Maternal microbiota *Bifidobacterium* promotes placental morphogenesis, nutrient transport and fetal growth in mice

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Author Contributions: JL-T, ZS, ANS-P, LJH designed research; JL-T, ZS, RK, GLG conducted research, JL-T, ZS, RK, MJD contributed analytic tools and performed analysis; DvS contributed reagents; JL-T, ZS, ANS-P, LJH wrote the paper with feedback from all the authors.

Competing Interest Statement: The authors declare that they have no competing interests.

Classification: Biological Sciences – Physiology

Keywords: Pregnancy, Placenta, Microbiota, Fetus, *Bifidobacterium*

This file includes:

Main Text: 5,454 (introduction, results, discussion and methods)

Main Figures: 3

Tables: 1

Supplementary Figures: 4

Supplementary Tables: 3

References: 55

Significance: Metabolism is highly influenced by the gut microbiota, which is particularly important during gestation, when key metabolites are used for feto-placental growth. However, the contribution of the maternal gut microbiota (and microbiota-generated metabolites) in determining fetal outcomes is largely unexplored. Here, we show that maternal gut communities and specific microbiota members are key modulators of placental phenotype with important consequences for fetal development. We have revealed novel roles for a maternal *Bifidobacterium* strain, that include the control of placental capillary morphogenesis and nutrient transporters (glucose and lipids), which affect fetal metabolism and growth. Our work has important implications for the establishment of novel therapeutic strategies to treat pregnancy complications.

Abstract

The gut microbiota plays a central role in regulating host metabolism. However, while substantial progress has been made in discerning how the microbiota influences host functions post birth and beyond, little is known about how key members of the maternal gut microbiota can influence feto-placental growth. Here, using germ-free and specific-pathogen-free mice, we demonstrate that the bacterium *Bifidobacterium breve* UCC2003 modulates maternal body adaptations, placental structure and nutrient transporter capacity, with implications for fetal metabolism and growth. The effects of *B. breve* UCC2003 on feto-placental growth are mediated, in part, by changes in the maternal and placental metabolome (*i.e.* acetate and carnitine). Histological analysis of the placenta confirmed that *Bifidobacterium* modifies placental structure via changes in *Igf2P0*, *Dlk1*, *Mapk1* and *Mapk14* expression. Additionally, *B. breve* UCC2003, acting through *Slc2a1* and *Fatp3-4* transporters, was shown to restore fetal glycaemia and fetal growth in association with changes in the fetal hepatic transcriptome. This study provides knowledge towards a novel and safe therapeutic strategy for treating pregnancy disorders via modulation of the maternal gut microbiota.

Main Text

Introduction

All nutrients and metabolites required for feto-placental growth are provided by the mother, which in turn is thought to be influenced by the maternal gut microbiota through breakdown of complex dietary components (1). During gestation, liberated metabolites may be used by the placenta for morphogenesis, and transported across the placenta for use by the fetus for growth and development (2, 3). This is highly important across gestation, particularly at later stages, when fetal growth is maximal. Notably, there are also alterations in the maternal microbiota throughout pregnancy with levels of the beneficial bacterial genus *Bifidobacterium*

rising from trimester 1 onwards (4–6). Failure of the mother to provide nutrients and metabolites to the fetus can result in pregnancy complications including small for gestational age (SGA), fetal loss and stillbirth. However, the contribution of the maternal microbiota in determining fetal outcomes is largely unexplored. Knowledge in this area would be highly valuable for developing treatments to improve fetal growth, with benefits for population health.

Studies performed with Germ free (GF) mice have identified that the microbiota is a key regulator for adequate development, early immune education and metabolism (7–11). However, little is known about how maternal microbiota influences fetoplacental growth and more specifically, placental structure and function. Here, we hypothesized that the maternal gut microbiota, and specific microbiota members, regulate fetal growth by modulating placental development and nutrient supply. We tested this hypothesis by comparing conceptus growth in three different maternal gut microbiota conditions by using conventional specific-pathogen-free (SPF) mice (used as a base line to define correct fetoplacental growth) (11), GF mice, and maternal GF mice colonized with *Bifidobacterium breve* UCC2003 (12). As a keystone microbiota member and ‘probiotic’ species, *B. breve* may represent a suitable option for treating pregnancy complications by exerting beneficial metabolic effects on maternal physiology and associated fetoplacental growth. Indeed, *B. breve* induced changes in maternal physiology, placental morphogenesis and the abundance of placental glucose and lipid transporters, which were associated with improvements in growth and metabolism of the fetus.

Results

Germ-free mice treated with *B. breve* have altered body composition and caecum metabolic profile

To assess whether maternal microbiota is able to influence fetoplacental growth, GF mice were treated orally with *B. breve* UCC2003 from day 10 of gestation (treatment on days 10, 12 and 14; i.e. BIF group), and compared to GF and SPF dams (for experimental overview see Figure S1). Timing and dosing were based on the fact that levels of *Bifidobacterium* rise throughout pregnancy (5) (colonization levels during pregnancy can be found in Figure S2). Moreover, previous work has indicated three consecutive doses of *B. breve* UCC2003 facilitates stable gut colonization, with the advantage of also avoiding repeated handling of the mice, which may induce spontaneous abortions (13, 14). In addition, from a translational point of view, we also wanted to correlate with potential future supplementation studies in women at the point pregnancy is confirmed.

Maternal body composition differed between groups with GF and BIF mice showing increased digestive tract weight and lower pancreas mass compared to SPF mice. GF and BIF mice had similar circulating concentrations of glucose and insulin to SPF mice (assessed in fed

conditions; Table 1). Compared to SPF mice, treatment with *B. breve* reduced maternal gonadal fat depot, liver, and spleen weights in BIF mice. No differences were observed in the circulating concentrations of leptin, cholesterol, triglycerides, or free fatty acids in maternal serum (Table 1).

Metabolomics analysis in maternal caecum samples indicated that the concentration of 13 out of 115 metabolites were significantly altered (Table 1 and Table S1). Acetate was significantly influenced by *B. breve* (Table 1), with BIF dams having intermediate concentrations compared to SPF and GF mice (the low levels of acetate detectable in GF mice, most likely originated from the diet and/or are host-derived). These findings suggest that acetate producing *B. breve* and the wider gut microbiota may exert selective effects on maternal metabolic (gonadal fat depot and liver) and immune organs (spleen).

Maternal gut microbiota and *B. breve* regulate fetal growth by controlling fetal glycaemia

Although the three experimental groups had similar numbers of viable fetuses per litter, the GF group had higher numbers of resorptions within the litter (defined as large or small hemorrhagic-to-black implantation sites) compared to SPF and BIF mice (Figure 1A-B). Similarly, when this parameter was calculated as resorption rate, and corrected for litter size, we found similar results (Figure S3). In fact, all GF dams had at least one resorption, whilst *B. breve* reduced the appearance of resorptions by 50% (Figure 1B). No evidence of intrauterine death, defined as a lack of fetal vascular flow in the yolk sac vessels, were found. Compared to SPF and BIF mice, GF fetuses were growth restricted, hypoglycaemic and had reduced liver weight, but had preserved brain size (Figure 1C-E). These findings highlight that the maternal microbiota influence fetus viability and that *B. breve* is a strong modulator of fetal growth and metabolism.

Maternal *B. breve* modulates fetal hepatic transcriptome

The liver is a key organ for glucose storage and production. As fetuses from BIF mice had enlarged livers and improved glycaemia, we next determined if there were changes in the fetal hepatic transcriptome (livers were collected on GD18.5, when fetal liver function is particularly active prior to term; Figure 1F). A total of 602 genes were differentially expressed, with 94 significantly up-regulated and 508 down-regulated genes in BIF group, when compared to GF group (Figure 1G-H). Functional enrichment analysis indicated many metabolic pathways were regulated in the fetal livers of BIF mice including carboxylic acid and lipid metabolic processes, steroid hydroxylase activity, fatty acid metabolism and response to glucocorticoid (Figure 1I; Table S2). Therefore, maternal *B. breve* appears to exert beneficial alterations in fetal hepatic function to support fetal growth.

Maternal gut microbiota and *B. breve* control placental morphogenesis

To further understand the links between the maternal microbiota and the regulation of fetal growth, we assessed placental structure. When compared to SPF mice, placentas were lighter in GF and BIF mice (Figure 2A). Placental efficiency, defined as the grams of fetus produced per gram of placenta, was significantly improved in the BIF group compared to SPF and GF mice (Figure 2B). Analysis of placental compartments showed that lack of maternal gut microbiota significantly hampered growth of the placental labyrinth transport zone (Lz), without compromising the endocrine junctional zone or decidua volumes (Figure 2C). It also did not affect placental glycogen storage (Figure 2D) or the volume of the trophoblast (Figure E-F). Analysis of maternal blood spaces revealed that the BIF group had reduced spaces compared to SPF mice, while the volume and the length of fetal capillaries were significantly reduced in the GF compared to SPF (Figure 2F-G). Similarly, surface area for exchange of the Lz was significantly decreased in GF compared to SPF mice (Figure 2H). The barrier between maternal and fetal blood was also determined to be thinner in BIF *versus* GF mice (Figure 2I). Lz apoptosis levels were similar between groups (Figure 2J).

To define the molecular mechanisms behind the changes in the Lz, we quantified the expression of select genes in micro-dissected Lz. Common angiogenic factors *Vegf*, *Ang1*, *Ang2* were similarly expressed between groups (Figure 2K). However, the expression of signaling pathways involved in cell proliferation and growth, namely the MAPK pathway, was significantly altered by changes in maternal gut microbiota; *Mapk1* was shown to be increased in both GF and BIF, while *Mapk14* (also known as *p38Mapk*) was revealed to be specifically up-regulated in the Lz of BIF mice. In addition, *Dlk1* and *Igf2P0*, which are key genes implicated in metabolism and Lz formation, were significantly up-regulated in the BIF group compared to GF mice. The expression of *Akt* did not vary with group (Figure 2K). As informed by western blotting, activation of ERK was reduced in the placental Lz of GF compared to SPF mice, and this effect was reversed by BIF (Figure 2L). p38MAPK protein activity was similar between groups. DLK1 protein level was also lower in GF compared to SPF mice. However BIF increased DLK1 protein levels when compared to both SPF and GF mice (Figure 2L). Further analysis by qPCR showed that the mRNA levels of *Nras*, but not *Kras*, were significantly elevated in the BIF compared to the SPF group (Figure 2M). Overall, these findings suggest that the maternal gut microbiota, and *B. breve*, regulate the development of the mouse placental Lz via modulation of specific cell growth and metabolic genes/pathways.

Maternal gut microbiota and *B. breve* affect placental glucocorticoid handling and controls key placental nutrient transporters

Glucocorticoids are a class of steroid hormones that can affect both fetal and placental formation. To control glucocorticoid actions on the conceptus, the placenta expresses 11 β -hydroxysteroid dehydrogenase type 1 and 2 (*11Hsd1* and *11Hsd2*). We found that the

expression of *11Hsd1* was significantly elevated in the BIF group compared to the GF (no change in *11Hsd2*; Figure 2N). Moreover, to better understand the changes in fetal growth and glycemia between groups, we quantified the expression of selected amino acid, glucose and lipid transporters in the Lz. We found no difference in the expression of system A amino acid transporters (*Slc38a1*, *Slc38a2*, *Slc38a4*) between groups (Figure 2O). However, the key glucose transporter *Slc2a1* was up-regulated in the Lz of BIF mice compared to GF mice (*Slc2a3* mRNA levels were similar between groups; Figure 2O). Fatty acid transporters were also altered, with high expression levels of *Fatp1* in the GF *versus* the SPF group, and increased levels of *Fatp3* and *Fatp4* in the BIF group compared to the GF (Figure 2O; *Cd36* expression levels were unaltered). Collectively, these data suggest that maternal gut microbiota, and *B. breve*, regulate fetal growth by inducing changes in the expression of key nutrient transporters within the placenta.

Differences in placental labyrinth growth are linked to an altered placental metabolome

To gain further mechanistic understanding of the changes observed in the placental Lz and fetal liver, we analysed >80 metabolites at GD16.5 (Figure 3 and Table S1). We found 5 metabolites significantly altered in the placental Lz (Figure 3). Amino adipic acid in the Lz was very low in GF/BIF groups as well as in fetal livers (Figure 3A). Treatment with *B. breve* significantly reduced the concentrations of acetylcarnitine and carnitine in Lz tissue compared to SPF placentas, but not in fetal livers (Figure 3B-C). Levels of formate in placental Lz were significantly elevated in both GF and BIF compared to SPF mice (Figure 3D), with a similar trend (although not significant) in fetal liver samples. Acetate was also altered in the Lz (Figure 3E), with concentrations significantly lower in the SPF compared to the GF group, whilst BIF samples showed intermediate levels (although these levels were much lower than observed in the maternal caecum). Similar to formate, concentrations of acetate in fetal liver followed similar directions to the Lz, yet were not statistically different between groups. These data suggest that maternal gut microbiota, and *B. breve*, regulate the fetal and placental growth via modulation of the placental Lz metabolome.

Discussion

In this study, we provide evidence that the maternal microbiota and the microbiota member *B. breve* regulate maternal body composition during gestation and beneficially modulate fetoplacental growth. To the best of our knowledge, this is the first demonstration of a maternal gut bacterium remotely controlling placental structure and nutrient transporters, with important implications for fetal glycaemia and fetal growth. We observed that the effects of *Bifidobacterium* are partially mediated by altered metabolites in maternal caecum and in placental Lz tissue, with alterations in the expression of key genes in the placental Lz and fetal liver.

Bifidobacterium is the dominant microbiota member in vaginally delivered, breast fed infants, with certain species and strains known to stimulate and aid the maturation of the immune system (15). *B. breve* UCC2003 also regulates responses at the gut barrier, inducing homeostatic epithelial cell programming, and protecting against inflammatory insults (14, 16). Importantly, pregnancy is accompanied by increasing *Bifidobacterium* abundance in the gut of women and mice (5), which is associated with changes in progesterone concentrations during pregnancy. Additionally, alterations in the abundance of *Bifidobacterium* are also linked to the development of serious pregnancy complications like preeclampsia (17). Our study shows that *B. breve* improves pregnancy outcomes in mice, as indicated by the lower number of resorptions, elevated placental efficiency and restored fetal glycaemia and weight. *B. breve* UCC2003 was also shown to induce changes in the metabolite milieu, including acetate, acetylcarnitine and carnitine in the mother and placenta, which were likely key for the beneficial effects on fetal growth. Acetate is a major bifidobacterial fermentation by-product, which directly mediates epithelial cell responses but also exerts systemic beneficial metabolic effects (18, 19). More generally, microbial-derived short-chain fatty acids (SCFAs) modulate multiple host physiological systems and during pregnancy are associated with maternal gestational weight, neonatal length and body weight, and protection against allergic airway disease in the developing fetus (20, 21). Acetate crosses the placenta (21), so in our model, the elevated maternal *B. breve*-derived acetate may exert beneficial effects on feto-placental growth via (i) maternal effects, through interactions within the maternal gut mucosa as evidenced by higher maternal caecum acetate concentrations in *B. breve* supplemented (and SPF dams vs. GF) and the subsequent impact on maternal organs (liver, adipose and spleen) and nutrient handling, (ii) effects on the placenta, through use of acetate for cellular metabolism, growth and function, and/or (iii) effects on the fetal metabolism following transport of acetate across the placenta to the fetus. Compared to the maternal caecum, levels of acetate were relatively low in the placental Lz and fetal liver (for all 3 groups). This suggests that *B. breve* (and SPF microbiota-derived acetate) may be used to support anabolic processes *in utero* (hence the very low levels detected), and together with changes in acetylcarnitine and carnitine, may be supported by our observations of elevated fatty acid transporter expression in the placenta and differentially regulated genes in the fetal liver. The observed modulation of immune-associated pathways in the fetal liver, including those associated with G protein-coupled receptor signalling (e.g. *Dusp9*), also indicates a role for direct acetate-associated responses (22). *B. breve* supplementation also restored fetal glycaemia and weight in BIF mice, achieving similar values to that seen for SPF fetuses. Previous *in vivo* studies indicate different strains of *Bifidobacterium* (including *B. breve*) modulate glucose handling (23), with this genus also consistently associated with potential protection against human metabolic disorders e.g. type 2 diabetes (24, 25). In addition, our observations of reduced maternal gonadal fat mass and maternal liver weight in *B. breve*-treated dams compared to SPF dams, suggest that *Bifidobacterium*, or *B. breve* metabolites,

could affect maternal metabolic responses in key organs of the mother. In fact, previous work in adult mice suggests elevation of gut acetate levels due to *Bifidobacterium* treatment plays a key role in regulating glucose handling systemically and reduces visceral fat accumulation (26).

Maternal microbiota significantly affected the placental vascular bed by modifying the elongation of their fetal capillary network, which is required for adequate fetal growth (27). Moreover, *B. breve* significantly reduced the interhaemal membrane barrier thickness of the placenta, which may facilitate exchange of nutrients and gases. The mechanisms governing these structural changes could be partially mediated by changes in the expression of two imprinted genes (28), *Igf2* (namely the placenta-exclusive isoform, *Igf2P0*) and *Dlk1*, and via MAPK upregulation. In this regard, deletion of *Igf2P0* results in fetoplacental growth restriction in association with reduced placental surface area for exchange, fetal capillary volume and increased barrier thickness (reviewed by (29)); parameters that were partially improved by *B. breve* UCC2003 in line with the *Igf2P0* mRNA upregulation. Interestingly, *Igf2P0* is important for the structural and transport adaptations occurring in the placenta in response to maternal nutritional restriction (30) and recent work has shown that *Igf2* in the placenta interacts with MAPK signaling (31). *Dlk1*, a non-canonical ligand of the Notch signalling pathway localized to the endothelial cells of fetal capillaries in the placental LZ, also regulates placental vascularisation and branching morphogenesis (32). In addition, IGF2 and DLK1 can both mediate cellular actions via the MAPK pathway (33, 34). *Mapk14*, also known as p38MAPK, forms part of a signal transduction pathway that has been linked to environmental stresses and inflammatory cytokines (35). However, p38MAPK also regulates many normal cellular processes, including proliferation and cytoskeletal organisation. Furthermore, research in mice has demonstrated that *Mapk14* is essential for embryogenesis and placental LZ angiogenesis and vascular remodeling (36). In addition, placentas exposed to *B. breve* UCC2003, had lower concentrations of acetylcarnitine and carnitine compared to SPF, suggesting a greater reliance on these compounds for energy production, or enhanced transfer of these fatty acids to the fetus (37). Carnitine is well-known for mediating the transport of fatty acids into mitochondrial matrix for fatty acid β -oxidation. Carnitine can also promote p38MAPK signalling activation in cardiac tissue (38). Taken together, placental structural, transport and metabolic changes seen with *B. breve* UCC2003 treatment may link to the altered metabolites/nutrient milieu in the mother and would have been beneficial in providing additional substrates for fetal growth and development (when compared to GF; Figure S4).

While our study has clear strengths and strong translational implications for pregnancy complication treatments, it also has certain limitations. Unsurprisingly, we did not see a full 'rescue' of placental phenotype in the monocolonised GF *B. breve* (BIF) group, compared to complex microbiota found in SPF dams. However, structural and functional adaptations of

these placentas exposed to *B. breve* were adequate enough to ‘rescue’ fetal weight and fetal glycaemia. An array of gut-associated signaling and a diverse metabolite pool are expected to provide more complete placental development. Indeed, other or additional *Bifidobacterium* species and/or strains may be required for placental and fetal development, given strain-specific host physiology responses (16, 39). Further studies should allow the relative contributions of other microbial- and *Bifidobacterium*-derived factors to be elucidated. In addition, future work should evaluate the role of *B. breve* across gestation using larger cohorts of mice. Nonetheless, this study has revealed novel roles for *Bifidobacterium* and provides a safe therapeutic strategy for treating pregnancy complications, suggesting an opportunity for *in utero* programming through maternal *Bifidobacterium* and associated metabolites.

Overall, although our study was performed in mice and is not representative of a clinical scenario, our study highlights the importance of the maternal gut microbiota during gestation and demonstrates that *B. breve* modulates maternal physiology, placental structure and nutrient transporter capacity with impact on fetal glycaemia and fetal growth (Figure S4). Our findings prompt an in-depth investigation into how additional members of the gut microbiota impact on pregnancy outcomes. These future studies are important for the design of novel therapies to combat fetal growth restriction and other pregnancy complications.

Materials and Methods

Bifidobacterium breve UCC2003/pCheMC

B. breve UCC2003/pCheMC was generated by introducing the plasmid pCheMC to electrocompetent *B. breve* UCC2003 as described previously (40). In brief, *B. breve* UCC2003 was grown until mid-log phase, chilled on ice and washed twice with ice cold sucrose citrate buffer (1mM citrate, 0.5M sucrose, pH5.8) and then electroporation of cells was carried out under the following conditions; 25 MF, 200 Ohms, 2 KV. Transformed cells were incubated for 2 hours in Reinforced Clostridial Medium (RCM) at 37°C in a controlled anaerobic chamber then plated (41) on RCM agar plates with selective antibiotics. Colonies were sub-cultured 3 times on RCM agar plates with selective antibiotics. Antibiotics were used at the following final concentrations erythromycin 2µg/mL.

Lyophilised *B. breve*

B. breve was grown in De Man, Rogosa and Sharpe agar (MRS) under anaerobic conditions overnight. The bacterial cell pellet was resuspended in 10% milk powder and lyophilised in 200 ml quantities. Lyophilised *B. breve* was reconstituted with 500µl PBS. Concentration of *B. breve* was 10¹⁰CFU/ml. All batches were tested for contamination upon reconstitution on Luria-Bertani (LB) and Brain-Heart Infusion (BHI) plates under anaerobic and aerobic conditions at 37°C. No contamination of *B. breve* was detected.

Mice

All mouse experiments were performed under the UK Regulation of Animals (Scientific Procedures) Act of 1986. The project license PDADA1B0C under which these studies were carried out was approved by the UK Home Office and the UEA Ethical Review Committee. All mice were housed in the Disease Modelling Unit at the University of East Anglia, UK. Animals were housed in a 12:12 hour light/dark, temperature-controlled room and allowed food and water ad libitum. Female Germ Free C57BL/6J (GF) and Specific Pathogen Free (SPF) mice aged 6-8 weeks were used for the study. GF mice were bred in germ free isolators (2 females to 1 male) and on gestational day (GD) GD9.5, pregnant mice (confirmed by weight) were removed from the GF isolator and transferred to individually ventilated cages. The sterility of these cages was previously tested and found to be suitable for housing GF mice for 1 week. Sterile water was changed every 2 days.

***B. breve* colonisation levels**

Mice were given 100µL of reconstituted lyophilised *B. breve* UCC2003 by oral gavage (containing 10^{10} CFU/mL) at GD10, GD12 and GD14 or 100µL vehicle control (PBS, 4 % skimmed milk powder), with this dosing regimen reflecting a more realistic time frame for women who are more likely to start taking probiotics once their pregnancy is confirmed. At GD16.5 and GD18.5, mice were sacrificed by cervical dislocation and samples collected for molecular and histological analysis. The experimental design can be found in Figure S1.

Faecal samples were checked for contamination and *B. breve* colonization at GD12 and GD14 and GD16. Briefly, faecal samples from GF and GF treated with *B. breve* were diluted in 500 µL of PBS and agitated for 30 mins at 4°C on an Eppendorf MixMate 5353 Digital Mixer Plate Shaker. The faecal solution was passed through a 0.45µm syringe filter. Faecal solution was diluted 1 in 100 and 20 µL was added to a De Man, Rogosa and Sharpe agar plate with erythromycin and incubated for 48 hours in an anaerobic chamber at 37°C. Colony forming units were counted using a click counter.

Blood hormones and circulating metabolites

Maternal blood was obtained by cardiac exsanguination immediately after cervical dislocation. Blood was centrifuged and serum collected and stored at -80°C until further analysis. Blood glucose and plasma concentrations of leptin, insulin, triglycerides, cholesterol, and free fatty acids were determined as previously reported (42). Fetal blood glucose levels were measured with a handheld glucometer (One Touch Ultra; LifeScan) immediately after decapitation of the fetus.

Placental histology

Placentas were cut in half and fixed in 4% paraformaldehyde overnight at 4°C. Samples were

washed 3 times with PBS for 15 minutes each and storage in 70% ethanol until embedding in wax. Embedded placentas were cut at 5µm thickness and stained with haematoxylin and eosin for gross morphology. Placental layer volumes were calculated using the Computer Assisted Stereological Toolbox (CAST v2.0). For analysis of labyrinth components, sections were stained with lectin for identification of fetal endothelial vessels and with cytokeratin for trophoblasts. Further details of the double-labelling immunohistochemistry and the analysis performed can be found elsewhere (43). For the analysis of placental glycogen, sections were stained with Periodic acid–Schiff (Sigma-Aldrich) previous incubation with 0.5% periodic acid (Thermo Fisher Scientific). Sections were counterstained with Fast-green (Sigma-Aldrich) and digitalized with the nanozoomer scanner (Hamamatsu). Analysis of placental glycogen accumulation was performed with Image J and conducted blinded to experimental groups. TUNEL staining for placental cell death was performed using the TUNEL Assay Kit - HRP-DAB (Abcam, ab206386) following manufacturer instructions except for the counterstaining which was substituted for Nuclear Fast Red (Vector). Sections were digitalized using a nanozoomer scanner (Hamamatsu) and the amount of apoptosis in the labyrinth zone was calculated in 5 random areas (x20 magnification) and analysed by Image J software.

Western blotting

Protein extraction was performed with RIPA buffer as described previously (44). Lysates were separated by SDS-PAGE and incubated with antibodies against p-MAPK (Thr202/Tyr204) (Cell Signalling, 4370; 1/1000), t-MAPK 44/42 (Cell Signalling, 4695; 1/1000), DLK-1 antibody (Abcam, ab21682; 1/1000), p-P38MAPK (Cell Signalling, 4511; 1/1000) and t-P38MAPK (Cell Signalling, 8690; 1/1000). Reactive bands were detected by chemiluminescence (Thermo Scientific, Scientific SuperSignal West Femto) and quantified by Image J software. Proteins were normalized to Ponceau S Staining (45).

RNA extraction and qPCR

Extraction of RNA from micro-dissected placental labyrinth zones was performed with RNeasy Plus Mini Kit (Qiagen) and reverse transcribed using the High Capacity cDNA RT Kit minus RT inhibitor (Applied Biosystems) according to manufacturer's instructions. Samples were analysed using MESA Blue SYBR (Eurogentec) and primers (See Supplementary Table 3) were synthesized by Sigma-Aldrich. The expression of each gene was normalized to the geometric mean expression of two reference genes *Hprt* and *Ubc*, which remained stably expressed across the groups. Analysis was performed using the 2-ΔΔCt method (46).

Sequence pre-processing, Differential Gene Expression (DGE) analysis and Functional enrichment analysis

Fetal liver RNA on GD18.5 was extracted using the RNeasy Plus Mini Kit (Qiagen). RNA sequence pre-processing and DGE analysis was performed as previously described with slight

modifications (14). Briefly, FASTQ reads were initially quality-filtered using fastp v0.20.0 with options -q 10 (sequence reads with phred quality <10 were discarded). Subsequently, sequence reads for each sample were merged (merge-paired-reads.sh) and followed by rRNA sequence filtering via SortMeRNA v2.1 based on SILVA rRNA database optimised for SortMeRNA software (47, 48). Filtered reads were then unmerged (unmerge-paired-reads.sh) and ready for transcript quantification. Transcript mapping and quantification were then performed using Kallisto v0.44.0 (49). *Mus musculus* (C57BL/6 mouse) cDNA sequences (GRCm38.release-98_k31) retrieved from Ensembl database were indexed with Kallisto utility *index* at default parameter and was used for following transcript mapping and abundance quantification via Kallisto utility *quant* at 100 bootstrap replicates (-b 100) (50).

RNA raw counts were subjected (Kallisto outputs) to DGE analysis, which was performed using R library *Sleuth* (v0.30.0) (51). Transcripts were then mapped to individual genes using Ensembl BioMart database (GRCm38.p6) with function *sleuth_prep* and option *gene_mode* = *TRUE*. Genes with an absolute log₂ (fold change) >1.0 and q value <0.05 (p-adjusted value; based on Wald test statistics) were considered to be differentially regulated (52). DGE statistics were plotted via functions within package *Sleuth*. Finally, functional enrichment analysis was performed using g:Profiler webtool g:GOst based on organism *Mus Musculus* species (53). Briefly, a list of DGEs (Ensembl IDs) was uploaded to g:GOst, then selected 'GO molecular function', 'GO biological process' and 'Reactome' in the 'data sources'. Significance threshold was set at 0.001 (g:SCS threshold).

Metabolite extraction, Nuclear Magnetic Resonance (NMR) spectroscopy and metabolite quantification

Extraction of metabolite from liver, placenta, caecum contents and culture (spent) media samples were performed as previously described as a standard protocol (54). For caecal samples, frozen materials (stored at -80°C prior to analysis) were weighed ~50 mg before the addition of 600 µL of faecal water phosphate buffer solution. The faecal water phosphate buffer was prepared as follows: add 0.51g NaH₂PO₄.H₂O and 2.82g K₂HPO₄ to 200 mL D₂O (Deuterium Oxide; Merck). To this, 34.5 mg TSP (Trimethylsilyl propanoic acid; used as NMR standard) and 100 mg NaN₃ (Merck) were added (55). Next, the mixture was centrifuged for 10 min at 17,000 x g before transferring the mixture to an NMR tube (Merck) for subsequent NMR analysis.

For liver and placenta samples (stored at -80°C prior to analysis), frozen fresh tissue (~20-45 mg) was placed into a 2 ml sterile microcentrifuge tube pre-loaded with ~15-20 glass beads (Merck) while 200 µL of ice-cold methanol (Fisher Scientific) and 42.5 µL of ultra-pure cold water were added to it and vortexed. Tissue was disrupted via a tissue lyser (Qiagen) for 2 x 2 mins. 100 µL of ice-cold chloroform (Merck) was then added and vortexed. 100 µL of ice-cold

chloroform and 100 µL of ultra-pure cold water were added to the mixture, and kept on ice for 15 min. Liquid was then transferred into a new sterile microcentrifuge tube and centrifuged for 3 min at 17,000 x g. The top aqueous phase was transferred into a new microcentrifuge tube and speed-vacuumed for 30 min at 50°C and 30 min without heating prior to reconstitution with faecal water phosphate buffer solution at 600 µL. The mixture was then moved to an NMR tube (Merck) for subsequent NMR analysis. Metabolites from culture media Brain Heart Infusion (BHI; Oxoid) and spent media (BHI cultured with *B. breve* UCC2003 for 48 h) were extracted as follows: 400 µL of medium was transferred into a sterile microcentrifuge tube with the addition of 200 µL faecal phosphate buffer and mixed well. The mixture was then moved to an NMR tube (Merck) for further NMR analysis.

Samples in NMR tubes were subsequently subjected to NMR spectroscopy. The ¹H NMR spectra were recorded at 600 MHz on a Bruker AVANCE spectrometer (Bruker BioSpin GmbH, Germany) running Topspin 2.0 software. The metabolites were then quantified using the software Chenomx® NMR Suite 7.0™.

Statistical analysis

All statistical analysis and sample size are shown in each figure/table and in the corresponding figure/table legends. Only samples from viable fetuses were analysed. No statistical analysis was used to pre-determine sample size and samples were assigned code numbers and analysis was performed in a blinded fashion. Statistical calculations were performed using the GraphPad Prism software (GraphPad, San Diego, CA), SAS/STAT (Statistical System Institute Inc. Cary, NC, USA) and RStudio Version 1.4.1106 (RStudio Boston, MA) with R Version 4.0.3 (Vienna, Austria). Morphometric parameters of mother and number of viable/resorption per litter were analysed by one-way ANOVA followed by Tukey post hoc test. Resorption rate was calculated with a general linear model with total litter size as a covariate. Feto-placental weights, placental stereological measurements and placental Lz gene expression levels were analysed with a general linear mixed model, taking into account viable litter size as a covariate and taking each fetus as a repeated measure. Differences between individual metabolites between the three groups were tested with a Kruskal-Wallis test using the `kruskal.test` function with correction for multiple comparisons applied using the Benjamini & Hochberg false discovery rate method using the `p.adjust` function. Pairwise comparisons between the three groups were carried out with a Dunn's test on individual metabolites significantly different after correction for multiple comparisons using the `dunnTest` function in the FSA package. ROUT test was used for identification of outlier values. A probability value of < 0.05 was considered significantly different.

Acknowledgments

JL-T currently holds a Sir Henry Wellcome Postdoctoral Fellowship (220456/Z/20/Z) and previously a Newton International Fellowship from the Royal Society (NF170988 / RG90199). LJH is supported by Wellcome Trust Investigator Awards (100974/C/13/Z and 220876/Z/20/Z); the Biotechnology and Biological Sciences Research Council (BBSRC), Institute Strategic Programme Gut Microbes and Health (BB/R012490/1), and its constituent projects BBS/E/F/000PR10353 and BBS/E/F/000PR10356. ANS-P is supported by a Lister Institute of Preventative Medicine Research Prize (RG93692). Authors would like to thank Dr Ruben Bermejo-Poza (Complutense University of Madrid) for statistical advice and the Ferguson-Smith laboratory (University of Cambridge) for providing the DLK1 antibody.

Data availability

The fetal liver RNA-Seq raw sequencing data are deposited at the National Center for Biotechnology Information (NCBI) under BioProject PRJNA748000. Relevant data are within the manuscript and its Supporting Information files. Scripts for differential gene expression analysis can be accessed at GitHub, <https://github.com/raymondkiu/Maternal-foetal-microbiota-paper/>

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Figure 1. Effects of maternal gut microbiome and *B. breve* supplementation during pregnancy on fetal viability, growth and hepatic transcriptome. (A) Number of viable fetuses per litter, (B) Number of resorptions per litter (left) and number of dams with presence of resorptions within the litter (right), (C) Fetal weight, (D) Fetal blood glucose concentrations, (E) Fetal organ weights. All data shown in A to E were obtained on GD16.5 from: SPF (49 fetuses/6 dams), GF (33 fetuses/5 dams), BIF (34 fetuses/6 dams). (F-I) RNA-Seq analysis of fetal liver samples obtained at GD18.5, (F) PCA plot and (G) volcano plots showing up and down-regulated DEGs in BIF group (compared to GF group), (H) Heat map of the 20 most up and down-regulated DEGs (BIF group), (I) Functional profiling (g:Profiler) on 602 DEGs. Key enriched GO terms and REACTOME pathways are shown in the figure (significant threshold: $P_{adj} < 0.001$). RNA-seq was performed on fetal livers obtained at GD18.5 from a total of 3 GF and 4 BIF pregnant dams/litters. Statistical analysis for data shown in A-B was performed by one-way ANOVA Tukey multiple comparisons test. Data in C-E was analysed with a general linear mixed model, taking into account litter size as a covariate and taking each fetus as a repeated measure followed by Tukey multiple comparisons test. RNA-Seq data analysis is described in the material and methods section. Different superscripts indicate significant differences between groups (a versus b and/or c: $P < 0.05$; mean $\pm$ SEM).

Figure 2. Effects of maternal gut microbiome and *B. breve* supplementation during pregnancy on placental structure and placental gene expression on GD16.5. (A) Placenta weight, (B) Placental efficiency determined by dividing fetal by placental mass, (C) Placental regional analysis, (D) Representative staining of placental glycogen with PAS and glycogen abundance, (E) Representative image of lectin and cytokeratin staining for labyrinth zone structural quantification, (F-I) Stereological parameters determined in placental labyrinth zone, (J) Representative image of TUNEL staining for apoptosis quantification in labyrinth zone (K-O), Gene expression levels in micro-dissected labyrinth. Data were analyzed by general linear mixed model, taking into account litter size as a covariate and taking each fetus as a repeated measure followed by Tukey multiple comparisons test. ROUT test was used for identification of outlier values. Different superscripts indicate significant differences between groups (a versus b: P<0.05; mean±SEM). Placental weight-efficiency was obtained from: SPF (49 fetuses/6 dams), GF (33 fetuses/5 dams), BIF (34 fetuses/6 dams). Laboratorial analysis was performed with: SPF (13-15 placentas/6 dams), GF (10-11 placentas/5 dams) and BIF (14-11 placentas/6 dams).

Figure 3. Metabolomic profiling of placental labyrinth zone and fetal liver on GD16.5. Data were analysed by Kruskal-Wallis test followed by multiple comparisons using the Benjamini & Hochberg false discovery rate method and Dunn's test. ROUT test was used for identification of outlier values. Different superscripts indicate significant differences between groups (a versus b, P<0.05). Data presented as mean±SEM. Number of placentas – livers analysed per group: SPF (8-8/4 dams), GF (6-8/4-5 dams), BIF (6-7/5 dams).

Supplementary Figure 1. Schematic representation of the experimental design.

B. breve was administrated by oral gavage on gestational day (GD) 10, 12 and 14 to germ-free mice. Abbreviations: LZ (placental labyrinth zone); SPF (Specific-Pathogen-Free mouse); GF (Germ-Free mouse); BIF (Germ-Free mouse treated with *B. breve* UCC2003).

SPF group not analysed as cannot detect *B.breve* in SPF mice due to abundance of other bacteria

Supplementary Figure 2. Colonization levels of *B. breve* determined in maternal faecal samples on gestational day (GD), 12 and 14. Analysis performed by two-ways ANOVA (****P<0.0001). Data displayed as mean ± SEM. Number of dams for GF and BIF groups are 5 and 6, respectively.

Resorption rate

Supplementary Figure 3. Resorption rate defined as the number of resorptions divided by the sum of resorptions and viable fetuses within the litter. Data displayed as mean ± SEM and analysed by general linear with total litter size as a covariate. Different superscripts indicate significant differences between groups.

Supplementary Figure 4. Summary illustration showing how the maternal gut bacteria and *B. breve* affects mother, placenta and fetus during gestation. Abbreviations: LZ (placental labyrinth zone); SPF (Specific-Pathogen-Free mouse); GF (Germ-Free mouse); BIF (Germ-Free mouse treated with *B. breve* UCC2003); MBS (Maternal Blood Spaces); FC (Fetal Capillaries); SA (Surface Area).

Body composition				
	SPF (n=6)	GF (n=5)	BIF (n=6)	
Hysterectomy weight (g)	26.01±0.91	27.87±0.78	27.17±0.80	NS
Digestive Tract (g)	2.76±0.03 ^a	6.83±0.32 ^b	7.25±0.63 ^b	****
Caecum (g)	0.66±0.03 ^a	3.47±0.25 ^b	3.96±0.41 ^b	****
Small intestine (g)	1.66±0.03 ^a	2.65±0.11 ^b	2.59±0.14 ^b	****
Pancreas (mg)	315.40±30.12 ^a	183.40±24.74 ^b	190.60±38.71 ^b	*
Gonadal fat (mg)	433.10±43.20 ^a	297.0±37.02 ^{ab}	272.0±27.35 ^b	*
Liver (g)	2.09±0.10 ^a	1.79±0.05 ^{ab}	1.55±0.08 ^b	**
Spleen (mg)	117.90±2.80 ^a	91.76±10.60 ^{ab}	83.03±6.72 ^b	*
Circulating metabolites and metabolic hormones				
	SPF (n=6)	GF (n=5)	BIF (n=6)	
Glucose (mmol/L)	8.08±0.78	8.38±1.18	8.88±0.74	NS
Insulin (µg/L)	0.12±0.004	0.19±0.05	0.20±0.06	NS
Leptin (pg/mL)	2465±177.1	2739±486	2425±303	NS
Cholesterol (mmol/L)	1.33±0.03	1.56±0.08	1.41±0.09	NS
Triglycerides (mmol/L)	1.54±0.08	1.79±0.14	1.50±0.11	NS
Free Fatty Acids (µmol/L)	890.6±101.3	1440±362	1092±114.5	NS
Caecum Metabolites				
	SPF (n=3)	GF (n=4)	BIF (n=4)	
2.Methylbutyrate (mmol/Kg)	0.05±0.01 ^a	0±0.0 ^b	0±0.0 ^b	*
Isobutyrate (mmol/Kg)	0.41±0.24 ^a	0±0.0 ^b	0±0.0 ^b	*
Butyrate (mmol/Kg)	12.48±7.97 ^a	0±0.0 ^b	0±0.0 ^b	*
5.Aminopentanoate (mmol/Kg)	0.33±0.14 ^a	0±0.0 ^b	0±0.0 ^b	*
Propionate (mmol/Kg)	4.48±1.99 ^a	0±0.0 ^b	0±0.0 ^b	*
Citrulline (mmol/Kg)	0.30±0.07 ^a	0±0.0 ^b	0±0.0 ^b	*
Fucose (mmol/Kg)	0.08±0.02 ^a	0±0.0 ^b	0±0.0 ^b	*
Trimethylamine (mmol/Kg)	0.06±0.006 ^a	0±0.0 ^b	0±0.0 ^b	*
Isovalerate (mmol/Kg)	0.09±0.01 ^a	0±0.0 ^b	0±0.0 ^b	*
Methylamine (mmol/Kg)	0.05±0.02 ^a	0±0.0 ^b	0±0.0 ^b	*
Malonate (mmol/Kg)	0.09±0.02 ^a	0±0.0 ^b	0±0.0 ^b	*
Valerate (mmol/Kg)	0.57±0.27 ^a	0±0.0 ^b	0±0.0 ^b	*
Acetate (mmol/Kg)	35.49±20.66 ^a	0.55±0.06 ^b	3.79±1.64 ^{ab}	*

Table 1. Effects of maternal gut microbiome and *B. breve* administration during pregnancy on maternal body composition, circulating metabolites and hormones in maternal serum, and metabolites in caecum. Body composition and metabolites/hormones in serum were analyzed by one-way ANOVA followed by Tukey multiple comparisons test. Metabolites in maternal caecum were analysed by Kruskal-Wallis test followed by multiple comparisons using the Benjamini & Hochberg false discovery rate method and Dunn's test. ROUT test was used for identification of outlier values. Different superscripts indicate significant differences between groups (a versus b, P<0.05) (*P<0.05; **P<0.01; ***P<0.001;

****P<0.0001; NS: not significant). Data presented as mean±SEM. The number of dams used for each group is annotated on the table.

Supplementary Table 1. List of differentially expressed genes and pathways detected in the liver RNA-Seq on day 18.5 of gestation.

Supplementary Table 2. List of metabolites analysed in maternal caecum, placental labyrinth zone and fetal liver on day 16.5 of gestation.

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	<i>Forward</i>	<i>Reverse</i>
<i>Hprt</i>	CAGGCCAGACTTTGTTGGAT	TTGCGCTCATCTTAGGCTTT
<i>Ubc</i>	GGAGTCGCCCCGAGGTCA	AAAGATCTGCATCGTCTCTCTCAC
<i>Vegf</i>	GAAGCTACTGCCGTCCGATT	CTTCATCGTTACAGCAGCC
<i>Ang1</i>	GAAGCAACTTCTCAACAGACA	TTCTTTGTGTTTTCCCTCCATT
<i>Ang2</i>	CTTCTACCTCGCTGGTGAAGAG	GCTAAAATCACTTCCTGGTTGG
<i>Akt</i>	GCCGCCTGATCAAGTTCTCC	TTCAGATGATCCATGCGGGG
<i>Mapk1</i>	TGCTTTCTCTCCCGCACAAA	GGCCAGAGCCTGTTCAACTT
<i>Mapk14</i>	AGCTGTGCGAGACCGTTTCAG	GATGGGTCACCAGGTACACG
<i>Dlk1</i>	GAAAGGACTGCCAGCACAAAG	CACAGAAGTTGCCTGAGAAGC
<i>Igf2P0</i>	GAGGAAGCTCTGCTGTTTGG	CAAAGAGATGAGAAGCACCAAC
<i>Hsd11b1</i>	GAGGAAGGTCTCCAGAAGGTA	ATGTCCAGTCCGCCCCAT
<i>Hsd11b2</i>	GGCTGGATCGCGTTGTC	CGTGAAGCCCATGGCAT
<i>Slc38a1</i>	CGGCGCCTTTCCCTTTATTTTC	CCGTAACTCGAGGCCACTT
<i>Slc38a2</i>	TTCTGATTGTGGTGATTTGCAAGAA	CAGGATGGGCACAGCATACA
<i>Slc38a4</i>	AAGGTAGAGGCGGGAAAGGG	AGGAACTTCTGACTTTTCGGCA
<i>Slc2a1</i>	GCTTATGGGCTTCTCCAAACT	GGTGACACCTCTCCCACATAC
<i>Slc2a3</i>	GA TCGGCTCTTTCCAGTTTG	CAA TCA TGCCACCAACAGAG
<i>Cd36</i>	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
<i>Fatp1</i>	GGCTCCTGGAGCAGGAACA	ACGGAAGTCCCAGAAACCAA
<i>Fatp3</i>	GAGAACTTGCCACCGTATGC	GGCCCCTATATCTTGGTCCA
<i>Fatp4</i>	GATTCTCCCTGTTGCTCCTGT	CCATTGAAGCAAACAGCAGG
<i>Fatp6</i>	AACCAAGTGGTGACATCTCTGC	TCCATAAAGTAAAGCGGGTCAG
<i>Kras</i>	ACAGGCTCAGGAGTTAGCAAG	ACACAGCCAGGAGTCTTTTCT
<i>Nras</i>	CAAAGCAAGCCCACGAACTG	CATCGTCACTGCTGTTGAGC

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Supplementary Table 3. List of primers used for placental labyrinth zone qPCR