also influence odds of remission to normoglycemia, meaning that probiotic therapy may be most effective if implemented when individuals are diagnosed.

The main knowledge users of our study include healthcare practitioners, researchers in the field and patients with prediabetes. We plan to disseminate our findings through a number of methods including study publication in a peer-reviewed endocrinology or diabetes-related journal as well as through oral and poster presentations in endocrinology and gastroenterology conferences and patient symposiums. We will also leverage dissemination through the professional and research networks of the study investigators who are dietitians, endocrinologists and a gastroenterologist, all of who are trained and involved in clinical epidemiology research.

4.3. GAPS IN KNOWLEDGE AND FUTURE DIRECTIONS

Many gaps remain in our understanding of the interactions between probiotics, gut microbes and health. The functions of many microbiota and the pathways through which these functions are carried out remain largely undetermined. There is an essential need for additional large, sufficiently-powered trials to be conducted with the aim of understanding the activities of gut microbiota, particularly in relation to metabolic disorders.

Regardless of whether our results show a benefit or not, further research is required to confirm the role of probiotics in the prevention of T2D as well as in remission to normoglycemia. Future trials should consider using a multi-arm design to test various strain combinations, concentrations, forms and durations. It is also necessary for trials to attempt to link health outcomes to microbiome changes in order to enhance our understanding of probiotic strains and their impact on host microbiome and health. Understanding the way different strains function will enable probiotic therapeutics to be tailored to specific diseases and populations and

will enhance their application to disease treatment and prevention. The potential for therapeutic probiotic use is limited to the strains that have been most frequently studied, namely *Lactobacillus* and *Bifidobacteria* due to their common and historical use in foods[207]. More attention should be centered on less commonly used strains that have shown promise rather than only focusing on the most commonly available strains.

The impact of lifestyle factors such as diet and physical activity on the host's microbiota is also not well understood. Understanding how shifts in microbiota can be manipulated to infer health benefits is vital to therapeutic developments.

If found effective for inducing prediabetes remission, probiotics should be investigated as an adjunct therapy in not only the management of T2D but also in T2D remission efforts. A number of trials have demonstrated the possibility of T2D remission using an intensive pharmacological approach in combination with lifestyle modification[208–210]. The addition of probiotics may act synergistically to propel and augment the diabetes remission process.

4.4. CONCLUSION

With the incidence of type 2 diabetes continuing to rise, there is an urgent need for heightened efforts to prevent the disease from occurring. Those with prediabetes are at highest risk of developing T2D and the current strategies to prevent the progression of prediabetes to T2D are not always feasible at the population level. The focus of diabetes prevention should be remitting individuals back to normoglycemia rather than maintaining them in prediabetes status. The need for alternative or adjunct methods to induce remission to normoglycemia is essential. The role of microbiota in metabolic processes presents the potential for therapeutic applications of probiotics. To the best of our knowledge, our trial is the first of its kind, investigating the effect of a multi-species probiotic on induction of drug-free prediabetes remission to normoglycemia. We hope that the results, regardless of what they may be, will prompt additional investigations that will add to the knowledge within this field. If successful,

probiotics would offer a therapeutic option for the remission of prediabetes and possibly the prevention of T2D that is simple, cheap and easy to incorporate into standard clinical care.

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APPENDIX 1: Changes in the intestinal microbiota associated with type-2 diabetes in human

Study group	Methodology	Microbiota change[a]		Reference
		Phylum or class	Genus or species	
Diabetics vs. nondiabetics ($n = 18$ vs. $n = 18$)	Real-time PCR	↑Betaproteobacteria		35
		↓Firmicutes (Clostridia)		
Diabetics vs. nondiabetics ($n = 16$ vs. $n = 12$)	Real-time PCR		↓*Bifidobacterium*	36
			↓*Bacteroides vulgatus*	
Diabetics vs. nondiabetic ($n = 71$ vs. $n = 74$)	Shotgun sequencing		↑*Bacteroides caccae*	
			↑*Clostridium hathewayi*	
			↑*Clostridium ramosum*	
			↑*Clostridium symbiosum*	
			↑*Eggerthella lenta*	
			↑*Escherichia coli*	37
			↑*Akkermansia muciniphila*	
			↑*Desulfovibrio*	
			↓*Clostridiales* sp. SS3/4	
			↓*Eubacterium rectal*	
			↓*Faecalibacterium prausnitzii*	
			↓*Roseburia intestinalis*	
			↓*Roseburia inulinivorans*	
Diabetics vs. nondiabetics ($n = 53$ vs. $n = 43$)	Shotgun sequencing		↑*Lactobacillus* spp.	38
			↓*Clostridium* spp.	
			↑*Clostridium clostridioforme*	
			↓*Roseburia*	
Prediabetics vs. nondiabetics ($n = 64$ vs. $n = 44$)	16S rDNA sequencing		↓*Akkermansia muciniphila*	39
			↓*Faecalibacterium prausnitzii*	
Diabetics vs. prediabetics ($n = 13$ vs. $n = 64$)	16S rDNA sequencing		↓*Bacteroides*	39

[a]Arrows indicate increases or decreases of each bacterial group in the disease subject group compared with the control group (either nondiabetic or prediabetic).

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APPENDIX 2: Randomized Controlled Trials of Probiotics in Prediabetes and Diabetes

Randomized controlled trials assessing effects of probiotics on glycemic outcomes in adults with prediabetes and type 2 diabetes						
Author, Year	**Population, Sample (n)**	**Duration**	**Intervention strain, concentration and duration**	**Control regimen**	**Outcomes**	**Statistically Significant Comparisons Within and Between Groups (control group - probiotic group)**
Andreasen, 2010	T2D/IGT/NGT n=44	4 weeks	Capsules L.acidophilus NCFM - 1g 10^{10} CFU/g	Placebo capsules	Insulin sensitivity (euglycemic clamp)	Insulin sensitivity decreased from baseline in the placebo group (p=0.03). Insulin sensitivity increased from baseline non-significantly in the probiotic group (p=0.09). Figures not reported.
Asemi, 2013	T2D n=54	8 weeks	1 Capsule/d 7 viable and freeze-dried strains: L. acidophilus (2 ×10^9CFU) L. casei (7x10^9CFU) L. rhamnosus(1.5 × 10^9CFU) L. bulgaricus (2×10^8 CFU) B.breve(2×10^{10} CFU) B. longum (7×10^9 CFU) S. thermophilus (1.5×10^9CFU) 100 mg fructo-oligosaccharide	Placebo capsules	HbA1c FPG Insulin HOMA-IR	**DIFFERENCE IN MEAN CHANGE IN FPG FROM BASELINE:** **Within control group** = 1.6 mmol/L (SD=2.44), (p=0.002) **Between groups**= 1.51 mmol/L (SE=0.57), (p=0.01) **DIFFERENCE IN MEAN CHANGE IN HOMA-IR FROM BASELINE:**

						Within probiotic group: 0.78 (SE=0.31), (p=0.02) **Within control group:** 2.38 (SE=0.65) (p=0.001) **Between groups**= 1.60 (SE=0.72) (p=0.03) **DIFFERENCE IN MEAN CHANGE IN INSULIN FROM BASELINE:** **Within probiotic group=** 2.04 ulU/ml (SE=0.82) (p=0.02) **Within control group=** 4.11 ulU/ml (SE=0.91) (p<0.001) No significant changes between groups.
Ejtahed, 2011	T2D n=60	6 weeks	Yogurt 300g/d L. bulgaricus Streptococcus thermophilus Enriched with: L. acidophilus La5 - $7.23x10^6$ and B. lactis Bb12 - $6.04x10^6$	Conventional yogurt with L. bulgaricus and Streptococcus thermophilus	FPG HbA1c Insulin	**DIFFERENCE IN MEAN CHANGE IN FPG FROM BASELINE:** **Within probiotic group=** -0.70 mmol/L (SD=1.38), (p<0.01) **Between groups=** 0.88 mmol/L (SD=0.26), (p<0.05)

						DIFFERENCE IN MEAN CHANGE IN HBA1C FROM BASELINE: **Within control group:** 0.30% (SD= 0.77), (p<0.05) **Between groups:** 0.42% (SD=0.2), (p<0.04) Results for FPG and HbA1c adjusted for duration of diabetes, polyunsaturated fat intake, and baseline values
Hove, 2015	T2D n=42	12 weeks	Yogurt 300ml/d L. helveticus	Yogurt that was artificially acidified with glucono-lactone 1.75%	FPG HbA1c HOMA-IR	**DIFFERENCE IN MEDIAN CHANGE FROM BASELINE IN FPG:** **Between groups =** 0.9 mmol/L,(p=0.022) Adjusted for age, esomeprazole use, baseline value. IQR not reported P-values for within group change were not reported, only between groups.
Judiono, 2014	Prediabetes and T2D n=106	30 days	Standard diet + 200 ml/d of clear kefir	Standard diet (not specified)	FPG 2hrBG	Mean difference in change between groups not reported, only p-values.

			35 probiotic bacteria in the kefir, strains not specified 10^7 CFU/g		Hba1c	**DIFFERENCE IN MEAN CHANGE IN FPG FROM BASELINE:** **Between groups:** p=0.015 **DIFFERENCE IN MEAN CHANGE IN HBA1C FROM BASELINE:** **Within probiotic group:** -0.13% (SD=0.15) (p<0.05) **Between groups:** (p=0.001)
Jung, 2013	Obese adults with FPG>5.55mmol/L n=62	12 weeks	2 capsules with each meal (TID) L. gasseri BNR17 10^{10} CFU	Placebo capsules	HbA1c FPG	**MEAN CHANGE IN HBA1C FROM BASELINE:** **Within control group=** -0.1% (SD=0.2) (p<0.05) No significant changes between groups.
Mazloom, 2011	T2D n=34	6 weeks	Capsules 1500mg capsule BID (after lunch and dinner) L. acidophilus, L. bulgaricus, L. bifidum, and L. casei CFU not specified	Placebo capsules	FPG HOMA-IR	No significant changes within and between groups.

Mohamadshahi,2014	T2D with BMI>25kg/m^2 n=42	8 weeks	Yogurt 300g/d Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus. ENRICHED WITH Bifidobacterium animalis subsp. lactis Bb12 (DSM 10140) and Lactobacillus acidophilus strain La5 3.7 × 10^6 CFU/g	Yogurt with Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus.	FPG HbA1c	**MEAN CHANGE IN HBA1C FROM BASELINE:** **Within Probiotic group:**-1.15% (SD= 0.27), (p=0.032) **Between groups=** 1.39 % (SE= 0.095), (p<0.001)
Ostadrahimi, 2015	T2D with FBG.6.9mmol/L, no insulin n=60	8 weeks	Fermented milk (Kefir) 600ml, lunch and dinner S. thermophilus L. casei, L. acidophilus and B. lactis 3.25 × 10^6 CFU	600ml conventional fermented milk (dough), lunch and dinner Streptococcus thermophilus Lactobacillus bulgaricus	FPG HbA1c	**DIFFERENCE IN MEAN CHANGE FROM BASELINE IN FPG:** **Within probiotic group:** -1.25mmol/L (4.10), (p=0.05) **Between groups =** 1.18mmol/L (SE=1.30), (p=0.03) Adjusted for baseline difference. **DIFFERENCE IN MEAN CHANGE IN HBA1C FROM BASELINE:** **Between groups=** 1.23mmol/L (SE=0.51),

						(p=0.02) Adjusted for serum levels of glucose, baseline values of HbA1c and energy intake.
Tonucci, 2015	T2D n=45	6 weeks	Goat milk 120g/d at breakfast l.acidophilus La-5, bifidobacterium animalis subsp lactis BB-12 (added to the placebo to make the intervention) 10^9 CFU/120g	Conventional fermented goat milk fermented with streptococcus thermophilus TA-40	FPG HbA1c HOMA-IR	**DIFFERENCE IN MEDIAN CHANGE IN HBA1C FROM BASELINE:** **Between groups =** 0.78%, (p=0.02) IQR not reported
Tripolt, 2014	Metabolic syndrome n=28	12 weeks	Milk drink (Yakult light ®) containing Lactobacillus casei Shirota, 65mL TID 10^8 CFU/mL	None	FPG 2hrPG HOMA-IR HOMA-B	No significant changes within or between groups.

FPG = fasting plasma glucose; HOMA-IR = homeostasis model assessment for insulin resistance; HOMA-β = homeostasis model assessment for β-cell function; 2hrPG= 2-hour post-prandial glucose

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APPENDIX 3: Study Visit Schedule

	STUDY VISIT SCHEDULE																
	Screening	Run-in week 1	Run-in week 2	Randomization	V1	V2		V3		V4		V5		V6			V7
Weeks after randomization:		-2	-1	0	2	4	6	8	10	12	14	16	18	20	24	28	32
ACTIVITY/ASSESSMENT																	
Voluntary signed consent	X																
Screening for eligibility criteria	X																
Urine BhCG*	X																
Serum creatinine	X																
Clinic visit	X	X	X	X		X		X		X		X		X			X
RD visit and assessment		X	X	X		X		X		X		X		X			X
3-day diet diary		X		X				X						X			X
Provide placebo sachets		X	X														
Randomization				X													
Start intervention				X													
Provide study sachets				X		X		X		X		X					
Stop intervention														X			
Anthropometrics (height**, weight, waist circumference)		X		X		X		X		X		X		X			X
Medical events and change in medications CRF		X		X		X		X		X		X		X			X
Activity questionnaire (GPAQ)				X				X						X			X
Telephone follow-up					X		X		X		X		X		X	X	
Adherence check			X	X	X	X	X	X	X	X	X	X	X	X			
Adverse event check			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
HbA1c	X													X			X
Fasting glucose and insulin				X										X			X
Fecal sample collection				X										X			X

*Urine BhCG test for women of child-bearing potential only

**Height will be measured at baseline only

APPENDIX 4: Sample Size Calculation

Test Comparing Two Proportions from https://www.stat.ubc.ca/~rollin/stats/ssize/b2.html

Inference for Proportions: Comparing Two Independent Samples

(To use this page, your browser must recognize JavaScript.)

Choose which calculation you desire, enter the relevant population values (as decimal fractions) for p1 (proportio
(assumed the same for each sample). You may also modify α (type I error rate) and the power, if relevant. After m

- (•) Calculate Sample Size (for specified Power)
- ( ) Calculate Power (for specified Sample Size)

Enter a value for p1: .08

Enter a value for p2: .16

- ( ) 1 Sided Test
- (•) 2 Sided Test

Enter a value for α (default is .05): .05

Enter a value for desired power (default is .80): .80

The sample size (for each sample separately) is: 258

APPENDIX 5: Alternative Sample Size Calculations

	Control group remission proportion	Probiotic group remission proportion	Power	n per arm	Total N (without 10% attrition)
Sample size calculation 1	5%	10%	80%	435	870
			90%	582	1164
Sample size calculation 2	8%	16%	80%	258	516
			90%	345	690
Sample size calculation 3	10%	20%	80%	199	398
			90%	266	532