The Effects of Myo-inositol and Probiotic Supplementation in a High Fat Fed Preclinical Model of Glucose Intolerance in Pregnancy

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Shortened title: Inositol and probiotics in pregnancy

Keywords: gestational diabetes, glucose intolerance, pregnancy, diabetes, mice, high fat diet, myo-inositol, inositol, probiotics

Abstract

Glucose intolerance during pregnancy – a major driver of gestational diabetes mellitus (GDM) – has significant short and long-term health consequences for both the mother and child. As GDM prevalence continues to escalate, there is growing need for preventative strategies. There is limited but suggestive evidence that myo-inositol (MI) and probiotics (PB) could improve glucose tolerance during pregnancy. This study tested the hypothesis that MI and/or PB supplementation would reduce the risk of glucose intolerance during pregnancy. Female C57BL/6 mice were randomised to receive either no treatment, MI, PB (*Lactobacillus rhamnosus* and *Bifidobacterium lactis*), or both (MIPB) for four weeks. They were then provided *ad libitum* access to high fat diet (HFD) for one week before mating commenced and throughout mating and gestation, while remaining on their respective treatments. An oral glucose tolerance test occurred at gestational day (GD) 16.5 and euthanasia and tissue collection at GD18.5. Neither MI or PB, separately or combined, improved glucose tolerance. However, MI and PB both independently increased adipose tissue expression of *Ir*, *Irs1*, *Akt2*, and *Pck1*, and PB also increased *Pparγ*. MI was associated with reduced gestational weight gain, whilst PB was associated with increased maternal fasting glucose, total cholesterol and pancreas weight. These results suggest that MI and PB may improve insulin intracellular signalling in adipose tissue but this did not translate to meaningful differences in glucose tolerance in this experiment. The absence of fasting hyperglycaemia or insulin resistance suggests this is a very mild model of GDM, which may have affected our ability to assess the impact of these nutrients.

Introduction

Gestational diabetes mellitus (GDM) – defined as hyperglycaemia diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation[1] – is a common obstetric complication, affecting an estimated 16.5% of pregnancies worldwide[2] . In the majority (~80%) of cases, GDM is the result of β cell dysfunction on a background of chronic insulin resistance, leading to glucose intolerance[3]. Risk factors include a family history of diabetes (either type 1, type 2, or gestational diabetes), advanced maternal age, and overweight and obesity[4–6]. Although GDM usually resolves following delivery, it is associated with a number of short- and long-term health consequences for both the mother and child. The mother is at increased risk of further pregnancy complications, surgical delivery, and of developing future type 2 diabetes (T2DM) and cardiovascular disease (CVD)[7] . The child is at increased risk of being born large for gestational age (LGA), experiencing shoulder dystocia and respiratory distress, as well as developing obesity, T2DM and CVD in later life[8] . This perpetuates an intergenerational cycle of disease that further escalates the obesity epidemic. To break this cycle, it would be beneficial to generate therapies that prevent GDM from developing[9]. Current treatments include diet and lifestyle interventions, followed by insulin treatment or oral agents such as metformin. Although women are able to maintain adequate glycaemic control using these treatment strategies, they can be difficult to implement, and concerns remain regarding the long-term effects of oral agents on the developing fetus. Further, prevention is preferred over treatment because GDM is typically diagnosed after 24 weeks of gestation, when the fetus may have already been exposed to hyperglycaemia. For these reasons, it would be beneficial to develop novel, safe, and effective strategies for GDM risk reduction.

A growing body of research suggests that myo-inositol (MI) – a simple carbohydrate produced in the body and available in foods such as fruits and cereals – can facilitate insulin signalling and reduce blood glucose concentrations in individuals with T2DM and GDM[10]. This is because MI forms the structural basis of phosphatidylinositol (PI) and the phosphatidyl phosphate lipids (PIP2/PIP3), in the insulin signalling pathway[11]. Furthermore, probiotic (PB) supplementation has been associated with improved glucose metabolism and reduced risk of GDM[12]. While the mechanisms linking PB supplementation to metabolic health are poorly understood, PBs modify the intestinal microbiome and stimulate production of short-chain fatty acids (SCFAs). SCFAs affect the expression of a number of proteins that have been demonstrated to increase insulin sensitivity and decrease gut permeability [13,14]. So far, the evidence that MI or PB supplementation should be recommended before or during pregnancy to reduce the risk of GDM is limited[15,16]. Further, it is unknown if the combination of MI and PB – which are both easy-to-administer and safe nutritional supplements that appear to affect

glucose regulation via different mechanisms – would have additive effects. The purpose of this study was to assess if MI and PB, both separately and in combination, would improve glucose tolerance and other measures related to GDM – including lipidaemia, hepatic steatosis, and intestinal permeability – in a preclinical mouse model.

Experimental methods

All animal procedures were approved by the University of Auckland Animal Ethics Committee in accordance with the New Zealand Animal Welfare Act, 1999. Eighty 7-week-old nulliparous female C57BL/6 mice were acquired from the Vernon Jansen Unit (VJU) at the University of Auckland, New Zealand, and housed in groups of four within individually-ventilated cages with woodchip bedding. Mice were maintained in a 12-hour light cycle environment with an ambient temperature of 22°C and 40–45% humidity. After one week of acclimatisation, mice were randomly assigned to either receive control diet (AIN-93G, Research Diets Inc. NJ, USA; 20% kcal protein, 63.9% kcal carbohydrate, 15.8% kcal fat; 3.9 kcal/g), control diet with MI added to the diet (AIN-93G, Research Diets Inc.; with 2% added MI (Sigma-Aldrich, St Louis, MO)), control diet with PB mix added to drinking water (*Lactobacillus rhamnosus* (*L. rhamnosus*) and *Bifidobacterium Lactis* (*B. lactis*) at 6 g/L, for 10^9 CFU per day), or control diet with MI added to the diet and PB mix added to drinking water. MI dosage was based on previous rodent studies[17], and was below the maximum dose tested and tolerated in human studies (20g/day)[18], while PB dosage was based on that used previously in humans[19]. 5 weeks of exposure to the treatments prior to the onset of HFD and throughout mating and gestation (for a total of ~10 weeks exposure) was chosen in order to maximize the potential preventive effects of the supplements. This duration is beyond the length of exposure utilized in other studies of these supplements in pregnant mice, and was therefore determined to be sufficient[20,21]. Because the probiotic included maltodextrin (DE 12) as a binding agent, the non-probiotic groups received an equal dose (5 g/L) of the same form of maltodextrin (Glucidex IT12, Axieo Specialties, Auckland, NZ) in their drinking water. Drinking solutions were measured and changed daily. Preventive measures were taken when handling cages and mice to prevent contamination between probiotic and non-probiotic groups, including the use of separate equipment as well as changing probiotic cages and drinking solutions last. Body weight and food intake were measured weekly. After four weeks (12 weeks of age), mice were switched onto HFD (D12451, Research Diets Inc., 20% kcal protein, 35% kcal carbohydrate, 45% kcal fat; 4.73 kcal/g), with or without added 2% MI. One additional group remained on control diet and acted as a reference group. The groups were thereby labelled as follows: control diet (CD – reference only), high fat diet (HFD), HFD with MI added to the diet (HFMI), HFD with probiotic added to drinking water (HFPB), and HFD with MI added to the diet and probiotic added to drinking water (HFMIPB). Groups and their definitions are summarised in Figure 1. A profile of the diets used in this study is provided in Table 1.

After one week of HFD exposure, mice were placed with unrelated males for a period of one week. They remained on their allocated diet/treatment throughout mating and pregnancy. HFD

one week prior to and throughout pregnancy has been previously demonstrated to produce an effective mouse model of gestational-specific glucose intolerance[22]. Mice were checked daily by inspection of the vagina for a cervical plug. Upon its detection (denoted gestational day 0.5: GD0.5), female mice were separated from males and were pair-housed with food and water intake monitored throughout pregnancy. If after one week a mouse did not become pregnant, it was removed from the study. Although initial groups consisted of 16 mice, the numbers that became and remained pregnant for the study period are presented as the final numbers per group in Figure 1.

Oral glucose tolerance test

Glucose tolerance was measured at GD16.5. At 0800 h, food was removed and, following a six hour fasting period, blood glucose was measured by slicing 1 mm from the tip of the tail, dabbing the first resulting drop on a paper towel, and measuring the second drop with a glucometer (FreeStyle Optimum Neo, Abbott Diabetes Care, Alameda, CA). Mice were then dosed with 2 g/kg glucose solution via oral gavage, and blood glucose was measured at 15, 30, 60 and 120 minutes[23]. Blood was also collected in heparinised capillary tubes at 0, 15 and 60 minutes and plasma prepared for later insulin analysis.

Gut permeability procedure

Gut permeability was assessed because it has been associated with the development of metabolic disease[24] and because probiotic are thought to affect glucose tolerance in part by reducing gut permeability[25]. At GD18.5, mice were again fasted for six hours and then dosed with 4000-Da fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich, St Louis, MO) via oral gavage (600 mg/kg body weight), in order to later assess gut permeability, outlined in the plasma analysis section.

Tissue collection

At GD18.5, following a six hour fast, mice were anesthetised using isoflurane and ~1 mL blood was acquired via cardiac puncture. Fasting blood glucose was measured from the tail tip as described above. Mice were then culled by cervical dislocation, and the uterine horns with fetuses were removed and placed into ice-cold saline. The maternal pancreas, liver, adipose tissue (retroperitoneal, gonadal, perirenal and mesenteric), and kidneys were removed, weighed and either snap-frozen and stored at -80°C or fixed in 10% neutral buffered formalin for later histological analysis. In addition, the digestive tract was removed, flushed with saline, cut into sections (oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum and faecal

147 samples) and snap-frozen. Fetuses and their placentae were sexed, weighed and length
148 measured.

149 **Plasma analysis**

150 Tail and cardiac puncture blood were collected in EDTA-coated tubes and centrifuged at
151 2500 rpm for 10 minutes. Plasma was then aliquoted and stored in light-protected tubes at
152 −20ºC. Commercially available mouse-specific ELISAs were used to measure plasma insulin
153 (Ultrasensitive Mouse Insulin ELISA, Crystal Chem., Chicago, IL, USA #90080; sensitivity: 50
154 pg/mL), leptin (Mouse Leptin ELISA, Crystal Chem. #90030; sensitivity: 200 pg/mL) and
155 adiponectin (Mouse Adiponectin ELISA, Crystal Chem. #80569; sensitivity: 8 pg/mL). HOMA-
156 IR was calculated as (fasting glucose (mmol/L) x fasting insulin (mU/L))/14.1[26]. Matsuda
157 index was calculated as: 10000/(√ [fasting glucose (mmol/L) x fasting insulin (mU/L) x mean
158 glucose over OGTT (mmol/L) x mean insulin over OGTT (mU/L)])[27]

159 A COBAS automated analyser (Roche Diagnostics, Switzerland) was used to measure plasma
160 total cholesterol (Roche 04718917190; sensitivity: 9.7 mg/dL), high density lipoprotein (HDL;
161 Roche 05401488190), low density lipoprotein (LDL; Roche 05401682190), and free fatty acids
162 (FFAs; Wako WA243491795).

163 For gut permeability analysis, 25 µL plasma was diluted in 25 µL phosphate-buffered saline
164 (PBS, pH 7.4). Two standard curves were obtained by serial two-fold dilution of FITC-D stock
165 solution at 10 mg/mL, covering a wide range (first range 800–12.5 µg/mL; second range 50–
166 0.78 µg/mL). Samples, standards and blanks were transferred to a clear 96-well microplate
167 (Corning, NY, USA) and were protected from light at all times. FITC-D concentration was
168 detected using an InfiniteF200 fluorescence spectrophotometer (Tecan, Männedorf,
169 Switzerland) and Tecan I-control software (Tecan, Männedorf, Switzerland) at an excitation
170 wavelength of 485 nm and emission wavelength of 528 nm[28].

171 **Histology**

172 Gonadal adipose, liver and placental tissue samples were fixed in 10% neutral buffered formalin,
173 and were paraffin embedded and sectioned (10 µm) using a Leica RM 2135 rotary microtome
174 (Leica Instruments, Nussloch, Germany). Haematoxylin and eosin (H&E) staining was
175 performed, and sections were mounted using DPX (Sigma-Aldrich, NZ). Slides were visualised
176 under a light microscope (Nikon Eclipse E800, Tokyo, Japan), and images were captured with
177 NIS Elements-D software by an individual blinded to the study groups. For adipose histology,
178 four representative images were captured per sample at 20x magnification, and images were
179 analysed using ImageJ 1.50v software (US National Institutes of Health, Bethesda, USA) to

determine adipocyte size. Four sections has been determined sufficient in previous studies from our group[29]. For placentae, two images were captured under 4x magnification, and the width, labyrinth zone, junctional zone and decidua lengths were determined using ImageJ. For liver, sections were examined under 20x magnification, and foci of lobular inflammation were counted and graded (small foci = 1, medium foci = 2, large foci = 3). Ten random images per animal at 40x magnification were evaluated for general steatosis (score of 0–3) and microvesicular steatosis (score of 0–2). NAFLD score (NAS) was calculated as the unweighted sum of general steatosis, microvesicular steatosis and lobular inflammation scores[30].

Gene expression analysis

Maternal gonadal adipose tissue was studied because it is the most directly associated with the development of metabolic disease in mice[31] and generates the largest RNA yield. RNA was extracted using Trizol reagent (ThermoFisher Scientific, New Zealand) and a bead homogeniser (TissueLyser; Qiagen, Hilden, Germany) according to the manufacturers' instructions. Minor modifications were made to the protocol to maximise RNA yield, namely: (1) sample was centrifuged and the lipid layer was removed by pipette following homogenisation, and (2) samples were left with isopropanol for 2 hours at −20ºC rather than 10 minutes at room temperature to aid precipitation of RNA. RNA was suspended in nuclease-free water, and concentrations were measured using a NanoPhotometer N60 (Implen, Munich, Germany). Only RNA samples with a 260/280 nm ratio of ~2.0 and 260/230 nm ratio of 1.7-2.2 were used further. RNA quality was validated by gel electrophoresis[32]. mRNA was reverse transcribed using High-capacity cDNA kit (Applied Biosystems, Warrington, UK). Taqman Fast Advanced Master Mix (Applied Biosystems, Warrington, UK) and pre-designed Taqman probes (Applied Biosystems, Warrington, UK) were pipetted into microplates using an epimotion automated pipetting robot (Eppendorf, Hamburg, Germany), and qPCR was performed using the Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (ThermoFisher, New Zealand). Taqman probes examined are outlined in Supplementary Table 1. Gene expression was normalised to *Atpaf1* (ATP synthase mitochondrial F1 complex assembly factor 1; Mm00619286_g1) and *Tbp* (TATA-box binding protein; Mm01277042_m1), according to previous optimisation studies and in-house testing[32]. The $2^{-\Delta\Delta C_T}$ method was used for analysis[33].

Statistical analysis

Statistical analysis was performed using SPSS Statistics 24 (IBM, Armonk, NY), and graphs were generated in Prism 7 (GraphPad, San Diego, CA). Sample size was based on in-house pilot data of the model and previous studies of the effects of MI and PB[24,25] with the primary

outcome being the peak of OGTT (delta = 20% $Cmax_{HFD}$). With an alpha = 0.5 and power = 0.80, and taking into account the fertility rate of C57BL/6 mice on HFD (70%), a recruitment size of n = 16 per group was required, in order to generate a final number of at least n = 10 per group. For each outcome, CD (reference) and HFD were compared using unpaired student's t-test, in order to determine the effects of the short-term HFD model. Within-HFD treatment groups were then compared using two-way ANOVA, with repeated measures in the case of growth/food intake curves and OGTT results[34]. Boxplots, Shapiro-Wilk's test and Levene's test were used to assess outliers, normality, and homogeneity of variances, respectively. If outliers were determined to be genuine and not the result of input or measurement error, the outlier value was winsorized[35]. Where data failed Shapiro-Wilk's or Levene's test, data were appropriately transformed[34]. Where both the non-transformed and transformed data yielded the same result (i.e. degrees of significance), non-transformed data are presented for clarity. Where a significant two-way interaction between MI and PB was present, multiple comparison differences were detected using Tukey post-hoc test. Where there were significant differences between treatment groups, Hedges' g statistic (the recommended measure for sample sizes < 20) was used to calculate the effect size of these differences[36]. Data are presented as mean ± SEM.

Results

Pre-pregnancy measurements

Body weights did not differ amongst the groups until after the commencement of HFD, at which point groups fed HFD weighed more than CD ($p = 0.030$); no differences were observed across treatment groups (Figure 2.A). Similarly, food and calorie intake did not differ amongst the groups until after the commencement of HFD, at which point HFD groups had increased calorie intake compared with CD, including adjustment for energy intake from fluids (Figure 2.B–D; $p = 0.028$). However, again there were no effects of treatment.

Pregnancy measurements

There were no differences between CD and HFD in gestational weight gain or litter size. MI was associated with reduced gestational weight gain ($g = 1.91$, $p = 0.013$), which was not explained by any difference in litter size (Table 2). While groups fed HFD had reduced food intake over pregnancy compared with CD ($p = 0.020$), this difference disappeared when expressed as energy intake. No effects of treatment were observed (Table 2).

OGTT and fasting plasma measurements

HFD one week before and throughout pregnancy successfully induced glucose intolerance at GD16.5, as demonstrated at 30 ($p < 0.0001$) and 60 ($p = 0.003$) minutes of the OGTT, and in the area under the curve ($p = 0.0004$; Figure 3.A; B). However, there was no effect of any of the treatments on oral glucose tolerance (Figure 3.A; B). There were also no differences between any of the groups in plasma insulin during the OGTT (Figure 3.C; D).

HFD decreased fasting plasma insulin ($p = 0.033$) and increased fasting plasma LDL at GD18.5 compared with CD ($p = 0.014$, Table 3). There were no differences between HFD and CD in fasting blood glucose, plasma leptin, adiponectin, Matsuda index, or HOMA-IR at GD18.5. PB was associated with increased fasting blood glucose ($g = 0.64$; $p = 0.043$) and plasma total cholesterol ($g = 0.85$; $p = 0.015$) at GD18.5 (Table 2). Treatments had no effect on fasting plasma insulin, leptin, HOMA-IR, Matsuda index, adiponectin, HDL, LDL, or FFAs at GD18.5 (Table 3).

Gut permeability

There were no significant differences between any of the groups in plasma FITC-D concentration at GD18.5, indicating no differences in gut permeability (Supplementary Figure 1.).

Organ weights

HFD increased retroperitoneal (p = 0.011) and gonadal (p = 0.023), but not perirenal and mesenteric adipose deposition, compared with CD (Table 4). HFD also resulted in decreased pancreas weight (p = 0.042), but had no impact on average kidney or liver weight (Table 4). There were no effects of MI or PB on adipose deposition in any depot or on average kidney weight. PB was associated with increased pancreas weight (g = 0.68; p = 0.042, Table 4). Further, an interaction between MI and PB treatment was observed when liver weight was assessed (p = 0.048); mice receiving PB and MI simultaneously showed a slight reduction in relative liver weight when compared to the single treatments (Table 4). There were, however, no significant multiple comparison differences.

Adipocyte histology

HFD increased average adipocyte size (p = 0.036; Figure 4.A-B), and increased the proportion of adipocytes measured at >15000 μm compared with CD (p = 0.024; Figure 4.C). Neither MI nor PB had any effect on adipocyte histology (Figure 4).

Gonadal adipose tissue gene expression

HFD was associated with reduced gonadal adipose gene expression of *Pck1* (p = 0.028) and *Pparg* (p = 0.0007) compared with CD (Figure 5.D; E). There were significant MI/PB interactions observed when expression of *Ir* (p = 0.002), *Irs1* (p = 0.002), *Akt2* (p < 0.0001), and *Pck1* (p = 0.0012) was analysed. In all of these cases, HFMI and HFPB increased expression compared with HFD alone, but HFMIPB did not (Figure 5). There was also a significant increase in *Akt2* expression amongst MI groups overall (g = 1.06; p = 0.023; Figure 5.C), and a significant increase in *Pparg* expression amongst PB groups overall (g = 2.04; p = 0.024; Figure 5.E). No differences were observed in *Slc2a4*, *Igf1r*, *Fas*, *Lepr*, *Tnf*, *Mcp1*, *Il6*, *Angptl4*, *Nlrp3*, *Nfκb*, *Il1β, Cd11,* or *Ccr5* (Supplementary Figure 2).

Hepatic histology

HFD increased hepatic non-alcoholic steatosis score (NAS) compared with CD (p = 0.015; Figure 6.C). None of the treatments had any effects on hepatic histology (Figure 6).

Fetal measurements

HFD reduced male fetal weight (p = 0.037), female fetal weight (p = 0.0016), male abdominal circumference (p = 0.0043), and female abdominal circumference (p < 0.0001) compared with CD (Table 5). PB was associated with increased male fetal weight (g = 0.68; p = 0.035) and

293 increased female placental weight ($g = 0.89$; $p = 0.021$) in HFD fed animals, making them more
294 similar to those from CD dams (Table 5). An interaction between MI and PB treatment was
295 observed when male abdominal circumference was measured, in which PB tended to increase
296 abdominal circumference in the absence of MI, but tended to decrease it in the presence of MI,
297 although there were no significant multiple comparison differences (Table 5).

298

Discussion

The aim of this study was to determine whether MI and PB, taken together or separately before and during pregnancy, would impact the development of HFD-induced glucose intolerance during pregnancy[22]. This mouse model allowed a factorial design to determine the interaction of treatments, as well as more thorough examination of potential mechanistic pathways and whole-tissue analysis, which would not be possible in human trials.

Suitability of the mouse model

GDM is an incredibly difficult condition to model in small animals, as reviewed by Pasek and Gannon (2013)[37]. This is because GDM, by definition, only develops after the onset of pregnancy[1]. The model used in this study is an adaptation of a mouse model previously developed by Pennington *et al.* (2017) [22]. We chose this model because our original model of choice, the heterozygous $LepR^{db/+}$ mouse, did not display glucose intolerance, as discussed in detail in two of our previous publications[38,39]. Pennington *et al.* demonstrated that acute exposure to HFD one week prior to and during pregnancy impaired islet cell proliferation, therefore reducing insulin secretion and resulting in gestational glucose intolerance. We similarly demonstrated glucose intolerance compared with CD – our primary outcome. Like Pennington et al., we also saw reduced plasma fasting insulin. This result is in contrast with most longer-term HFD studies in mice and in women with GDM, where fasting insulin is usually raised due to insulin resistance[40,41]. We also did not observe leptin resistance in our model – another typical trait in long-term HFD rodent studies and in GDM[42,43]. It is likely that our acute exposure to HFD was not sufficient for insulin and leptin resistance to develop, and that longer exposure to HFD would have resulted in a more pronounced metabolic phenotype. However, such an extended exposure would've negated the pregnancy-specific aspect of the study. Our model did demonstrate increased body weight, increased adipose tissue deposition, increased LDL cholesterol, increased adipocyte size, and reduced fetal weight compared with CD. Each of these outcomes are associated with GDM, except for reduced fetal weight (GDM usually results in macrosomia[44]). However, reduced fetal weight is commonly observed in pregnant mice fed HFD[45,46], which is one of the limitations of using HFD-induced models of GDM. Overall, our model was effective at inducing our primary outcome (glucose intolerance), but not many of the secondary characteristics of GDM, including insulin resistance, and our results should be viewed in light of these limitations.

Effects of myo-inositol

MI supplementation did not significantly improve glucose tolerance, our primary outcome. However, MI did increase adipose gene expression of key members of the insulin signalling pathway – *Ir, Akt2* and *Pck1*. Mice in the HFMI group ate on average 3 g/day, meaning they consumed about 60 mg MI/day, which is beyond the 36 mg/day recently reported to show beneficial effects in pregnant mice[20]. Previous studies have similarly demonstrated beneficial effects of MI at doses ranging from 0.08 mg/day–48 mg/day in mouse models of neural tube defects[47,48]. Therefore, it is unlikely that the lack of effect on glucose tolerance in this study was due to an insufficient dose of MI. Although the dietary model used in this study did demonstrate impaired glucose tolerance, the absence of fasting hyperglycaemia or insulin resistance in this current study suggests that it is a very mild model of GDM, which may have prevented us from demonstrating some of the benefits of MI. This is consistent with the results of Ferrari *et al*. (2016), which reported very little effect of MI in HFD-fed pregnant mice, but did see benefit in a mouse model of metabolic syndrome (HFD + eNOS$^{-/-}$)[20]. One explanation offered by the authors was that the metabolic syndrome model displayed fasting hyperglycaemia and hyperleptinaemia, while the HFD-only model did not. Also consistent with Ferrari *et al*., we demonstrated reduced gestational weight in MI-fed mice. This finding is notable, as excessive gestational weight gain is a significant risk factor for poor pregnancy outcomes, including GDM[49]. However, we saw no effect of MI on adipose deposition (i.e. fat mass) or adipose or liver histology. Increased fat deposition, adipocyte hyperplasia, and liver steatosis are all indicators of metabolic disease[50–52]. In contrast, Croze *et al*. (2015) reported reduced adipose deposition in HFD-fed male mice supplemented with MI, although they similarly saw no improvement in liver steatosis with MI[53]. These discrepancies may simply represent differences between sexes and during pregnancy. Further, MI did not improve the growth restriction observed in the HFD group in the current study, which aligns with the results of Ferrari *et al*. (2016) and with a previous study from our group[29,33]. However, it should be noted that GDM is more frequently associated with macrosomia, which is difficult to replicate in rodents. In human trials, MI is associated with reduced rates of fetal macrosomia[10,54].

Effects of probiotics

Like MI, PB did not affect glucose tolerance, but did increase adipose gene expression of insulin signalling mediators *Ir, Irs1*, *Akt2* and *Pck1* compared with HFD alone. PB was also surprisingly associated with increased fasting blood glucose and plasma total cholesterol concentrations. A recent meta-analysis of thirty-two randomised controlled trials (RCT) of various strains of probiotics noted a significant reduction in total cholesterol concentration [55]. However, one RCT investigating the effects of *Lactobacillus salivarius* in GDM did report an increase in total and

LDL cholesterol during pregnancy[56]. Therefore, it may be the case that probiotics have unintended consequences for lipid metabolism during pregnancy, and this warrants further investigation. The most recent systematic review/meta-analysis of the use of various probiotics for management of GDM found that probiotics do not decrease fasting glucose or LDL, which is consistent with our results[13].

PB also had a significant effect on adipose *Pparγ* expression (Hedges' *g* was 2.0, where a Hedges' *g* of 0.8 is considered a large effect size[36,57]). Probiotic supplementation (*Lactobacillus reuteri, Lactobacillus crispatus, Bacillus subtilis*[58] and *Lactobacillus casei, B. bacterium longum*[59]) has previously been associated with enhanced PPARγ activation in HFD- and STZ-induced rodent models of obesity and diabetes[58,59]. Several studies similarly suggest that the probiotic compound VSL#3 exerts its beneficial effects through PPARγ-dependent mechanisms[60–62]. However, to our knowledge, the probiotic strains used in the current study (*Lactobacillus rhamnosus* and *Bifidobacterium lactis*) have not been previously linked to PPARγ. Typically, upregulated *Pparγ* expression results in improved adipogenic capacity and a reduction in ectopic fat deposition, such as in the liver. However, we did not observe any effects of PB on measures of liver steatosis. Therefore, while our results further support a *Pparγ*-inducing effect of probiotic supplementation, we have not confirmed any physiological benefit from this.

PB also increased maternal pancreas weight, male fetal weight, and female placental weight. Typically, increased pancreatic mass will be accompanied by reduced blood glucose; however, in this study, we saw the opposite[63]. These data could illustrate a feedback mechanism, whereby pancreatic mass increased to compensate for increased blood glucose. The observed increase in fetal and placental weight following PB treatment could be interpreted as an improvement of HFD-induced growth restriction when compared with the CD group. However, our study was not powered for this outcome, and it should be noted that the available data on probiotic use in human pregnancy has not reported any meaningful changes in fetal weight or growth[12].

Effects of combined myo-inositol and probiotics

In most cases, the combination of MI and PB did not result in an additive, beneficial effect compared to the effects seen when the ingredients were administered separately. Indeed, in some cases the combination negated beneficial effects of the individual components. This was most pronounced with adipose gene expression: while MI and PB individually enhanced expression of *Ir* and *Akt2,* the HFMIPB group was not different than HFD alone. As ours is the first study to examine MI and PB together, the reasons for this are unknown. However, our data suggest

399 that the combination of MI and PB might not be more effective than the individual components
400 for managing glucose intolerance and associated metabolic outcomes during pregnancy.

401 Strengths of this study include the balanced two-way factorial ANOVA design, allowing for
402 pooled analysis of treatments, and the onset of supplementation before pregnancy, allowing an
403 investigation into the preventative effects of MI and PB. As discussed earlier, a limitation of
404 this study is that the chosen mouse model – short-term HFD feeding – did not result in many of
405 the metabolic dysfunctions that usually accompany glucose intolerance. It is possible that longer
406 exposure to HFD would have resulted in a more severe phenotype (albeit, one not limited to
407 pregnancy), and that in this case we may have seen some effect of MI and/or PB. This is
408 especially true given that both supplements had effects on adipose gene expression. Another
409 limitation of this study is that we did not examine the supplements in normal control-fed animals
410 during pregnancy. We recognise that supplements such as these may have exhibit different
411 effects in healthy animals. It is also important to note that fathers were exposed to the same diets
412 as the mothers during mating, and that therefore paternal effects may have contributed to the
413 fetal and placental outcomes.

414 In conclusion, neither MI, PB, nor the combination of the two, had an impact on HFD-induced
415 glucose intolerance. The effects of the treatments on other measures of metabolic health during
416 pregnancy were also minimal. MI and PB did separately affect adipose tissue gene expression
417 of insulin signalling mediators (both increased *Ir* and *Akt2*), but this effect was ameliorated in
418 the combination. PB was also associated with increased fasting glucose and total cholesterol,
419 which are of unknown consequence. It is possible that the lack of some key features of GDM in
420 the model used in this study (fasting hyperglycaemia, hyperinsulinaemia, hyperleptinaemia and
421 adipose inflammation) may have prevented a full demonstration of the effects of MI and PB.

Acknowledgements

The authors would like to thank Axieo Specialties, who donated the maltodextrin used for control purposes. They would also like to acknowledge Satya Amirapu, who was responsible for histological processing, sectioning, and staining, Thashma Ganapathy for assistance with animal experiments, and Michael Pellizzon for formulating the diets used in this study.

Financial Support

This work was funded by the Nestle Research Center (NRC). The funders contributed to the design of the study and approved the final version of the manuscript, but did not conduct the study, analyse the data, or write the manuscript. The maltodextrin used for control purposes was donated by Axieo Specialties, Auckland, New Zealand.

Conflict of Interest

This research was funded by Nestlé Research. J. M. Ramos Nieves, F. Budin, K. Mace and I. Silva-Zolezzi are full time employees of Nestlé Research.

Authorship

J.P. helped design the study, collected data, analysed data, and primarily wrote the manuscript. J.R.N., F.B., K.M., and I.S.Z. helped design the study. C.R. helped to supervise the study and edit the manuscript. M.V. and P.B. helped design and supervise the study, and edit the manuscript. J.S. helped design the study, supervised the study, collected data, and edited the manuscript.

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611 **Tables**

612 **Table 1. Composition of the four diets used in this study**

	Control Diet (D10012G)		Control Diet with 2% Myo-inositol added		HFD (D12451)		HFD with 2% Myo-inositol added	
	gm	Kca;	gm	kcal	gm	kcal	gm	kcal
Protein	20	20.3			24	20	23	20
Carbohydrate	64	63.9			41	35	41	35
Fat	7.0	15.8			24	45	23	45
Total		100				100		100
Kcal/gm	3.9				4.7		4.6	
Ingredient								
Casein	200				200	800	200	800
L-Cystine	33				3	12	3	12
Corn Starch	397				72.8	291	72.8	291
Maltodextrin 10	132				100	400	100	400
Sucrose	100				172.8	691	172.8	691
Cellulose	50				50	0	50	0
Soybean Oil	70				25	225	25	225
Lard					177.5	1598	177.5	1598
Mineral Mix S10026	35				10	0	10	0

DiCalcium Phosphate					13	0	13	0
Calcium Carbonate					5.5	0	5.5	0
Potassium Citrate, 1 H2O					16.5	0	16.5	0
Vitamin Mix V10001	10				10	40	10	40
Choline Bitartrate	2.5				2	0	2	0
Myo-inositol	0				0	0	17.51	0
FD&C Yellow Dye #5					0	0	0	0
FD&C Red Dye #40					0.05	0	0.025	0
FD&C Blue Dye #1					0	0	0.025	0
Total	1000	4000			858.15	4057	875.66	4057
Myo-inositol (g/kg)	0				0		20	

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Table 2. Maternal weight gain, energy intake and litter size at GD 18.5 (cull)

Data analysed by Student's t-test (CD vs. HFD) or two-way ANOVA (all HFD groups) followed by Tukey post-hoc test and expressed as mean ± SEM. * indicates a significant difference between HFD and CD (reference).; n = 10–13 mice per group.

	CD (reference)	**HFD**	**HFMI**	**HFPB**	**HFMIPB**	*Effect of MI*	*Effect of PB*	*Interaction*
Gestational weight gain (g)	13.6 ± 0.5	14.2 ± 0.3	13.5 ± 0.7	14.3 ± 0.5	12.0 ± 0.8	***p = 0.013***	**NS**	**NS**
Litter size	7.8 ± 0.4	8.7 ± 0.2	8.6 ± 0.5	8.4± 0.3	7.9 ± 0.4	**NS**	**NS**	**NS**
Average total food intake over pregnancy (g)	60.9 ± 1.5	54.8 ± 2.1*	63.5 ± 3.5	57.4 ± 1.5	56.3 ± 2.2	**NS**	**NS**	**NS**
Average total energy intake over pregnancy (kcal)	237 ± 6	257 ± 10	292 ± 16	270 ± 7	253 ± 11	**NS**	**NS**	**NS**

Table 3. Maternal plasma profile at GD 18.5 (cull)

Data analysed by Student's t-test (CD vs. HFD) and two-way ANOVA (all HFD groups) followed by Tukey post-hoc test and expressed as mean ± SEM. * indicates a significant difference between HFD and CD (reference); n = 10–13 mice per group.

	CD (reference)	**HFD**	**HFMI**	**HFPB**	**HFMIPB**	*Effect of MI*	*Effect of PB*	*Interaction*
Fasting glucose (mmol/L)	7.3 ± 0.3	7.1 ± 0.5	7.0 ± 0.4	8.2 ± 0.5	7.8 ± 0.5	**NS**	***p = 0.043***	**NS**
Fasting insulin (ng/mL)	1.1 ± 0.1	0.7 ± 0.1*	1.0 ± 0.6	0.9 ± 0.1	0.8 ± 0.2	**NS**	**NS**	**NS**
HOMA-IR	17.7 ± 2.0	11.4 ± 2.3	15.3 ± 2.6	14.4 ± 1.1	14.3 ± 3.7	**NS**	**NS**	**NS**
Matsuda index	34.1 ± 4.0	45.3 ± 5.9	35.1 ± 4.2	31.9 ± 2.8	44.5 ± 11.0	**NS**	**NS**	**NS**
Fasting adiponectin (ng/mL)	7890 ± 575	6778 ± 404	6542 ± 426	6335 ± 424	6495 ± 364	**NS**	**NS**	**NS**
Fasting leptin (ng/mL)	15.4 ± 2.5	34.3 ± 9.7	41.3 ± 5.1	39.5 ± 7.7	26.9 ± 5.4	**NS**	**NS**	**NS**
Total cholesterol (mmol/L)	0.69 ± 0.07	0.81 ± 0.07	0.71 ± 0.06	0.88 ± 0.09	1.05 ± 0.09*#	**NS**	***p = 0.015***	**NS**
Plasma HDL (mmol/L)	0.59 ± 0.08	0.76 ± 0.07	0.68 ± 0.10	0.86 ± 0.11	0.96 ± 0.09	**NS**	**NS**	**NS**
Plasma LDL (mmol/L)	0.10 ± 0.01	0.15 ± 0.02*	0.10 ± 0.01	0.15 ± 0.01	0.16 ± 0.01#	**NS**	**NS**	**NS**
Plasma FFA (mmol/L)	0.39 ± 0.08	0.26 ± 0.02	0.51 ± 0.13	0.30 ± 0.00	0.27 ± 0.02	**NS**	**NS**	**NS**

Table 4. Maternal organ weights at GD 18.5 (cull)

Data analysed by Student's t-test (CD vs. HFD) or two-way ANOVA (all HFD groups) followed by Tukey post-hoc test and expressed as mean ± SEM. * indicates a significant difference between HFD and CD (reference).; *n* = 10–13 mice per group.

	CD (reference)	**HFD**	**HFMI**	**HFPB**	**HFMIPB**	*Effect of MI*	*Effect of PB*	*Interaction*
Retroperitoneal fat (% BW)	0.18 ± 0.01	0.25 ± 0.02*	0.30 ± 0.03	0.27 ± 0.03	0.32 ± 0.07	**NS**	**NS**	**NS**
Gonadal fat (% BW)	0.55 ± 0.06	0.92 ± 0.13*	1.19 ± 0.17	0.95 ± 0.12	1.01 ± 0.15	**NS**	**NS**	**NS**
Perirenal fat (% BW)	0.18 ± 0.02	0.26 ± 0.06	0.21 ± 0.02	0.23 ± 0.04	0.25 ± 0.04	**NS**	**NS**	**NS**
Mesenteric fat (% BW)	0.62 ± 0.03	0.60 ± 0.06	0.54 ± 0.04	0.62 ± 0.06	0.63 ± 0.06	**NS**	**NS**	**NS**
Pancreas (% BW)	0.46 ± 0.03	0.40 ± 0.02	0.42 ± 0.02	0.45 ± 0.02	0.44 ± 0.01	**NS**	***p = 0.042***	**NS**
Kidney (av. % BW)	0.45 ± 0.02	0.42 ± 0.01	0.43 ± 0.01	0.42 ± 0.01	0.41 ± 0.01	**NS**	**NS**	**NS**
Liver (% BW)	4.39 ± 0.06	4.30 ± 0.09	4.34 ± 0.09	4.34 ± 0.10	3.99 ± 0.06	**NS**	**NS**	***p = 0.048***

Table 5. Fetal measurements

Data analysed by Student's t-test (CD vs. HFD) or two-way ANOVA (all HFD groups) followed by Tukey post-hoc test, data presented as mean ± SEM * indicates a significant difference between HFD and CD (reference). *n* = 10–13 mice per group.

	CD	HFD	HFMI	HFPB	HFMIPB	*MI*	*PB*	*Interaction*
Male fetal weight (g)	1.19 ± 0.020	1.12 ± 0.019*	1.10 ± 0.019	1.18 ± 0.017	1.14 ± 0.031	**NS**	***p = 0.035***	**NS**
Female fetal weight (g)	1.18 ± 0.019	1.07 ± 0.023*	1.04 ± 0.018	1.10 ± 0.012	1.07 ± 0.048	**NS**	**NS**	**NS**
Male crown–rump length (mm)	28.54 ± 0.32	28.64 ± 0.32	28.43 ± 0.18	28.43 ± 0.31	27.93 ± 0.87	**NS**	**NS**	**NS**
Female crown–rump length (mm)	28.85 ± 0.25	28.1 ± 0.32	27.89 ± 0.39	28.40 ± 0.39	27.94 ± 0.53	**NS**	**NS**	**NS**
Male circumference (mm)	24.68 ± 0.27	23.41 ± 0.29*	23.80 ± 0.45	24.65 ± 0.33	23.09 ± 0.83	**NS**	**NS**	***p = 0.049***
Female circumference (mm)	24.58 ± 0.27	22.52 ± 0.29*	22.62 ± 0.20	23.67 ± 0.38	22.54 ± 0.57	**NS**	**NS**	**NS**
Male placental weight (g)	0.12 ± 0.00	0.11 ± 0.01	0.12 ± 0.00	0.12 ± 0.00	0.12 ± 0.01	**NS**	**NS**	**NS**
Female placental weight (g)	0.12 ± 0.01	0.11 ± 0.00	0.11 ± 0.00	0.11 ± 0.00	0.12 ± 0.01	**NS**	***p = 0.021***	**NS**
Male fetal:placental ratio	10.31 ± 0.37	10.36 ± 0.50	9.87 ± 0.43	9.69 ± 0.36	10.33 ± 0.46	**NS**	**NS**	**NS**
Female fetal:placental ratio	10.61 ± 0.38	10.63 ± 0.41	10.17 ± 0.49	9.41 ± 0.15	10.10 ± 0.42	**NS**	**NS**	**NS**
Male % labyrinth zone	55.19 ± 3.01	46.67 ± 3.18	50.42 ± 3.42	50.15 ± 2.33	54.22 ± 10.51	**NS**	**NS**	**NS**
Female % labyrinth zone	43.70 ± 0.85	53.28 ± 3.22	54.52 ± 4.40	55.84 ± 2.10	52.61 ± 3.76	**NS**	**NS**	**NS**

Male % junctional zone	22.43 ± 1.44	25.96 ± 1.37	25.46 ± 1.86	23.87 ± 2.35	20.49 ± 1.30	**NS**	**NS**	**NS**
Female % junctional zone	27.68 ± 2.41	22.50 ± 2.67	21.24 ± 1.77	19.58 ± 1.58	18.40 ± 0.97	**NS**	**NS**	**NS**

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Figure legends

Figure 1. Experimental design

Timeline of experiment. Probiotic mix contained *L. rhamnosus* and *B. lactis* at 10^9 CFU per day.

Figure 2. Pre-pregnancy measurements

Fluid intake was measured daily and body weights and food intake were measured weekly. (A) Body weights per mouse per week prior to mating; (B) Food intake per mouse per week prior to mating; (C) Energy intake from fluid per day per mouse prior to mating; (D) Energy intake per week per mouse accounting for fluid energy. Data analysed by repeated measures ANOVA and expressed as mean ± SEM, where $*p < 0.05$ all groups compared with CD; $n = 16$ mice per group.

Figure 1. Glucose tolerance and plasma insulin concentrations at GD16.5

(A) OGTT curves following oral gavage dose of 2 g/kg D-glucose at GD16.5; (B) AUCs of OGTT curves at GD16.5; (C) Plasma insulin concentration during OGTT at GD16.5; (D) Insulin AUCs at GD16.5. Data analysed by two-way repeated measures, Student's t-test (CD vs. HFD) or two-way ANOVA followed by Tukey post-hoc test (all HFD groups), and expressed as mean ± SEM. $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$ when HFD is compared with CD, $n = 10–13$ mice per group.

Figure 4. Adipocyte histology

(A) Representative H&E stained sections of gonadal adipose tissue. Scale bar = 10 μm; (B) Average adipocyte area per group; (C) Percentage of adipocytes per area bracket. Data analysed by Student's t-test (CD vs. HFD) or two-way ANOVA followed by Tukey post-hoc test (all HFD groups), and expressed as mean ± SEM, where $*p < 0.05$, HFD vs. CD; $n = 10–13$ mice per group.

Figure 5. Gonadal adipose tissue gene expression

Adipose mRNA expression determined by qPCR. Differences were seen in: (A) *Ir;* (B) *Irs1*; (C) *Akt2*; (D) *Pck1*; (E) *Pparγ*. Data analysed as Student's t-test (CD vs HFD) or two-way ANOVA followed by Tukey post-hoc test (all HFD groups), and presented as mean ± SEM,

where *$p < 0.05$ and ****$p < 0.0001$ when HFD is compared with CD. Dissimilar letters denote a significant difference between groups according to Tukey. n = 6–10 mice per group.

Figure 6. Hepatic histology

H&E stained sections of liver. Each section was examined under 20x magnification to evaluate lobular inflammation. Ten random 40x magnification fields per animal were evaluated for general steatosis and microvesicular steatosis. (A) Representative micrographs from each experimental group at 20x magnification; (B) Representative micrographs from each experimental group at 40x magnification. Scale bars = 10μM; (C) NAFLD activity score (NAS) in table format. Data analysed as Student's t-test (CD vs HFD) or two-way ANOVA followed by Tukey post-hoc test (all HFD groups), and presented as mean ± SEM, where *$p <$ 0.05 when HFD is compared with CD; n = 10 – 13 mice per group.

Supplementary Table 1. Taqman probes used for adipose gene expression analysis

Gene	Taqman gene expression assay code
Ir	Mm01211875_m1
Irs1	Mm01278327_m1
Igf1r	Mm00802831_m1
Akt2	Mm02026778_g1
Slc2a4	Mm00436615_m1
Pck1	Mm01247058_m1
Lepr	Mm00440181_m1
G6pc	Mm00839363_m1
Fas	Mm01204974_m1
Pparγ	Mm00440940_m1
Ccr5	Mm01963251_s1
Nlrp3	Mm00840904_m1
Nfkb	Mm00479807_m1
Il1b	Mm00434228_m1
Cd11c	Mm00498701_m1
Tnf	Mm00443258_m1
Mcp-1	Mm00441242_m1
Il-6	Mm00446190_m1
Angptl4	Mm00480431_m1

Supplementary Figure 1. No differences in gut permeability were observed across groups. Mice were dosed with 600 mg/kg FITC-D after five hours fasting on GD18.5, and concentrations one hour later, at cull, were measured by fluorescence spectrophotometry.

Data presented as mean ± SEM; *n* = 8–12 mice per group.

Supplementary Figure 2. Genes examined in gonadal adipose tissue in which no differences in expression were observed

Assessed by qPCR.

Data expressed as mean ± SEM; *n* = 6 –10 mice per group.