Proposed mechanisms of probiotic *Lactobacillus rhamnosus* HN001 in prevention of gestational diabetes mellitus

A profile of dietary intake and metabolites among PiP study women

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Abstract

Background: Recent studies have reported significant beneficial effects of probiotics for prevention of Gestational Diabetes Mellitus (GDM). However, few studies have evaluated such effects in the context of background diet, specifically dietary fibre. Additionally, to date no studies have assessed the metabolome response to *Lactobacillus rhamnosus* HN001 supplementation in relation to GDM status and dietary fibre intakes. Therefore, this thesis will assess the dietary intake and metabolite profiles of women who participated in the Probiotics in Pregnancy (PiP) study.

Aims: To evaluate, 1) whether the dietary macronutrient intake and, 2) metabolite profiles of PiP study participants differed by probiotic HN001 exposure, dietary fibre intake during pregnancy and GDM development.

Methods: Participants were randomised at 14-16 weeks' gestation to receive HN001 ($6x10^9$ CFU) or placebo supplementation daily. Three-day food diaries and blood plasma samples were collected from participants at 26-28 weeks' gestation. The macronutrient content of the diets was determined using Foodworks 9. Metabolomics analysis was conducted by gas chromatography-mass spectrometry.

Results: Protein, fat, and carbohydrate (as % total energy) of the total PiP cohort were within the recommended guidelines for pregnancy. The dietary fibre intake of women diagnosed with GDM was significantly lower compared to women without GDM ($p = 0.027$). While statistically insignificant, the mean dietary fibre intake was lowest for women who were supplemented with HN001, yet still developed GDM (22.5±8.5g/d, $p = 0.637$) compared to all other groups. The GDM metabolome was associated with biomarkers of branch chain amino acid catabolism, the tricarboxylic cycle and inflammation. The HN001 metabolome was associated with biomarkers of tryptophan metabolism. High dietary fibre intake (≥25g/d) was associated with histidine, an anti-inflammatory biomarker.

Discussion: The success of probiotic supplementation in preventing GDM may depend on sufficient dietary fibre intake. Analysis of the metabolome of PiP study participants indicates that probiotic HN001 supplementation and high dietary fibre intake may prevent GDM by generation of anti-inflammatory metabolites including histidine and signalling of tryptophan metabolism.

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Statement of Contribution

This thesis utilised food diaries and plasma samples from the Probiotics in Pregnancy study.

The food diaries were entered into Foodworks 9 by Gabrielle Orr, who is the author of this thesis. Associate Professor Lindsay Plank assisted development and application of the statistical plan and where specified conducted the statistical analysis using SAS software.

Dr Elizabeth McKenzie conducted the metabolomics (GC-MS) assay for this thesis. The description of methods for the metabolomics analysis was provided by Raphael Bang. Statistical plans for the metabolomics analysis were determined by Raphael Bang and Associate Professor Rinki Murphy, in collaboration with the author. Raphael Bang conducted the statistical analysis and guided interpretation of the data for application in this thesis. Interpretation of the metabolites, in relation to GDM, dietary fibre and probiotic HN001 supplementation was completed by the author.

Statistical analysis (unless otherwise stated), graphs (with the exception of the heat maps) and tables were produced by the author.

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Abbreviations

AI	Adequate intake
ANOVA	Analysis of variance
BCAA	Branch chain amino acid
BMI	Body Mass Index
CFU	Colony Forming Units
CFU/d	Colony Forming Units per day
CHO	Carbohydrate
CI	Confidence interval
d	day
g	Gram
g/d	grams per day
GC-MS	Gas chromatography-mass spectrometry
GDM	Gestational Diabetes Mellitus
GI	Glycaemic index
GLUT-1	Glucose Transporter-1
GPR	G-coupled Protein Receptor
HUMBA	Healthy Mums and Babies trial
IADPSG	International Association of Diabetes in Pregnancy Study Group
IL-6	Interluekin-6
kJ	Kilojoule
LC-MS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharide
LysoPC	Lysophosphatidylcholine
MCF	Methyl Chloroformate
NGT	Normal glucose tolerance
NMR	Nuclear magnetic resonance

NOD	Nucleotide-binding Oligomerisation domain
NRV	Nutrient Reference Value
OGTT	Oral Glucose Tolerance Test
OR	Odds Ratio
PiP	Probiotics in Pregnancy
PRR	Pattern Recognition Receptor
PUFA	Polyunsaturated Fatty Acid
PYY	Peptide Tyrosine Tyrosine
RiPE	Research in Pregnancy Ethics study
RYGB	Roux-en-Y gastric bypass
SCFA	Short chain fatty acid
Spp.	Species
SPRING	Study of Probiotics in Gestation
TCA	Tricarboxylic acid cycle
T2DM	Type 2 Diabetes Mellitus
TE	Total energy
TLR	Toll Like Receptor
TMS	Trimethylsilyl
TNF-α	Tumour necrosis factor-α
%TE	Percentage total energy

Chapter 1 Introduction

1.1 Scope: Probiotics in Pregnancy study

This thesis is a mechanistic sub-study of the Probiotics in Pregnancy (PiP) study which focusses on diet and metabolomic profiling to evaluate the how *Lactobacillus rhamnosus* HN001 interacts with the host metabolism to prevent gestational diabetes mellitus (GDM). The PiP study was a two-centre, randomised, double-blind, placebo-controlled trial including 423 pregnant women, conducted in Wellington and Auckland, New Zealand (1,2). The aim of the PiP study was to assess whether probiotic supplementation by *Lactobacillus rhamnosus* HN001 ($6x10^9$ CFU/d) administered to mothers daily from 14-16 weeks' gestation until six-months postpartum, 1) reduces the prevalence of infant eczema and atopic sensitisation and, 2) reduces the prevalence of GDM, maternal bacterial vaginosis and maternal postpartum depression and anxiety (1). No specific dietary advice was given to participants. At 24-30 weeks' gestation participants were assessed for GDM using 75g oral glucose tolerance test glucose thresholds as per both the International Association of Diabetes in Pregnancy Study Group (IADPSG) (3) and New Zealand guidelines (4). As per the higher glucose thresholds by the New Zealand diagnostic criteria, the prevalence of GDM was significantly lower in the probiotic supplementation group (2.1%; 95% CI 0.6, 5.2) compared to the placebo group (6.5%; 95% CI 3.5, 10.9) (p = 0·03). When GDM was classified according to lower glucose thresholds by the IADPSG diagnostic criteria, there was a trend towards lower relative rates of GDM in women supplemented with HN001, however this result was not statistically significant (p = 0.08). When evaluating the sub-group of women at high risk of GDM, Wickens et al. (2) reported significantly lower relative rates of GDM, in women supplemented with HN001, with a history of GDM compared to the placebo (RR 0·00; 95% CI 0·00, 0·66, p = 0·004) and in women aged ≥35 years (RR 0·31; 95% CI 0·12, 0·81, p = 0·009). However, there were no significant differences in apparent protective effect of HN001 among those with a higher BMI ≥30kg/m^2 (2). The PiP study demonstrated that probiotic HN001 supplementation from 14-16 weeks' gestation may reduce the prevalence of GDM in New Zealand women, particularly among older women and those with a history of GDM during previous pregnancies (2). Therefore, by utilising the food diaries and blood plasma samples collected at 26-28 weeks' gestation this thesis will, 1) evaluate whether the dietary macronutrient intake and, 2) metabolite profiles of PiP study participants differed by GDM status, probiotic HN001 exposure and dietary fibre intakes in order to answer the question "*How does Lactobacillus rhamnosus HN001 protect against GDM and is this protection dependent on background dietary fibre intake?*"

1.2 Thesis Aims / Objectives

1. Dietary analysis
    a. Determine the dietary macronutrient (carbohydrate, fat and protein) and energy intake of women who participated in the PiP study in comparison to *Nutrient Reference Values for New Zealand and Australia* for pregnant women (19-50 years).
    b. Determine whether women with GDM had altered macronutrient intake compared with those who did not develop GDM (with or without probiotic HN001 supplementation).
    c. Determine whether those who received probiotic HN001 supplementation but still developed GDM had lower dietary fibre intake compared to those who did not develop GDM (with or without probiotic HN001 supplementation).
2. Metabolomics analysis
    a. To determine metabolites associated with GDM compared to pregnant woman with normal glucose tolerance (NGT) at 24-30 weeks' gestation.
    b. To determine metabolites associated with probiotic HN001 supplementation compared to placebo control.
    c. To determine metabolites associated with dietary fibre intake.
    d. To identify whether dietary fibre intake and probiotic HN001 supplementation produced metabolites that are associated with mechanisms that may contribute to prevention of GDM.

Chapter 2 Literature Review

2.1 Nutrition during pregnancy

Pregnancy is a period of time when maternal nutrition and lifestyle choices have the potential to impact the offspring's health during childhood and later in adulthood (5,6). It has been proposed that common non-communicable diseases such as type 2 diabetes mellitus (T2DM) originate in response to undernutrition in foetal life and infancy (7). Thus, the aim of nutrition during pregnancy is to foster a healthy intrauterine environment, such that overconsumption is limited for the mother and undernutrition is prevented for the foetus (7). This can be achieved by eating a varied healthy diet, the use of appropriate supplements (folic acid, iodine, iron (as required)), exercise, and avoidance of alcohol, smoking and high risk foods (5).

The Ministry of Health developed the *Food and Nutrition Guidelines for Healthy Pregnant and Breastfeeding Women* with the aim to provide evidence-based policy advice on nutrition, lifestyle and environmental determinants to ensure that all women in New Zealand maintain the best possible health for themselves and their infant during their pregnancy and while breastfeeding (8). The guidelines were developed with a priority to identify health inequalities that exist for women so that policies can be developed to address health inequities for New Zealand children from before birth (8).

The guidelines state the nutrient reference values (NRV) for key nutrients, including energy, fat, carbohydrate, protein and dietary fibre. These reference values are based on the *Nutrient Reference Values for New Zealand and Australia* determined in 2006 by a joint project with the *New Zealand Ministry of Health* and *Australian Commonwealth Department of Health and Ageing Project* (8). The research that informed these dietary guidelines largely focused on individual nutrients and related health outcomes (5,8). A brief outline of these recommendations and their rationale is outlined in Table 1.

As our diet consists of a large variety of nutrients from a range of foods, dietary information available to women during their pregnancy, such as the Ministry of Health *Eating for Healthy Pregnant Women* resource (9), provides practical advice for achieving the recommended intakes of nutrients. While the national healthy eating for pregnancy guidelines are available online and by lead maternity carers (9), data is lacking to demonstrate the level of nutritional input that women in New Zealand receive during their pregnancy.

Table 1 A brief outline of the New Zealand energy and macronutrient recommendations for women during their pregnancy.

	Nutrient Reference Value (8)				
Macronutrient	**1st trimester**	**2nd Trimester**	**3rd Trimester**	**Rationale**	**Practical Advice for Pregnancy** (8,9)
Energy	9200-9700kJ/d	Additional 1400kJ/d	Additional 1900kJ/d	Energy requirements increase during pregnancy for the synthesis of new tissue and the growth of existing tissue to support pregnancy and breast-feeding. While excess energy intake during pregnancy is associated with adverse outcomes (increased risk of GDM, preeclampsia, macrosomia) energy restriction also results in adverse outcomes for the mother and the foetus (e.g. congenital abnormalities, low birthweight, and metabolic syndrome conferred through foetal programming) (8,10).	Energy intake should be tailored individually according to pre-pregnancy BMI, gestational weight gain targets and daily physical activity .
Carbohydrate	45 - 65%TE or 175g/d†	45 - 65%TE or 175g/d†	45 - 65%TE or 175g/d†	Glucose, derived from carbohydrates, is the primary energy source for both the mother and the foetus. During normal pregnancy, the maternal metabolism adapts to maintain maternal euglycaemia and provide sufficient glucose to the foetus for growth and to prevent ketosis. Therefore, sufficient carbohydrate intake is vital to provide enough energy to the foetus (8).	- Eat at least six servings of breads and cereals each day (preferably wholegrain). - Also eat at least six servings of vegetables and fruit each day. - Increase fluid along with dietary fibre to maintain regular bowel habits.
Fibre	28g/d	28g/d	28g/d	Dietary fibre is increased in relation to increased energy needs during pregnancy of approximately 12% and for laxation. During pregnancy, high progesterone levels affect the smooth muscle tone of the gastrointestinal tract, resulting in reduced transit times. While this mechanism enhances nutrient absorption (as the contents are in the intestinal lumen for longer), it can result in constipation. Therefore, intake of adequate dietary fibre helps to maintain regular bowel habits during pregnancy (8).	

Macronutrient	Nutrient Reference Value (8) 1st trimester	2nd Trimester	3rd Trimester	Rationale	Practical Advice for Pregnancy (8,9)
Fat	20-35%TE (≤10% SFA)	20-35%TE (≤10% SFA)	20-35%TE (≤10% SFA)	During early pregnancy the foetus uses fatty acids supplied by the mother for fuel. Later in pregnancy, the foetus is able to produce its own fatty acids for fuel (8). In the third trimester, dietary intake of long chain PUFA are vital as nerve tissue growth is at a maximum (8). Dietary fat also enhances absorption of fat-soluble vitamins A, D, E and K (8).	- Limit intake of foods high in SFA, including red meats, fried foods, full fat dairy, coconut oil and butter. Instead replace with foods containing omega-3 long chain PUFA such as, tuna, sardines, salmon, wharehou and kahawai. - Choose low fat dairy products (≥3 servings per day).
Protein	15-25%TE or 0.75g/kg/d	15-25%TE or 1.0g/kg/d	15-25%TE or 1.0g/kg/d	Protein requirements increase during the second and third trimester of pregnancy to support foetal growth and maternal tissue synthesis (8).	- Eat at least two servings of lean meat, poultry, seafood, eggs, nuts and seeds or legumes per day. - Women who follow a plant-based diet should also ensure they eat wholegrain breads and cereals to provide sufficient amino acid intake, as plant-based protein sources are often lacking the amino acid present within wholegrain breads and rice.

Abbreviations: kJ/d = kilojoule per day; g/d = grams per day; %TE = percentage of total energy; BMI = body mass index; GDM = gestational diabetes mellitus; SFA = saturated fat; PUFA = polyunsaturated fatty acids; g/kg/d = number of grams per kilogram body weight per day.
†Set by the Institute of Medicine (11)

Diet is also heavily influenced by environmental, community and cultural aspects (12). Qualitative research from the *Research in Pregnancy Ethics* study (RiPE study), conducted in a small sample of women (*n* = 20) who participated in the PiP study, reported that women experience anxiety and worry around decisions about what they should eat and drink during pregnancy (12). This stems from the extensive amount of information available for pregnant women through social media, the internet or word of mouth; a proportion of which is misleading or unsupported by evidence (12).

Up-to-date data for the macronutrient intake of New Zealand women during their pregnancy is lacking; with (at the time of writing) the most recent data published by Watson et al. (13) who assessed the dietary intake of 504 Polynesian and European women from urban and rural, northern New Zealand. However, the primary objective of this study was to investigate the association of infant birthweight with dietary intakes and nutritional supplement use. Therefore, comparisons of the macronutrient intakes to the NRV's were not made in detail (13). In recent years, there has been a shift to assess dietary intakes as patterns rather than assessment of individual nutrients (5,14–16). This takes into account that food is consumed in various combinations and is heavily impacted by the environment (5). Surveys of the dietary patterns of New Zealand pregnant women have been conducted as part of the longitudinal *Growing Up in New Zealand* study (5,14). Dietary data was collected using a semi-quantitative 44-item food frequency questionnaire to evaluate the retrospective intake of certain food items over four weeks during the third trimester of pregnancy (5,14). Morton et al. (14) found that only 3% of pregnant women in New Zealand (*n* = 5664) adhere to the recommendations for all four food groups as stated by the dietary guidelines. The recommended daily number of servings of vegetables and fruit (≥6) were met by 25% of women; 26% met the recommendation for breads and cereals (≥6 servings); 58% met the milk and milk products recommendation (≥3 servings) and 21% of women met the recommendation for lean meat, meat alternatives and eggs (≥2 servings) (14). Further research conducted by Wall et al. (5) determined that adherence to dietary guidelines by participants in the *Growing Up in New Zealand* study was heavily dependent on education, BMI, and socio-demographic characteristics, including ethnicity. Participants who ate a "health conscious" dietary pattern, including high quantities of vegetables, wholemeal food items and high fibre cereal, were older women with a higher health literacy (5). In comparison, a "junk" food dietary pattern was significantly associated with decreasing maternal age, lower education levels, poor health prior to pregnancy and this dietary pattern was also more prevalent amongst Māori or Pacifica participants (5). The results of these New Zealand studies are in line with research exploring the nutrient intake of pregnant women in Australia and Canada. Whereby, the existing dietary recommendations do not align with the contemporary diets of women during their pregnancy (15,16).

The current evidence shows that a significant proportion of women are not adhering to dietary recommendations for pregnancy as outlined by the national guidelines (5,14–16). However, data is lacking to illustrate whether women in New Zealand are achieving the required macronutrient intakes for pregnancy regardless of their dietary pattern. Moreover, nutrition studies conducted by food frequency questionnaires must be interpreted with caution as they are prone to bias. This is due to reliance on retrospective collection of dietary information and are limited to recall of the foods as specified on the questionnaire (17). Therefore, this thesis will analyse 3-day food diaries completed by women during 26-28 weeks' gestation to determine whether women who participated in the PiP study are meeting the macronutrient requirements, regardless of the foods consumed, as specified by the *Nutrient Reference Values for New Zealand and Australia* for pregnant women (19-50 years) (8).

2.2 Introduction to gestational diabetes mellitus

GDM is commonly defined as "hyperglycaemia that is first detected in pregnancy" (4,18–20). However, according to the American Diabetes Association (19), GDM specifically refers to hyperglycaemia that is first detected in the second or third trimester of pregnancy, usually between 24-28 weeks' gestation. This latter definition takes into account the increasing prevalence of women with obesity during childbearing age, and consequently the number of women with undiagnosed T2DM pre-pregnancy (19). Contrary to GDM which develops during the second trimester of pregnancy and is expected to resolve after delivery, women who are screened for hyperglycaemia in the first trimester are diagnosed with T2DM, which is expected to remain after pregnancy (19,20).

2.2.1 Disease aetiology

Pregnancy induces changes to maternal carbohydrate, lipid and amino acid metabolism to ensure adequate energy and nutrients are continuously provided to the foetus (18). As such, progressive maternal insulin resistance naturally occurs, between 14 and 27 weeks' gestation (18,21–23). In healthy pregnancies, the pancreas responds by increasing secretion of insulin from β-cells by 200-250% to maintain maternal euglycaemia (21). However, in women with GDM, the capacity of the pancreas to produce and secrete enough insulin, relative to the degree of insulin resistance, is overwhelmed and maternal hyperglycaemia (GDM) ensues and typically manifests by 28 weeks' gestation (18,21).

2.2.2 Screening / diagnosis

There is no consistent international standard for screening and diagnosis of GDM (24). However, diagnosis is typically made following an oral glucose tolerance test (OGTT) at 24-28 weeks' gestation, i.e. second trimester of pregnancy (4). Table 2 describes the screening and diagnostic criteria most commonly used for population diagnosis and in research. As per the New Zealand clinical practice guidelines for pregnancy all women are also screened for elevated HbA1c (≥48mmol/mol) at their first antenatal bloods, ideally before 20 weeks' gestation, to rule out undiagnosed T2DM (4). Of relevance to this thesis, it is important to note that fasting plasma glucose thresholds for diagnosis of GDM are higher in New Zealand (4) compared to the IADPSG guidelines (3). Thus, the PiP study assessed GDM outcomes based on both of these thresholds and consequently detected a greater number of GDM cases using the lower IADPSG diagnostic thresholds (2). As such, GDM may be under detected in NZ by the use of the current fasting plasma glucose thresholds, therefore the New Zealand diagnostic guidelines for GDM are under debate (25).

Table 2 Diagnosis and screening criteria for GDM.

	New Zealand Guidelines (4)	**IADPSG** (3)	**WHO-1999** (20)	**Carpenter-Coustan** (26)	**ADA** (19)
Method of screening	Polycase test: 50g oral glucose challenge - Plasma glucose ≥7.8mmol/L referred for 75g OGTT. - Women with values ≥ 11.0 mmol/L diagnosed with GDM	-	-	-	Screen for pre-existing undiagnosed diabetes first prenatal visit for those with risk factors of GDM using OGTT
Diagnostic test	75g OGTT	75g OGTT	75g OGTT	50g OGTT	75g OGTT or 50g (non-fasting) measure followed by 100g OGTT for those who screen positive
Diagnosis of GDM					
Fasting plasma glucose	≥5.5mmol/L	5.1mmol/L	≥7.0mmol/l	≥5.0mmol/L	≥5.1mmol/L
1-hour post 75g OGL	-	≥10mmol/L	-	10mmol/L	≥10 mmol/L
2-hour post 75g OGL	≥9.0mmol/L	≥8.5mmol/L	≥11.1mmol/l	≥8.6mmol/L	≥8.5mmol/L
3-hour post 75g OGL	-	-	-	≥7.8mmol/L	-

Abbreviations: IADPSG = International Association of Diabetes in Pregnancy Study Group; WHO = World Health Organisation; ADA = American Diabetes Association; OGTT = oral glucose tolerance test; OGL = oral glucose load.

2.2.3 Epidemiology

International trends suggest that the prevalence of GDM is increasing globally, with prevalence spanning up to 45% in some populations, depending on ethnicity, genetics and diagnostic criteria used (24). The latest national statistics from 2014 observed the prevalence of GDM as occurring in 4.9% of all pregnancies in New Zealand, indicating a 13.9% increase ($p \leq 0.01$) between 2001-2013 (4). However, a recent study utilising data from the *Growing up in New Zealand* study found that GDM prevalence is likely higher at 6.2% of the population when taking into account multiple data sources (27). Of concern, this study also found that a third of women who were diagnosed with GDM as per their medical records were unaware of their GDM diagnosis (27). In New Zealand, the highest rates of GDM are recorded in women of non-European ethnicity, such as Asian (median 8.1%), Pacific (median 7.2%), Middle Eastern, Latin American and African (median 7.5%) ethnicities (4). A median rate of 3.3% is observed for Māori women, however this is likely an underestimate of the true prevalence (4). A recent study demonstrated that diagnostic screening for GDM is significantly lower in Māori and Pacific women compared to NZ European, European (other) or Asian women (28). A lack of screening or awareness of GDM diagnosis may negatively impact the health of both the mother and her infant. Therefore, accurate estimation of the prevalence of GDM is vital to ensure policy development, funding allocation and health service delivery, provides equitable care across New Zealand (27).

2.2.4 Maternal, postnatal and infant health risks

If left unmanaged, GDM can have adverse outcomes for both the mother and the foetus. In pregnancy, glucose is transferred from the mother to the foetus across the placenta via facilitated diffusion (18). Therefore with maternal hyperglycaemia, the foetus is also exposed to a hyperglycaemic environment *in utero*. The foetus adapts to the increased glucose load by upregulating insulin, however this response can lead to hyperinsulinemia in the foetus, resulting in a number of short term and long term health complications (18,29).

In the short term, increased energy availability to the foetus results in macrosomia, a condition whereby the infant is large-for-gestational age. This increases the risk of delivery complications including the need for caesarean section and shoulder dystocia (29,30). Infants are also placed at higher risk of respiratory distress syndrome, as insulin inhibits the production of surfactant in the foetus so they are born with underdeveloped lungs (18). The infant may also experience hypoglycaemia and hyperbilirubinemia at birth (18,29). In the long term, metabolic dysregulation *in utero* can cause damage to the foetal pancreatic β-cells and increases the risk of metabolic syndrome (obesity, diabetes, and cardiovascular disease) for the infant later in life conferred through foetal

programming (7). This perpetuates the “vicious diabetes cycle” (31), whereby the offspring is placed at higher risk of developing T2DM or GDM. This trans-generational effect of GDM places significant emotional, physiological and economic burden on both the mother and her offspring (31).

Women with GDM also have increased risk of preeclampsia which can lead to further complications for both the mother and the infant including acidosis and premature birth (18,29). Additionally, due to the metabolic dysfunction driven by GDM, over half of mothers with GDM during their pregnancy develop T2DM within 10 years and are at higher risk of GDM in subsequent pregnancies (32).

2.2.5 Risk factors for GDM

While the exact pathogenesis of GDM remains to be clearly defined, it is thought that GDM is caused by a combination of abnormal pregnancy-induced factors, low-grade inflammation related to excess adipose tissue, genetics, and the environment (21). Maternal insulin production is placed under immense strain during pregnancy. Therefore, women who are diagnosed with “pre-diabetes” or with obesity, who are overweight BMI ≥25, or whom experience excessive gestational weight gain are at greatest risk of developing GDM (4,18,21). There are several other known risk factors for GDM including, being a member of a high risk ethnic-group (Indo-Asian, Māori, Pacific, Middle Eastern), increasing maternal age (≥35 years) and a family history of diabetes (4,18). However, some women will be diagnosed with GDM without exhibiting any of the known risk factors (21).

There is an abundance of literature demonstrating that a poor diet, preconception and during pregnancy, increases the risk of GDM. This includes high consumption of sugar-sweetened beverages, fried food, animal fat, dietary cholesterol, red and processed meat, foods with a high-glycaemic load, and a low intake of cereal fibre or carbohydrates (33). Likewise, a collection of studies have investigated the relationship between key macronutrients and risk of GDM (see Table 3). High intakes of protein and fat from animal products, especially red and processed meat, were associated with an increased risk of GDM (34–36) Conversely, high intakes of vegetable proteins and an increase of dietary fibre lowered the risk of GDM (35,37).

A large body of this research is from dietary data collected by food frequency questionnaire as part of the Nurses’ Health Study II (35–38). While, this comprehensive prospective cohort study enabled the investigation of macronutrient intakes of a large cohort of women, it is limited in its application to other ethnicities as the majority of participants were of American Caucasian ethnicity. Furthermore, this study assessed the diet of women preconception, thus was not able to account for changes (if any) to the dietary intake of women during their pregnancy (35–38). However, notably, three studies assessed the macronutrient intake of pregnant women in the second trimester of pregnancy and

reported consistent results (34,39,40). Therefore, based on this research, we hypothesised that women who developed GDM in the PiP study had high fat intake and low dietary fibre intake, compared to women who did not develop GDM, irrespective of probiotic HN001 supplementation.

Table 3 Prospective cohort studies evaluating the association between macronutrient intakes and risk of GDM.

Ref.	Macronutrient	Participants	Study Design	Macronutrient and GDM Risk
Pang et al. (34)	Protein	980 multi-ethnic Asian women from the Growing Up in Singapore Toward health Outcomes study.	Dietary Assessment: 24-hour recall and 3-day food diary at 26-28 weeks' gestation. GDM Diagnosis: WHO-1999 criteria	Animal protein (OR 2.87; 95% CI 1.58-5.20; p = 0.001) and vegetable protein (OR 1.78; 95% CI 0.99-3.20; p = 0.009) both associated with increased risk of GDM.
Bao et al. (35)	Protein	15,294 women from the Nurses' Health Study II cohort between 1991-2001	Dietary Assessment: Pre-pregnancy dietary assessment using semi-quantitative FFQ GDM diagnosis: self-reported	High intake (~18.58% TE) of animal protein was associated with increased risk of GDM (OR 1.49; 95% CI 1.03–2.17; p = 0.013). High intake (~6.36% TE) of vegetable protein associated with lower risk of GDM (OR 0.69 95% CI 0.50–0.97, p = 0.034). Substituting a red meat protein source for a plant-based protein source reduced the risk of GDM by 51% for nuts and 33% for legumes (p <0.05).
Bowers et al. (36)	Fat	13,475 women from the Nurses' Health Study II cohort between 1991-2001	Dietary Assessment: Pre-pregnancy dietary assessment using semi-quantitative FFQ GDM diagnosis: self-reported	Higher intakes of animal fat and cholesterol was significantly associated with increased GDM risk (p <0.05). PUFA, MUFA and trans-fat intake was not associated with GDM risk.
Zhang et al.(37)	Fibre	13,110 women from the Nurses' Health Study II cohort between 1991-2001	Dietary Assessment: Pre-pregnancy dietary assessment using semi-quantitative FFQ GDM diagnosis: self-reported	Every 10g/day increase of dietary fibre reduced the risk of GDM by 26%. Every 5g/day increase in dietary fibre from fruit and cereal was associated with 26% and 23% reduction in risk of GDM, respectively.
Bao et al. (38)	CHO	21,411 women from the Nurses' Health Study II cohort between 1991-2001	Dietary Assessment: Pre-pregnancy dietary assessment using semi-quantitative FFQ GDM diagnosis: self-reported	Low CHO with concurrent high protein and fat from animal sources associated with increased risk of GDM (p <0.05). Low CHO with concurrent high protein and fat from plant sources not associated with risk of GDM.

Ref.	Macronutrient	Participants	Study Design	Macronutrient and GDM Risk
Saldana et al. (39)	Fat CHO Protein	1698 women from the Pregnancy, Infection and Nutrition Study	Dietary Assessment: FFQ (24-29 weeks' gestation) GDM Diagnosis: Carpenter& Coustan criteria	CHO intake of approx. 51 $\pm$ 7.1%TE positively associated with GDM (p <0.001) Total fat intake of approx. 35 $\pm$ 5.9%TE positively associated with GDM (p = 0.001) Protein and energy not associated with GDM.
Ley et al. (40)	Fat CHO Protein	205 pregnant women in Toronto, Canada	Dietary Assessment: FFQ (24-29 weeks' gestation) GDM Diagnosis: National Diabetes Data group	CHO intake of approx. 49.6 $\pm$ 6.2%TE and total fat intake of approx. 37 $\pm$ 5.2%TE positively correlated with GDM risk (p <0.05). SFA and low intakes of fruit and vegetable fibre positively correlated with fasting glucose (p <0.05).

Abbreviations: GDM = gestational diabetes mellitus; OR = odds ratio; CI = confidence interval; FFQ = food frequency questionnaire; CHO = carbohydrate; SFA = saturated fat; TE = total energy.

2.2.6 Current clinical guidelines for treatment of GDM

In New Zealand, women diagnosed with GDM are offered ongoing treatment throughout the remaining duration of their pregnancy and as required postpartum through the *Diabetes in Pregnancy* services (4). The first line of treatment is nutrition counselling by a registered dietitian (4). Research has shown that nutrition counselling combined with physical activity is effective for promoting euglycaemia (41,42). Thus, reducing the need for pharmacological treatment and improving perinatal outcomes (41–43). Dietitians focus on recommending dietary and lifestyle adjustments that are achievable by the patient to meet their glycaemic targets, receive adequate nutrition and achieve appropriate weight changes or maintenance (4,18,42). Currently, evidence-based nutrition practice guidelines for management of New Zealand women with GDM are unavailable. Therefore, a cross-sectional online survey completed by New Zealand registered dietitians whom provide nutrition counselling for women with GDM was undertaken in 2015 (44). The survey examined the nutrition interventions and management of GDM provided by dietitians in New Zealand, to guide the development of specific New Zealand evidence-based nutrition practice guidelines. The study found that a significant proportion of dietitians in New Zealand felt that the service within which they worked offered inadequate dietetic input for women with GDM (44). Despite national (4) and international (45) guidelines emphasising the importance of nutritional counselling in the GDM management, not all women diagnosed with GDM or whom were referred were seen for dietetics services in New Zealand (44). Additionally, 50% of dietitians reported that they only saw women once during their pregnancy after diagnosis of GDM (44). However, international evidence based guidelines emphasised that women should be seen within one week of diagnosis and at least three times post diagnosis (44,45).

The current nutrition recommendations by the Ministry of Health, New Zealand (4,46), state that women with diabetes during pregnancy (including GDM) should:

- Consume ≥175g of carbohydrate per day, spread evenly throughout the day.
- Reduce their intake of saturated fats
- Consume lean protein
- Energy intake should be no less than 1800kcal/day (7530kJ) to avoid malnutrition for both mother and foetus
- Maintain gestational weight gain in accordance with Ministry of Health recommendations.

Despite the stated nutrition recommendations, Lawrence et al. (44) found through their survey that there were inconsistencies in the dietetic management of GDM. As reported by New Zealand dietitians, topics frequently covered during nutrition consults include general healthy eating advice, pregnancy weight gain recommendations and label reading (44). There is some evidence to suggest that high glycaemic index (GI) foods contribute to elevated maternal glucose (18). Therefore, in some cases nutrition education regarding the role of carbohydrate quantity, quality and distribution (including low / high GI foods) in GDM may be appropriate (18,45). It is important to note that dietitians in New Zealand are trained to provide culturally appropriate, patient-centred nutrition care, thus some variability between nutrition interventions is to be expected. However, the survey by Lawrence et al. (44) highlighted the need for locally relevant and a consistent systematic approach to dietary interventions to protect the health of pregnant women and their infants. When GDM cannot be controlled with dietary and lifestyle intervention alone, women should be offered additional pharmacological therapy with oral hypoglycaemics and/or insulin (4).

2.3 Current prevention strategies for GDM

As described in section 2.2.4, the health effects of GDM have the potential to place a considerable long-term burden on individuals, communities and the New Zealand health care system (4). Therefore, the development of effective and sustainable preventative measures for GDM is crucial. As previously mentioned, all women in New Zealand are offered screening for diabetes (i.e., an HbA1c test) at their 'booking' antenatal bloods, within the first trimester (4). Women with an HbA1c between 41-49mmol/mol are considered to have prediabetes and are at high risk of developing GDM, therefore are offered tailored dietary and lifestyle advice through the *Diabetes in Pregnancy* service (4,47). All other women are provided standard pregnancy care and are encouraged to maintain a healthy diet during their pregnancy based on the Ministry of Health food and nutrition guidelines for pregnancy (Table 1) (4).

However, with the increasing prevalence of GDM (24) current standard pregnancy care is arguably inadequate for preventing GDM. As previously discussed (section 2.2.5), poor diet including high intakes of protein and fat (34–36) and low intakes of carbohydrates, including dietary fibre (37), are significant dietary risk factors for GDM. As dietary intervention is deemed the cornerstone of management for diagnosed GDM (4), it is logical that dietary interventions would also be effective for primary prevention of GDM.

Pregnancy is considered a powerful "teachable moment" for diet and lifestyle behaviour change as pregnant women are generally concerned about the health and well-being of their offspring (48). Additionally, dietary modification during pregnancy is also deemed safe, is accessible and is well-accepted by women (48). A comprehensive meta-analysis of 20 randomised control trials (n = 6444 women) assessed diet intervention or mixed-approach (diet and exercise) for prevention of GDM. Diet interventions, included individualised healthy eating advice according to national guidelines, calorie restriction and limiting glycaemic load. Diet interventions alone showed a 33% reduction in GDM, however, the trend was statistically insignificant (p = 0.15). When controlled for studies that assessed diet intervention alone, in high-risk groups, a significant reduction in GDM was observed for women who were obese or overweight (p = 0.05) (49). Furthermore, a Cochrane systematic review assessed 23 randomised control trials (n = 6633 women) and concluded that there was *moderate*-quality evidence supporting the role of diet and exercise combined for prevention of GDM compared to standard pregnancy care (50). However, it is likely that the success of these trials is due to the provision of personalised nutrition counselling by trial dietitians and nurses in 16 of the included studies. Tailored exercise advice was also provided by physiotherapists in two of the included trials (50). Collectively, these studies support the effectiveness of individually tailored nutrition and fitness

advice compared to standard care for prevention of GDM (50). Another recent Cochrane systematic review of 11 randomised control trials (*n* = 2786) reported that evidence for dietary advice alone compared with standard care was regarded as *very-low* to *low* grade evidence (33). Dietary advice compared in these reviews included low GI vs moderate-to-high GI foods and inclusion of high fibre foods compared to standard dietary advice (33). One study investigated inclusion of food items high in dietary fibre, however this intervention was not provided until 27 weeks' gestation. As diagnosis of GDM is made between 24-28 weeks' gestation, this study provided dietary intervention too late to have an preventative effect for GDM (51).

The lack of *high*-quality evidence for interventions to prevent GDM is likely owing to a number of factors. Firstly, many studies included within these systematic reviews were limited by issues with study quality including blinding, selective reporting and attrition (49,50). Moreover, dietary and lifestyle intervention studies are prone to non-compliance and often rely on subjective, self-reported data (49). Finally, while dietary modification during pregnancy is deemed well-accepted by women (48), dietary and lifestyle modification is heavily influenced by BMI, socio-demographic characteristics, food availability, and often requires a significant level of behaviour change (14,50,52).

Personalised nutrition and exercise counselling has been shown in a number of studies to reduce the risk of developing GDM (33,49). However, our current New Zealand public health care system is unable to support personalised nutrition counselling for all women during their pregnancy. Therefore, there is an urgency for prevention strategies that are accessible to all women, yet also take into account individual and community needs. The results of the PiP study indicated that probiotics may be a novel accessible approach for prevention of GDM which was achieved without any specific dietary intervention.

2.4 Novel preventative target for GDM: the gut microbiota

2.4.1 Introduction to the gut microbiota

The human gut is host to a diverse and dynamic community of trillions of micro-organisms, including bacteria, fungi, archaea, viruses and protozoans (53). These microbes outnumber the cells of the human host by a factor of ten to one and their collective genomes contain greater than 100 fold more genes than the human genome (53). This complex ecosystem constitutes the gut microbiota (53,54). Colonisation of the gut microbiota is strongly influenced by 1) mode of delivery (caesarean or vaginal) due to exposure to vaginal microbes during labour; 2) whether the infant is breast-fed or formula-fed; and 3) administration of antibiotics or probiotics early in life (55,56). Studies also suggest that diet and exposure to certain microbes during the perinatal period may influence the offspring's metabolic and immunologic profiles *in utero* (55). The transition to the "adult" microbiome begins as the infant transitions to solid food and by 2-5 years of age the unique microbiome for an individual is established (53,56). Although the number and diversity of the "adult" gut microbiota remains relatively constant throughout life, the environment, diet, antibiotic and/or probiotic use is thought to impact the composition of the gut microbiome (53,57).

During the past two decades there has been an increasing recognition that a mutualistic host-microbe relationship exists, such that a significant body of research is exploring the role of the gut microbiota in health and disease (53). Gut bacteria have been found to play an important role in the modulation of inflammatory processes and the immune system, maintenance of the gut mucosal barrier, nutrient and drug metabolism, energy homeostasis, lipid metabolism and protection against pathogens (54). Evidence suggests that an imbalance or maladaptation of the intestinal microbiota, i.e. 'dysbiosis', contributes to various human diseases including inflammatory bowel diseases and irritable bowel syndrome, metabolic diseases such as obesity and diabetes, allergies, neurological disorders and mental illness, and some cancers (53,54,57). Therefore, research exploring gut microbiota profiles that are causally attributed to prevention and treatment of disease is an active area of research.

The human gut microbiota is dominated by five major bacterial phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia*) and one Archaea (*Euryarchaeota*); however greater than 5000 bacterial species have been identified (54,58). Studies in both mouse models and humans have reported that changes in the ratio of *Firmicutes* to *Bacteroidetes* are significantly associated with obesity and glucose regulation (59,60). Two studies comparing the gut microbial profiles of individuals with T2DM compared to individuals with normal glucose tolerance showed that individuals with T2DM had significantly lower levels of *Bifidobacterium (Actinobacteria* phylum*), Firmicutes* and class

Clostridia (60,61). Furthermore, metagenomic analysis of the microbiome of women with GDM (*n* = 43) compared to women without GDM (*n* = 81), at 21-29 weeks' gestation, demonstrated that women with GDM have less *Bifidobacterium* spp., *Alistipes* spp., *Roseburia* spp., and *Eubacterium* sp. present in their microbiome (62). Significantly, gram-negative bacteria (e.g. *Bacteroidetes* and *Klebsiella variicola*) were found in higher abundance in women with GDM and were positively correlated with high blood glucose levels (62). Functional analysis of the microbial pathways of women with GDM demonstrated that these bacteria were associated with lipopolysaccharide (LPS) biosynthesis and energy metabolism pathways, including regulation of glucose levels (62). However, this study only analysed one stool sample and had a larger number of women without GDM affecting statistical comparisons (62).

Collectively, this research suggests that dysbiosis of the gut microbiota, i.e. reductions of *Bifidobacterium* spp. and *Firmicutes* and an increase in *Bacteroidetes*, is detrimental to glucose regulation and may contribute to the pathogenesis of GDM and T2DM (60–62). The *Firmicutes* phylum contains the well-studied genera *Lactobacillus*, several strains of which are probiotics (58). As both *Bifidobacterium spp.* and *Firmicutes* are reportedly lower in people with GDM and T2DM, research investigating the therapeutic role of *Lactobacillus* and *Bifidobacterium* as probiotic supplements is ongoing.

2.4.2 Manipulating the gut microbiota

2.4.2.1 *Probiotics*

Probiotics are defined as "live microorganisms that when administered in adequate doses confer a benefit to the host" (63). With growing evidence linking dysbiosis with insulin resistance, probiotics are an attractive option for prevention of GDM as compliance has been shown to be better compared to dietary change (64). Probiotics are naturally present in fermented food, such as yoghurt, kefir, kimchi and sauerkraut; and supplements are now also readily available (65). Supplements containing *Bifidobacterium* sp. and *Lactobacillus* sp. are the most common commercial and researched probiotic supplements used due to their safety, efficacy and ability to reach the colon intact (65). These species are also well-tolerated and not linked to adverse pregnancy outcomes (64). However, as the composition of the "adult" gut microbiota is thought to remain relatively constant throughout life and has enormous inter-individual variation (56), the impact of probiotic supplements on the gut microbiota and the hosts metabolism is under debate.

For a probiotic supplement to have an effect, it must first be able to withstand gastric acid, biliary and pancreatic secretions in order to reach the intestines intact (66). Currently, screening of faecal samples is the only method available to assess whether probiotics have survived the journey through the gastrointestinal tract (65). However, a recent systematic review reported that probiotic supplementation did not have an effect on the faecal microbiota composition in healthy individuals in six of the seven included randomised control trials (66). However, there is some evidence to suggest that probiotic supplements may have a restorative effect when dysbiosis is present (66,67). This effect has been demonstrated by shifts in the composition of the faecal microbiota and measurable effects on the hosts physiology following probiotic supplementation in disease states (67). Furthermore, studies in humans and a murine model demonstrated that the gut mucosal microbiome does not entirely correlate with the faecal microbiome (68). These studies indicate that probiotics may make subtle changes to the gut mucosal microbiome that are not able to be detected by metagenomic sequencing of stool samples (66,68).

Another major challenge of probiotic supplementation is the ability of the probiotic to compete with existing bacteria within the gut to establish a niche (69,70). This phenomenon is referred to as *colonisation resistance* or *competitive exclusion* (69). While research suggests that colonisation may not be necessary for a probiotic to confer benefits to host metabolism, probiotic intervention studies tend to have positive results with longer intervention periods (69). This may be due to the ability of the probiotic to interact with an individuals' gut microbiota to produce an accumulative effect on the hosts metabolism (69).

Finally, it is important to note that the biological effects of probiotics are *strain specific*; such that research of one strain cannot be extrapolated to another (70). Douillard et al. (71), demonstrated this by comparing 100 strains of *Lactobacillus rhamnosus* obtained from both human and dairy sources. Genetic differences between one strain to the next, displayed very different phenotypic characteristics such as bile acid resistance and carbohydrate transport and metabolism (71). This emphasises that the physiological effects of one specific strain of probiotic should not be generalised to all probiotics of the same species name.

As previously discussed, studies have suggested that individuals with T2DM or GDM have lower proportions of *Firmicutes* phyla (*Lactobacillus* spp.) and *Bifidobacterium* spp. in their gut microbiota (60–62). Therefore, several randomised control trials have investigated the effect of specific strains of *Lactobacillus* and *Bifidobacterium* probiotic supplements for prevention and treatment of GDM, as discussed in section 2.5. Of these studies, Wickens et al. (2) demonstrated that *Lactobacillus rhamnosus* HN001 supplementation reduced the risk of GDM in the PiP study. As previous studies

have reported that metagenomic sequencing is potentially inadequate to demonstrate the functional capacity of HN001 within the gut microbiome (as the effects of its supplementation may be too subtle) (72). Metabolomics is a novel approach to investigate the effect of probiotic supplementation by systematic measurement of collective metabolites in the host (73), potentially produced by interaction of HN001 with the gut microbiota. This thesis will therefore use a metabolomics based approach to determine the net effect of HN001 on the plasma metabolome of PiP study participants.

2.4.2.2 *Diet*

Globally, different diets among different populations, have led to divergence in the taxonomy of the gut microbiota. Thus, diet is considered the most important determinant for the diversity and composition of an individual's gut microbiota throughout their lifetime (54,56,57). In a landmark study, De Filippo et al. (74) compared the faecal microbiota of a small group of children living in a rural African village in Burkina Faso (n = 15) with those living in Florence, Italy (n = 15). The gut microbiota of Burkina Faso children showed significant enrichment in phyla of bacteria (*Prevotella*, *Xylanibacter*) that are known to contain bacterial genes for cellulose and xylan hydrolysis. In addition, the researchers found significantly higher levels of short-chain fatty acids (a by-product of the fermentation of dietary fibres) in Burkina Faso children ($p \leq 0.001$) (74). Therefore, it is reasonable to conclude that the gut microbiota of Burkina Faso children has evolved to digest a diet rich in plant-based foods allowing them to maximise energy intake from dietary fibre; compared with European children who consume a "Western" diet, high in animal protein, sugar, and fat, and low in dietary fibre (74).

Both animal models and human studies have shown that even short-term changes in one's habitual diet can have a substantial impact on the composition of the gut microbiota (53,54,75). Turnbaugh et al. (75) used a humanised gnotobiotic mice model to demonstrate that switching from a low-fat, plant polysaccharide rich diet to a "Western" diet, high in fat and sugar, shifted the composition of the gut microbiota within a single day (75). Studies in humans have produced similar results. Lawrence et al. (76) conducted a short-term diet intervention cross-over trial, whereby participants were provided a precisely controlled diet based on animal products or a plant-based diet for four days. Four day administration of an animal product based diet increased the abundance of bile-tolerant microbes (*Alistipes*, *Bilophila* and *Bacteroides*) and decreased the levels of *Firmicutes*. Interestingly, consuming a plant-based diet produced opposite effects (76). Furthermore, a 10-week cross-over trial whereby 14 overweight men received precisely controlled diets high in resistant starch and non-starch polysaccharides or a reduced carbohydrate weight loss diet, showed that changes to the gut microbiota occurred rapidly and were reversed equally rapidly by changes in dietary intervention (77).

While the composition of the gut microbiota changes in response to diet, it is likely that physiological changes induced by the gut microbiome occur over longer periods of time driven by changes to the hosts metabolome.

2.4.2.3 *Prebiotics*

While the majority of studies investigating the influence of diet on the gut microbiota have been conducted in animal studies or small, controlled, intervention studies, clear indication exists that a diet rich in fruits, vegetables and wholegrains is associated with a "*healthy*" gut microbiota (54). This is owing to the dietary fibres present in plant-based foods, i.e. *the prebiotic concept* (78). Prebiotics are defined as "non-digestible plant-derived carbohydrates that act as a fermentation substrate within the colon, stimulating the preferential growth and activity of a limited number of microbial species that confer health benefits on the host" (78). To date, all known prebiotics are dietary fibres, primarily inulin-type fructans (inulin, oligofructose and fructo-oligosaccharides) and galactans (galacto-oligosaccharides) (78). Animal studies have shown that prebiotics promote the proliferation of bacteria that effectively metabolise dietary fibres, e.g. *Bifidobacterium* and *Lactobacillus* (57,79). Furthermore, studies have shown that concurrent administration of prebiotics enhances the efficacy of the probiotic (79). The end products of fermentation of prebiotics by certain microbes are carbon dioxide (CO_2), hydrogen (H_2) and short chain fatty acids (SCFA) (79). Animal and human studies have shown that production of SCFA play a major role in diabetes (GDM and T2DM) (79).

2.4.3 Mechanisms underpinning the efficacy of probiotics and prebiotics in preventing diabetes

Based on research in animal models and humans it is hypothesised that concurrent administration of probiotics and prebiotics enhances the abundance of SCFA (79). This results in changes to energy harvest and satiety, inflammation and intestinal permeability and ultimately, the metabolic regulation of glucose by insulin (79). Figure 1 summarises the proposed mechanisms of prebiotics and probiotics in relation to the development of T2DM and GDM.

2.4.3.1 *Energy harvest and hormone regulation*

Fermentation of prebiotics by resident gut microbiota produces SCFA: lactate, acetate, propionate, and butyrate (79). Butyrate provides energy for intestinal cells; and acetate and propionate are used as substrates for gluconeogenesis and lipogenesis by the liver and peripheral organs (57,58). Furthermore, SCFA play a role in metabolic regulation through signalling G-protein-coupled receptors, namely GPR41 and GPR43. Activation of GPR41 and GPR43 signals intestinal expression of peptide

tyrosine tyrosine (PYY) and glucagon-like peptide-1 (GLP-1). Both PYY and GLP-1 are gut hormones that participate in the regulation of appetite and glucose metabolism, respectively. PPY slows intestinal transit increasing feelings of satiety, thus decreasing energy intake. GLP-1 stimulates proliferation of β-cells in the pancreas and enhances secretion of insulin (57,58).

2.4.3.2 *Inflammation and intestinal permeability*

It is recognised that systemic inflammation accompanies T2DM and GDM (21). Maintenance of the intestinal epithelium is thought to be integral to this. The intestinal epithelium is one cell layer thick, with cells bound together by tight junctions (80). When there are "gaps" between the cells, components of the gut microbiota, including LPS and peptidoglycans, can translocate into systemic circulation (58,79,80). LPS are a component of gram negative bacterial cell walls, that are known to activate macrophages and increase proinflammatory cytokines (IL-6 and TNF-α) (79). Similarly, peptidoglycan (another component of bacterial cell walls) bind to NOD (nucleotide-binding oligomerization domain) receptors, also signalling proinflammatory cascades (58). The activation of various immune cascades results in apoptosis, β-cell dysfunction and consequently insulin resistance in peripheral tissues (58,79,81). Several studies have demonstrated that individuals with T2DM have serum endotoxin levels (i.e. LPS) 2-fold greater than individuals without T2DM ($p \leq 0.001$) (82), thus supporting this mechanism as an integral component of diabetes pathogenesis.

Significantly, intestinal permeability is influenced by diet. Mice fed with high-fat diets exhibited significant levels of serum endotoxins and pro-inflammatory cytokines. However, when administered prebiotics the integrity of the gut epithelium improved and the circulating levels of LPS and pro-inflammatory cytokines reduced (57). These findings are supported by human studies, whereby individuals with T2DM and GDM who were administered prebiotics exhibited reduced inflammatory cytokines (83). It is hypothesised that the production of SCFA, as a result of microbial fermentation in the gut, improves the integrity of the intestinal epithelium by upregulation of transcription of tight junction proteins (53,58,79). SCFA signal glucagon-like peptide 2 (GLP-2) to increase proliferation of crypt cells, while PPAR-γ activation reduces inflammation in colonic epithelial cells (58,79). As a result, maintenance of the intestinal epithelium prevents inflammatory components, such as LPS and peptidoglycan, being released into circulation thus reducing systemic inflammation (80).

Based on these mechanisms, this thesis will explore whether the beneficial effect of probiotic HN001 supplementation, observed in the PiP study, was dependent on concurrent high dietary fibre (i.e., prebiotic) intake.

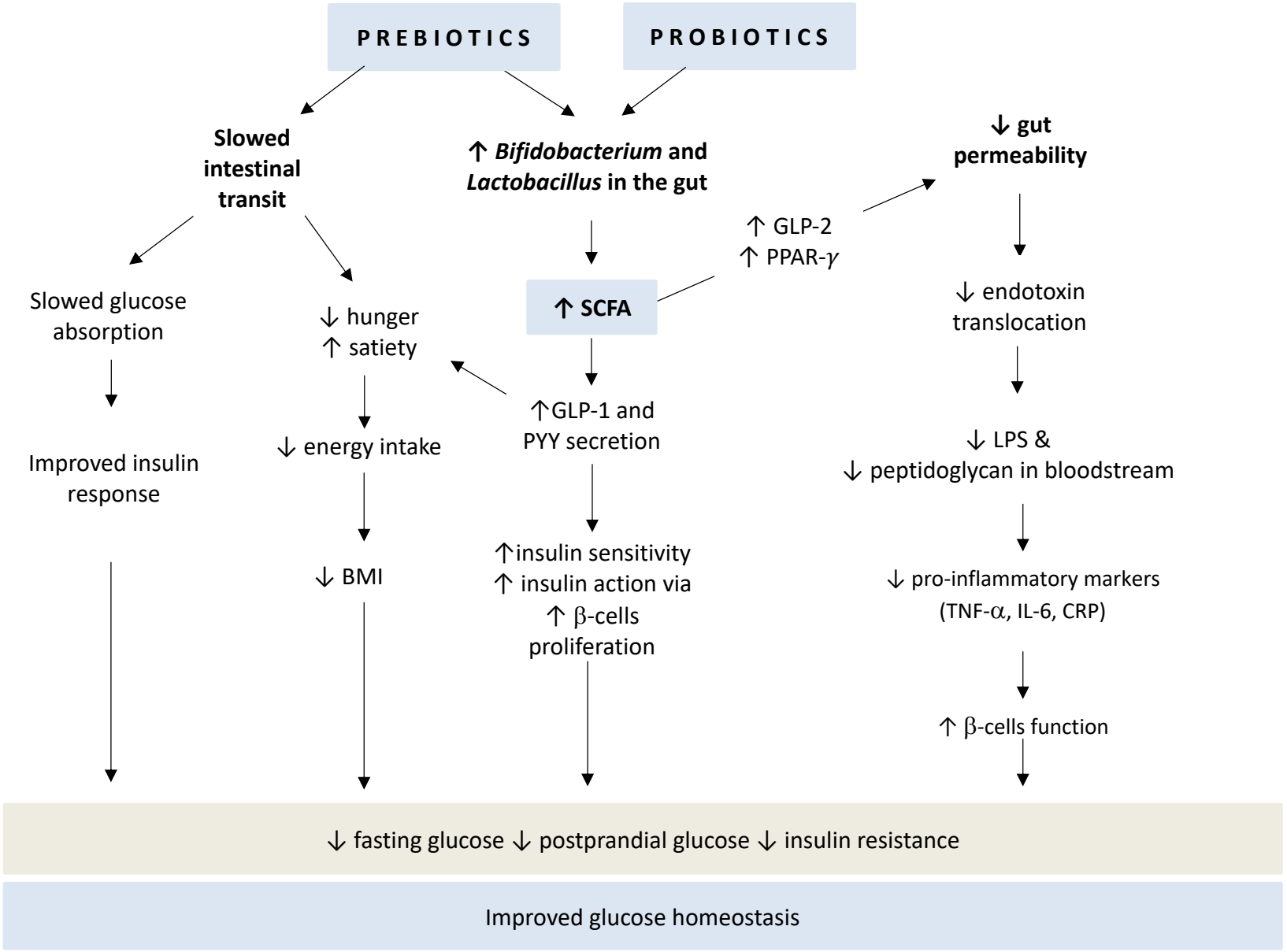


Figure 1 Proposed mechanisms of probiotics and prebiotics for prevention of GDM. Modified from O'Connor et al. (79).

2.5 Probiotic intervention studies for prevention of GDM

A landmark study conducted in Finland was the first trial to identify the efficacy of probiotics for prevention of GDM (Table 4). Luoto et al. (84) compared probiotic supplementation, *Lactobacillus rhamnosus* GG (10^{10} CFU/d) and *Bifidobacterium lactis* Bb12 (10^{10} CFU/d), with intensive dietary counselling for prevention of GDM and other adverse perinatal outcomes in a double-blind, placebo controlled randomised trial (84). Pregnant women (*n* = 256) were randomised at their first maternal welfare clinic appointment, in the first trimester of pregnancy, to either receive dietary counselling and probiotics (*n* = 85), dietary counselling and placebo (*n* = 86) or no intervention (*n* = 85) (84). Significantly, probiotic intervention was most effective for prevention of GDM when combined with dietary intervention, reducing the frequency of GDM to 13%, compared to 36% (diet/placebo) and 34% (no intervention) (*p* = 0.03) (84). Dietary intervention was based on Nordic Nutrition Recommendations, with additional personalised recommendations for food products with favourable fat and dietary fibre content delivered by a nutritionist. However, according to the Finnish National Program all women in Finland receive nutrition counselling based on the Nordic Nutrition recommendations, therefore, Luoto et al. (84) did not assess the efficacy of probiotic intervention in pregnant women without dietary counselling. The PiP study (2) (described previously in section 1.1.), supported the results by Luoto et al. (84) and of note assessed the efficacy of probiotic intervention without nutrition counselling.

In contrast, other probiotic intervention studies for prevention of GDM in women with obesity/overweight have been shown to be ineffective (Table 4). Lindsay et al.'s (85) Probiotics in Pregnancy study conducted in Ireland assessed the effect of 4-week supplementation (24-28 weeks' gestation) of *Lactobacillus salivarius* UCC118 (10^9 CFU/d) in a cohort of pregnant women with obesity (*n* = 138) (85). No changes were observed for any of the biomarkers of insulin resistance or glucose regulation in the probiotic group compared to placebo (85). However, these results are likely owed to the short-term intervention period and the timing of the probiotic intervention (69,70). As diagnosis of GDM is generally made between 24-28 weeks' gestation (24), it is likely that the probiotic was administered too late to have a preventative metabolic effect. The recent Australian SPRING (Study of PRobiotics IN Gestation) study explored the effect of *Lactobacillus rhamnosus* (LGG) (>$1x10^9$ CFU/d) and *Bifidobacterium animalis* subspecies *lactis* (BB12) (>$1x10^9$ CFU/d) for prevention of GDM in a group of high-risk, obese/overweight women (*n* = 411) (86). All participants received standard nutrition advice as per Australian nutrition guidelines for pregnancy, however, to the best of our knowledge this did not include specific dietary fibre advice. In accordance, with Lindsay et al. (85), probiotics did not protect against GDM. However, due to issues with hospital routines affecting

recruitment 14.4% of participants in the SPRING study had 1-4 weeks less supplementation period compared to participants in the New Zealand PiP study (2,86). Finally, the Healthy Mums and Babies (HUMBA) trial is the most recent double-blind, randomised placebo controlled probiotic intervention trial conducted in South Auckland, New Zealand (87). Pregnant women with obesity (72% of participants are Māori and Pacific women) were randomised to receive culturally tailored diet intervention or standard dietary care in combination with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB12 ($7x10^9$ CFU/d) or placebo. Dietary intervention included delivery of nutrition education by community health workers, usually of Māori and Pacific ethnicity. Nutrition education included culturally tailored advice about healthy food and drink choices, limiting energy dense foods, healthy recipes, label reading, portion control and managing cravings. In comparison, women randomised for routine dietary advice received the New Zealand Ministry of Health *Eating for Healthy Pregnant Women* resource. No significant reduction of GDM was observed for either nutrition intervention or probiotic supplementation (87). However, unlike Luoto et al. (84), to the best of our knowledge neither dietary intervention in the HUMBA study focused specifically on increasing dietary fibre intake (87).

Significantly, participants of the negative probiotic GDM prevention studies (HUMBA, SPRING and Irish PiP study) had a relatively high median BMI of 38.8kg/m^2, ≥25 kg/m^2 and 33.6 ± 2.6kg/m^2, respectively (85–87). In contrast, women in the positive probiotic GDM preventions studies (New Zealand PiP and Finnish study) had a median BMI of 25-26kg/m^2 and 23.6kg/m^2, respectively (2,84). This supports the bias of the results for application in lean populations (84). Furthermore, as obesity is linked to dysbiosis it is possible that higher doses of probiotics are required to influence the gut microbiota in combination with dietary interventions, specifically dietary fibre, in high-risk groups.

Currently, no probiotic intervention studies have taken into account the effect of concurrent dietary fibre intake with probiotics. Therefore, the first part of this thesis will explore the hypothesis that the efficacy of *Lactobacillus rhamnosus* HN001 probiotic supplementation in preventing GDM in the PiP study was dependent on sufficient dietary fibre intake. The second part of this thesis will determine whether concurrent high dietary fibre intakes and HN001 supplementation produced a unique protective GDM metabolome.

Table 4. Randomised control trials investigating the effect of probiotic supplementation for prevention of GDM.

Study, Country (Ref)	Participants	Intervention	GDM Diagnostic Criteria[1]	GDM Outcome
HUMBA, New Zealand (87)	Pregnant women with obesity (*n* = 230) Median BMI = 38.8kg/m^2	*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB12(≥6.5x10^9 CFU/d) or placebo and culturally tailored or standard dietary advice as per New Zealand nutrition guidelines.	IADPSG and NZ	No significant differences between intervention groups for GDM outcome.
SPRING, Australia (86)	Pregnant women with obesity or overweight (*n* = 411) Median BMI = ≥25kg/m^2	*Lactobacillus rhamnosus* (LGG) and *Bifidobacterium animalis* subspecies *lactis* (BB-12) (>1x10^{9}CFU/d) or placebo from 15.9±1.5 weeks gestation until delivery. All participants received standard dietary advice as per the Australian nutrition guidelines	IADPSG	GDM prevalence: 12.3% in placebo group vs. 18.4% in probiotic group (*p* = 0.10)
PiP, New Zealand (2)	Pregnant women with a personal and partner history of atopic disease (*n* = 423) Median BMI = 25-26kg/m^2	Probiotic (*Lactobacillus rhamnosus* HN001 (6×10^9 CFU/d) or placebo from 14-16 weeks' gestation until 6-months post-partum. No dietary advice given.	IADPSG and NZ	↓ GDM prevalence (by NZ definition) (*p* = 0.03) ↓ prevalence (by IADPSG definition) in women with history of GDM (*p* = 0.004) or ≥35 years (*p* = 0.009)
PiP, Ireland (85)	Pregnant women with obesity (*n* = 138) Median BMI = 33.6 ± 2.6kg/m^2	Lactobacillus salivarius UCC118 (10^{9}CFU/d) or placebo from 24 to 28 weeks' gestation (four week intervention). Research dietitian provided brief information about healthy eating using the food pyramid as a guide to all participants.	Carpenter Coustan	No significant changes in maternal fasting glucose between baseline and 28 weeks' gestation or GDM prevalence
NAMI, Finland (84).	Pregnant women with at least one family member having an allergic disease (*n* = 256) Median BMI = 23.6 ± 3.8kg/m2	*Lactobacillus rhamnosus* GG (10^{9}CFU/day) and *Bifidobacterium lactis* Bb12 (10^{9}CFU/day) or placebo from first trimester u*ntil the end of exclusive breastfeeding with dietary counselling; o*r no intervention (control). All participants received dietary advice as per the Finland National Program. The diet intervention cohort received intensive dietary counselling that included recommendations for food items low in fat and high in dietary fibre.	Finland 2002-2005 clinical guideline[2]	GDM prevalence (*p* = 0.003): 13% diet/probiotics 36% diet/placebo 34% control

Abbreviations: GDM = gestational diabetes mellitus, HUMBA = Healthy Mums and Babies; SPRING = Study of Probiotics IN Gestation, BMI = body mass index, CFU/d = colony forming units per day, IADPSG = International Association of Diabetes and Pregnancy Study Group, NZ = New Zealand, PiP = Probiotics in Pregnancy, NAMI = Nutrition, Allergy, Mucosal immunology and Intestinal microbiota.

[1]Outlined in table 2 (except Finland 2002-2005 clinical guideline as specified below)

[2]Finland 2002-2005 GDM diagnostic criteria: ≥4.8mmol/l at baseline, ≥10.0mmol/l at 1h or ≥8.7mmol/l at 2h following 75g OGTT.

2.6 Introduction to metabolomics

The second part of this thesis aims to explore the metabolite profile of probiotic *Lactobacillus rhamnosus* HN001 supplementation during pregnancy, dietary fibre intake in pregnancy, and GDM outcomes.

The aim of metabolomics is to evaluate the biomarkers (or *metabolites*) in a biological system at a given time point to provide a "metabolic snapshot" (73). It involves measurement and identification of endogenous and exogenous molecules (molecular weight <1.5kDa) that are the substrates and products of metabolic systems (88). Thus, producing comprehensive results that illustrate metabolites of disease processes and nutritional or environmental exposures (73,89).

Metabolomic studies can involve a "targeted" or "non-targeted" experimental approach (88,90). A targeted analysis involves quantification of a subset of metabolites in order to test a hypothesis. Thus, the success of a targeted screen depends on a strong hypothesis and *a priori* knowledge of the metabolic enzymes, their associated metabolites and the biochemical pathways involved (88). In contrast, a non-targeted metabolomics approach involves analysis of thousands of unknown metabolites within a given biological sample. This enables relative differences across two conditions or a population to be measured (88). In this way, non-targeted studies are extremely useful for identifying metabolic biomarkers involved in nutrition intervention studies whereby the pathways involved are undetermined (91). However, a limiting factor of a non-targeted approach is the variation across experimental approaches, such as choice of analytical platform, sample preparation and statistical validation (91). This variation presents a challenge for comparing and producing reproducible results across research groups as each process enables detection of certain molecules. Therefore, it is challenging to unify "gold standard" metabolomic protocols as the methods adopted by research groups depend on the biological sample, context of intervention and the aim of the experiment (91). This thesis adopted a non-targeted approach, as metabolomic research in GDM and associated nutrition intervention studies are still in their infancy so, few candidate metabolites for GDM, probiotics or diet are known. A non-targeted approach enabled the identification of potential novel metabolites and associated pathways involved in the pathogenesis of GDM, probiotic HN001 supplementation and dietary fibre intake.

Biological samples for analysis by metabolomics must also be carefully considered and their selection based on known disease processes or the intervention to be measured. For example, to compare health or disease states with a nutrition intervention, measuring the metabolites present in serum or plasma is a reasonable reflection of pathways involved since the serum/plasma metabolome is the

net effect of a whole-body response to diet and the environment (73). To measure metabolites within tissues would provide a different interpretation as the tissue metabolome reflects the cell autonomous effect (73,88). On the other hand, urine is also commonly analysed in metabolomic studies. While urine represents a rich summation of metabolites present during the hours directly prior to sampling; it poses analytical challenges due to the fact that urine is an excretory product that is affected considerably by physiological factors and fluid intake (73). Therefore, for the purpose of the metabolomic analysis in this thesis, plasma was selected as the biological sample to provide a snapshot of the participants metabolic status at the time of sampling in association with GDM status, dietary fibre intakes and probiotic supplementation.

As previously mentioned, there are multiple analytical platforms for conducting metabolomic research. Each platform is biased towards detecting certain chemical classes or biological compounds due to the differences in range (from pico – to millimolar) of metabolites within a metabolome (92). The two main analytical approaches selected for the generation of metabolomic data in nutrition research are Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) coupled to a chromatography technique (88,92). A brief description of each approach, as well as the limitations and advantages of each technique is described in the following sections.

2.6.1 Nuclear magnetic resonance

NMR measures the intrinsic magnetic property of atomic nuclei to encode information about the structural and chemical properties of a molecule (93). ^{1}H (proton) NMR is most commonly used for clinical research as the majority of known metabolites contain hydrogen atoms. NMR has minimal preparation requirements as it does not require separation or ionisation of metabolites. Therefore, the biological sample can be recovered for further analyses (93). While NMR provides high reproducibility, this technique is less commonly used in non-targeted studies as it is relatively insensitive compared to MS (88,93).

2.6.2 Mass spectrometry

The most commonly used platform for non-targeted studies is MS coupled with separation techniques such as Gas Chromatography (GC) and Liquid Chromatography (LC). MS is the most popular analytical method chosen for metabolomics research as it is highly sensitive, efficient and reproducible at a relatively low cost. (89). MS involves three steps 1) ionisation - converting metabolites from a biological sample into gas phase ions; 2) fragmentation - separating the ions according to their mass-to-charge ratio (m/z); and 3) detection - moving the ions through an electric field whereby the abundance/intensity of respective ions are measured (89). The identification of certain metabolites is

then achieved by comparing the m/z measurement or fragmentation pattern of a certain compound to existing metabolomic databases or libraries, e.g. KEGG database (89). Several MS analysers are used in research, including Quadrupole, Time of Flight and Orbitrap (73,91). Each mass analyser measures ions using different methods, thus producing variable results due to accuracy and range of the analyser and resolving power, i.e. how well the fragmentation steps can be performed and measured (91). Therefore, the mass analyser selected for particular studies depends on the context of the research. Furthermore, the choice of chromatography techniques must also be carefully considered as each analytical method is restricted to detection of certain classes of metabolites (91).

2.6.3 Liquid chromatography – mass spectrometry

Liquid chromatography – mass spectrometry (LC-MS) is useful for detecting large polar, weakly polar and neutral metabolites at low concentrations. LC-MS is often followed by electrospray ionisation techniques which operate in both positive and negative ions modes (92). This enables wider coverage of the metabolome as metabolites are detected as either positive or negative ions, not both (92). A major advantage of LC-MS is that there is no need for chemical derivatisation of metabolites as LC is able to separate non-volatile and soluble compounds. Furthermore, as the analysis temperature is lower than in GC-MS thermally labile compounds can also be detected (92). However, the data produced by LC-MS has limited libraries for identifying compounds, therefore identification of metabolites is time consuming and limiting (92).

2.6.4 Gas chromatography – mass spectrometry

Gas chromatography – mass spectrometry (GC-MS) is a highly sensitive metabolomics method with high analytical reproducibility at a relatively low cost. However, GC-MS is restricted to measuring volatile, thermally and energetically stable compounds. Therefore, as many metabolites are not thermally labile or volatile enough in their native state within biological samples, they must be converted to volatile derivatives (89,94). Two techniques can be used for derivatisation including silylation (TMS) and alkylation by methyl chloroformate (MCF) (94). MCF derivatisation is preferable for analysis of amino acids, nucleotides and organic acids, while TMS is more useful for sugars and their derivatives (94). For the purpose of this thesis MCF derivatisation followed by GC-MS is the most suitable metabolomics method since published findings have indicated that amino acids and organic compounds are central to the metabolic pathways involved in GDM (73). However, GC-MS will limit identification of known molecules associated with pro/pre-biotics such as inflammatory biomarkers and molecules involved in the regulation of intestinal permeability, as GC-MS can only detect molecules ≤1.5kDa (molecular weight) (88).

2.7 Thesis Hypotheses

Based on the comprehensive review of the literature (Chapter 2) and the following systematic review (Chapter 3) of previous metabolomic studies we hypothesised that:

1. Women who developed GDM would have altered macronutrient intake (e.g. high fat intakes and low dietary fibre intake) compared to women with normal glucose tolerance at 26-28 weeks' gestation.
2. High dietary fibre intake was required for the prevention of GDM and for probiotic HN001 supplementation to have a benefit in further reducing GDM risk.
3. The metabolome of PiP study participants will be differentiated by GDM status, probiotic HN001 supplementation, and dietary fibre intake.
4. Probiotic HN001 supplementation and high dietary fibre intakes would produce metabolites that may contribute to prevention of GDM.

Chapter 3 Systematic review of GDM, probiotic supplementation and dietary fibre metabolome

3.1 Introduction

GDM is defined as hyperglycaemia with onset occurring during the second trimester of pregnancy and which is expected to resolve after pregnancy (4,18–20). International trends suggest that the prevalence of GDM is increasing globally, with prevalence spanning up to 45% in some populations (24). Uncontrolled or poorly controlled GDM can cause severe short-term complications (macrosomia and delivery complications) and can have long-term metabolic consequences for both the mother and the infant later in life (18,29). Therefore, development of effective preventative strategies would not only reduce the risk of adverse pregnancy outcomes, but also reduce the prevalence of postpartum T2DM for the mother and metabolic syndrome later in life for the infant conferred through foetal programming (7). Metabolomics provides a methodology that allows us to gain a mechanistic understanding of GDM by assessment of all metabolites involved in the disease pathogenesis, rather than simply from a glucose and insulin perspective (73). Improved understanding of the pathogenesis of GDM could therefore assist in the development of more targeted preventative and therapeutic guidelines (73).

Extensive research in both animal and human studies have indicated that manipulating the gut microbiota by dietary interventions, i.e. diets high in dietary fibre (prebiotics), and probiotics may protect against GDM (57). However, the mechanisms by which this may occur is yet to be determined. Metabolomics provides a methodology to evaluate these interventions by determining metabolites that are altered as a result (73). Furthermore, metabolomics provides a supplementary method to traditional dietary recall methods for quantifying dietary fibre intake, as such analytical approaches can be used to identify biomarkers of dietary fibre fermentation detectable in plasma (91). Furthermore, it is unknown whether probiotics need to colonise the gut to produce an effect (66), therefore analysis of the metabolome of participants receiving probiotic supplementation compared to a placebo could highlight metabolic changes induced by certain species of bacteria. Thus, assessment of the metabolome in probiotic intervention studies for prevention of GDM could provide further mechanistic insights into how probiotics work and in which subgroup of women, particularly with reference to concurrent dietary fibre intake.

The purpose of this systematic review is to critically examine the clinical studies that characterise the metabolites associated with GDM to distinguish the metabolomic profiles of women with GDM from those without. Furthermore, this systematic review aims to assess probiotic supplementation studies and dietary fibre intervention studies to identify known metabolomic biomarkers of probiotic supplementation and dietary fibre intakes. The patterns of metabolites identified within this systematic review will be used to guide the analysis of the metabolomic investigations for this thesis in relation to GDM outcomes, probiotic *Lactobacillus rhamnosus* HN001 supplementation during pregnancy, and dietary fibre intake in pregnancy.

3.2 Method

3.2.1 Aims / Objectives

1. To determine metabolites associated with GDM compared to pregnant woman with normal glucose tolerance at 23-30 weeks' gestation.
2. To determine metabolites associated with probiotic supplementation compared to a placebo control.
3. To determine metabolites associated with dietary fibre intake.

3.2.2 Search strategy

Literature searches of MedLine (Ovid), MedLine (Embase 1980-present) and Scopus were conducted in May 2019. The following key terms were used: "*metabolomics*", "*Gestational Diabetes*", "*GDM*", "*probiotics*", "*dietary fibre."* Proper Boolean operators "AND" and "OR" were also included to be as comprehensive as possible. The review was limited to studies written in English. Specific details of the search strategy conducted for each database and research aim can be found in appendix A. The literature search strategy was discussed with the thesis supervisor, however, conducted individually by the author. Therefore, some selection bias may exist.

3.2.3 Eligibility criteria

Articles were included or excluded on the basis of title and abstract (first screening), full-text articles (second screening) and finally data extraction (third screening). The inclusion / exclusion criteria for each research aim is detailed in Table 5. Studies presented as abstracts with no subsequent full report of study results were also excluded at retrieval of full-text articles.

3.2.4 Data extraction and analysis

Data on population characteristics, study design, metabolomics approach and study results were extracted. A quantitative meta-analysis of the data was not appropriate owing to; the limited number of metabolomic studies relevant to GDM, dietary fibre or probiotic supplementation, the clinical heterogeneity of the probiotic and dietary fibre intervention studies, methodological heterogeneity of all studies and the overall purpose of the systematic review.

Table 5 Eligibility criteria for systematic review.

Literature Search	Inclusion Criteria	Exclusion Criteria
GDM Metabolome	- Metabolites were examined in maternal plasma or serum - Metabolomic techniques LC-MS, GC-MS or NMR were used to construct metabolite profiles - Blood samples for metabolomics assessed at 23-30 weeks' gestation - Comparison of metabolites between GDM (test) vs women with NGT(control)	- Analysed metabolite profiles in animal model - Inadequate/no control group
Probiotic Metabolome	- Metabolites were examined in plasma or serum - Metabolomic techniques LC-MS, GC-MS or NMR were used to construct metabolite profiles - Probiotic intervention vs placebo control - RCT	- Analysed metabolite profiles in animal model - Inadequate/no control group - Postprandial assessment of metabolites only
Dietary Fibre Metabolome	- Metabolites were examined in plasma or serum - Metabolomic techniques LC-MS, GC-MS or NMR were used to construct metabolite profiles - RCT or cross-over trial - High dietary fibre as an intervention vs low dietary fibre control - Habitual high dietary fibre intakes vs low dietary fibre intakes	- Analysed metabolite profiles in animal model - Inadequate/no control group - Postprandial assessment of metabolites only

Abbreviations: GDM = gestational diabetes mellitus; GC-MS = gas chromatography-mass spectrometry; LC-MS = light chromatography-mass spectrometry; NMR = nuclear magnetic resonance; NGT = normal glucose tolerance, RCT = randomised control trial.

3.3 Results

3.3.1 GDM metabolome literature search

The selection algorithm for the nine studies that met the inclusion/exclusion criteria for GDM studies is detailed in Figure 2. The metabolome of a total of 450 women with GDM and 655 women with normal glucose tolerance (NGT) was analysed using gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) or nuclear magnetic resonance (NMR) based approaches. Five studies explored the metabolome using plasma samples and four studies used serum samples. The methodologies, including diagnostic criteria and metabolomic platform, and study results are summarised in Table 6. Branch chain amino acids (BCAA) (leucine, isoleucine and valine) and aromatic amino acids (tyrosine and phenylalanine) were upregulated in three studies (95–97). Other amino acids associated with GDM are alanine (96,97), glycine (97,98), arginine (97,98), aspartate (97) and proline (97). These studies suggest that amino acid metabolic pathways are associated with the pathogenesis of GDM. Furthermore, upregulation of bile salts was detected in one study (99). Finally, biomarkers of fatty acid metabolism including fatty acid oxidation (96,100) and downregulation of polyunsaturated fatty acids (101) were also identified in three studies.

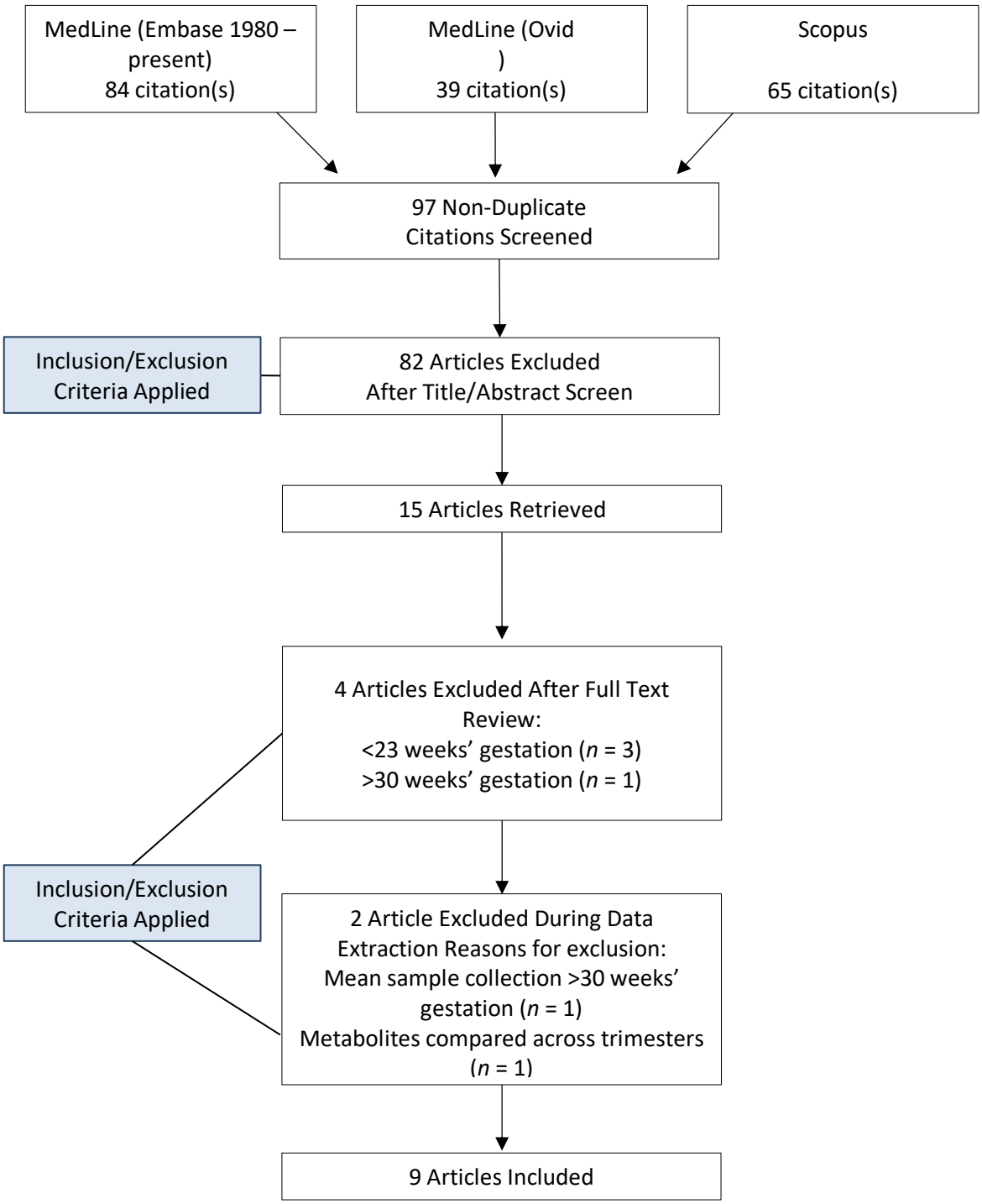


Figure 2 Selection algorithm for metabolomic studies associated with GDM.

Table 6 Studies investigating metabolites associated with GDM.

Ref.	Methods & analytical platform(s)	Diagnostic criteria for GDM	Population		Gestational age (weeks)	Metabolites of GDM compared to control*	
			Women with GDM	Women with NGT		Upregulated (% change)†	Downregulated (% change)†
Metabolites assessed in fasting plasma sample							
Law et al. (101)	LC-MS (untargeted)	IADPSG	34	27	23-27	N/A	Polyunsaturated or chemically modified phospholipids
Dudzik et al. (100)	GC-Q/MS (untargeted)	WHO-1998	24	24	24-28	3-hydroxybutyrate (81%) 2-hydroxybutyrate (51%) Stearic acid (34%) Glycerol (29%) Linoleic acid (25%) Oleic acid (21%) Palmitoleic acid (37%) Palmitic acid (13%)	N/A
Lehmann et al. (95)	LC-MS, GC-MS (targeted)	IADPSG	9	15	25-26	Acylcarnitine C18:0 Leucine	Glycine
Park et al. (97)	HPLC (targeted)	Carpenter–Coustan criteria	64	25	24-28	Aspartate Glycine Arginine Alanine Proline Lysine Isoleucine Tyrosine Valine	N/A

Ref.	Methods & analytical platform(s)	Diagnostic criteria for GDM	Population		Gestational age (weeks)	Metabolites of GDM compared to control*	
			Women with GDM	Women with NGT		Upregulated (% change)†	Downregulated (% change)†
Dudzik et al. (102)	LC–QTOF-MS, GC–Q-MS (untargeted) Metabolites presented were identified by GC-MS	WHO-1998	20	20	23-28	3-hydroxybutyric acid (75%) 2-hydroxybutyric acid (68%) Linoleic acid (19%) Glycerol (19%) Fumaric acid (15%)	Pyruvic acid (54%) Creatinine (49%) Glycine (39%) L-tryptophan (24%) Lauric acid (24%) L-glutamic acid (14%)
Metabolites assessed in fasting serum sample							
Chen et al. (103)	LC-MS & GC-MS (untargeted)	IADPSG	32	44	26-28	2-aminobutyric acid	N/A
Gao et al. (99)	UPLC/Q-TOF MS (targeted)	IADPSG	38	27	25-28	2 dihydroxy conjugated, 1 trihydroxy unconjugated and 5 sulfated bile acids.	N/A
White et al. (96)	NMR (targeted)	IADPSG	198	448	23-30	Valine Leucine Isoleucine Phenylalanine Tyrosine Alanine Pyruvate Palmitoleic acid	Polyunsaturated fatty acids
Rahimi et al. (98)	HPLC	ADA	31	25	>25 weeks	Arginine Glycine Methionine	None

Abbreviations: NGT = normal glucose tolerance; LC-MS = Liquid Chromatography-Mass Spectrometry; GC-QTOF/MS = Gas-Chromatography-Quadruple/Mass Spectrometry; LC–QTOF-MS = Liquid Chromatography- Quadruple Time of Flight/Mass Spectrometry; NMR = nuclear magnetic resonance; UPLC/Q-TOF-MS = Ultraperformance Liquid Chromatography- Quadruple Time of Flight/Mass Spectrometry; HLPC = high-performance liquid chromatography; HOMA-IR = homeostatic model assessments for insulin resistance; WHO-1998 = World Health Organisation – 1998; IADPSG = International Association of Diabetes and Pregnancy Study Group; ADA = American Diabetes Association.
*All metabolites significant $p < 0.05$, or $p < 0.01$
†Percentage change specified if available.

3.3.2 Probiotic supplementation metabolome literature search

The selection algorithm for the three studies that met the inclusion/exclusion criteria is detailed in Figure 3. All included studies (involving 268 adult participants) compared metabolites in participants who took a probiotic supplement compared to a placebo control. All studies included *Lactobacillus* sp. and one study included *Bifidobacterium* sp. in addition to a *Lactobacillus* sp. probiotic. The methodologies, including intervention and metabolomic platform, and study results are summarised in Table 7. One study detected upregulation of medium chain acylcarnitine's in overweight adults supplemented with two strains of *Lactobacillus* sp. for 12 weeks (104). Biomarkers of fatty acid metabolism, including lysophosphatidylcholine's (LysoPC's), fatty acid primary amides and their acid derivatives were upregulated by probiotic supplementation in a cohort of adults with normal BMI (105). Probiotic supplementation in women with irritable bowel syndrome (IBS) was also observed to restore serum tyrosine and glucose to baseline levels comparative to healthy individuals (106). With the simultaneous upregulation of lactate produced by supplementation of a lactic acid producing bacteria, this study indicates that probiotics may influence energy homeostasis (106).

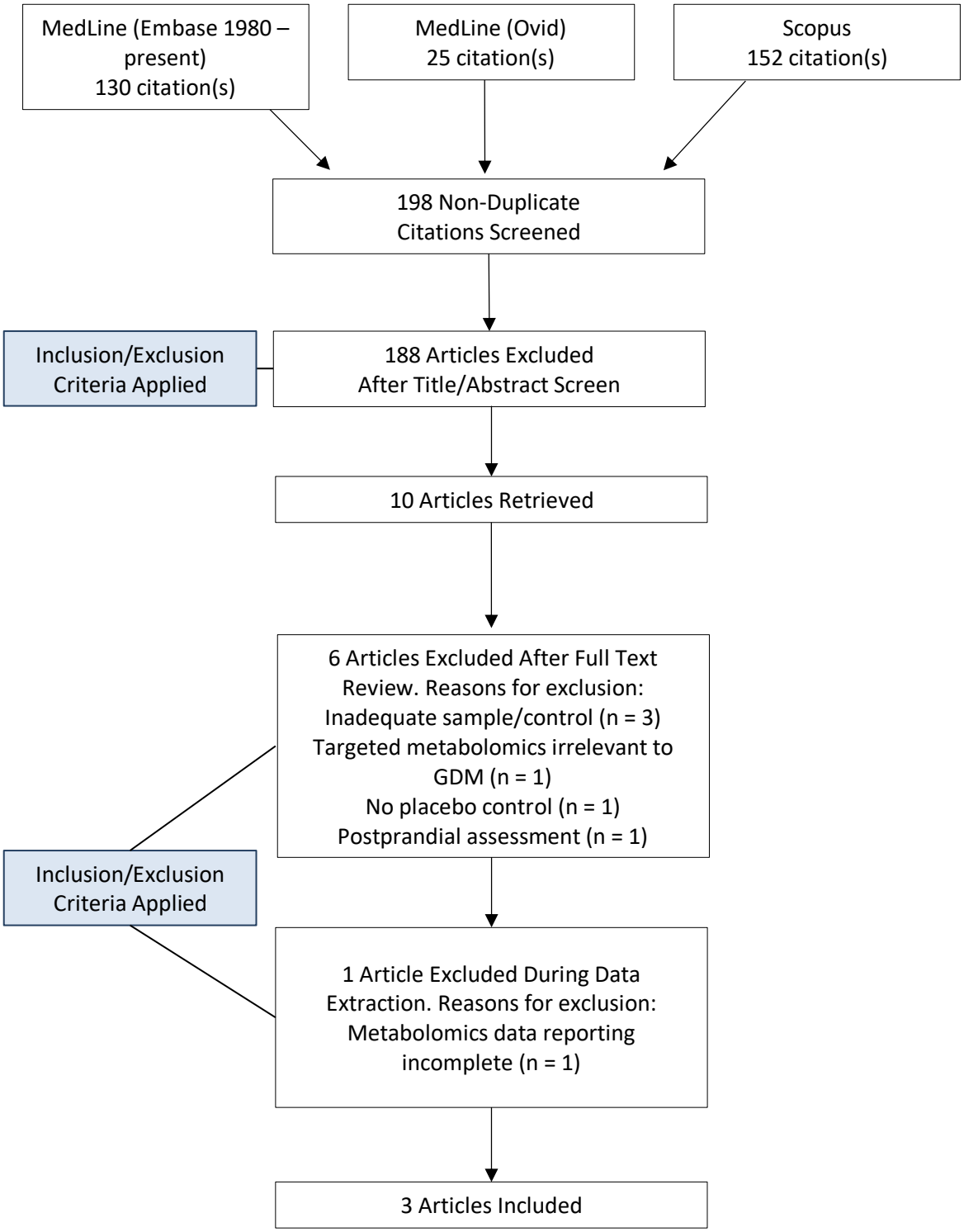


Figure 3 Selection algorithm for metabolomic studies associated with probiotic supplementation.

Table 7 Randomised control trials investigating metabolites associated with probiotic supplementation.

Ref.	Participant features	Study design and intervention	Metabolomics platform (sample)	Metabolites of probiotic supplementation compared to control		Associated Pathways
				Upregulated	Downregulated	
Kim et al. (104)	Overweight adults (*n* = 66) Age = 51 – 54 years Mean BMI = 26 - 27kg/m^2	Participants were randomised to receive a probiotic powder containing *Lactobacillus curvatus* HY7601 (5x10^9 CFU/d) and *Lactobacillus plantarum* KY1032 (5x10^9 CFU/d) (*n* = 32) or a placebo powder (*n* = 34) for 12-weeks. Participants were instructed to follow their habitual dietary intake.	Nontargeted UPLC-LTQ/ Orbitrap MS (fasting plasma)	Medium-chain acylcarnitine's (*q* <0.001): Octenoylcarnitine (C8:1), Tetradecenoylcarnitine (C14:1), Decanoylcarnitine (C10), Dodecenoylcarnitine (C12:1)	None	Increase in *AC* correlated with reduction in adiposity in probiotic group. Suggests probiotic induced increase in *AC* associated with increased lipolysis during body fat reduction.
Ahn et al. (105)	Healthy Adults with mild-to-moderate hypertriglyceridemia (without diabetes) (*n* = 92) Age = 40 - 65 years Mean BMI = 24 - 25kg/m^2	Participants were randomised to receive a probiotic powder containing *Lactobacillus curvatus* HY7601 (5x10^9 CFU/d) and *Lactobacillus plantarum* KY1032 (5x10^9 CFU/d) (*n* = 46) or a placebo powder(*n* = 46) for 12-weeks.	Nontargeted UPLC-LTQ/ Orbitrap MS (fasting plasma)	None	Palmitole amide Palmitic amide Oleamide LysoPC's (C14:0 .C16:1, C16:0, C17:0, C18:3, C18:2, C18:1, C20:3) (*q* < 0.01 or <0.05)	*Palmitole amide*, *palmitic amide*, oleamide are fatty acid primary amides. *LysoPC's* are intermediates in the metabolism of lipids. Reduction in these metabolites is linked to reduction of triglycerides observed in probiotic group.
Hong et al. (106)	Women with IBS (*n* = 73) Age = 21 – 55 years	Women with IBS were randomised to consume a fortified yoghurt drink 3x per day containing 4x10^9 CFU of *Lactobacillus* sp. HY7801, *Bifidobacterium longum* HY8004, and *Lactobacillus brevis* HY7401 (or a placebo yoghurt drink for 8-weeks.	^{1}H NMR (fasting serum)	Lactate (*p* < 0.001)	Glucose (*p* < 0.05)* Tyrosine (*p* < 0.05)* ** Glucose and tyrosine were elevated at baseline in individuals with IBS and then normalised to those of healthy individuals with probiotic supplementation.*	Upregulated *of Lactate* is likely due to supplementation of lactic acid producing bacteria. *Glucose* is a marker of energy homeostasis. *Tyrosine* is an amino acid.

Abbreviations: BMI = body mass index, CFU/d = colony forming units per day, UPLC-LTQ/ Orbitrap MS = ultra-performance liquid chromatography- linear-trap quadrupole-Orbitrap mass spectrometry, AC = acylcarnitine, GC-TOF/MS = gas chromatography-time of flight/mass spectrometry, BCAA = branch chain amino acid, ^{1}H NMR = proton nuclear magnetic resonance, MUFA = monounsaturated fatty acid.

3.3.3 Dietary fibre metabolome literature search

The selection algorithm for the four studies that met the inclusion/exclusion criteria is detailed in Figure 4. The studies (involving 83 adult participants) were randomised, cross-over controlled studies that compared metabolites associated with high and low intakes of dietary fibre. The methodologies, including metabolomic platform and details of the dietary interventions, as well as study results are summarised in Table 8. Biomarkers of tryptophan metabolism (kynurenate, ribitol, ribonic acid and indoleacetic acid) were significantly linked to dietary fibre intakes ≥47g/d (107,108). However, pathway analysis did not render any significant results (108). Furthermore, high dietary fibre intake was associated with a reduction of circulating BCAA - leucine and isoleucine (109). Other detected metabolites include betaine and N,N-dimethylgycine, 2,6-dihydroxybenzoic acid and 2-aminophenol sulfate which are biomarkers of rye intake (109,110). Therefore, no consistent physiological pathways are yet identified in relation to these metabolites.

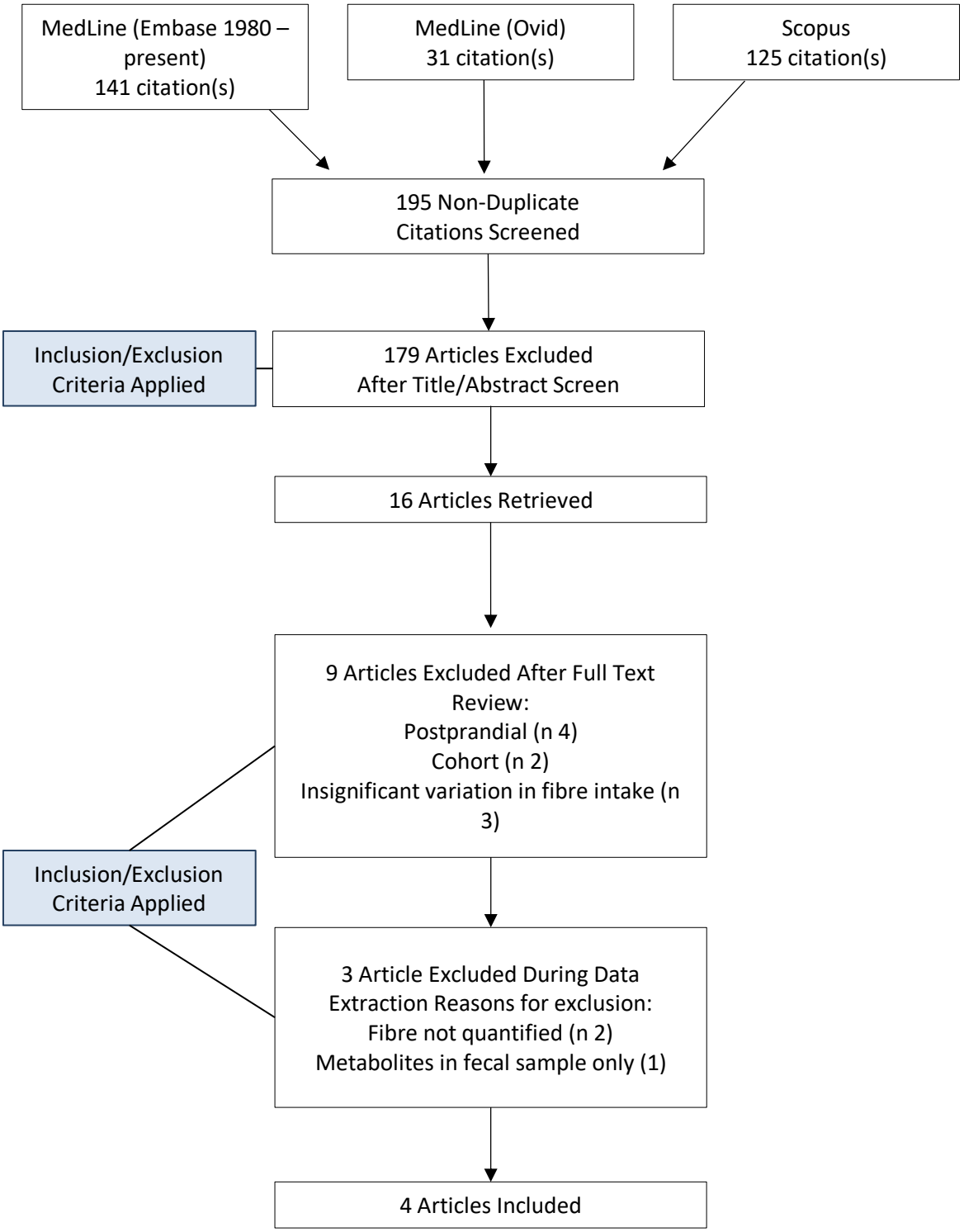


Figure 4 Selection algorithm for metabolomic studies associated with dietary fibre.

Table 8 Randomised controlled crossover trials investigating metabolites associated with dietary fibre.

Ref.	Participant feature (s)	Description of intervention	Metabolomics platform (sample)	Metabolites associated with high DF intake		
				Upregulated	**Downregulated**	**Associated pathways**
Barton et al. (108)	Healthy adults (*n* = 19) Age = 18 – 45 years BMI = <25 kg/m^2 or >28 kg/m^2	Participants were provided a high GL (DF = 28g / day) and low GL (DF = 56g / day) controlled diet. Each intervention was 28-days long with a 28-day wash out period between each study diet. The low GL diet included food items such as all-bran, pumpernickel bread and larger quantities of fruit and vegetables.	Targeted LC/MS (fasting plasma)	Kynurenate (40% ↑, $p < 0.05$)	None	Tryptophan metabolism / NMDA receptor antagonist; inhibits pro-inflammatory mechanisms
Johansson-Persson et al. (110)	Healthy adults (*n* = 25) Age = 49 – 66 years Mean BMI = 26.6kg/m^2	Participants followed a high-fibre (~48g / day) intervention or low-fibre (~30.2g / day) for 5-weeks, with a 3-week wash-out period between interventions. The high fibre intervention included one ready meal, one bread roll and two beverages fortified with oat bran, rye bran and sugar beet fibre to be consumed every day. The 5-week low fibre period included one ready meal, one bread roll and two beverages without fortification.	Untargeted LC-QTOF/MS	2,6-dihydroxybenzoic acid ($p < 0.01$) 2-aminophenol sulfate ($p < 0.01$)	None	Biomarkers associated with dietary fibre intake. No significant physiological pathway association reported.
Moazzami et al. (109)†	Postmenopausal women (*n* = 39) with total cholesterol 5.0-8.5mmol/L BMI = 20-33kg/m^2	During the 8-week intervention periods participants were required to consume 20% of their energy intake from a high fibre rye bread or low fibre wheat bread. During the high fibre test period participants consumed ~47g DF / day. During the low fibre test period participants consumed ~15.2g DF / day. An 8-week wash-out period was followed between interventions.	Targeted NMR (fasting serum & plasma)	Betaine N,N-dimethylgycine	Leucine Isoleucine	High DF may reduce insulin resistance by reducing circulating BCAA
Lankinen et al. (107)†	As described for Moazzami et al. (2012)		Targeted LC/MS & GC/MS (fasting plasma)	Ribitol Ribonic acid Indoleacetic acid	Myristoleic acid ($p = 0.002$) Oleic acid ($p = 0.002$)	*Ribitol* / *ribonic* acid precursor of tryptophan. Indoleacetic acid is a breakdown product of tryptophan. Myristoleic and oleic acid = FA metabolism

Abbreviations: DF = dietary fibre, BMI = body mass index, GL = glycaemic load, LC/MS = light chromatography/mass spectrometry, LC-QTOF/MS = light chromatography-quadruple time of flight/mass spectrometry; FA = fatty acid. †Both studies used blood samples from the same study.

3.4 Discussion

3.4.1 Summary of findings

This systematic review identified a number of metabolites associated with GDM pathogenesis, probiotic supplementation and dietary fibre interventions. These metabolites include biomarkers of BCAA and aromatic amino acid catabolism, fatty acid oxidation and tryptophan metabolic pathways.

3.4.2 Amino acid metabolic pathways

BCAA (leucine, isoleucine and valine), and aromatic amino acids (tyrosine and phenylalanine) were consistently upregulated in women with GDM compared to women with NGT (95–97). Specifically, tyrosine and valine were also positively associated with HOMA-IR (homeostatic model assessments for insulin resistance) (97). A number of studies have associated BCAA and aromatic amino acids with risk factors of GDM, including obesity and inflammation (111). Additionally, BCAA have been associated with impaired glucose tolerance in people with T2DM (97,111). Especially compelling, is the metabolomics study conducted as part of the large-cohort Framingham Study (112). This study illustrated that BCAA and aromatic amino acids were elevated in the metabolome of individuals who developed T2DM up to 12 years prior to onset; indicating that dysregulation of BCAA and aromatic amino acid metabolism may occur well before the onset of diabetes (112). These studies suggest that dysregulation of BCAA and aromatic amino acid metabolic pathways plays a key role in the development of insulin resistance, and consequently the development of GDM (102,111).

In contrast to GDM-related upregulation of BCAA, metabolomic studies of probiotic and dietary fibre interventions demonstrate a reduction in BCAA (isoleucine and leucine) and aromatic amino acids (tyrosine). The randomised controlled cross-over trial by Moazzami et al. (109) demonstrated that high fibre intake (~47g dietary fibre per day, controlled by supplementation of rye bread) reduced the levels of circulating isoleucine and leucine in a group of post-menopausal women (with high cholesterol). However, no significant differences between fasting insulin or glucose were associated with the lower plasma levels of BCAA (109). Despite this, reduction of BCAA in the high dietary fibre group were consistent with metabolites identified in the FUNGENUT study, whereby, a reduction of isoleucine was observed in relation to dietary fibre intakes of 27g per day (113). Furthermore, tyrosine was found to reduce to normal levels in women with IBS who were supplemented with probiotics (106). It is yet to be established whether the metabolites altered by high dietary fibre or probiotic supplementation are as a result of manipulation of the gut microbiota. However, it is tempting to suggest that dietary fibre and probiotic interventions may be a potential preventative measure for

GDM by offsetting the increased circulating BCAA and aromatic amino acids identified in the GDM metabolome.

3.4.3 Fatty acid metabolic pathways

Dysregulation of fatty acid metabolic pathways is recognised as a key aspect of insulin resistance (21,102). Monounsaturated fatty acids, palmitoleic acid and oleic acid, both are significantly higher in women with GDM compared to controls (96,100). In comparison, fatty acid amides of palmitoleic acid, and oleic and myristoleic acid were reduced by probiotic supplementation and dietary fibre, respectively. Furthermore, increased fatty acid oxidation is also reflected by an increase of 3-hydroxybutyrate, a biomarker associated with oxidative stress and dysregulation of BCAA metabolic pathways (100,102). Moreover, previous studies in individuals with NGT demonstrated that medium-chain acylcarnitine's, associated with impaired fatty acid oxidation, decrease with the progression of glucose intolerance (102,114). Notably, probiotic supplementation increased the concentration of medium-chain acylcarnitine's in women with obesity. Indicating that probiotic supplementation may be an effective intervention for impaired fatty acid oxidation.

3.4.4 Tryptophan metabolism

Notably, two studies investigating metabolites associated with high dietary fibre intake (≥47g per day) identified enrichment of biomarkers involved in tryptophan metabolism, including kynurenate (109), ribitol, ribonic acid and indoleacetic acid (107). Ribitol and ribonic acid are both precursors of tryptophan, while indoleacetic acid is a breakdown product (107). Tryptophan metabolism is associated with numerous physiological functions including regulation of gastrointestinal functions, including satiety and intestinal permeability (115). Furthermore, kynurenate is an NMDA antagonist associated with anti-inflammatory mechanisms (109). Significantly, Dudzik et al. (102) found that L-tryptophan was decreased by 24% in women with GDM. The involvement of tryptophan is yet to be established in GDM pathogenesis (116), however dietary fibre interventions could have a positive effect for prevention of GDM by increasing satiety, thus lowering BMI, and by activation of anti-inflammatory pathways (107,108).

3.4.5 Strengths and limitations

Strengths of this systematic review include the stringent selection criteria and the systematic and comprehensive selection and evaluation of the literature. GDM metabolome studies were selected based on gestational age reflective of the period of sample collection in the PiP study and all metabolites were identified in serum or plasma samples. However, as the literature review was

conducted independently by the author, some selection bias may exist. Metabolomics is a relatively new area of research, thus gold-standard protocols have not yet been developed (91). Therefore, there was variation in analytical platforms, sample processing methods and statistical analysis between studies. These variations, along with the variation in GDM diagnostic criteria used, may have contributed to the variation in results. Furthermore, the majority of studies had small sample sizes. This reduced the power to detect all associated metabolic changes and the data was highly susceptible to random effects. However, many of the included studies matched participants based on BMI, age and weeks' gestation or removed confounding variables. Furthermore, this systematic review included studies from different countries, presenting metabolites from a range of ethnic backgrounds. Therefore, genetic, cultural, and dietary factors must be considered when applying the findings to different ethnic populations.

3.4.6 Concluding remarks

The purpose of this systematic review was to evaluate current literature investigating the metabolome of women with GDM and metabolites associated with dietary fibre and probiotic supplementation; in order to aid the interpretation of the metabolomics analysis in this thesis. In this systematic review, the metabolites most consistently associated with GDM include biomarkers of BCAA and aromatic amino acid metabolic pathways. Of clinical relevance, leucine, isoleucine, tyrosine, and biomarkers of fatty acid metabolism were also identified (with opposite effect) in probiotic and dietary fibre intervention studies. Significantly, biomarkers of tryptophan metabolism were associated with high dietary fibre intakes.

Collectively, these studies indicate that probiotic supplementation and high dietary fibre intakes may prevent GDM by manipulation of BCAA, aromatic amino acid, fatty acid and tryptophan metabolic pathways. To the best of our knowledge, this thesis is the first study that will explore the metabolic interactions between probiotic HN001 supplementation, dietary fibre and GDM using a metabolomics based approach.

Chapter 4 Method

4.1 Experimental design

This thesis utilises 3-day food diaries and blood plasma samples collected in the *Probiotics in Pregnancy* study (1). The experimental workflow has been outlined in Figure 5.

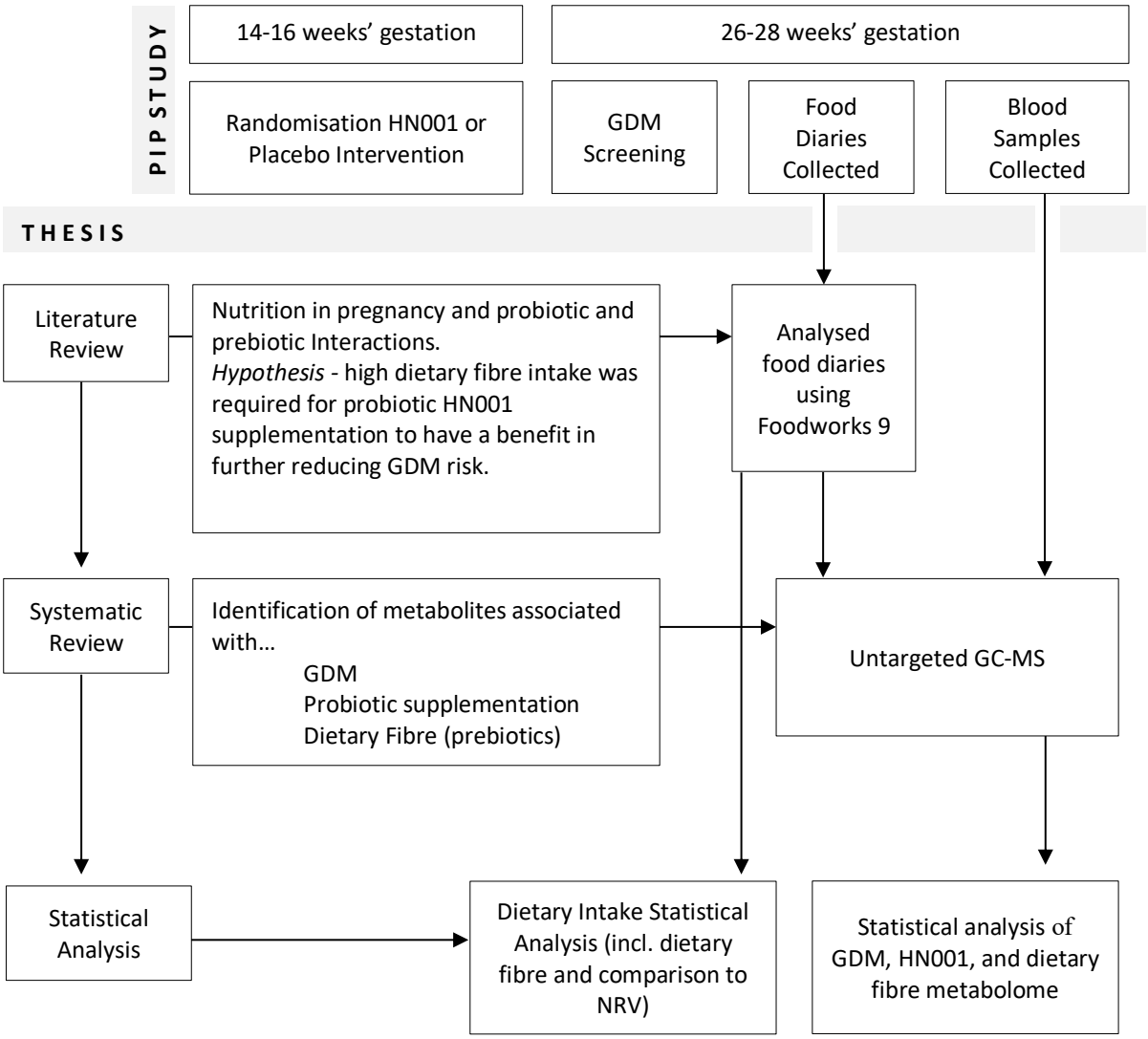


Figure 5 Flow diagram outlining the experimental design, broadly divided into 4 parts: 1) Probiotic in Pregnancy study; 2) dietary data analysis; 3) systematic review and 4) analysis of metabolites using appropriate statistical methods to identify potential biomarkers of GDM, dietary fibre and probiotic HN001 supplementation.

Abbreviations: GDM = gestational diabetes mellitus; NRV = Nutrient Reference value; GC-MS = gas chromatography-mass spectrometry.

4.2 Probiotics in Pregnancy study

4.2.1 Ethics

The PiP Study was granted ethical approval by the New Zealand Multi-region Ethics Committee (MEC/11/09/077). Prior to commencing participation in the study, participants were informed of all necessary information in relation to the study. Written consent was obtained (1,2).

4.2.2 Participants

Participants were recruited from the Wellington and Auckland region via clinicians and lead maternity carers. Women who met the below criteria were enrolled into the study at 14–16 weeks' gestation. Gestation was determined on the basis of the earliest first-trimester scan or the date of the last menstrual period (1,2).

The inclusion criteria for the PiP study included:

- English-speaking
- They or the child's biological father have a history of asthma, eczema, or allergic rhinitis requiring treatment
- The participant intends to stay in either Auckland or Wellington for 18 months following study enrolment

The exclusion criteria for the PiP study included:

- Women under age 16
- Women with type 1 or type 2 diabetes were excluded from GDM analysis
- Diagnosed with serious immunological disorder or taking immune suppressant drugs, known cardiac valve disease or history of transplant or HIV
- Required IVF to conceive current pregnancy
- The pre-enrolment scan showed major foetal abnormalities
- Is taking or intending to take probiotic supplements or drink during the pregnancy
- Already enrolled in another randomised control trial
- A severe allergy to cow's milk

4.2.3 Study capsules

Participating women were randomised to receive capsules containing either HN001 (6×10^9 CFU/d) or placebo (maize-derived maltodextrin, identical in appearance and smell to the probiotic) daily from enrolment (14-16 weeks' gestation) until 6 months postpartum. Fonterra Co-operative Group Ltd (Fonterra) developed the HN001 study capsules and tested their viability over time. Loss in viability was <0·1 log, and within the limit of uncertainty of the counting method. Participants were instructed to keep the study capsules in the refrigerator and to avoid taking the capsule within 10 minutes of consuming hot food or fluid (1,2).

Randomisation was conducted externally by a statistician at Fonterra. All staff involved in the research project and participants were blinded to the study allocation (1,2).

4.2.4 GDM diagnosis

Assessment of GDM was conducted at 24-30 weeks' gestation using plasma glucose results obtained after OGTT following a 12h overnight fast. GDM diagnosis was defined according to the IADPSG guidelines: a fasting plasma glucose ≥5·1 mmol/l, or 1h post 75g glucose load ≥10mmol/l or at 2h ≥8·5 mmol/l (3). A secondary analysis was conducted using New Zealand diagnostic thresholds ≥5·5mmol/l fasting or ≥9mmol/l at 2h to define GDM (4). For the purpose of this thesis, all women who were diagnosed with GDM by the IADPSG guidelines were included in the study. Women who were negative for GDM as per the New Zealand guidelines, however, were not assessed for GDM as per the IADPSG guidelines were excluded from the analysis (1,2).

4.2.5 Food diary collection

Participants were required to complete one 3-day food diary between 26-28 weeks' gestation. Participants were requested to record all food and drink eaten, at the time of eating. Written instructions provided to the participants requested that they include specific details of portion size, method of cooking (e.g. fried, grilled, boiled, roasted), specific details of the food (e.g. breakfast cereal = weet-bix, milk = whole or trim), and any additions to the food (e.g. sauces, dressings, spreads). Records of any dietary supplements taken during the 3-day period including the study capsule were also requested. Food diaries were collected at the time of the participants OGTT.

4.2.6 Blood sample collection and processing

A fasting blood sample was collected at the time of the OGTT from participants enrolled in the PiP study. The samples were stored at -80°C for future analysis (1,2).

4.3 Food diary analysis

Food diaries were entered into Foodworks 9 Professional (Xyris™ software) by the author (*n* = 348). Foodworks 9 professional uses nutritional data from FOOD files, a reference food composition table developed by the *Ministry of Health* and *Plant and Food Research*. Due to the limitations associated with food diaries, i.e. unspecific descriptions of amounts taken or missing details of foods, informed assumptions were made by the author based on published nutritional guidelines or manufacturer recommendations. These included: *Eating and Activity Guidelines for New Zealand Adults* (117) The *2008/09 New Zealand Adult Nutrition Survey* (118), and *The Concise New Zealand Food Composition Table* (119). Details of the assumptions made by the author during data entry are detailed appendix B.

The author was blinded to the participants intervention during food diary data entry to eliminate bias.

4.3.1 Nutrient reference values

The Food and Nutrition Guidelines for Healthy Pregnant and Breastfeeding Women (19-50 years) were referred to for reference values for energy, protein, fat, carbohydrate and dietary fibre (8). These reference values are based on the *Nutrient Reference Values for New Zealand and Australia* determined in 2006 by a joint project with the *New Zealand Ministry of Health* and *Australian Commonwealth Department of Health and Ageing Project* (8).

4.3.2 Statistical analysis

Descriptive analyses for participant characteristics were conducted using IBM® *SPSS*® Statistics (version 25) (by the author). Food diaries were analysed (by the author) to ascertain the mean and standard deviation of the macronutrient intake of participants also using IBM® *SPSS*® Statistics (version 25). Two-way analysis of variance (ANOVA) and Fisher's exact test were conducted using SAS version 9.4 (SAS Institute, Cary, NC) to determine the statistical impact of HN001 intervention and the effect of GDM. Individual means were compared when a significant interaction effect was detected with the two-way ANOVA (by Associate Professor Lindsay Plank). Figures were constructed (by the author) using SigmaPlot (Systat Software, Inc., San Jose, CA).

4.4 Untargeted metabolomics analysis

The protocol for gas chromatography – mass spectrometry (GC-MS) and data pre-processing was provided by Mr Raphael Bang, PhD Candidate, School of Biology, University of Auckland.

4.4.1 Gas chromatography – mass spectrometry

GC-MS was used for relative quantification and identification of metabolites in MCF derivatised human plasma. The instrument used was an Agilent 7890B GC coupled to a 5977A inert MS with a split/splitless inlet. The column used was a fused silica TG-1701, 30m long, 250µm (internal diameter), 0.15µm stationary phase (86% dimethylpolysiloxane, 14% cyanopropylphenyl). One microlitre of the derivatised sample was injected directly to the GC system using an Agilent autosampler into a glass split/splitless 4mm (internal diameter) straight inlet liner packed with deactivated glass wool (Supelco). The inlet temperature was fixed at 280°C. The samples were injected in splitless mode using ultra-high purity grade Helium as carrier gas at a column flow rate of 1mL/min. The GC oven temperature setting are outlined in Table 9. The transfer line to the mass selective detector was maintained at 250 °C, the ion source at 200°C, and quadrupole at 200°C. The ion source was operated in electron impact ionization mode at 70eV. Compounds were detected using mass spectra acquired in scan mode in the range of 38 to 550m/z.

Table 9 Sample processing

Oven stage	**Ramp rate (°C /min)**	**Final Temperature (°C)**	**Hold time (min)**
Initial	-	45	2
1	9	180	5
2	40	220	5
3	40	240	11.5
4	40	280	10

4.4.2 Data pre-processing

Raw GC-MS data generated from sample analysis was processed through the software Automated Mass spectral Deconvolution and Identification System (AMDIS) for deconvolution. Identification of compounds was based on an in-house MCF mass spectral library, only considering those with a match quality above 65%. An in-house R package was used for automated integration of reference ion peak height. Each identification was individually screened, and manual retention time correction and subsequent re-integration was applied where required. The resulting peak abundance values were normalized by the internal standard d4-Alanine. Generalized log transformation was first applied to the normalised data followed by Pareto scaling to make features within the data more comparable

prior to applying analytical methods. Identification of compounds was based on an in-house MCF mass spectral library, only considering those with a match quality above 65%.

4.4.3 Statistical analysis

Statistical plans were determined by Raphael Bang in collaboration with the author (Gabrielle Orr) and primary supervisor (Rinki Murphy). Shapiro Wilk's normality test was first applied across all metabolite identifications to assess normality of data. The non-parametric Wilcoxon-Mann-Whitney two sample test was applied for group comparisons as the dataset displayed non-normal distribution of peak intensities – a typical feature of metabolomics data-sets. Where stated, a two-way ANOVA was used for modelling differences of metabolites between study groups followed by a Tukey's HSD post-hoc test (conducted by Raphael Bang). Fold change of significant metabolites identified from statistical analyses were calculated (as % change) (by the author). All tables and figures (with the exception of the heat map's – constructed by Raphael Bang) were constructed by the author for presentation. Each significant compound and its associated pathways were classified using The Human Metabolome Database (Version 4.0) to improve the understanding and interpretation of the findings.

Chapter 5 Results

5.1 Participant characteristics

Figure 6 shows the inclusion of participants through the phases of the PiP study and for inclusion in the dietary and metabolomics analysis for this thesis. As the IADPSG guidelines (3) have lower glucose thresholds for detection of GDM, participants were excluded if GDM was classified as negative by the New Zealand guidelines (4) and IADPSG screening was not conducted (*n* = 8). Of the participants who completed GDM screening by both the IADPSG and New Zealand diagnostic criteria, food diaries were missing for 35 participants. Therefore, 352 food diaries were available for entry into Foodworks 9. Of these food diaries, four were incomplete. Incompletion of the food diary was classified as inadequate details of the food consumed (e.g. breakfast = "cereal" or lunch = "sandwich") or incompletion of all three days. As a result, 348 dietary records were available for analysis. Of the participants with completed GDM screening and food diaries, 328 blood plasma samples were available for metabolomics analysis.

Baseline summary data for the participants at 14-16 weeks' gestation, for whom dietary records were available (*n* = 348) are detailed in Table 10. Mean age of 33-34 years was similar within all groups, except for the GDM/placebo group who had a mean age of 37.3 $\pm$ 4.4 years. NZ European was the predominant ethnicity in this study (*n* = 281, 81%), followed by Māori (*n* = 41, 12%). Food diaries were available for 19 (6%) Asian participants, and only 6 (2%) Pacific people and 1 (0.3%) person identifying as other. There was a significant difference between the mean baseline BMI, such that participants who did not develop GDM had a lower BMI irrespective of the study intervention (GDM; 30.8 $\pm$ 7.5kg/m^2 vs noGDM; 26.2 $\pm$ 4.5 kg/m^2; p <0.001). This finding is in line with results published by Wickens et al. (2). No significant differences were apparent in the BMI or weight between the included participants (*n* = 348) and those who did not complete 3-day food diaries (*n* = 35). However, participants who did not complete food diaries were statistically significantly younger (31.5 $\pm$ 5.3 vs 33.8 $\pm$ 4.2 years; p = 0.002) and 56.4% were of non-European ethnicity (26% Māori, 21% Asian and 10% Pacific people).

Baseline summary data for participants included in each metabolomics analysis (*n* = 328) are detailed in Table 11. There were no significant differences between participants included in the food diary analysis compared to the metabolomics analysis.

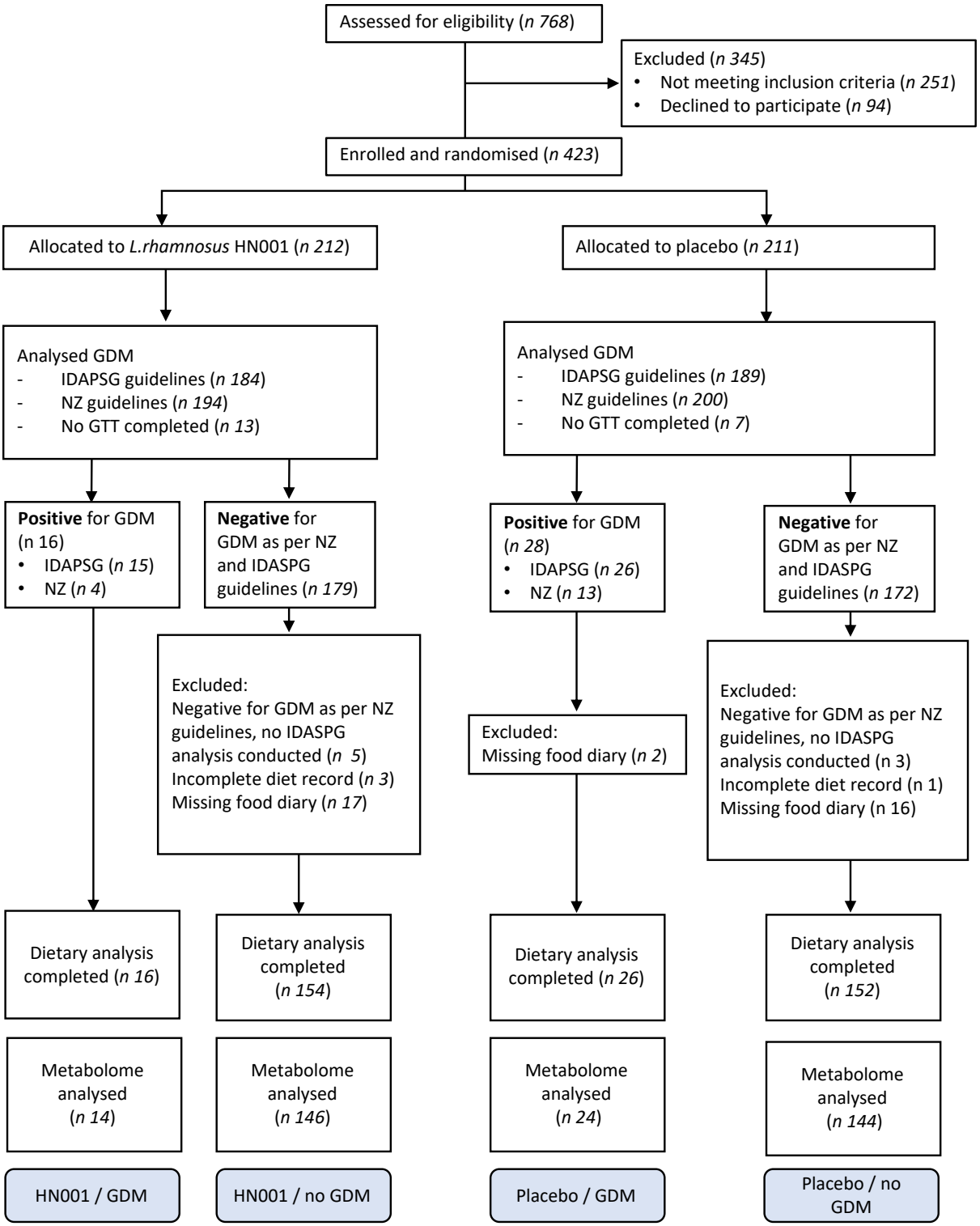


Figure 6 Flow chart of participants included in this thesis.

Table 10 Characteristics of the study population at baseline (14-16 weeks' gestation) for participants with completed 3-day food diaries.

	GDM		no GDM	
	HN001	**Placebo**	**HN001**	**Placebo**
n	16	26	154	152
Age (years)*	33.0 ± 3.7	37.2 ± 4.4	33.4 ± 4.2	33.7 ± 3.9
Weight (kg)*	82.9 ± 19.2	83.9 ± 20.6	71.7 ± 12.1	72.4 ± 13.5
BMI (kg/m^2)*	30.4 ± 7.2	31.1 ± 7.6	26.0 ± 4.3	26.4 ± 4.7
Ethnicity†				
Māori	3 (19%)	4 (15%)	14 (9%)	20 (13%)
Pacifica	1 (6%)	1 (4%)	3 (2%)	1 (1%)
Asian	1 (6%)	2 (8%)	8 (5%)	8 (5%)
European	11 (69%)	19 (73%)	129 (84%)	122 (80%)
Other	0 (0%)	0 (0%)	0 (0%)	1 (1%)
GDM Diagnosis†				
NZ guidelines	4 (25%)	12 (46%)	-	-
International guidelines	16 (100%)	26 (100%)	-	-

*Data presented as mean ± standard deviation

†Data presented as frequency (%)

Table 11 Characteristics of the study population at baseline (14-16 weeks' gestation) for participants included in the metabolomics analysis.

	GDM		no GDM	
	HN001	**Placebo**	**HN001**	**Placebo**
n	14	24	146	144
Age (years)*	32.8 ± 3.9	37.0 ± 4.4	33.3 ± 4.1	33.6 ± 4.0
Weight (kg)*	83.4 ± 20.4	83.1 ± 21.2	71.4 ± 12.2	72.6 ± 13.6
BMI (kg/m^2)*	30.7 ± 8.0	30.4 ± 7.6	25.9 ± 4.3	26.5 ± 4.8
Ethnicity†				
Māori	3 (21%)	3 (13%)	12 (8%)	17 (12%)
Pacifica	1 (7%)	1 (4%)	3 (2%)	1 (1%)
Asian	1 (7%)	2 (8%)	8 (5%)	7 (5%)
NZ European	9 (64%)	18(75%)	123(84%)	118 (81%)
Other	-	-	-	1 (1%)

*Data presented as mean ± standard deviation

†Data presented as frequency (%)

5.2 Food diary analysis

5.2.1 Comparison to nutrient reference values

The mean and standard deviation of the dietary macronutrient (protein, fat and carbohydrate) and energy intake of PiP study participants was determined by the author and compared to *New Zealand and Australia Nutrient Reference Values (NRV) for Pregnancy* (19-50 years) (8) (Table 12). Evaluation of all participants included in this thesis (*n* = 348) showed that the mean dietary intake of fat, protein and carbohydrate (as % total energy (%TE)) were within accordance with the recommended NRV. However, the mean saturated fat intake was greater than the NRV. Additionally, the mean total energy (8427 ± 5221kJ per day) was lower than the NRV (9600-12,500kJ per day) for pregnant women in their second trimester.

When evaluating by study group, mean dietary intake of energy, fat, protein and carbohydrate (%TE) were in accordance with the recommended nutrient reference range. With the exception of the carbohydrate intake of GDM/placebo participants which on average fell 1% below the recommended carbohydrate intake range (NRV = 45-65%TE).

Protein intake (as g per day (g/d)) during pregnancy is recommended as 1g per kg of body weight. Estimated mean dietary protein intake was higher than the estimated protein requirements for the total PiP study cohort. Mean protein intakes were also higher than estimated requirements for GDM/placebo and noGDM/placebo participants (Table 12).

Table 12 Average macronutrient content (mean ± SD) reported by PiP study participants using a 3-day food diary.

			GDM		No GDM		p-values		
	NRV	**PiP cohort**	**HN001**	**Placebo**	**HN001**	**Placebo**	**GDM**	**HN001**	**Interaction**
n	-	348	16	26	154	152	-	-	-
Energy (kJ/d)	9600-12500	8427 ± 5221	7338 ± 2091	7901 ± 1713	8240± 1827	8229 ± 1600	0.305	0.986	0.512
Estimated Protein req.[1]	-	73.43 ± 14.27	82.85 ± 19.12	71.69 ± 12.07	83.89 ± 20.58	72.42 ± 13.47	-	-	-
Protein (g/d)	-	80.69 ± 19.61	80.99 ± 16.62	81.50 ± 23.48	81.32 ± 20.06	79.88 ± 18.87	0.846	0.890	0.769
Protein (%TE)	15-25	17.04 ± 3.31	19.28 ± 3.51	17.98 ± 4.18	16.99 ± 3.15	16.68 ± 3.20	**0.001**	0.148	0.376
Carbohydrate (g/d)	>175g	229.4 ± 58.42	207.5 ± 86.09	206.5 ± 48.96	234.2 ± 62.75	230.4 ± 56.43	**0.013**	0.814	0.893
Carbohydrate (%TE)[2]	45-65	46.53 ± 6.04	46.19 ± 6.73	43.77 ± 6.74	46.80 ± 5.94	46.77 ± 5.89	0.077	0.229	0.240
Dietary Fibre (g/d)	28	25.73 ± 8.37	22.54 ± 8.48	23.40 ± 6.68	26.33 ± 8.79	25.86 ± 8.10	**0.027**	0.893	0.637
Fat (g/d)	-	72.6 ± 21.67	60.89 ± 18.56	76.19 ± 24.39†	72.42 ± 22.35†	73.40 ± 20.58†	0.232	**0.026**	**0.051**
Fat (%TE)	20-35	32.68 ± 5.58	30.84 ± 6.46‡	35.12 ± 5.70	32.36 ± 5.58‡	32.78 ± 5.39‡	0.665	**0.013**	**0.040**
SFA (%TE)	≤10	13.44 ± 3.87	12.20 ± 2.83	13.83 ± 3.47	13.42 ± 3.47	13.52 ± 4.39	0.487	0.188	0.242
% of total fat									
SFA	-	-	45.39 ± 7.53	44.66 ± 8.67	46.39 ± 7.70	45.62 ± 7.67	0.445	0.569	0.988
MUFA	-	-	38.41 ± 4.84	39.92 ± 4.62	38.24 ± 5.38	38.46 ± 4.48	0.331	0.302	0.441
PUFA	-	-	16.21 ± 3.73	15.42 ± 5.63	15.37 ± 4.65	15.68 ± 4.61	0.711	0.764	0.490

Abbreviation: SD = standard deviation; PiP = probiotic in pregnancy; NRV = nutrient reference value, g/d = grams per day, kJ/d = kilojoules per day, %TE = percent of total energy, SFA = saturated fatty acid, MUFA = monounsaturated fatty acid, PUFA = polyunsaturated fatty acid

[1]Estimated mean protein requirements based on 1g per kg body weight per day.

[2]Excludes dietary fibre

†Mean value significantly different from that of the HN001/GDM group ($p < 0.05$).

‡Mean value significantly different from that of the Placebo/GDM group ($p < 0.05$).

5.2.2 Dietary fat intake

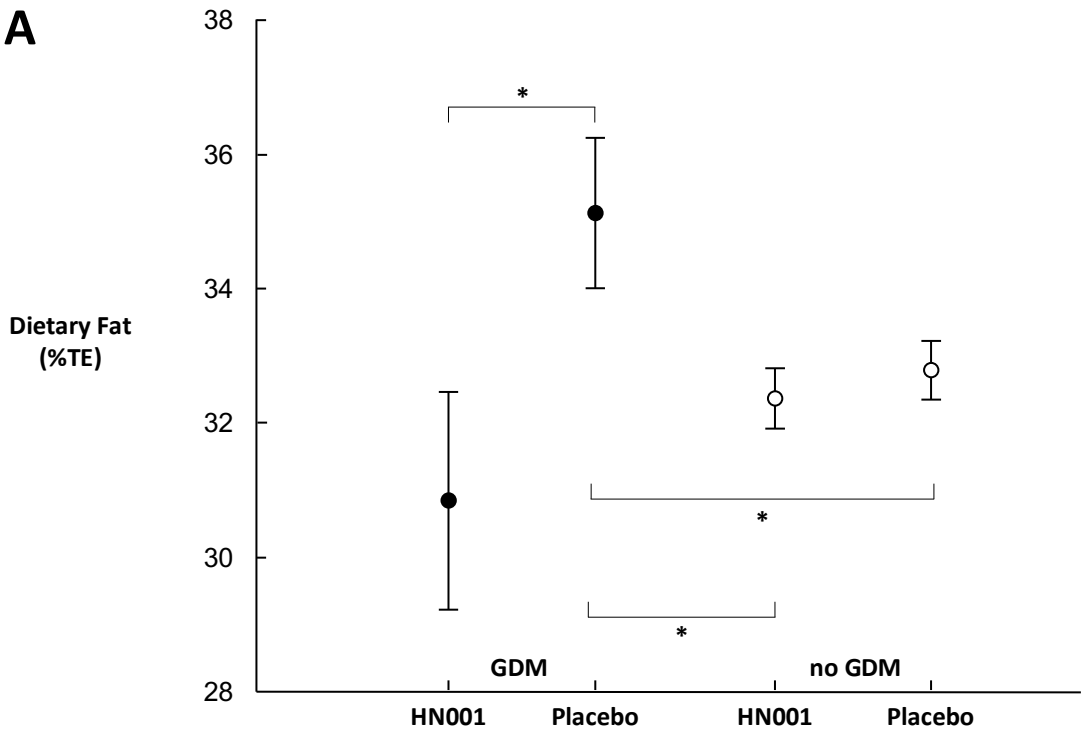
Two-way ANOVA revealed significant differences in dietary fat intake (as %TE and g/day) (Table 12; Figure 7, A and B). GDM/placebo participants had significantly higher reported intakes of fat compared to all other groups (GDM/placebo: 35.12 ± 5.70%TE; 76.19 ± 24.39g/d; p <0.05). GDM/HN001 participants had the lowest intakes of dietary fat compared to all other groups (GDM/HN001: 30.84 ± 6.46%TE; 60.89 ± 18.56g/d; p <0.05). There were no significant differences between groups when evaluating fatty acids (saturated, polyunsaturated and monounsaturated) as percentage of total fat.

5.2.3 Dietary protein intake

Estimated protein intake (as g/d) was not significantly different across study groups. However, when evaluated as percentage of total energy, women with GDM had significantly higher protein intake compared to women without GDM (GDM: 18.48 ± 3.95%TE vs. noGDM: 16.84 ± 3.17%TE; p = 0.001), irrespective of probiotic supplementation (Figure 8).

5.2.4 Dietary carbohydrate intake

Estimated carbohydrate intake (as %TE) was not significantly different across study groups. However, when evaluated as g per day women with GDM had significantly lower carbohydrate intake compared to women without GDM (GDM: 206.9 ± 64.60g/d vs. no GDM: 232.4 ± 56.94g/d; p = 0.013), irrespective of probiotic supplementation (Figure 9).The carbohydrate analysis does not include dietary fibre intake, which is discussed in section 5.2.5.



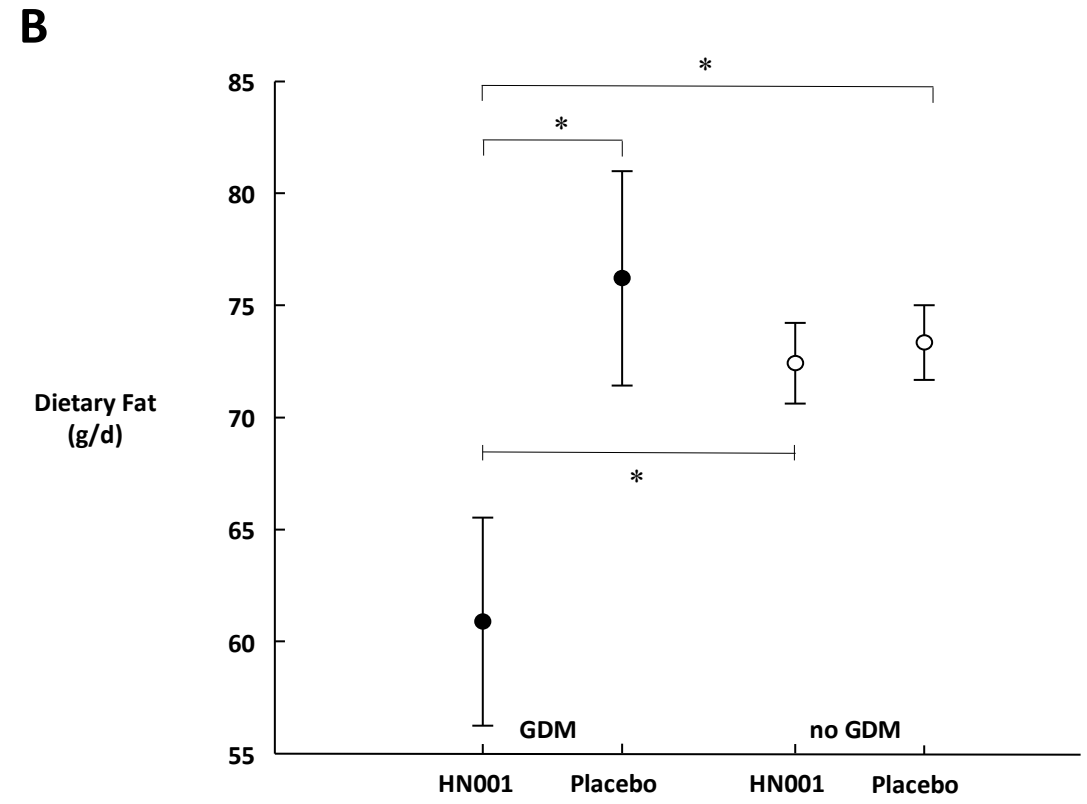


Figure 7 Estimated dietary fat intakes of women in the PiP study as **A**) % of total energy; **B**) g/d.
*$p < 0.05$

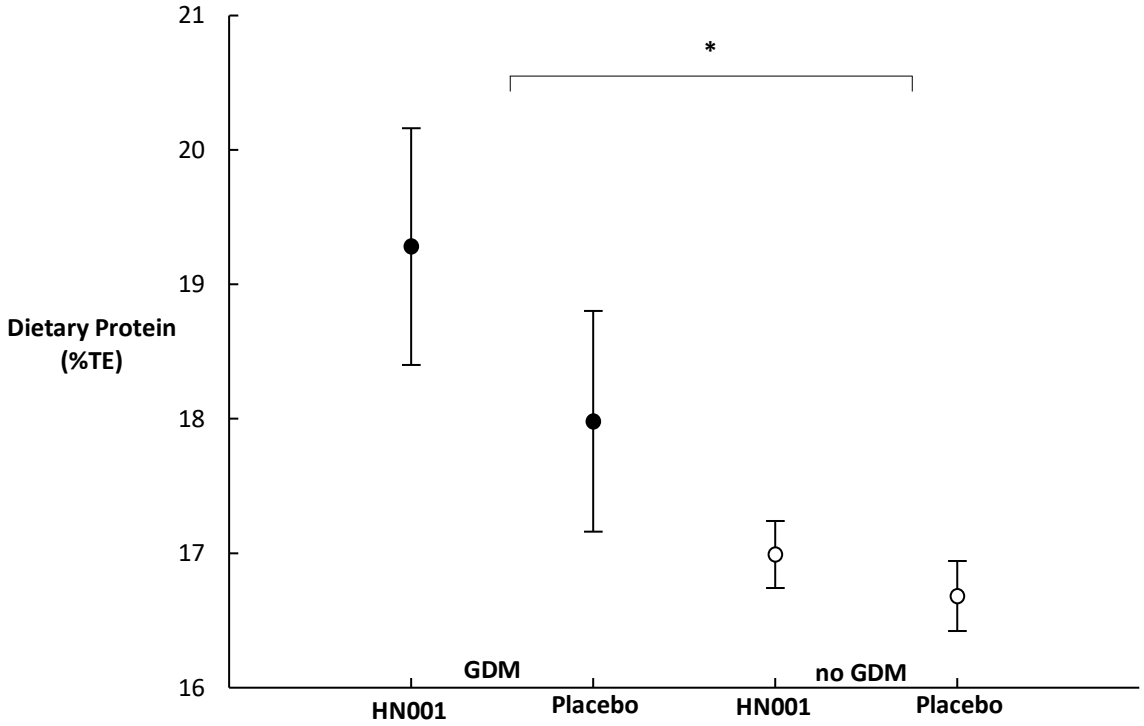


Figure 8 Estimated protein intakes of women in the PiP study as %TE.
*p = 0.001

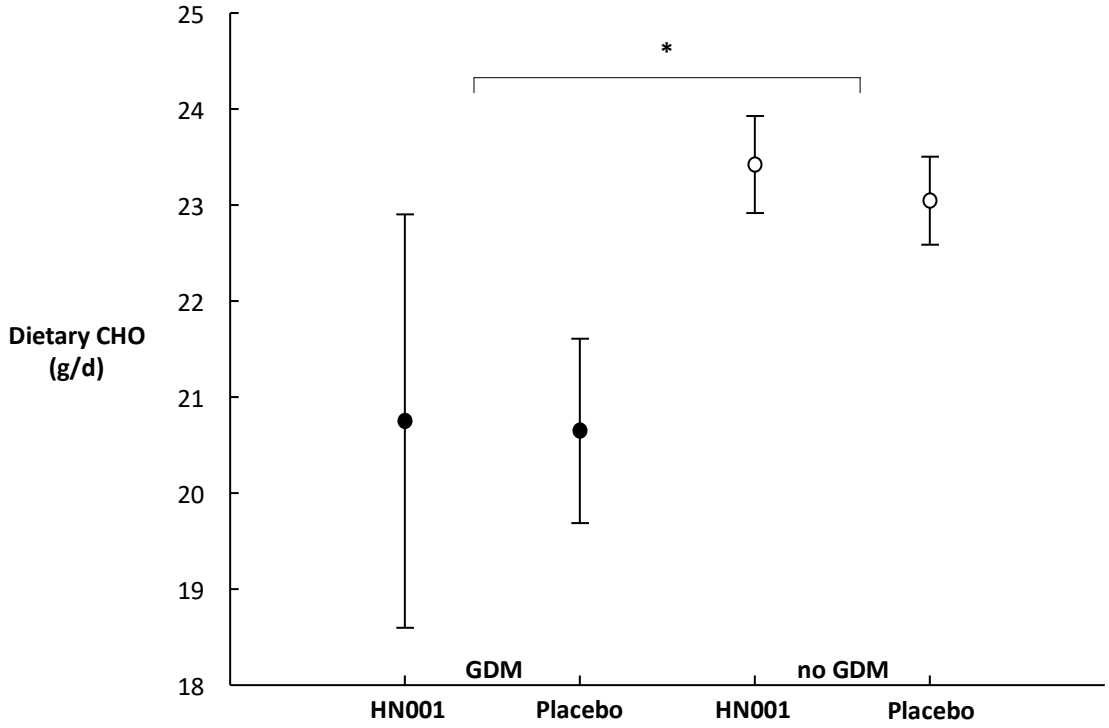


Figure 9 Estimated carbohydrate (CHO) intakes of women in the PiP study reported as g/d.
*p = 0.013

5.2.5 Dietary fibre intake

Irrespective of probiotic supplementation, women who developed GDM had significantly lower dietary fibre intake compared to women without GDM (GDM: 23.07 ± 7.33g/day vs. noGDM: 26.10 ± 8.45g/day; $p = 0.027$) (Table 12; Figure 10). The dietary fibre NRV states that during pregnancy women should aim for dietary fibre intake of 28g/d, compared to the recommended intakes of ≥25g/d for non-pregnant women (8). No significant association was found between either dietary fibre intake of ≥25g/d or ≥28g/d and GDM status in this cohort (Table 13). Adequate dietary fibre intake during pregnancy, ≥28g per day, was reported by only 37.4% of total participants. However, 52.3% of women without GDM reported dietary fibre intakes ≥25g per day. A greater proportion of women with GDM had dietary fibre intake <25g/d (61.9%).

To evaluate the impact of dietary fibre on the efficacy of probiotic HN001 supplementation, dietary fibre intakes of study groups were also compared (Table 12). noGDM/HN001 participants had the highest intake of dietary fibre compared to all other groups (noGDM/HN001: 26.33 ± 8.79 g/d). In contrast, dietary fibre intake was lowest for participants who were allocated to probiotic HN001 supplementation, yet still developed GDM (GDM/HN001: 22.54 ± 8.48g/d). However, no statistical interaction was detected for HN001 supplementation and GDM status.

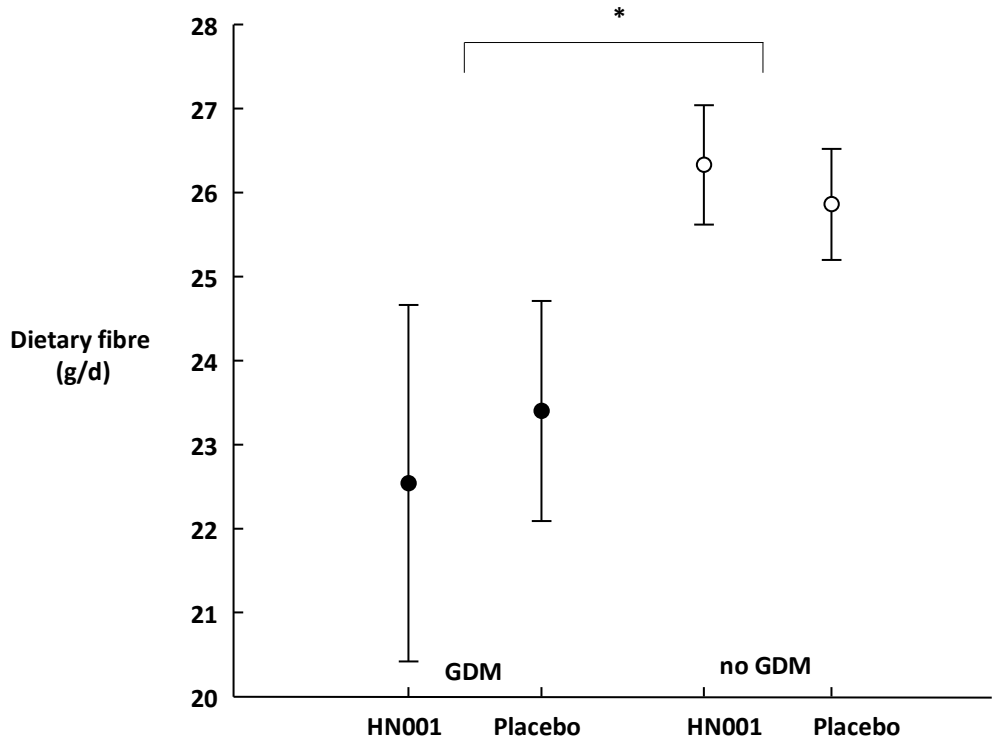


Figure 10 Estimated dietary fibre intakes of women in the PiP study reported as g per day.
*p = 0.027

Table 13 Number of participants *(n*) with adequate dietary fibre intake above recommended guidelines (≥28g/d or ≥25g/d) compared to poor dietary fibre intake (<28g/d or <25g/d), irrespective of study intervention (HN001 or placebo). *p*-value indicates significance of dietary fibre thresholds and GDM status.

Dietary Fibre Intake	**GDM** (*n)*	**No GDM** (*n)*	***p*-value**
Adequate (≥28g/d)	10	120	0.062
Poor (<28g/d)	32	186	
Adequate (≥25g/d)	16	160	0.100
Poor (<25g/d)	26	146	

5.3 Untargeted metabolomics analysis

5.3.1 PiP study cohort metabolome

A GC-MS based methodology was applied for plasma metabolomic analysis of 328 women who participated in the PiP study. Baseline summary data for the participants at 14-16 weeks' gestation, for who metabolomics analysis was completed is illustrated in section 4.2.2, Table 11. A total of 164 metabolites were detected and of these metabolites 107 were identified using the in-house mass spectral MCF library (57 are unknown metabolites). A complete list of the metabolites identified by GC-MS in the PiP study cohort metabolome is detailed in appendix C.

Tukey HSD method and two-way ANOVA were used for modelling differences of metabolites between study groups. Based on these analyses the mean normalised peak response (± standard deviation) of 31 significant metabolites (p <0.05) are detailed in Table 14. Salicylic acid, 2-oxovaleric acid and tryptophan were significantly associated simultaneously with GDM and probiotic HN001 supplementation (p <0.05). A total of 27 metabolites were significantly associated independently with GDM outcome. Three metabolites were significantly associated independently with probiotic HN001 supplementation (p <0.05).

The mean normalised peak response signifies which study groups had the most significant alteration of metabolite levels, however, without direct comparison the direction of the metabolites are unknown. Therefore, based on the mean normalised peak responses the percentage fold change of significant metabolites associated independently with GDM and HN001 were determined by direct comparison between GDM vs. no GDM (including HN001 and placebo) (Figure 11) and HN001 vs. placebo (GDM and no GDM) (Figure 12).

The analysis of the whole PiP study metabolome included GDM, probiotic HN001 supplementation and dietary fibre as confounding variables. Therefore, as detailed in the following sections further comparisons were conducted to determine the significance of metabolites associated with GDM, HN001 and dietary fibre intakes, independently.

Table 14 Normalised peak response of statistically significant metabolites identified in PiP study cohort metabolome.

	GDM		No GDM		*p* values		
Metabolite	**HN001** (*n* = 14)	**Placebo** (*n* = 24)	**HN001** (*n* = 146)	**Placebo** (*n* = 144)	**HN001**[1]	**GDM**[1]	**Interaction**[2]
Salicylic acid	$1.65x10^{-4} \pm 9.57x10^{-5}$	$1.07x10^{-3} \pm 4.14x10^{-3}$	$3.29x10^{-4} \pm 8.27x10^{-4}$	$2.32x10^{-4} \pm 2.61x10^{-4}$	0.850	0.976	**0.012**
2-Oxovaleric acid	$1.90x10^{-2} \pm 2.01x10^{-2}$	$1.03x10^{-2} \pm 3.53x10^{-3}$	$7.51x10^{-3} \pm 3.35x10^{-3}$	*$8.20x10^{-3} \pm 4.23x10^{-3}$*	0.324	**<0.001**	**0.012**
Tryptophan	$1.19x10^{-1} \pm 3.44x10^{-2}$	$1.32x10^{-1} \pm 2.13x10^{-2}$	*$1.41x10^{-1} \pm 3.43x10^{-2}$*	$1.34x10^{-1} \pm 3.20x10^{-2}$	0.232	0.120	**0.040**
2-Hydroxybutyric acid	$2.02x10^{-2} \pm 5.87x10^{-3}$	$1.82x10^{-2} \pm 4.65x10^{-3}$	$1.28x10^{-2} \pm 4.61x10^{-3}$	$1.21x10^{-2} \pm 4.36x10^{-3}$	0.490	**<0.001**	0.724
2-Hydroxyisobutyric acid	$1.94x10^{-2} \pm 5.59x10^{-3}$	$1.75x10^{-2} \pm 4.36x10^{-3}$	$1.25x10^{-2} \pm 4.48x10^{-3}$	$1.19x10^{-2} \pm 4.28x10^{-3}$	0.552	**<0.001**	0.687
3-Methyl-2-oxopentanoic acid	$1.44x10^{-2} \pm 4.25x10^{-3}$	$1.42x10^{-2} \pm 3.60x10^{-3}$	$1.22x10^{-2} \pm 3.24x10^{-3}$	$1.16x10^{-2} \pm 2.64x10^{-3}$	0.343	**<0.001**	0.631
cis-Aconitic acid	$3.69x10^{-3} \pm 9.86x10^{-4}$	$4.00x10^{-3} \pm 1.02x10^{-3}$	$3.27x10^{-3} \pm 9.35x10^{-4}$	$3.13x10^{-3} \pm 9.37x10^{-4}$	0.559	**<0.001**	0.193
4-Methyl-2-oxopentanoic acid	$1.49x10^{-2} \pm 4.19x10^{-3}$	$1.56x10^{-2} \pm 3.47x10^{-3}$	$1.33x10^{-2} \pm 3.31x10^{-3}$	$1.28x10^{-2} \pm 2.83x10^{-3}$	0.682	**<0.001**	0.282
Phenethyl acetate	$8.57x10^{-4} \pm 8.09x10^{-4}$	$1.20x10^{-3} \pm 7.81x10^{-4}$	$1.66x10^{-3} \pm 1.27x10^{-3}$	$1.58x10^{-3} \pm 1.05x10^{-3}$	0.958	**<0.001**	0.098
2-Aminobutyric acid	$3.81x10^{-2} \pm 1.08x10^{-2}$	$3.47x10^{-2} \pm 7.70x10^{-3}$	$3.12x10^{-2} \pm 1.11x10^{-2}$	$2.96x10^{-2} \pm 1.12x10^{-2}$	0.300	**0.001**	0.772
Itaconic acid	$7.15x10^{-4} \pm 1.95x10^{-4}$	$7.72x10^{-4} \pm 2.39x10^{-4}$	$6.51x10^{-4} \pm 2.17x10^{-4}$	$6.10x10^{-4} \pm 2.10x10^{-4}$	0.282	**0.001**	0.263
Citric acid	$1.44x10^{-3} \pm 4.18x10^{-4}$	$1.59x10^{-3} \pm 5.18x10^{-4}$	$1.33x10^{-3} \pm 4.36x10^{-4}$	$1.24x10^{-3} \pm 4.44x10^{-4}$	0.205	**0.001**	0.143
2-Oxoglutaric acid	$2.17x10^{-3} \pm 5.83x10^{-4}$	$1.85x10^{-3} \pm 4.08x10^{-4}$	$1.71x10^{-3} \pm 4.87x10^{-4}$	$1.73x10^{-3} \pm 4.87x10^{-4}$	0.864	**0.003**	0.104
Benzothiazole	$5.10x10^{-4} \pm 1.47x10^{-4}$	$5.08x10^{-4} \pm 1.52x10^{-4}$	$4.17x10^{-4} \pm 1.48x10^{-4}$	$4.53x10^{-4} \pm 2.16x10^{-4}$	0.108	**0.005**	0.612
Palmitelaidic acid	$2.94x10^{-2} \pm 1.96x10^{-2}$	$3.02x10^{-2} \pm 1.62x10^{-2}$	$2.30x10^{-2} \pm 1.16x10^{-2}$	$2.42x10^{-2} \pm 1.22x10^{-2}$	0.203	**0.007**	0.723
Phenylalanine	$1.22x10^{-1} \pm 2.27x10^{-2}$	$1.18x10^{-1} \pm 2.48x10^{-2}$	$1.08x10^{-1} \pm 2.00x10^{-2}$	$1.10x10^{-1} \pm 2.33x10^{-2}$	0.615	**0.009**	0.545
Adipic acid	$2.28x10^{-4} \pm 1.41x10^{-4}$	$2.11x10^{-4} \pm 1.45x10^{-4}$	$1.67x10^{-4} \pm 7.08x10^{-5}$	$1.72x10^{-4} \pm 7.65x10^{-5}$	0.471	**0.010**	0.424
2-Aminoadipic acid	$2.69x10^{-3} \pm 4.05x10^{-4}$	$2.57x10^{-3} \pm 4.99x10^{-4}$	$2.39x10^{-3} \pm 4.40x10^{-4}$	$2.42x10^{-3} \pm 5.19x10^{-4}$	0.730	**0.011**	0.415
Isocitric acid	$2.13x10^{-4} \pm 7.12x10^{-5}$	$2.31x10^{-4} \pm 7.40x10^{-5}$	$2.00x10^{-4} \pm 5.87x10^{-5}$	$1.92x10^{-4} \pm 6.56x10^{-5}$	0.438	**0.012**	0.268
Dehydroascorbic acid	$2.43x10^{-3} \pm 5.63x10^{-4}$	$2.72x10^{-3} \pm 1.20x10^{-3}$	$2.33x10^{-3} \pm 6.15x10^{-4}$	$2.28x10^{-3} \pm 5.61x10^{-4}$	0.964	**0.015**	0.318
Pyruvic acid	$6.51x10^{-3} \pm 3.59x10^{-3}$	$5.91x10^{-3} \pm 2.66x10^{-3}$	$5.14x10^{-3} \pm 2.03x10^{-3}$	$4.82x10^{-3} \pm 2.09x10^{-3}$	0.162	**0.015**	0.753

	GDM		No GDM				
						***p* values**	
Metabolite	**HN001** (*n* = 14)	**Placebo** (*n* = 24)	**HN001** (*n* = 146)	**Placebo** (*n* = 144)	**HN001**[1]	**GDM**[1]	**Interaction**[2]
Aspartic acid	$1.02x10^{-1} \pm 2.39x10^{-2}$	$8.48x10^{-2} \pm 2.34x10^{-2}$	$8.13x10^{-2} \pm 2.40x10^{-2}$	$8.14x10^{-2} \pm 2.91x10^{-2}$	0.602	**0.020**	0.109
Glutamic acid	$6.94x10^{-2} \pm 1.90x10^{-2}$	$5.81x10^{-2} \pm 1.85x10^{-2}$	$5.67x10^{-2} \pm 2.10x10^{-2}$	$5.50x10^{-2} \pm 2.31x10^{-2}$	0.259	**0.027**	0.265
Hippuric acid	$3.05x10^{-3} \pm 2.69x10^{-3}$	$3.24x10^{-3} \pm 1.81x10^{-3}$	$4.41x10^{-3} \pm 4.51x10^{-3}$	$4.51x10^{-3} \pm 4.17x10^{-3}$	0.476	**0.033**	0.691
Malonic acid	$3.38x10^{-4} \pm 9.45x10^{-5}$	$2.99x10^{-4} \pm 6.67x10^{-5}$	$2.90x10^{-4} \pm 8.35x10^{-5}$	$2.83x10^{-4} \pm 7.39x10^{-5}$	0.442	**0.033**	0.315
Creatinine	$8.63x10^{-3} \pm 2.45x10^{-3}$	$7.88x10^{-3} \pm 2.06x10^{-3}$	$7.44x10^{-3} \pm 1.84x10^{-3}$	$7.34x10^{-3} \pm 1.67x10^{-3}$	0.700	**0.033**	0.438
Trans-4-Hydroxyproline	$1.06x10^{-2} \pm 6.83x10^{-3}$	$8.15x10^{-3} \pm 5.00x10^{-3}$	$7.34x10^{-3} \pm 4.21x10^{-3}$	$6.80x10^{-3} \pm 3.78x10^{-3}$	0.234	**0.043**	0.608
DL-3-Aminoisobutyric acid	$3.46x10^{-3} \pm 1.61x10^{-3}$	$2.84x10^{-3} \pm 9.71x10^{-4}$	$2.70x10^{-3} \pm 1.13x10^{-3}$	$2.72x10^{-3} \pm 1.21x10^{-3}$	0.749	**0.045**	0.332
Malic acid	$1.78x10^{-3} \pm 6.96x10^{-4}$	$1.50x10^{-3} \pm 5.45x10^{-4}$	$1.44x10^{-3} \pm 5.58x10^{-4}$	$1.40x10^{-3} \pm 5.37x10^{-4}$	0.504	**0.045**	0.311
2-Aminophenylacetic acid	$2.56x10^{-4} \pm 9.17x10^{-5}$	$2.23x10^{-4} \pm 1.09x10^{-4}$	$2.23x10^{-4} \pm 9.84x10^{-5}$	$1.98x10^{-4} \pm 8.39x10^{-5}$	**0.013**	0.086	0.735
4-Hydroxycinnamic acid	$5.61x10^{-5} \pm 2.34x10^{-5}$	$6.47x10^{-5} \pm 2.91x10^{-5}$	$5.66x10^{-5} \pm 2.79x10^{-5}$	$6.33x10^{-5} \pm 3.68x10^{-5}$	**0.039**	0.599	0.855
Levulinic acid	$1.26x10^{-4} \pm 3.61x10^{-5}$	$1.32x10^{-4} \pm 4.32x10^{-5}$	$1.14x10^{-4} \pm 3.81x10^{-5}$	$1.22x10^{-4} \pm 4.04x10^{-5}$	**0.048**	0.138	0.778

[1]Tukey HSD method [2]two-way ANOVA

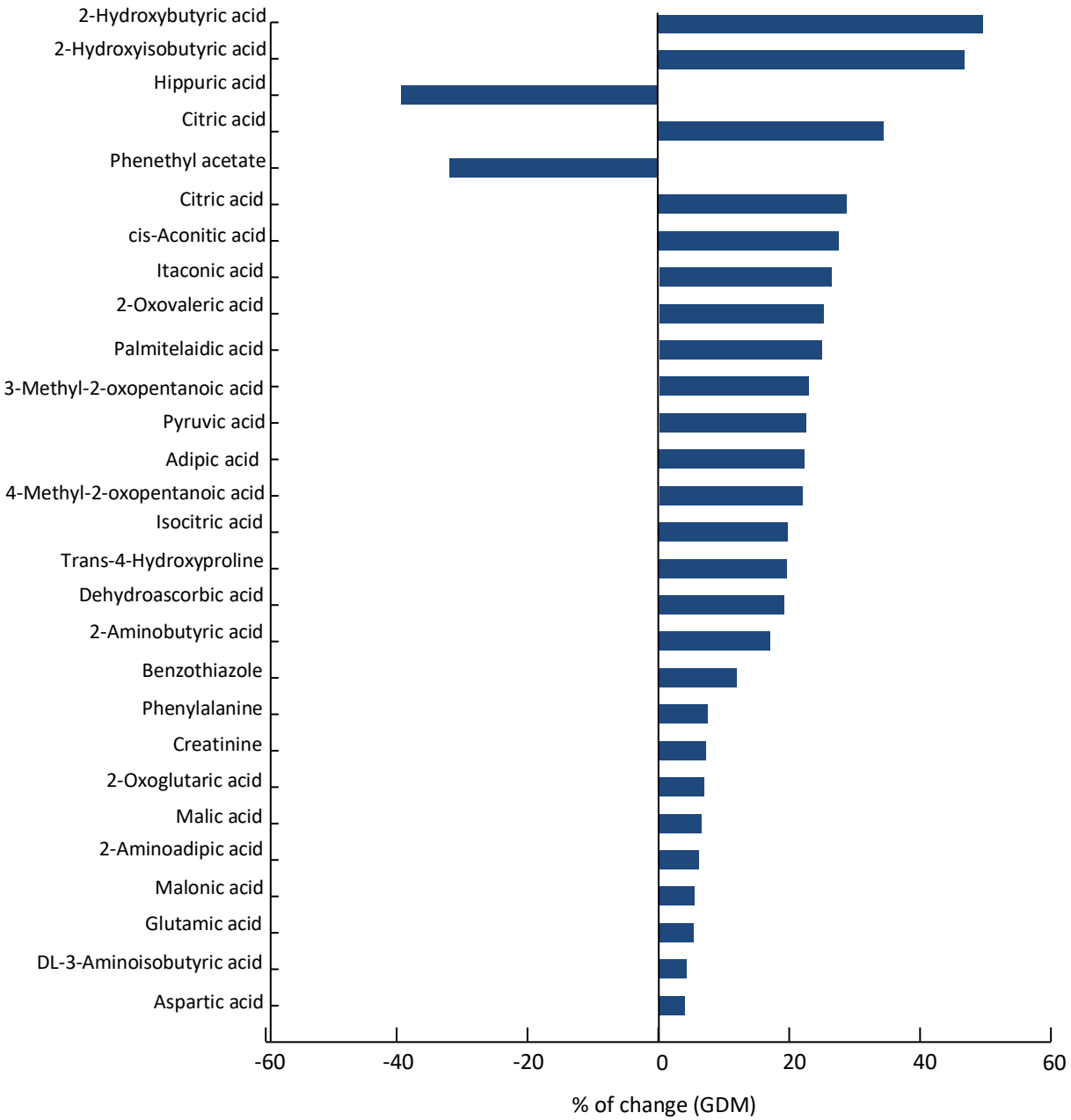


Figure 11 Percentage change of significant metabolites (p <0.05) associated with the GDM metabolome compared to the metabolome of women with NGT.

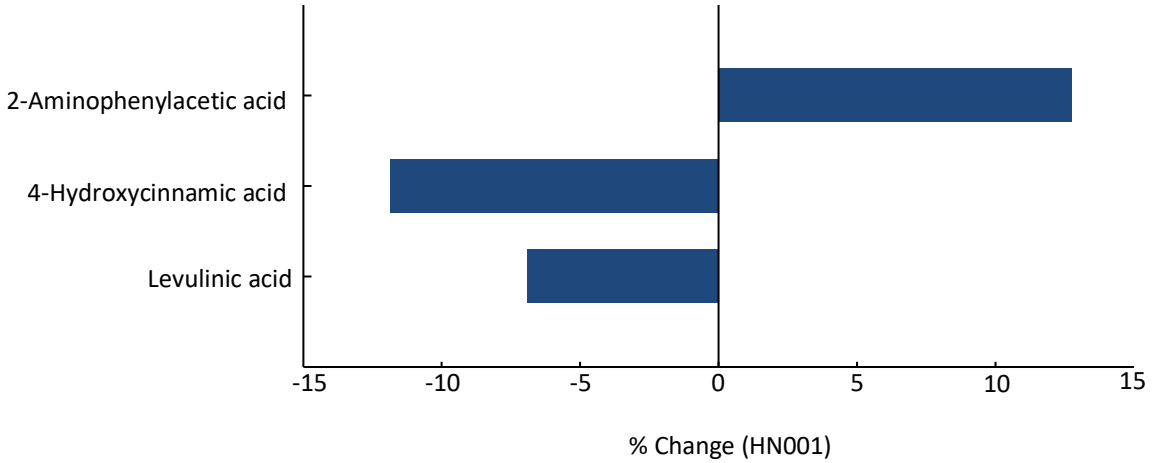


Figure 12 Percentage change of significant metabolites ($p < 0.05$) independently associated with HN001 supplementation compared to placebo.

5.3.2 GDM metabolome

To distinguish a GDM metabolome the plasma metabolome of women with GDM (*n* = 24) was compared to women who did not develop GDM (*n* = 144). Only women who were supplemented with placebo were included in this analysis, thus removing probiotic HN001 supplementation as a confounding variable. Characteristics of the participants included in this analysis is outlined in Table 15.

A Wilcoxon-Mann-Whitney two sample test to compare differences in metabolite levels between GDM cases and non-GDM controls resulted in 15 metabolites of significance (p <0.05). As detailed in Table 15, metabolites associated with amino acid metabolism, Tricarboxylic Acid Cycle (TCA), fatty acid metabolism and inflammation were significantly altered in women with GDM compared to no GDM.

5.3.3 HN001 metabolome

To determine metabolites associated with probiotic HN001 supplementation without the confounding variable of GDM metabolites; the metabolome of women who did not develop GDM who were allocated HN001 (*n* = 146) supplementation was compared to women without GDM who were allocated placebo supplementation (*n* = 144). Characteristics of the participants included in this analysis is outlined in Table 15. Two metabolites associated with tryptophan metabolism remained statistically significant (Table 15), 2-aminophenylacetic acid was significantly increased (5%, p = 0.03) and tryptophan was significantly decreased (0.49%, p = 0.021) in no GDM/HN001 participants compared to no GDM/placebo controls.

As tryptophan remained statistically significant, direct comparison of the normalised peak responses detailed in Table 14 showed that tryptophan was significantly increased by 18% in noGDM/HN001 participants compared to women with HN001/GDM (p = 0.040).

5.3.4 Dietary fibre metabolome

Heat maps (Appendix D, Figure 15 and Figure 16) were constructed to identify correlations between metabolites and dietary fibre intakes within each of the study groups. Correlation analysis of metabolite levels and dietary fibre intake revealed no statistical significance (p <0.05) for those showing strong correlations (i.e. correlation coefficient <0.75). Indicating that metabolites associated with dietary fibre intake were not simultaneously associated with HN001 and GDM.

To determine the metabolome of dietary fibre intakes independently, the metabolome of no GDM/placebo participants was analysed. Based on a potential protective metabolic effect of dietary

fibre between 25-26g per day (as determined by analysis of the 3-day food dairies), high dietary fibre intake was considered ≥25g per day. Therefore, the metabolome of noGDM/placebo participants who consumed dietary fibre ≥25g per day was compared to noGDM/placebo participants who consumed dietary fibre <25g per day. Of the 12 significant metabolites independently associated with high dietary fibre intakes, seven are dietary biomarkers and four are associated with amino acid metabolism (including BCAA), including the amino acid histidine – an anti-inflammatory biomarker (Table 15).

To identify which dietary fibre metabolites were augmented by HN001, the metabolites enriched in women supplemented with HN001 with dietary fibre intakes <25g per day were subtracted from the metabolome of women supplemented with HN001 with dietary fibre intakes ≥25g per day. All women included in this analysis had normal glucose tolerance, to remove confounding metabolites associated with GDM. The noGDM, HN001 supplemented, high fibre intake metabolome analysis revealed ten significant metabolites that are indicated as favourable for metabolic health. These include three biomarkers of fatty acid metabolism, five dietary biomarkers, and one amino acid. Significantly, lactic acid, a biomarker of gluconeogenesis, was downregulated in this analysis (Table 15).

Table 15 Metabolomes associated with GDM, probiotic HN001 supplementation and dietary fibre intakes.

Metabolome	Comparison	*n*	Age	Weight	BMI	Ethnicity	Dietary Fibre Intake (g/d)	Significant Metabolites (% change) Increased	Decreased
GDM Metabolome (placebo only)	**GDM** (test)	24	37.0 ± 4.4*	83.1 ± 21.2*	30.4 ± 7.6*	Māori: 3 (13%) Pacific: 1 (4%) Asian: 2 (8%) NZ European: 18(75%)	23.4 ± 6.68	*BCAA Pathways:* 2-hydroxyisobutyric acid (6%), 2-hydroxybutyric acid (5%), 3-methyl-2-oxopentanoic acid (6%), 4-methyl-2-oxopentanoic acid (2%). *Inflammation:* Dehydroabietic acid (37%), itaconic acid (10%). *TCA:* citric acid (9%), cis-aconitic acid (6%), fumaric acid (2%), isocitric acid (2%). *Fatty Acid Metabolism:* Palmitelaidic acid (10%). *Dietary Biomarker:* Benzothiazole (8%).	*BCAA Pathways:* 2-aminobutyric acid (3%), 2-oxovaleric acid (1%). *Vitamin C derivative:* dehydroascorbic acid (3%)
	No GDM (control)	144	33.6 ± 4.0	72.6 ± 13.6	26.5 ± 4.8	Māori: 17 (12%)Pacific: 1 (1%) Asian: 7 (5%) NZ European: 118 (81%) Other: 1 (1%)	25.9 ± 8.10		Ref.
HN001 Metabolome (no GDM only)	**HN001** (test)	146	33.3 ± 4.1	71.4 ± 12.2	25.9 ± 4.3	Māori: 12 (8%) Pacific: 3 (2%) Asian: 8 (5%) NZ European: 123(84%)	26.3 ± 8.79	*Tryptophan metabolism:* 2-aminophenylacetic acid (5%).	*Tryptophan metabolism:* Tryptophan (0.5%)
	Placebo (control)	144	33.6 ± 4.0	72.6 ± 13.6	26.5 ± 4.8	Māori: 17 (12%) Pacific: 1 (1%) Asian: 7 (5%) NZ European: 118 (81%) Other: 1 (1%)	25.9 ± 8.10		Ref.

Metabolome	Comparison	*n*	Age	Weight	BMI	Ethnicity	Dietary Fibre Intake (g/d)	Significant Metabolites (% change) Increased	Decreased
Dietary Fibre Metabolome (placebo, no GDM only)	**≥25g DF** (test)	74	33.9 ± 3.6	70.7 ± 11.6	25.6 ± 4.0*	Māori:7 (9%) Asian: 4 (5%) NZ European: 63 (86%)	32.0 ± 5.3	*Anti-inflammatory:* histidine (6%) *BCAA*: leucine (4%), isoleucine (4%). *AA:* asparagine (6%) *Biomarker of food (fruit, vegetables, wholegrains):* methylthioacetic acid (24%), hippuric acid, phenethyl acetate (34%), dehydroascorbic acid (11%), oxalic acid (12%), glyceric acid (12%).	*Biomarker of dairy fat:* X10-heptadeconoic acid (9%) *Other: t*rans-4-hydroxyproline (15%)
	<25g DF (control)	70	33.3 ± 4.3	74.6 ± 15.2	27.5 ± 5.3	Māori:10 (14%) Pacific:1 (1%) Asian: 3 (4%) NZ European: 55 (80%) Other:1 (1%)	19.1 ± 4.0		Ref.
Probiotic with Dietary Fibre metabolome (HN001, no GDM)	**HN001and ≥25g DF** (test)	77	33.7 ± 3.6	70 ± 11.1	25.4 ± 4.3	Māori: 6 (8%) Pacific: 1 (1%) Asian: 4 (5%) NZ European: 66 (86%)	33.1 ± 6.57	*Fatty Acid Metabolism:* myristoleic acid (16%), myristic acid (16%), decanoic acid (5%). *AA:* glyoxylic acid (7%) *Biomarker of food (fruit, vegetables, wholegrains):* phenethyl acetate (52%), methylthioacetic acid (51%), hippuric acid (42%), oxalic acid (19%).	*Gut Metabolite from digestion of polyphenols:* benzoic acid (18%). *Gluconeogenesis:* lactic acid (8%)
	HN001 and <25g DF (control)	69	32.9 ± 4.6	73.0 ± 13.2	26.5 ± 4.3	Māori: 6 (9%) Pacific: 2 (3%) Asian: 4(6%) NZ European: 57 (83%)	19.2 ± 4.1		Ref.

*Statistical variation between test and control groups; $p < 0.05$

Chapter 6 Discussion

6.1 Macronutrient intake of PiP study participants

While the primary focus of this thesis was to assess the presence of an interaction between dietary fibre and HN001 supplementation for prevention of GDM; assessment of 3-day food diaries also provided an opportunity to assess the dietary intakes of PiP study participants overall.

A recent survey of the dietary patterns of pregnant women in New Zealand reported that only 3% of women adhere to the Ministry of Health *Food and Nutrition Guidelines for Healthy Pregnant and Breastfeeding Women* (8,14). Analysis of 3-day food diaries collected at 26-28 weeks' gestation, suggests that the macronutrient and energy intake of women who participated in the PiP study were within accordance of the *Nutrient Reference Values for New Zealand and Australia* (NRV) for pregnancy (8). However, estimated saturated fat intake was approximately 3% higher than the NRV and surprisingly, estimated mean energy intake were slightly below the reference range for all study groups (8).

To the best of our knowledge, the latest assessment of individual macronutrient intakes of New Zealand women during their pregnancy was conducted by Watson et al. (13) prior to 2010. In this prospective cohort study, dietary information was collected by a 24-hour recall and a 3-day food diary from 424 New Zealand European and Polynesian pregnant women. Mean carbohydrate, total fat, and protein intakes were greater in the 4th month of gestation, compared to the reported intakes in this thesis (13). An earlier pilot study (52) assessed dietary intake of pregnant women (90% New Zealand European, 7% Māori and 3% other ethnicities, *n* = 196) by instructing participants to weigh all food eaten over a 8-day period in the 4th month and 7th month of pregnancy. In this study, protein intake was consistent with the estimated intakes of PiP study participants; however, total fat, saturated fat and carbohydrate intakes (as g/d) were higher than the intakes reported in this thesis (52). Total macronutrient intakes as %TE were not explicitly reported in either studies for comparison (13,52). The lower dietary macronutrient and energy intakes estimated in this thesis, compared to stated studies, may be due to a combination of underreporting, reliance on one 3-day dietary recall, and systematic errors owing to dietary record tabulation and food codification. Further discussion of the limitations of the dietary assessment in this thesis are described in section 6.7.2.1. However, it is important to note that the limitations exist across all reported studies.

The dietary intake results from this thesis suggest that participants in the PiP study were consuming macronutrient intakes within recommended nutrient reference ranges (as %TE) regardless of their dietary pattern. However, these results are biased towards New Zealand European women who are older than 30 years with a BMI of 25-30kg/m^2. Further exploration of the contemporary dietary intakes of New Zealand women during their pregnancy is warranted, especially for women of Māori, Pacific and Asian ethnicity. This should include quantification of specific foods commonly eaten during pregnancy to aid the development of practical food and nutrition resources for pregnancy that align with dietary intakes of individuals and communities.

6.2 Impact of diet on development of GDM

Previous prospective cohort studies have correlated an increased risk of GDM with diets high in fat, animal protein and low in dietary fibre (33); therefore the second aim of this thesis was to determine whether women with GDM had altered macronutrient intake compared with those who did not develop GDM, irrespective of probiotic HN001 supplementation. In support of our hypothesis, women who developed GDM had significantly lower dietary fibre intake (g/d) compared to participants with without GDM at 26-28 weeks' gestation (p = 0.027). Total fat intake and saturated fat, was not significantly different between total women (HN001 and placebo participants) who developed GDM compared with women without GDM. However, when comparing total fat intake (%TE) of women supplemented with placebo only, women who developed GDM had higher intakes of total fat compared to women without GDM (p <0.05). Additionally, higher total protein intake (%TE) was also reported for women with GDM compared to women without GDM (p = 0.001). Finally, women who developed GDM had lower intakes of carbohydrate (g/d) compared to women who did not develop GDM (p = 0.013). These results are consistent with previous studies that have demonstrated that diets high in fat and protein (34–36) and low in carbohydrate and dietary fibre (35,37) may contribute to an increased risk of GDM.

Several studies have established a relationship between high intakes of animal protein (from dairy and meat) with an increased risk of GDM (34–36,39,40). Due to the methodology used in this thesis, protein intake was not differentiated by animal and plant based sources. However, in New Zealand, according to the most recent Nutrition Survey (118), animal food products are a mainstream part of the diet with the average New Zealander eating 3-4 servings of red meat per week and including dairy products in their diet every day. Although BCAA's account for only approximately 20% of the total amino acid content in red meat; the levels of circulating BCAA have been shown to dramatically increase by approximately 200% after ingestion of an animal protein rich meal (120). Dysregulation of BCAA catabolism is thought to contribute to the development of GDM, as evidenced by previous metabolomics studies (95–97), and the metabolomic profile of GDM participants in this thesis. Significantly, studies aimed to reduce the dietary intakes of BCAA demonstrated an improvement in post-prandial insulin sensitivity (121). The proposed mechanisms of BCAA in GDM pathogenesis are discussed further in section 6.5. However, it is possible that increased intakes of animal protein contributes to the dysregulation of BCAA catabolism present in GDM.

Moreover, the negative effects of animal protein on GDM development could also be attributable to the high saturated fat content that generally co-exists in animal food products (i.e. full-fat dairy products and red meat) (35,36). It is proposed that high saturated fat intakes could contribute to

increased systemic inflammation by dysregulation of the intestinal epithelium driven by changes to the gut microbiota (79). Consistent with the findings of this thesis, high fat is associated with an increased risk of GDM (36).

Women with GDM also had significantly lower intakes of carbohydrates. It is understood that the type of carbohydrate is important in management of diabetes, as refined carbohydrates (e.g. from white bread, baked goods, etc) raise post-prandial glucose levels to a greater extent than complex carbohydrates from food high in dietary fibre (18). The lower carbohydrate intake (as %TE) of women with GDM as reported in this thesis, is thought to indicate that energy from carbohydrates was displaced by energy from fat and protein. Previous studies have demonstrated that increasing carbohydrate intakes, while simultaneously decreasing fat, reduces the risk of impaired glucose tolerance (40). Moreover, studies have shown that substitution of animal protein with plant based sources reduces the risk of GDM (35). Plant based protein sources include legumes, soy products, nuts and seeds. These products also provide physiological benefits by the dietary fibre, vitamins and anti-oxidants that they contain.

Notably, the analysis of carbohydrate in this thesis did not include dietary fibre intake. Therefore, assessment of dietary fibre intakes independently showed that women with NGT had significantly higher intakes of dietary fibre (approximately 26g/d) compared to women with GDM (approximately 23g/d). The current AI value for dietary fibre is recommended as 28g per day during pregnancy. The rationale for this AI is to compensate for the increased energy intakes during pregnancy and for laxation (8). However, in this thesis, only 37.4% of participants achieved dietary fibre intakes ≥28g/d. Previous studies have also reported that pregnant New Zealand women consume approximately 24g of dietary fibre per day (13,52), indicating that typically women in New Zealand do not achieve the AI for dietary fibre during their pregnancy. Research has indicated that for every 10g increase of dietary fibre, the risk of GDM is reduced by 26% (37). In this thesis, a protective effect of dietary fibre was indicated at ≥25g, therefore further studies are warranted to confirm whether dietary fibre ≥25g per day during pregnancy is adequate to contribute to a reduction in risk of GDM.

Due to the perceived benefits of a diet high in dietary fibre, and low in animal protein and fat for reducing the risk of GDM, further studies are warranted to investigate the role of plant-based diets for prevention. However, delivery of these interventions would need to consider socioeconomic status, ethnicity and food availability to ensure practicability and compliance to the interventions. As nutrition intervention studies are limited by recall bias, studies should also consider adopting a simultaneous metabolomics based approach to determine biomarkers of dietary patterns.

6.3 Impact of dietary fibre and efficacy of HN001 intervention

Studies have shown that the metabolic impact of probiotics is improved by concurrent administration of high dietary fibre intake, functioning as a prebiotic approach (79). Therefore, we hypothesised that high dietary fibre intake was required to improve the efficacy of probiotic HN001 supplementation for prevention of GDM. In support of our hypothesis, women who were supplemented with HN001 yet still developed GDM, did indeed have the lowest intake of dietary fibre (22.5 $\pm$ 8.4g/d) compared to all other groups. In contrast, women who were supplemented with HN001 and did not develop GDM had the highest intakes of dietary fibre (26.3 $\pm$ 8.8g/d). Although statistical significance was not detected, these results indicate that women in the PiP study who were protected against GDM had adequate dietary fibre for their endogenous gut microbiota or the supplemented probiotic to ferment to produce protective metabolic effects. To the best of our knowledge four other studies have investigated the use of probiotics for prevention of GDM with conflicting results (84–87). Based on the results of this thesis, we speculate that the discordance of previous studies is owing to background dietary fibre intake.

The first study to report a successful effect of probiotic supplementation (*Lactobacillus rhamnosus* GG (10^{10} CFU) and *Bifidobacterium lactis* Bb12 (10^{10} CFU)), for prevention of GDM was conducted in Finland (84). Due to the Finnish National Health Programme, all participants in this study received basic dietary counselling based on the Nordic Nutrition Recommendations (84). These guidelines state that adults should aim for a dietary fibre intake of 25-35g per day, including during pregnancy (122). A study investigating the nutrient intake of pregnant women at high risk of GDM (due to obesity or previous GDM, *n* = 394) from the Finnish Gestational Diabetes Prevention study reported that women in the first trimester of pregnancy were meeting the dietary fibre recommendation 2.9g/MJ (i.e., 25-35g per day) (123). Furthermore, a multi-cohort study assessing the dietary intake of the Finnish population (*n* = 12,342) reported that on average Finnish people consume dietary fibre intakes between 23.9 $\pm$ 7.2g - 29.5 $\pm$ 9.1g per day (124). Furthermore in Luoto et al.'s (84) study, the greatest reduction in GDM was observed for participants who received both intensive dietary counselling and probiotic supplementation (84). In comparison to the general dietary counselling that all participants received, intensive dietary counselling included personalised recommendations for food products with favourable fat and dietary fibre content delivered by a nutritionist (84). Therefore, it is likely that the nutrition counselling and the background high dietary fibre intake of Finnish women supported the effectiveness of probiotic supplementation in Luoto et al.'s (84) study.

In contrast, the SPRING (86), Irish PiP (85) and HUMBA (87) study reported no effect of probiotic supplementation on GDM risk. A recent study exploring dietary intakes of 402 pregnant women in

Ireland demonstrated that only 68.2% of participants had dietary fibre intakes greater than 25g per day (125). Furthermore, a survey of 7486 Australian women reported that on average pregnant women in Australia were consuming inadequate dietary fibre (19.6± 6.5g – 21.4±7.2g per day) (126). While dietary data is unavailable for study participants, these national dietary assessment studies indicate that it is possible that women in the SPRING and Irish PiP studies were not consuming adequate dietary fibre to support probiotic supplementation to confer a protective effect against GDM.

Moreover, studies in New Zealand women reported that low dietary fibre intakes are associated with ethnicity (Māori and Pacific), age (≤30 years), lower education levels (≤5 years high school and further education), and low occupation and welfare groups (5). Therefore, ethnicity, BMI and socioeconomic status are considered significant proxies for dietary fibre intake. Therefore, it is possible that the ineffectiveness of the probiotic *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB12 supplementation in the South Auckland based HUMBA trial is a reflection of this (87). In this trial, 72% of participants were Māori and Pacific women and all participants had a BMI ≥30kg/m^2 (87). The diet intervention included culturally tailored advice and label reading, which included identification of high fibre foods for the relief of constipation (127). However, unlike the Finnish study (84), to the best of our knowledge nutrition educators did not primarily focus increasing dietary fibre intake. Furthermore, as previously stated national data suggests that women in New Zealand consume 24g of dietary fibre per day during pregnancy (13,52) and results of this thesis showed that only 37.4% of participants of the PiP study were meeting the AI (≥28g/d). Therefore, it is plausible that along with the presence of other risk factors for GDM, participants in the HUMBA study were also consuming inadequate dietary fibre to support sufficient GDM protective effects to arise from probiotic supplementation.

The results of the New Zealand PiP study (2) are consistent with the protective GDM results of the Finnish study. However, in the New Zealand PiP study, participants did not receive specific nutrition counselling nor is dietary counselling a mainstream service for women during their pregnancy in New Zealand (1). However, the average intake of dietary fibre was greatest (approximately 26g per day) for women supplemented with HN001, and who did not develop GDM. These levels are assumed to be greater than the dietary fibre intake of SPRING, Irish PiP and HUMBA study participants. Therefore, it is likely that the participants that avoided GDM in both the Finnish and PiP study had adequate dietary fibre for their endogenous gut microbiota or supplemented probiotic to produce protective metabolic effects against GDM development.

Future probiotic intervention studies should consider controlling for dietary fibre intakes and/or nutrition education that focuses on an increasing dietary fibre (i.e., by increasing the quantity and variety of fruit, vegetables, wholegrains, legumes, nuts and seeds); in order to confirm whether high dietary fibre (≥25g/day) is essential to improve the efficacy of probiotic supplementation for prevention of GDM. Alternatively, as dietary interventions are often limited by non-compliance future studies could consider using a prebiotic supplement along with a probiotic (i.e. a synbiotic – a combination of a pre and probiotic (79)). Currently, synbiotics have only been evaluated for their therapeutic effect in women with existing GDM, with conflicting results (128,129).

To date, this thesis was the first study to assess the metabolome of probiotic intervention study participants. Metabolites associated with probiotic HN001 supplementation and dietary fibre intakes are discussed in section 6.6.

6.4 Potential impact of HN001 supplementation on fat palatability

Analysis of the 3-day food diaries revealed that dietary fat intake was significantly lower for women with GDM who were supplemented with HN001, compared to all other groups. Research in both animal and human studies have demonstrated that changes to the gut microbiota can influence satiety and food preferences (130). Therefore, it is plausible that probiotic HN001 supplementation impacted PiP study participants taste preference for dietary fat.

Studies evaluating the food preferences of people post Roux-en-Y gastric bypass (RYGB) surgery, whereby their gut microbiota is significantly altered, showed that participants had a decreased desire for high fat foods following surgery (131). While the decrease in desire for high fat foods could be attributable to nutrition counselling typically provided to people undergoing bariatric surgery, studies in animals have also shown that RYGB significantly shifts the feeding preference of rats to low fat foods (130,131). Moreover, a recent animal study demonstrated that germ-free mice had altered taste receptors on their tongue and in their intestines for fat compared to mice with an intact gut microbiome (132). A recent double-blind, randomised, placebo-controlled trial evaluated the impact of *Lactobacillus rhamnosus* LPR on appetite and eating behaviours in a group of obese men and women (n = 105) (133). In female participants (n = 60), supplementation of LPR significantly reduced, appetite sensations including food cravings, hunger and disinhibition (susceptibility to overeat) (p < 0.05) (133). Food cravings often lead to the consumption of energy-dense, high fat foods, therefore it was unexpected that no differences were observed between the macronutrient intakes (including fat) in this study (133). While, LPR supplementation did not reduce the intakes of dietary fat, the effects of probiotics are *strain specific* (71). Therefore, further research is warranted to detect specific gut microbes or probiotics that may influence changes to an individual's food preferences. If successful, probiotic intervention may be a novel approach for reducing an individual's consumption of energy-dense food and shifting food preferences towards foods of greater nutritional quality.

As previously discussed, the intake of dietary fat is greater for women with GDM compared to women with NGT, who were supplemented with placebo. It is plausible that the overall gut microbiota of women with GDM varies to the gut microbiota of women with NGT, influencing eating behaviours and food choices. Therefore, it is reasonable to suggest that probiotic HN001 altered the fat palatability of supplemented participants although, not sufficiently to prevent GDM. Assessment of a dietary fat metabolome was outside the scope of this thesis; however, future studies should consider using metabolomics based approaches to further explore the dietary fat metabolome in relation to HN001 efficacy and GDM pathogenesis.

6.5 GDM metabolome

The plasma metabolome of women who were supplemented with placebo revealed 15 metabolites that significantly differentiated the metabolome of women with GDM (*n* = 24) compared to women with NGT at 26-28 weeks' gestation (*n* = 144). These metabolites are biomarkers of BCAA catabolism, the TCA cycle, and pathways associated with inflammation and glucose transport. The pathways affected by GDM in this cohort, are consistent with the findings of published literature described in the systematic review (Chapter 3).

6.5.1 BCAA and the TCA cycle

A number of studies have observed alterations of metabolites involved in the biosynthesis and degradation of BCAA in GDM (95–97). In this thesis, while BCAA's (isoleucine, leucine and valine) were not significantly different between GDM and no GDM states; breakdown products of BCAA's including 3-methyl-2-oxopentanoic acid and 4-methyl-2-oxopentanoic acid were significantly increased in women with GDM compared to no GDM. Additionally, intermediates of BCAA catabolism – 2-aminobutyric acid and 2-oxovaleric acid, were significantly decreased in women with GDM compared to no GDM. Of note, reduction of 2-aminobutyric acid in the GDM metabolome was unexpected, as 2-aminobutyric acid was reported to increase in GDM states in previous research (103). Despite this, the metabolomic analysis of this thesis supports the involvement of BCAA catabolism in the pathogenesis of GDM. Breakdown of BCAA's ultimately provides acetyl-CoA and succinyl-CoA for the TCA cycle (134,135). Interestingly, metabolomics analysis in this thesis also demonstrated significantly higher TCA cycle intermediates in women with GDM compared to no GDM (fumaric acid, citric acid, isocitric acid, and cis-aconitic acid). These metabolites were also increased in the metabolome of women with GDM in previous studies (96,97).

One of the key hypotheses linking BCAA's and TCA cycle with the pathogenesis of diabetes is by increased fatty acid oxidation and disruption of the mitochondrial redox balance (NADH/NAD+) (see Figure 13) (134–136). T2DM and GDM is often accompanied by increased free fatty acids in the tissue and blood in combination with reduced insulin action, resulting in hyperglycaemia and low-grade inflammation (21). As a result, intra-mitochondrial and cystolic NADH levels rise, increasing the NADH/NAD+ redox balance (136). Degradation of BCAA's is catalysed by BCAA aminotransferases (BCAT) and α-keto acid dehydrogenase (134,135). Studies have shown that a high NADH/NAD+ ratio inhibits α-keto acid dehydrogenase resulting in incomplete degradation of BCAA and a build-up of BCAA derivatives. Thus, potentially explaining the identification of BCAA catabolism intermediates in the metabolome of women with GDM in this thesis. Incomplete degradation of BCAA may then explain

the rise in TCA cycle intermediates, detected in the GDM metabolome, as insufficient acetyl-CoA and succinyl-CoA are produced by BCAA degradation. It is also theorised that an increased NADH/NAD+ balance also inhibits the TCA cycle directly, which would also contribute to an increase in TCA cycle intermediates (136). Furthermore, a high NADH/NAD+ ratio upregulates glutathione synthesis resulting in increased 2-hydroxybutyrate levels (100,102,134,136). 2-hydroxybutyric acid and 2-hydroxyisobutyric acid were significantly increased in women with GDM in our cohort and previous metabolomics studies, further indicating that disruption of the NADH/NAD+ balance is an integral component to GDM pathogenesis (100,102).

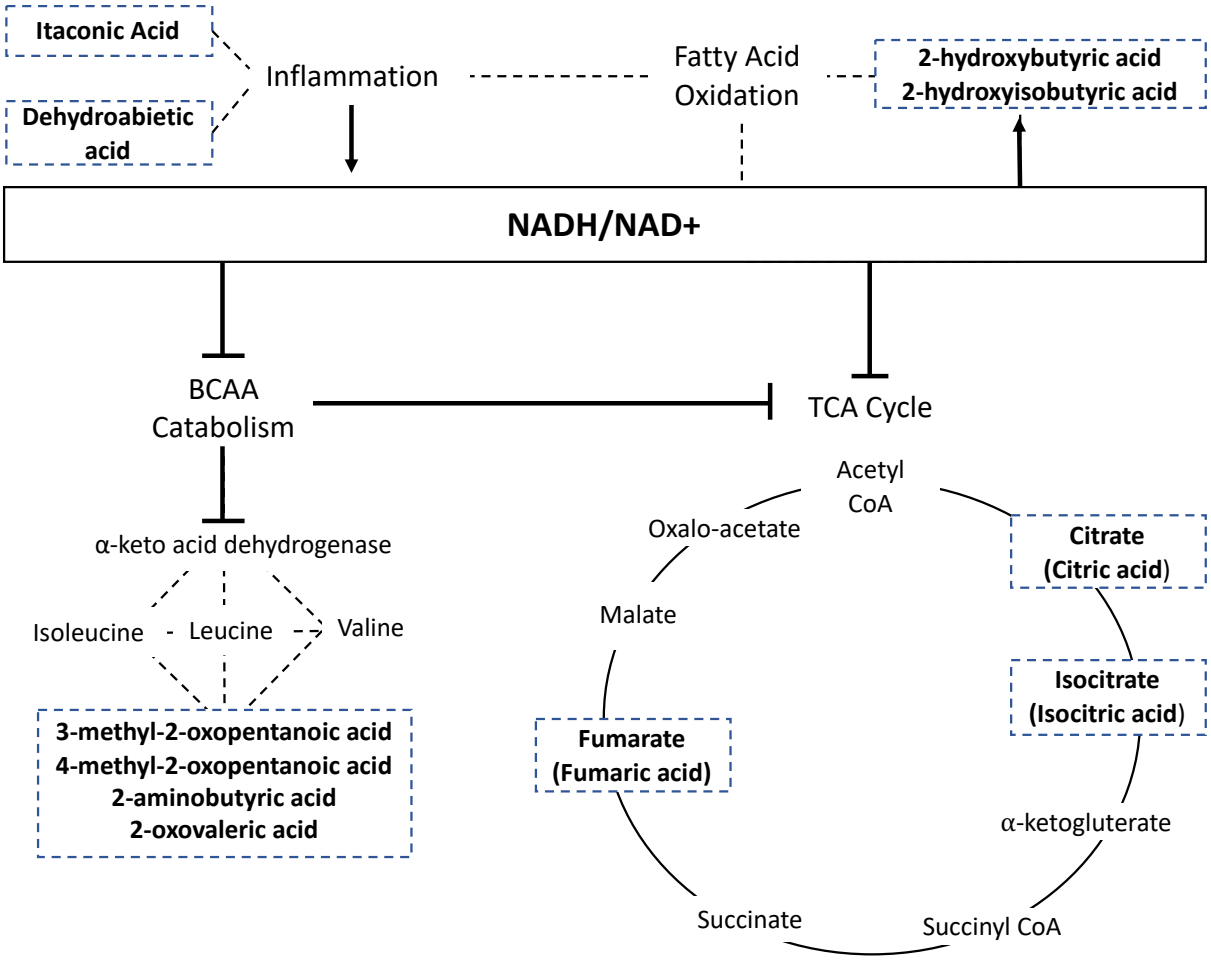


Figure 13 Proposed mechanisms of detected metabolites (in bold) in the GDM metabolome of PiP study participants.

6.5.2 Inflammation

Two metabolites associated with inflammation were identified in the metabolome of participants with GDM (see Figure 13). Itaconic acid, is a metabolite produced by macrophages in response to inflammation; and has previously been associated with GDM in a cohort of pregnant New Zealand women (137). Likewise in our analysis, itaconic acid was upregulated by 10% in women with GDM compared to women with NGT. Dehydroabietic acid was the greatest upregulated metabolite in women with GDM (37%). Dehydroabietic acid belongs to a class of organic compounds called diterpenoids, that are involved in lipid metabolism pathways. Studies in tissue culture and diabetic KK-Ay mice have shown that dehydroabietic acid is associated with anti-inflammatory effects and improvement in lipid metabolism (138,139). Therefore, the exact mechanism of dehydroabietic acid in GDM pathogenesis is yet to be determined.

6.5.3 Other metabolites

Dehydroascorbic acid was also found to be significantly downregulated (3%) in women with GDM compared to women with NGT. Dehydroascorbic acid is an oxidised form of vitamin C, that is able to cross the blood-brain barrier, where it is converted to ascorbic acid. Transport of dehydroascorbic acid is via the glucose transporter-1 (GLUT-1) (140). Therefore, in diabetic states dehydroascorbic acid is expected to increase in the blood as hyperglycaemia results in competition for the glucose transporter. This results in less ascorbic acid in cells, lowering the anti-oxidant capabilities of people with diabetes (140). Therefore, it was surprising that dehydroascorbic acid was reduced in women with GDM in this cohort. However, this could partially be explained by attributing the reduction of dehydroascorbic acid with reduced dietary intake of vitamin C containing foods, i.e. fruit, in women with GDM, reflected by low dietary fibre intakes of this cohort.

Finally, palmitelaidic acid is a long-chain trans fatty acid involved in fatty acid metabolism that significantly increased by 10% in women with GDM. Dysregulation of fatty acid oxidation is linked to GDM pathogenesis (102), however, it is unclear how palmitelaidic acid specifically has an effect on the GDM metabolome.

6.5.4 GDM metabolome – summary

The GDM metabolome identified in this thesis is in support of previous literature that demonstrates that dysregulation of BCAA catabolism and the TCA cycle is involved in GDM pathogenesis. Dysregulation of these pathways is driven by increased fatty acid oxidation and inflammation which

results in disruption of the mitochondrial redox balance (NADH/NAD+ ratio) (134–136). Further studies are warranted to investigate interventions that target these pathways.

6.6 Impact of HN001 and dietary fibre on the metabolome

This thesis aimed to determine metabolites augmented by HN001 supplementation and high dietary fibre intakes to establish the metabolic pathways that may be effected by these interventions. It was hypothesised that HN001 supplementation and high dietary fibre intakes would produce metabolites that impact biological pathways that are protective against development of GDM. Metabolomics analysis in this thesis demonstrated that biomarkers of tryptophan metabolism were associated with HN001 supplementation, independently and, simultaneously with GDM. Furthermore, anti-inflammatory metabolites and BCAA were independently associated with high dietary fibre intakes ≥25g per day. Metabolites enriched in the high dietary fibre (≥25g) / HN001 metabolome include biomarkers of fatty acid metabolism, amino acid metabolism and gluconeogenesis. The following sections discuss the proposed mechanisms of these metabolites in prevention of GDM.

6.6.1 Tryptophan metabolism

Analysis of the whole PiP cohort metabolome revealed that tryptophan was simultaneously dependent on HN001 supplementation and GDM status. Direct comparison of women supplemented with HN001, further revealed that tryptophan was significantly increased by 18% in noGDM/HN001 participants compared to GDM/HN001 participants. When GDM was removed as a confounding variable, 2-aminophenylacetic acid (a catabolite of the tryptophan-kynurenine pathway) and tryptophan remained significantly associated with HN001 supplementation. This indicates that probiotic HN001 supplementation may be a key regulator of tryptophan metabolism.

Tryptophan is an essential amino acid that accounts for 1-1.5% of total amino acids in both plant and animal proteins (141). Foods that contain relatively high levels of tryptophan include eggs, potatoes, cereal, broccoli, kiwifruit, bananas, fish, and meat (141). Additionally, studies in animal models have observed *Lactobacilli* as an important microbial species for catabolism of dietary tryptophan in the gut (142). Furthermore, previous studies have identified upregulation of tryptophan as a significant metabolite in the metabolome of infants (*n* = 19) following supplementation of probiotic *Lactobacillus paracasei* sp. *Paracasei F19* (LF19) (10^8 CFU/d) (143). However, in this thesis assessment of tryptophan in placebo participants showed that it only slightly decreased (0.5%). As the infant microbiome is more susceptible to changes than an adults (72), it is possible that HN001 was not able to perpetuate a change in tryptophan metabolism to the same extent as in infants. Additionally, metabolomic studies have identified biomarkers of tryptophan metabolism in association with high dietary fibre intakes (≥40g/d) (107,108). However, in this thesis, metabolites of tryptophan metabolism were not identified in association with dietary fibre intakes in our cohort. Firstly, the ability to observe such an association

may be impeded by the relatively low intakes of dietary fibre in the PiP study cohort compared to previous studies, indicating that dietary fibre intakes of 40-50g may be required to perpetuate a change in tryptophan metabolism. Furthermore, a study that monitored the changes to the microbiome and glucose tolerance of 800 participants over a week-long period, reported distinct inter-personal variation in the post-glucose response to meals (144). This study indicated that there are significant differences between an individuals' metabolism of specific meals, determined by the gut microbiota. In the dietary fibre interventions discussed in the systematic review (Chapter 3) the high dietary fibre interventions generally included food items such as rye and oat bran (107–109). It was beyond the scope of this thesis to quantify food items and dietary patterns. However, it was noted that foods that contain dietary fibre that were commonly eaten by PiP study participants included apples, potatoes, muesli bars and wholegrain bread. Therefore, it is possible that the types of foods eaten by our PiP study cohort did not confer a detectable response in tryptophan metabolism at the levels of dietary fibre that we approximated. Further studies are warranted to investigate specific food items high in dietary fibre or dietary patterns that influence a response in tryptophan metabolism and to confirm whether interpersonal variation exists.

Dudzik et al. (102) observed a significant reduction of L-tryptophan in the metabolome of women with GDM, however, the association of tryptophan with GDM pathogenesis is not yet established, Other studies have associated tryptophan metabolites with insulin resistance in T2DM, however these studies produced conflicting results as metabolomics studies are often limited in identifying the directionality of the metabolic pathways enriched (145,146). Tryptophan metabolism is associated with a number of physiological functions driven by catabolism by the gut microbiota (Figure 14) (141,146). Approximately, 4-6% of dietary tryptophan is catabolised by the gut microbiota to produce indole metabolites, including indoleacetic acid (146), found to increase following high dietary fibre intakes (107). Indole metabolites are associated with activation of anti-inflammatory pathways and enhance the secretion of GLP-1, a known regulator of satiety by suppression of gastrointestinal motility (141,146). GLP-1 is also associated with an increase in insulin secretion (57,58). Previous studies have found that inhibition of the production of indole metabolites in mouse models of obesity, lowers GLP-1 leading to increased intestinal permeability, increased LPS translocation and as a result systemic inflammation and insulin resistance (146). Furthermore, 1-2% of tryptophan is converted to serotonin whereby it contributes to satiety (146). As identified in the RCT by Sanchez et al. (133) probiotic supplementation (LPR) significantly increased feelings of fullness. Therefore, these results may be owing to activation of tryptophan metabolism by LPR supplementation (133,146). In theory, increasing satiety, reduces energy intakes, and as a result lowers BMI – a significant risk factor for GDM (79). Finally, approximately 95% of tryptophan is catabolised in the kynurenine pathway

(141,146). This pathway produces metabolites also involved in anti-inflammatory pathways (e.g. kynurenate (108)) and gastrointestinal functions (141,146).

Further studies are warranted to unravel the precise mechanisms by which HN001 may be influencing tryptophan metabolism. The directionality of the tryptophan metabolism pathways was unable to be distinguished in this thesis. However, there may be important associations between tryptophan metabolites perpetuated by high dietary fibre intakes (as recognised in the systematic review) and HN001 metabolites, as detected by analysis of the HN001 metabolome. Further studies are warranted to determine the extent of involvement of HN001 for influencing tryptophan metabolic pathways and whether it was enrichment of these pathways that conferred protection against GDM in our PiP study participants.

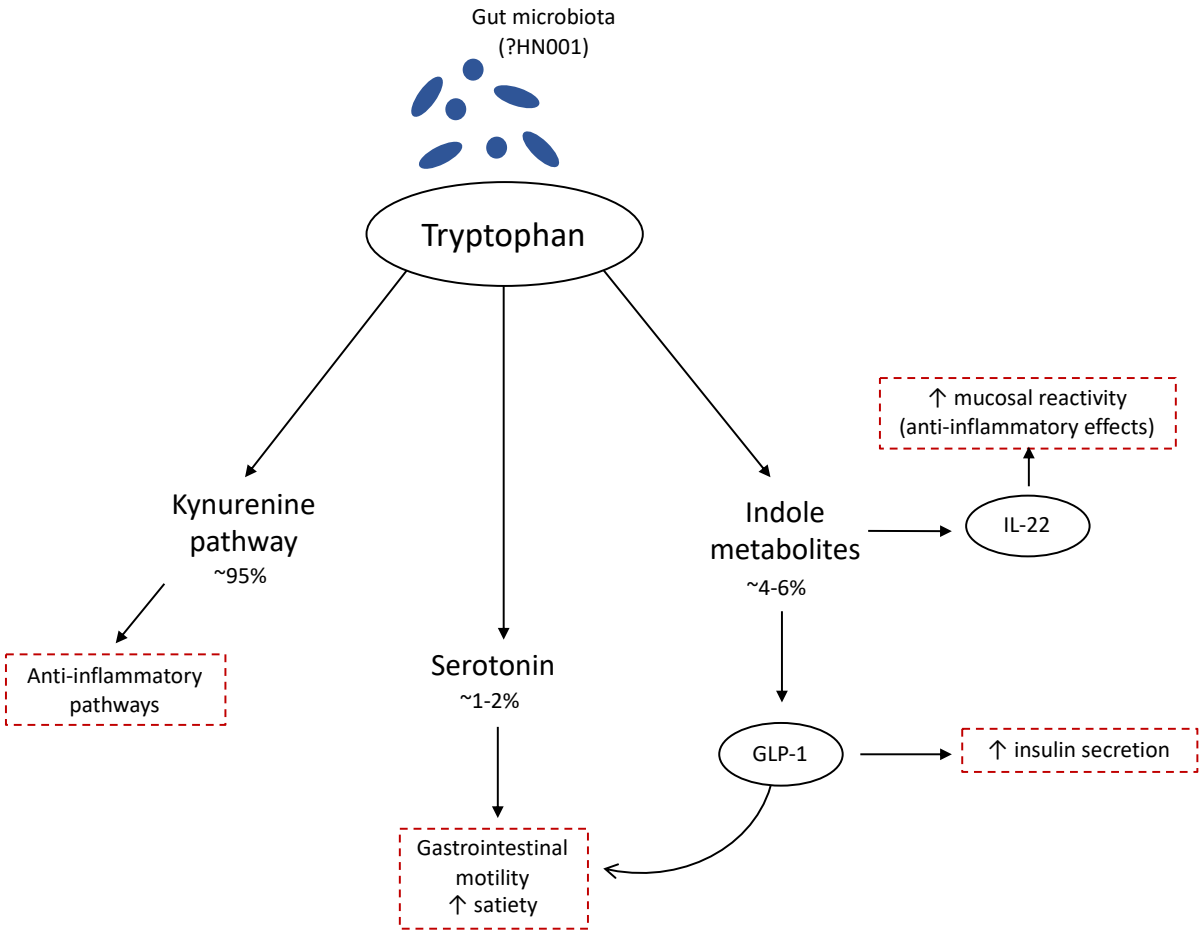


Figure 14 Proposed mechanisms of tryptophan metabolism for prevention of GDM by signalling of anti-inflammatory pathways, insulin secretion and increasing satiety.

6.6.2 Histidine

Significantly the high dietary fibre metabolome demonstrated enrichment of the anti-inflammatory molecule, histidine. Histidine is an important amino acid that has been documented to reduce inflammation by scavenging free radicals, thus reducing oxidative stress (147,148). However, the majority of research investigating histidine is in cell cultures and animal studies and few studies have evaluated the effect of histidine in diabetes (GDM or T2DM). One randomised control trial demonstrated that histidine supplementation reduced inflammation and oxidative stress in a study of obese women with metabolic syndrome (*n* = 100). As a result, a reduction in insulin resistance and a significant reduction in BMI was observed (147). As histidine supplementation has been associated with negative side effects (e.g. headaches, nausea and suppressed appetite) (147); further metabolomic studies are required to confirm whether high dietary fibre interventions are able to produce similar effects by activation of histidine anti-inflammatory signalling.

6.6.3 Other metabolites

Two biomarkers of fatty acid metabolism (myristoleic acid and oleic acid) were detected in the HN001, high dietary fibre metabolome. These metabolites have previously been suggested to decrease with high dietary fibre intakes (107). However, it is important to note that myristoleic acid and oleic acid are also biomarkers of dietary fat. Furthermore, inconsistent with previous studies (109), leucine and isoleucine were upregulated in women with dietary fibre intakes greater than ≥25g/d in this thesis. As previously discussed, intake of animal protein can increase circulating BCAA (120). Therefore, it is possible that leucine, isoleucine, myristoleic acid and oleic acid were raised due to intakes of protein and fat. Alternatively, the reduction of leucine and isoleucine in previous studies may be due specifically to intakes of rye (109). Further studies are required to determine the metabolite responses to certain food items. As expected, dietary biomarkers of fruit, vegetable and wholegrains were detected in the metabolome of women with high dietary fibre intakes. These metabolites could be used as robust measures of personalised nutrition. Furthermore, as metabolomics is still in its infancy it is yet to be discovered whether any of these biomarkers do in fact have therapeutic mechanisms.

Finally, lactic acid was decreased in the plasma metabolome of noGDM/HN001, high dietary fibre (≥25g/d) participants. Lactic acid is produced by fermentation of dietary fibre by the gut microbiota and is a substrate for gluconeogenesis. It is possible that lactic acid was altered in this cohort by increased fermentation of dietary fibre by HN001, as *Lactobacillus* is a lactic-acid producing microbe (79). However, previous metabolomic studies indicated that lactate increased following

supplementation of *Lactobacillus* (106). Pathway analysis of lactic acid is required to further elucidate whether reduction of lactic acid conferred protection against GDM.

6.6.4 Summary

Metabolomics is a novel approach to understand how nutrition and probiotic interventions influence the metabolism by measurement of net metabolites within a biological system (73). Results from this thesis indicate that probiotic HN001 supplementation and high dietary fibre intakes may be associated with a significantly reduced risk of GDM through generation of tryptophan metabolites and histidine. Generation of these metabolites is likely influenced by effects on the gut microbiota. Further studies are warranted to confirm this association.

6.7 Strengths and limitations

6.7.1 Strengths

To the best of our knowledge, this thesis was the first study to explore the metabolome of women with GDM in relation to probiotic HN001 supplementation and dietary fibre intake. Metabolites were identified in plasma by GC-MS which is a good reflection of net metabolism and is known to illustrate changes secondary to nutrition interventions. Additionally, identifying metabolites at the time of diagnosis by an untargeted screen produced hypotheses that can now be tested using a targeted GC-MS approach. Furthermore, collecting blood samples at 26-28 weeks' gestation allowed HN001 supplementation to have a metabolic effect as previous studies (69) have indicated that positive results of probiotic supplementation are achieved by extended supplementation periods.

The major strength of this thesis was the use of 3-day food diaries and plasma samples collected from a large group (*n* = 348) of pregnant women from the robust, two-centre, randomised, double-blind placebo-controlled PiP trial. Inclusion of participants who were diagnosed by the IADPSG criteria increased the number of participants included in this thesis, rather than limiting inclusion to women diagnosed by the higher New Zealand glucose thresholds.

Another strength was the inclusion of a systematic review in combination with an untargeted metabolomics analysis. Conducting a systematic review ensured that all literature between 2009-May 2019, investigating the plasma and serum metabolome of women with GDM, probiotic supplementation or high dietary fibre intake, was captured. This facilitated the interpretation of the metabolomics analysis of this thesis.

6.7.2 Limitations and future directions

Despite the strengths discussed in section 6.7.1, we acknowledge several limitations to this study.

6.7.2.1 *Food diaries as dietary quantification tool*

A key strength of this study is that all food diaries (*n* = 348) were entered into Foodworks 9 by the author for consistency. However, assessing nutrient intake by means of food dairies is understood to be affected by biased reporting of food consumption by research participants and systematic errors by researchers owing to dietary record tabulation and food codification (17). Therefore, the 3-day food records collected in the PiP study should be interpreted with caution. Many participants failed to record clear portion sizes, weights, or specifics of their meals, such as brand or composition of recipes. During data entry, reference guidelines were used and informed assumptions were made

where details were not provided by the participant (as outlined in appendix B). However, it is likely that the use of these guidelines led to an underestimation of the amount of food consumed by the participants, thus compounding potential existing underreporting.

The knowledge that foods must be recorded is also known to alter dietary behaviours (17). This is of particular relevance to this study as it is possible that participants may not have wanted to report food that is recommended to be avoided during pregnancy. Additionally, dietary intake is affected by the environment (17). As the PiP study recruited participants over an extended period of time some participants completed the dietary records at Easter, while others completed the dietary record during the Christmas/New Year period. As a result, notes were made by participants on the food records that the record did not reflect their habitual diet due to the holidays.

While the use of a 3-day food diary is intended to capture all food consumed by participants without researcher bias, future studies may benefit by providing more in-depth instructions for completion of the food diaries. For example, requesting that participants weigh food items would provide more precise estimates. However, it is recognised that this is often considered burdensome by research participants (17). Alternatively, a food frequency questionnaire tailored towards dietary fibre intake may have produced quantifiable data appropriate for the research aims of this thesis. Healey et al. (149) have developed and validated a habitual dietary fibre intake short food frequency questionnaire to assess the intake of dietary fibre. The tool was shown to be quick and reproducible in classifying individuals as having low, moderate or high dietary intakes (149). Future studies could also consider the use of developing smart phone applications (apps) for assessment of diet. A study investigating acceptance and validity of an app compared to 24-h dietary recalls reported that 83% of participants (n = 50) preferred using the app compared to completing the paper dietary record (150). However at present, most available app-based dietary assessment tools don't differ substantially from paper-based methods, such that dietary recall apps still rely on the input of the data by the research participant. However, development of software to enable transfer of data between research participant and the researcher could improve data quality (150). The development of validated dietary assessment toolkits by researchers that take into account participant and researcher burden is necessary. This will enable collection of the highest-quality of dietary data possible to ultimately produce evidence-based claims to benefit women during their pregnancy.

6.7.2.2 *Metabolomics study design*

Notably, when comparing the metabolome of HN001 and dietary fibre interventions, BMI, age and weight were not statistically different between comparison groups, with the exception of the BMI of

high dietary fibre participants being higher than the BMI of low dietary fibre participants. However, analysis of the GDM metabolome compared women of high BMI and weight (GDM) compared to women of lower BMI and weight (no GDM). As a result, it is understood that metabolites associated with obesity are present in our sample. Future studies, should consider removal of these confounding variables or match samples based on age, BMI, and ethnicity. Moreover, as food is not consumed in isolation it is difficult to distinguish the metabolites that are derived from dietary fibre alone. Therefore, future metabolomics studies could consider assessment of dietary patterns. Furthermore, there were large differences in sample numbers for the GDM metabolome analysis (GDM: *n* = 23 vs. no GDM: *n* = 144), this may have affected the power to detect all metabolites associated with GDM.

Blood samples were taken at the time of diagnosis of GDM. This limits the identification of *predictive* metabolites of GDM that could be targeted for prevention. However, with the identification of a GDM metabolome at 26-28 weeks' gestation, targeted metabolomics studies can now be conducted to identify the time-points during pregnancy at which plasma biomarkers (e.g. itaconic acid, BCAA and TCA cycle derivatives) successfully predict women who develop GDM. Therefore, collecting plasma samples at multiple time-points throughout pregnancy for analysis by GC-MS would, 1) measure how and when the metabolome changes in response to GDM, and 2) when the effect of diet (e.g. dietary fibre or fat) or probiotic supplementation interferes with the trajectory of GDM pathogenesis. This will enable the development of interventions to prevent the dysregulation of metabolic pathways associated with the progression of GDM.

Chapter 7 Thesis Summary

7.1 Conclusion

By a unique metabolomics based approach, in conjunction with assessment of 3-day food diaries, this thesis aimed to answer the question "*How does Lactobacillus rhamnosus HN001 protect against GDM and is this protection dependent on background dietary fibre intake?*" While not statistically significant, the mean dietary fibre intake was lowest in women from our study who were supplemented with HN001, yet still developed GDM. However, if our result is combined with the dietary fibre analyses of women in previous probiotic intervention studies, this may confirm our hypothesis that those participants who did not benefit had inadequate dietary fibre intake to support probiotic related beneficial impacts on preventing GDM. Assessment of the GDM metabolome indicated that dysregulation of BCAA catabolism, the TCA cycle and inflammation are key components of GDM pathogenesis. Therefore, by exploring the inter-relationship between significant metabolites detected in the GDM, HN001 and dietary fibre metabolomes of PiP study participants, it is hypothesised that HN001 and high dietary fibre intake may prevent GDM development by regulating anti-inflammatory pathways associated with tryptophan metabolism and histidine, respectively.

7.2 Practical advice for dietitians

Currently, there is inadequate evidence to recommend that women take probiotics during their pregnancy for prevention of GDM. For women who enquire as to the effectiveness of probiotic supplementation, it is important to remind them that while probiotic supplementation during pregnancy is considered safe, the effects of probiotics are strain specific such that the proposed effects of Lactobacillus rhamnosus HN001 for reducing risk of GDM, cannot be extrapolated to other probiotic strains; and that the research is still in its infancy. Alternatively, women should be encouraged to nurture a healthy gut microbiota by following a healthy dietary pattern; e.g. recommend the inclusion of a variety of fruit, vegetables, legumes and wholegrains.

Studies have shown that attainment of a healthy dietary pattern (as stated by national guidelines) is associated with sociodemographic attributes, such as age and higher levels of education, in conjunction with ethnic and cultural differences. Therefore, it is vital that dietitians advocate for equitable public health initiatives and provide tailored dietary advice to improve the nutritional status of all pregnant women in New Zealand.

Appendices

A. Search strategy for the systematic review

B. Assumptions made for Foodworks 9 data entry

C. Complete list of metabolites detected in the PiP study cohort metabolome.

D. Heat maps of metabolites associated with GDM, HN001 and dietary fibre.

Appendix A. Search strategy for the systematic review

Objective	MedLine (Ovid)	MedLine (Embase 1980-present)	Scopus
To determine metabolites associated with **GDM** compared to pregnant woman with normal glucose tolerance at 20-30 weeks' gestation.	Metabolomics/ Diabetes, Gestational/ gestational diabetes.mp or GDM.ti, ab, kw. 2 or 3 1 and 4 limit 5 to (humans and English language and yr="2009-Current")	Metabolomics/ Diabetes, Gestational/ gestational diabetes.mp or GDM.ti, ab, kw. 2 or 3 1 and 4 limit 5 to (humans and English language and yr="2009-Current")	(TITLE-ABS-KEY (metabolomic*) AND TITLE-ABS-KEY ("GDM" OR "gestational diabetes") AND AND (LIMIT-TO (PUBYEAR , 2019) OR LIMIT-TO (PUBYEAR , 2018) OR LIMIT-TO (PUBYEAR , 2017) OR LIMIT-TO (PUBYEAR , 2016) OR LIMIT-TO (PUBYEAR , 2015) OR LIMIT-TO (PUBYEAR , 2014) OR LIMIT-TO (PUBYEAR , 2013) OR LIMIT-TO (PUBYEAR , 2012) OR LIMIT-TO (PUBYEAR , 2011) OR LIMIT-TO (PUBYEAR , 2010) OR LIMIT-TO (PUBYEAR , 2009)) AND (LIMIT-TO (EXACTKEYWORD , "Humans") AND (LIMIT-TO (LANGUAGE , "English"))
To determine metabolites associated with **probiotic supplementation** compared to a placebo control	Metabolomics/ Probiotics/ 1 AND 2 limit 5 to (human AND English language)	Metabolomics/ Probiotics/ 1 AND 2 Limit 5 to (human AND English language)	(TITLE-ABS-KEY (metabolomic*) AND TITLE-ABS-KEY (probiotic*) AND (LIMIT-TO EXACTKEYWORD, "Humans')) AND (LIMIT-TO (LANGUAGE, "English"))
To determine metabolites associated with high **dietary fibre** intakes compared to low dietary fibre intake.	Metabolomics/ Dietary fiber/ "fiber" OR "fibre" OR "dietary fib*", ti, ab, kw. 2 or 3 1 and 4 limit 5 to (human AND English language)	Metabolomics/ Dietary fiber/ "fiber" OR "fibre" OR "dietary fib*", ti, ab, kw. 2 or 3 1 and 4 limit 5 to (human AND English language)	(TITLE-ABS-KEY (metabolomic*) AND TITLE-ABS-KEY ("fiber" OR "fibre" OR "dietary fib*") AND (LIMIT-TO (EXACTKEYWORD, "Humans")) AND (LIMIT-TO (LANGUAGE, "English"))

Appendix B. Assumptions made for Foodworks 9 data entry.

Food Description by Participant	Foodworks 9 Selection	Portion	Rationale			
			Nutrition Survey[1] (117)	Eating for healthy pregnant women (9)	Food Composition Tables (119)	Manufacturer
Dairy						
Milk *glass* / *cup*	Milk, cow, lite 1.5% fat, fluid	250ml	✓[2]	✓	-	-
Ice cream *scoop*	Ice cream, vanilla, standard	64ml	-	-	✓	-
Yoghurt *pottle*	Yoghurt, Fresh 'n Fruity, Lite, assorted fruits, non-fat, sweetened, fortified	150g	-	✓	-	✓
Cheese *slice*	Cheese, Cheddar	20g / slice	-	✓	-	-
Soy Milk	Soy milk, So Good Regular Soy Milk, Sanitarium, fortified	AS	-	-	-	-
Meat / Poultry						
Steak	Beef, hindquarter rump steak, separable lean, fast-fried	180g	✓	-	✓	-
Mince	Beef, mince, premium, simmered	195g	-	✓	-	-
Ham	Ham, sliced (slice 10.0 x 10.0 x 0.3cm)	2	-	-	✓	-
Egg	Egg, chicken, white & yolk, (cooking method specified)	Size 6	-	✓	✓	-
Chicken	Chicken, breast, grilled	1 single (107g)	-	✓	✓	-
Fish	AS, i.e. Fish, fillet, crumbed, frozen, baked	100g	-	✓	✓	-
Tuna	Tuna, canned in oil, assorted flavours, undrained	95g	-	-	-	✓
Sausage	Sausage, assorted meat & flavours, precooked	AS	-	-	-	-
Wholegrains						
Bread (type not specified)	Bread, mixed grain, light, sliced, prepacked[3]	AS	✓[3]	-	-	-
"Vogel's" bread	Bread, Mixed Grain, Ancient Grains, Vogel's	AS	-	-	-	-
Cereal *bowl*	AS i.e. *Skippy Cornflakes, Sanitarium, fortified*	1 cup (30g)	-	-	-	✓
Muesli	Toasted Muesli Golden Oats & Fruit, Sanitarium	½ cup (55g)	-	✓	-	✓
Porridge *bowl*	Porridge, prepared with milk & water, salt added	1 cup	-	-	✓	-
Rice *bowl*	Rice, white, Basmati, boiled, undrained	1 cup	-	✓	✓	-
Muesli Bar (type unspecified)	Muesli Bar	1	-	-	-	-
Cracker	AS, i.e. *cracker, rice, plain* or *cracker, wheat, assorted flavours, Arnott's*	AS	-	-	-	-

Food Description by Participant	Foodworks 9 Selection	Portion	Rationale: Nutrition Survey[1] (117)	Rationale: Eating for healthy pregnant women (9)	Rationale: Food Composition Tables (119)	Rationale: Manufacturer
Nuts *handful*	AS, i.e. *nut, cashew, roasted, salt added*	30g	-	✓	-	✓
Pasta	Pasta, white wheat flour, assorted shapes, regular, boiled, drained, no salt added	1 cup	-	✓	-	-
Cake *small slice*	AS, i.e. *cake, chocolate, baked, iced with butter icing*	100g	-	-	✓	-
Muffin	Muffin – blueberry / bran / chocolate	1	-	-	-	-
Meals						
Pizza *slice*	Flavour & number of slices specified by participant	100g / slice	-	-	✓	-
Soup *bowl*	AS, i.e. *soup, pumpkin, heated*	300ml	-	-	-	-
Pasta Sauce	Sauce, pasta, tomato based, heated, commercial	½ cup	-	-	-	-
Pasta Sauce with Veges	Sauce, pasta, chunky vegetable, tomato based, heated	½ cup	-	-	-	-
Fruit / Vegetables						
Apple	Apple, 'Braeburn', flesh & skin, raw	Medium	-	-	-	-
Banana	Banana, yellow, ripened, raw	Medium	-	-	-	-
Kumara	Kumara, orange, flesh, raw	135g	-	-	-	-
Potato	Potato, flesh & skin, baked without oil, no salt added, floury, new	1 medium	-	-	-	-
Fries	Fries, potato, straight cut, Independent Shops	10 fries / 69g	-	-	✓	-
Wedges	Potato, wedges, frozen, precooked in canola oil, baked	10 fries / 69g	-	✓	-	-
Oven Chips	Potato, fries, chunky cut, frozen, precooked in canola oil, baked	10 fries / 69g	-	✓	-	-
Mixed vegetables	Vegetables mix, carrots, corn, green beans & peas, frozen, boiled, drained, no salt added	½ cup	-	✓	-	-
Snacks						
Chips *handful*	Potato chip or crisp, assorted flavours, salted, fried in assorted oils	25g (10 chips)	-	-	✓	-
Chocolate *square*	AS, i.e. *chocolate, milk chocolate, Dairy Milk, Cadbury*	6g / square	-	-	-	✓
Pain au Chocolat	Croissant, plain (small 7-12cm long) and chocolate, milk chocolate, Dairy Milk, Cadbury (12g)	1	-	-	-	-

Food Description by Participant	Foodworks 9 Selection	Portion	Rationale: Nutrition Survey[1] (117)	Rationale: Eating for healthy pregnant women (9)	Rationale: Food Composition Tables (119)	Rationale: Manufacturer
Oils / Spreads						
"Olivani" / margarine	Margarine, monounsaturated, 75% fat, Olivani	AS	-	-	-	-
Butter	Butter, salted	AS	-	-	-	-
Jam	Jam, berry fruit	AS	-	-	-	-
Peanut Butter	Peanut butter, smooth & crunchy, no sugar or salt added	AS	-	-	-	-

Abbreviations: AS = as specified

[1]Results of Nutrition Survey published in the *Eating and Activity Guidelines for New Zealand Adults, [2]50% NZ adults use low-fat or trim milk, [3]25-30% NZ adults ate white bread, 50% ate light-grain breads.*

Appendix C. Complete list of metabolites identified in the PiP study cohort metabolome.

10-Heptadecenoic acid
11,14-Eicosadienoic
2-Aminoadipic acid
2-Aminobutyric acid
2-Aminophenylacetic acid
2-Hydroxybutyric acid
2-Hydroxyglutaramic acid MCF1
2-Hydroxyisobutyric acid
2-Methyloctadecanoic acid
2-Oxobutyric acid
2-Oxoglutaric acid
2-Oxovaleric acid
3-Acetoxy-3-Hydroxy-2-methylpropionic acid
3-Methyl-2-oxopentanoic acid
4-Hydroxycinnamic acid
4-Hydroxyphenylacetic acid
4-Methyl-2-oxopentanoic acid
Adipic acid
Alanine
Arachidic acid
Asparagine
Aspartic acid
Azelaic acid
Benzoic acid
Benzothiazole
beta-Alanine
BHT (Antioxidant)
bishomo-gamma-Linolenic acid
Caffeine
cis-4-Hydroxyproline
cis-Aconitic acid
cis-Vaccenic acid (C18_1n-7c)
Citric acid
Citric acid secondary peak
Creatinine
Cysteine
Decanoic acid
Dehydroabietic acid
Dehydroascorbic acid
DL-3-Aminoisobutyric acid
Dodecane
Dodecanoic acid
DPA
Ferulic acid
Fumaric acid
Gallic acid
gamma-Linolenic acid
Glutamic acid
Glutamine
Glutaric acid
Glyceric acid
Glycine
Glyoxylic acid
Gondoic acid
Heptadecane
Hexanoic acid
Hippuric acid
Histidine
Hydroxybenzoic acid
Isocitric acid
Isoleucine
Itaconic acid
Lactic acid
Leucine
Levulinic acid
Linoleic acid (
Lysine
Malic acid peak 1
Malic acid peak 2
Malonic acid
Margaric acid
Methionine
Methylthioacetic acid
Myristic acid
Myristoleic acid
NADP_NADPH
N-alpha-Acetyllysine
Nicotinamide
Nicotinic acid
Nonadecanoic acid
O-Acetylserine
Octanoic acid
Ornithine
Oxalic acid
Palmitelaidic acid
Palmitic acid
Pentadecane
Pentadecanoic acid
Phenethyl acetate
Phenylalanine
Proline
Pyroglutamic acid
Pyruvic acid
S-Adenosylmethionine
Salicylic acid
Serine
Stearic acid
Succinic acid
Threonine
trans-4-Hydroxyproline
trans-Cinnamic acid
trans-Vaccenic acid
Tricosane
Tridecane
Tryptophan
Tyrosine
Valine

Appendix D. Heat maps of metabolites simultaneously correlated with GDM, HN001 and dietary fibre.

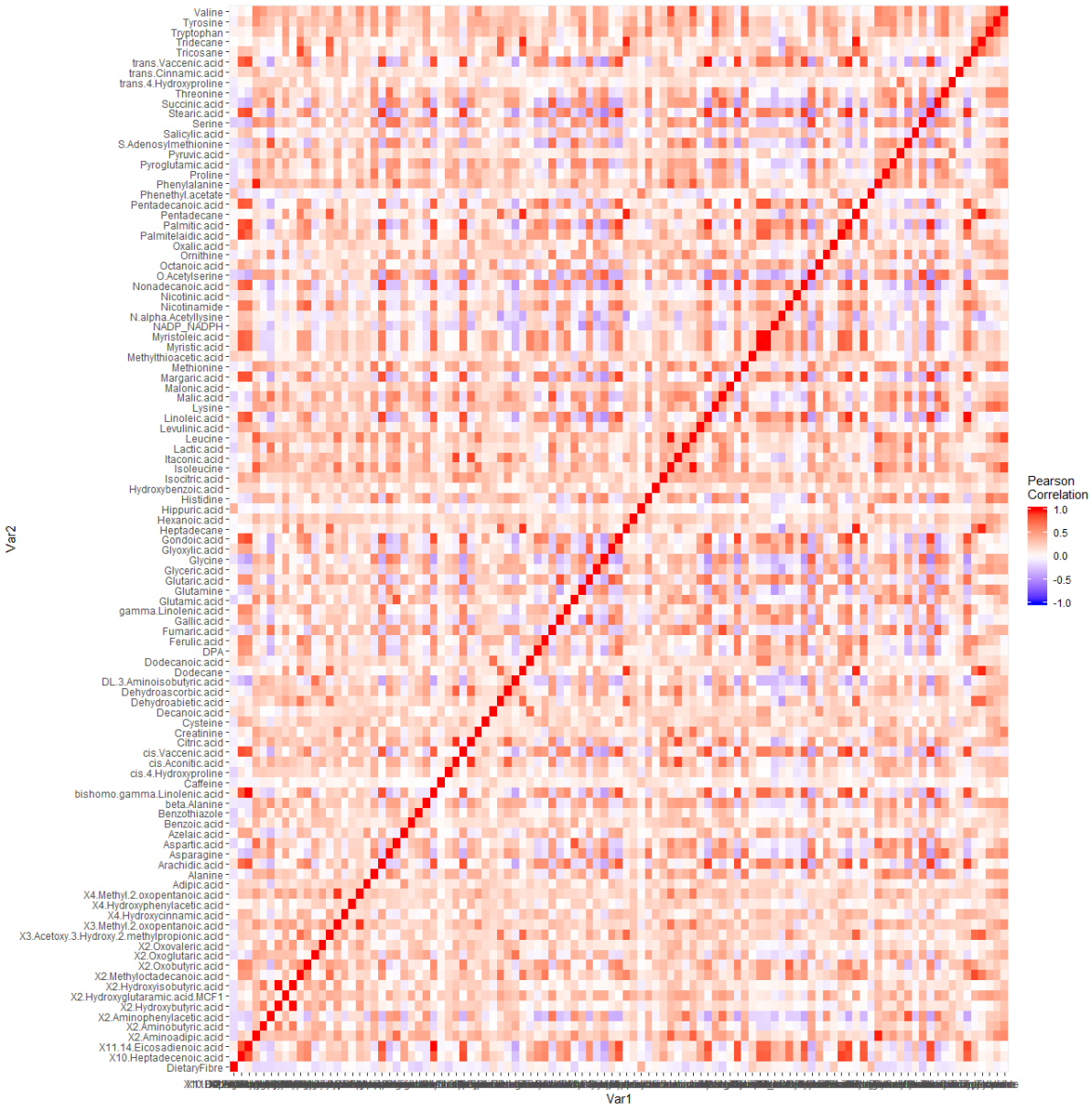


Figure 15 Heat map of metabolites correlated with GDM vs. no GDM (HN001 participants only). Correlation analysis of metabolite levels and dietary fibre intake revealed no statistical significance ($p < 0.05$) for those showing strong correlations (i.e. correlation coefficient < 0.75).

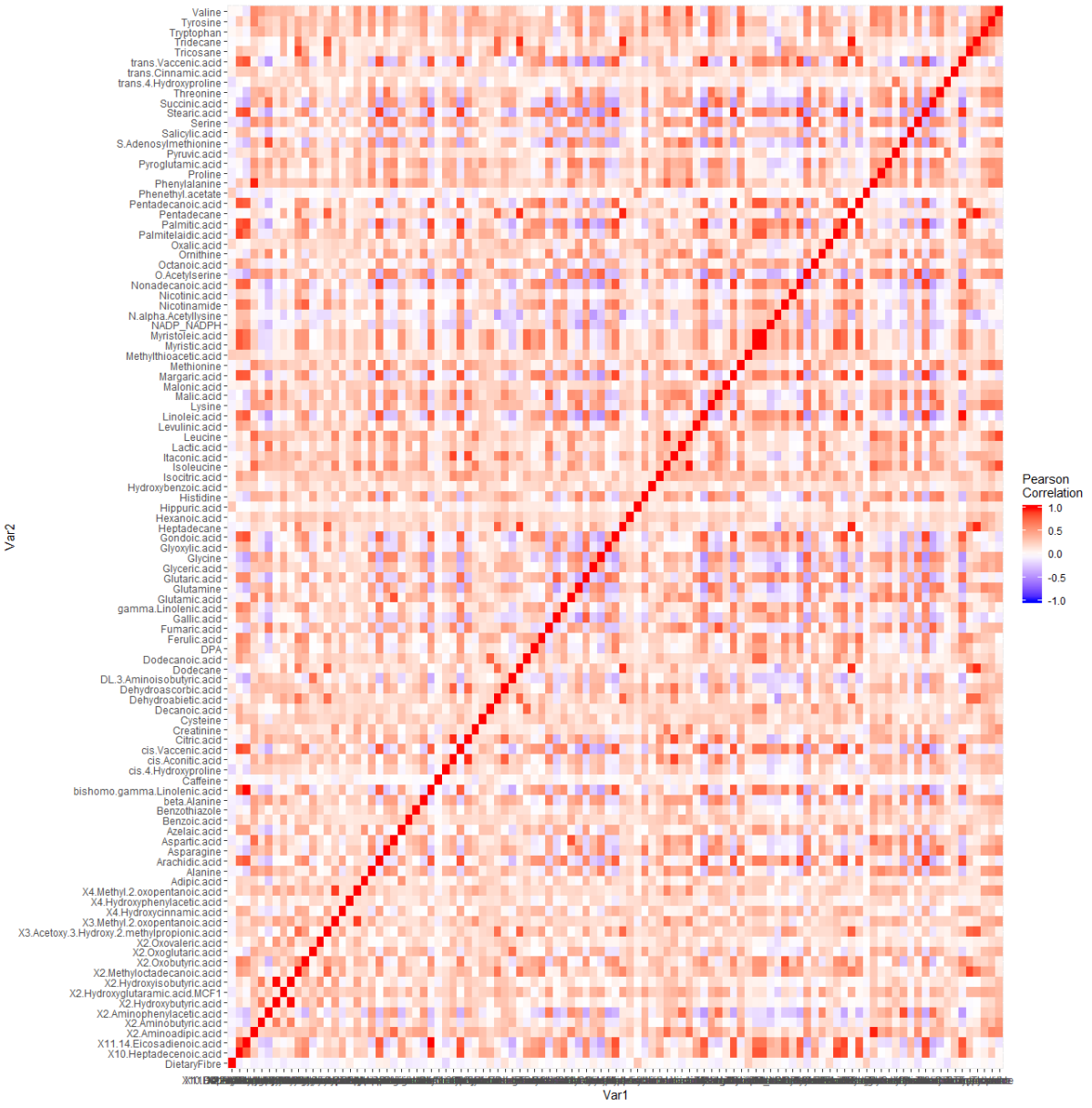


Figure 16 Heat map of metabolites correlated with HN001 vs. placebo (noGDM participants only). Correlation analysis of metabolite levels and dietary fibre intake revealed no statistical significance ($p < 0.05$) for those showing strong correlations (i.e. correlation coefficient < 0.75).

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