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

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Abstract

The gut microbiota plays a central role in regulating host metabolism. 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. Notably, in pregnant women, *Bifidobacterium* represents a key beneficial microbiota genus, with levels observed to increase across pregnancy. 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. Maternal and placental metabolome were affected by maternal gut microbiota (*i.e.* acetate, formate 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 *Fatp1-4* transporters, was shown to restore fetal glycaemia and fetal growth in association with changes in the fetal hepatic transcriptome. Our work emphasizes the importance of the maternal gut microbiota on feto-placental development and sets a foundation for future research towards the use of probiotics during pregnancy.

45 **Introduction**

46 All nutrients and metabolites required for feto-placental growth are provided by the mother, which in
47 turn is thought to be influenced by the maternal gut microbiota through breakdown of complex dietary
48 components [1]. During gestation, liberated metabolites may be used by the placenta for
49 morphogenesis, and transported across the placenta for use by the fetus for growth and development
50 [2, 3]. This is highly important across gestation, particularly at later stages, when fetal growth is
51 maximal. Notably, there are also alterations in the maternal microbiota throughout pregnancy with
52 levels of the bacterial genus *Bifidobacterium* rising from trimester 1 onwards [4–6]. Failure of the
53 mother to provide nutrients and metabolites to the fetus can result in pregnancy complications
54 including small for gestational age, fetal loss and stillbirth. However, the contribution of the maternal
55 gut microbiota in determining fetal outcomes is largely unexplored. Knowledge in this area would be
56 highly valuable for developing treatments to improve fetal growth, with benefits for population health.

57

58 Studies performed with germ free (GF) mice have identified that the microbiota is a key regulator for
59 adequate development, early immune education and metabolism [7–11]. However, little is known
60 about how maternal gut microbiota influences feto-placental growth and placental structure and
61 function. Here, we hypothesized that the maternal gut microbiota, and specific microbiota members,
62 regulate fetal growth by modulating placental development and nutrient supply. We tested this
63 hypothesis by comparing conceptus growth across a range of microbiome complexity; using
64 conventional specific-pathogen-free (SPF) mice as a model for standard microbial colonization, and as a
65 base line to define correct feto-placental growth [11]; GF mice which represent a completely clean and
66 naïve microbiome system; and a mono-colonized maternal GF model – GF mice colonized with
67 *Bifidobacterium breve* UCC2003 (group referred throughout the manuscript as BIF) [12].
68 *Bifidobacterium*, including *B. breve* UCC2003, is known to beneficially modulate the wider gut
69 microbiota and host responses [13–15]. It is defined as a probiotic “live microorganisms, which when
70 ingested or locally applied in sufficient numbers confer one or more specified demonstrated health
71 benefits for the host” (FAO/WHO; [16]). Therefore, *B. breve* may represent a suitable option for
72 treating pregnancy complications by exerting metabolic effects on maternal physiology and associated
73 feto-placental growth. Indeed, *B. breve* induced changes in placental morphogenesis and the
74 abundance of placental glucose and lipid transporters, which were associated with improvements in
75 growth and metabolism of the fetus.

76

77 **Materials and Methods**

78 ***Bifidobacterium breve* UCC2003/pCheMC**

79 *B. breve* UCC2003/pCheMC was generated by introducing the plasmid pCheMC to electrocompetent *B.*
80 *breve* UCC2003 as described previously to allow antibiotic tagging of *B. breve* for subsequent culture
81 studies [17]. In brief, *B. breve* UCC2003 was grown until mid-log phase, chilled on ice and washed
82 twice with ice cold sucrose citrate buffer (1mM citrate, 0.5M sucrose, pH5.8) and then electroporation
83 of cells was carried out under the following conditions; 25MF, 2000Ohms, 2KV. Transformed cells were
84 incubated for 2 hours in Reinforced Clostridial Medium (RCM) at 37°C in a controlled anaerobic
85 chamber then plated [18] on RCM agar plates with selective antibiotics. Colonies were sub-cultured 3
86 times on RCM agar plates with selective antibiotics. Antibiotics were used at the following final
87 concentrations erythromycin 2µg/mL.

88

89 **Lyophilised *B. breve***

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

96

97 **Mice**

98 All mouse experiments were performed under the UK Regulation of Animals (Scientific Procedures) Act
99 of 1986. The project license PDADA1B0C under which these studies were carried out was approved by
100 the UK Home Office and the UEA Ethical Review Committee. All mice were housed in the Disease
101 Modelling Unit at the University of East Anglia, UK. Animals were housed in a 12:12 hour light/dark,
102 temperature-controlled room and allowed food and water *ad libitum* (food/water intake was not
103 recorded). Female Germ Free C57BL/6J (GF) and Specific Pathogen Free (SPF) mice aged 6-8 weeks
104 were used for the study. GF mice were bred in germ free isolators (2 females to 1 male) and on
105 gestational day (GD) GD9.5, pregnant mice (confirmed by weight gain) were removed from the GF
106 isolator and transferred to individually ventilated cages. The sterility of these cages was previously
107 tested and found to be suitable for housing GF mice for 1 week. Sterile water was changed every 2
108 days. We assessed responses at 2 gestational phases – the majority of studies were carried out at
109 GD16.5, whilst the RNASeq studies utilized fetal livers harvested at GD18.5. A total of 6 SPF mice were
110 used for GD16.5 assessments (no SPF mice were studied on GD18.5). For the GF group a total of 5
111 (GD16.5) and 3 (GD18.5) dams were used. For the BIF mice, a total of 6 (GD16.5) and 4 (GD18.5) dams
112 were used.

113

114 ***B. breve* colonisation levels**

115 Mice were given 100 μ L of reconstituted lyophilised *B. breve* UCC2003 by oral gavage (containing
116 10¹⁰ CFU/mL) at GD10, GD12 and GD14 or 100 μ L vehicle control (PBS, 4 % skimmed milk powder), with
117 this dosing regimen reflecting a more realistic time frame for women who are more likely to take
118 probiotics once their pregnancy is confirmed. At GD16.5 and GD18.5, mice were sacrificed by cervical
119 dislocation and samples collected for molecular and histological analysis. The experimental design can
120 be found in Figure 1A.

121

122 Faecal samples were checked for contamination and *B. breve* colonization at GD12 and GD14 and
123 GD16. Briefly, faecal samples from GF and BIF mice were diluted in 500 μ L of PBS and agitated for 30
124 mins at 4°C on an Eppendorf MixMate 5353 Digital Mixer Plate Shaker. The faecal solution was passed
125 through a 0.45 μ m syringe filter. Faecal solution was diluted 1 in 100 and 20 μ L was added to a De Man,
126 Rogosa and Sharpe agar plate with erythromycin and incubated for 48 hours in an anaerobic chamber
127 at 37°C. Colony forming units were counted using a click counter. In SPF animals housed in the same
128 animal facility we have previously shown that *Bifidobacterium* represents ~1% of the total gut
129 microbiota [19].

130

131 **Blood hormones and circulating metabolites**

132 Maternal blood was obtained by cardiac exsanguination immediately after cervical dislocation. Blood

133 was centrifuged and serum collected and stored at -80°C until further analysis. Blood glucose and
134 serum concentrations of leptin, insulin, triglycerides, cholesterol, and free fatty acids were determined
135 as previously reported [20]. Fetal blood glucose levels were measured with a handheld glucometer
136 (One Touch Ultra; LifeScan) immediately after decapitation of the fetus (fetuses were selected at
137 random).

138

139 **Placental histology**

140 Placentas were cut in half and fixed in 4% paraformaldehyde overnight at 4°C. Samples were washed 3
141 times with PBS for 15 minutes each and storage in 70% ethanol until embedding in wax. Embedded
142 placentas were cut at 5µm thickness and stained with haematoxylin and eosin for gross morphology.
143 Placental layer volume densities (labyrinth zone, junctional zone and decidua) were calculated using
144 point counting and the Computer Assisted Stereological Toolbox (CAST v2.0) and converted to
145 estimated volumes by multiplying by the weight of the placenta. For analysis of labyrinth components,
146 sections were stained with lectin for identification of fetal endothelial vessels and with cytokeratin for
147 trophoblasts. Further details of the double-labelling immunohistochemistry can be found elsewhere
148 [21]. Structural analysis of the labyrinth was performed as previously described [22–24]. Briefly, fetal
149 capillaries, maternal blood spaces and trophoblast volume densities were calculated with a point
150 counting system in 16 random fields and their densities were then multiplied by the estimated volume
151 of the labyrinth zone to obtain the estimated component volume. To estimate the surface density of
152 the maternal-facing and fetal-facing interhaemal membranes, we recorded the number of intersection
153 points along cycloid arcs in a total 20 random fields of view. Both interhaemal membrane surfaces
154 were converted to absolute surface areas and the total surface area for exchange calculated by
155 averaging the two absolute surface areas. Fetal capillary length densities were obtained using counting
156 frames with two contiguous forbidden lines [24] and then converted to absolute capillary length by
157 multiplying by the volume of labyrinth zone. Fetal capillary diameter was estimated using the equation;
158 $d=2(\text{mean area}/\pi)^{1/2}$. The interhaemal membrane barrier thickness was determined using orthogonal
159 intercepts and measuring the shortest distance between fetal capillaries and the closest maternal
160 blood spaces at random starting locations (at least 99) within the labyrinth zone [24].

161

162 For the analysis of placental glycogen, sections were stained with Periodic acid–Schiff (Sigma-Aldrich)
163 previous incubation with 0.5% periodic acid (Thermo Fisher Scientific). Sections were counterstained
164 with Fast-green (Sigma-Aldrich) and digitalized with the nanozoomer scanner (Hamamatsu). Analysis of
165 placental glycogen accumulation was performed with Image J and conducted blinded to experimental
166 groups. TUNEL staining for placental cell death was performed using the TUNEL Assay Kit - HRP-DAB
167 (Abcam, ab206386) following manufacturer instructions except for the counterstaining which was
168 substituted for Nuclear Fast Red (Vector). Sections were digitalized using a nanozoomer scanner
169 (Hamamatsu) and the amount of apoptosis in the labyrinth zone was calculated in 5 random areas (x20
170 magnification) and analysed by Image J software.

171

172 **Western blotting**

173 Protein extraction was performed with RIPA buffer as described previously [25]. Lysates were
174 separated by SDS-PAGE and incubated with antibodies against p-MAPK (Thr202/Tyr204) (Cell
175 Signalling, 4370; 1/1000), t-MAPK 44/42 (Cell Signalling, 4695; 1/1000), DLK-1 antibody (Abcam,
176 ab21682; 1/1000), p-P38MAPK (Cell Signalling, 4511; 1/1000) and t-P38MAPK (Cell Signalling, 8690;

177 1/1000). Reactive bands were detected by chemiluminescence (Thermo Scientific, Scientific
178 SuperSignal West Femto) and quantified by Image J software. Proteins were normalized to Ponceau S
179 Staining [26].
180

181 **RNA extraction and qPCR**

182 Extraction of RNA from micro-dissected placental labyrinth zones was performed with RNeasy Plus
183 Mini Kit (Qiagen) and reverse transcribed using the High Capacity cDNA RT Kit minus RT inhibitor
184 (Applied Biosystems) according to manufacturer's instructions. Samples were analysed using MESA
185 Blue SYBR (Eurogentec) and primers (See Table S1) were synthesized by Sigma-Aldrich. The expression
186 of each gene was normalized to the geometric mean expression of two reference genes *Hprt* and *Ubc*,
187 which remained stably expressed across the groups. Analysis was performed using the 2- $\Delta\Delta Ct$ method
188 [27].
189

190 **Sequence pre-processing, Differential Gene Expression (DGE) analysis and Functional enrichment** 191 **analysis**

192 Fetal liver RNA on GD18.5 was extracted using the RNeasy Plus Mini Kit (Qiagen). Purified RNA was
193 quantified, and quality controlled using RNA 6000 Nano kit on a 2100 Bioanalyser (Agilent). Only
194 samples with RIN values above 8 were sequenced. RNA sequencing was performed at the Wellcome
195 Trust Sanger Institute (Hinxton, UK) on paired-end 75 bp inserts on an Illumina HiSeq 2000 platform.
196 Isolated RNA was processed by poly-A selection and/or Ribo-depletion. RNA sequence pre-processing
197 and DGE analysis was performed as previously described with slight modifications [28]. Briefly, FASTQ
198 reads were initially quality-filtered using fastp v0.20.0 with options -q 10 (sequence reads with phred
199 quality <10 were discarded). Subsequently, sequence reads for each sample were merged (merge-
200 paired-reads.sh) and followed by rRNA sequence filtering via SortMeRNA v2.1 based on SILVA rRNA
201 database optimised for SortMeRNA software [29, 30]. Filtered reads were then unmerged (unmerge-
202 paired-reads.sh) and ready for transcript quantification. Transcript mapping and quantification were
203 then performed using Kallisto v0.44.0 [31]. *Mus musculus* (C57BL/6 mouse) cDNA sequences
204 (GRCm38.release-98_k31) retrieved from Ensembl database were indexed with Kallisto utility *index* at
205 default parameter and was used for following transcript mapping and abundance quantification via
206 Kallisto utility *quant* at 100 bootstrap replicates (-b 100) [32].
207

208 RNA raw counts were subjected (Kallisto outputs) to DGE analysis, which was performed using R library
209 *Sleuth* (v0.30.0) [33]. Transcripts were then mapped to individual genes using Ensembl BioMart
210 database (GRCm38.p6) with function *sleuth_prep* and option *gene_mode = TRUE*. Genes with an
211 absolute \log_2 (fold change) >1.0 and q value <0.05 (p-adjusted value; based on Wald test statistics)
212 were considered to be differentially regulated [34]. DGE statistics were plotted via functions within
213 package *Sleuth*. Finally, functional enrichment analysis was performed using g:Profiler webtool g:GOst
214 based on organism *Mus Musculus* species [35]. Briefly, a list of DGEs (Ensembl IDs) was uploaded to
215 g:GOst, then selected 'GO molecular function', 'GO biological process' and 'Reactome' in the 'data
216 sources'. Significance threshold was set at 0.001 (g:SCS threshold).
217

218 **Metabolite extraction, Nuclear Magnetic Resonance (NMR) spectroscopy and metabolite** 219 **quantification**

220 Extraction of metabolites from fetal liver, placenta and maternal caecum contents were performed as
221 previously described as a standard protocol [36]. For caecal samples, frozen materials (stored at -80°C
222 prior to analysis) were weighed ~50mg before the addition of 600µL of faecal water phosphate buffer
223 solution. The faecal water phosphate buffer was prepared as follows: add 0.51g NaH₂PO₄.H₂O and
224 2.82g K₂HPO₄ to 200 mL D₂O (Deuterium Oxide; Merck). To this, 34.5mg TSP (Trimethylsilyl propanoic
225 acid; used as NMR standard) and 100mg NaN₃ (Merck) were added [37]. Next, the mixture was
226 centrifuged for 10 min at 17,000 x g before transferring the mixture to an NMR tube (Merck) for
227 subsequent NMR analysis.

228
229 For liver and placenta samples (stored at -80°C prior to analysis), frozen fresh tissue (~20-45 mg) was
230 placed into a 2ml sterile microcentrifuge tube pre-loaded with ~15-20 glass beads (Merck) while 200µL
231 of ice-cold methanol (Fisher Scientific) and 42.5µL of ultra-pure cold water were added to it and
232 vortexed. Tissue was disrupted via a tissue lyser (Qiagen) for 2 x 2 mins. 100µL of ice-cold chloroform
233 (Merck) was then added and vortexed. 100µL of ice-cold chloroform and 100µL of ultra-pure cold
234 water were added to the mixture, and kept on ice for 15 minutes. Liquid was then transferred into a
235 new sterile microcentrifuge tube and centrifuged for 3 minutes at 17,000 x g. The top aqueous phase
236 was transferred into a new microcentrifuge tube and speed-vacuumed for 30 minutes at 50°C and 30
237 minutes without heating prior to reconstitution with faecal water phosphate buffer solution at 600 µL.
238 The mixture was then moved to an NMR tube (Merck) for subsequent NMR analysis. Metabolites from
239 culture media Brain Heart Infusion (BHI; Oxoid) and spent media (BHI cultured with *B. breve* UCC2003
240 for 48 h) were extracted as follows: 400µL of medium was transferred into a sterile microcentrifuge
241 tube with the addition of 200µL faecal phosphate buffer and mixed well. The mixture was then moved
242 to an NMR tube (Merck) for further NMR analysis.

243
244 Samples in NMR tubes were subsequently subjected to NMR spectroscopy. The ¹H NMR spectra were
245 recorded at 600 MHz on a Bruker AVANCE spectrometer (Bruker BioSpin GmbH, Germany) running
246 Topspin 2.0 software. The metabolites were then quantified using the software Chenomx® NMR Suite
247 7.0™.

248
249 **Statistical analysis**
250 All statistical analysis and sample size are shown in each figure/table and in the corresponding
251 figure/table legends. Only samples from viable fetuses were analysed. No statistical analysis was used
252 to pre-determine sample size and samples were assigned code numbers and, were possible, analysis
253 was performed in a blinded fashion. Statistical calculations were performed using the GraphPad Prism
254 software (GraphPad v9, San Diego, CA), SAS/STAT 9.0 (Statistical System Institute Inc. Cary, NC,
255 USA) and RStudio Version 1.4.1106 (RStudio Boston, MA) with R Version 4.0.3 (Vienna, Austria). Data
256 reported as mean±SEM. Morphometric parameters of mother, litter size and western blot data were
257 analysed by one-way ANOVA followed by Tukey post hoc test. Feto-placental weights, placental
258 stereological measurements and placental Lz gene expression levels were analysed with a general
259 linear mixed model, taking into account viable litter size as a covariate and taking each fetus as a
260 repeated measure of the mother. In this statistical analysis, fetuses and placentas per litter are nested
261 within litters[38]. Identification of outliers was performed with ROUT Method. For metabolomics,
262 differences between individual metabolites between the three groups were tested with a Kruskal-
263 Wallis test using the kruskal.test function with correction for multiple comparisons applied using the

264 Benjamini & Hochberg false discovery rate method using the *p.adjust* function. Pairwise comparisons
265 between the three groups were carried out with a Dunn's test on individual metabolites significantly
266 different after correction for multiple comparisons using the *dunnTest* function in the *FSA* package. The
267 level of significance for all statistical tests used in this study was set at $P < 0.05$. All figures in the
268 manuscript show individual values (raw data). However, P values and $\text{mean} \pm \text{SEM}$ within the graphs
269 analysed by the general linear mixed model were corrected for repeated measures. Graphs containing
270 the individual dots and graphs with corrected $\text{mean} \pm \text{SEM}$ were generated with Graphpad and merged
271 with Adobe Illustrator.

272

273 Results

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

275 To assess whether maternal microbiota can influence feto-placental growth, GF mice were treated
276 orally with *B. breve* UCC2003 from day 10 of gestation (treatment on days 10, 12 and 14; i.e. BIF
277 group), and compared to GF and SPF dams (for experimental overview see Figure 1A). Timing and
278 dosing were based on the fact that levels of *Bifidobacterium* rise throughout pregnancy (5)
279 (colonization levels during pregnancy can be found in Figure S1). Previous work has indicated three
280 consecutive doses of *B. breve* UCC2003 facilitates stable gut colonization, with the advantage of also
281 avoiding repeated handling of the mice, which may induce spontaneous abortions [28, 39]. In addition,
282 from a translational point of view, we also wanted to correlate our animal model with potential future
283 supplementation studies in women at the point pregnancy is confirmed.

284

285 Maternal body composition differed between groups with GF and BIF mice showing increased digestive
286 tract weight and lower pancreas mass compared to SPF mice. GF and BIF mice had similar circulating
287 concentrations of glucose and insulin to SPF mice (Table 1). Compared to SPF mice, treatment with *B.*
288 *breve* reduced maternal gonadal fat depot, liver, and spleen weights in BIF mice. No differences were
289 observed in the circulating concentrations of leptin, cholesterol, triglycerides, or free fatty acids in
290 maternal serum (Table 1).

291

292 Metabolomics analysis in maternal caecum samples indicated that the concentration of 13 out of 81
293 metabolites were significantly altered (Table 1 and Table S2). Acetate was influenced by *B. breve* (Table
294 1), with BIF dams having intermediate concentrations compared to SPF and GF mice (the low levels of
295 acetate detectable in GF mice, most likely originated from the diet and/or are host-derived). These
296 findings suggest that acetate producing *B. breve* and the wider gut microbiota may exert selective
297 effects on maternal metabolic (gonadal fat depot and liver) and immune organs (spleen).

298

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

300 ***hepatic transcriptome***

301 The three experimental groups had similar numbers of viable fetuses per litter, although GF and BIF
302 groups showed a higher variability compared to the SPF group (Figure 1B). Compared to SPF and BIF
303 mice, GF fetuses were growth restricted, hypoglycaemic and had reduced liver weight, but had
304 preserved brain size (Figure 1C-E). As the liver is a key organ for glucose storage and production and
305 fetuses from BIF mice had heavier livers and improved glycaemia, we next determined if there were
306 changes in the fetal hepatic transcriptome (livers were collected from a small cohort of mice on
307 GD18.5, when fetal liver function is particularly active prior to term. Indeed, mouse fetal hepatocytes

308 are mature from GD18.5, when they present a similar gene expression pattern to those in the
309 postnatal liver [40]). A total of 602 genes were differentially expressed, with 94 significantly up-
310 regulated and 508 down-regulated genes in BIF group, when compared to GF group (Figure 1F-H).
311 Functional enrichment analysis indicated that pathways involved in haemoglobin and oxygen
312 transport-binding were significantly upregulated in the fetal livers of BIF mice (Figure 1I and Table S3).
313 In contrast, many metabolic pathways were downregulated in response to *B. breve* administration,
314 including carboxylic acid and lipid metabolic processes, steroid hydroxylase activity, fatty acid
315 metabolism and response to glucocorticoid (Figure 1H; Table S3). Therefore, maternal *B. breve* appears
316 to exert changes in fetal hepatic function with implications for fetal growth.
317

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

319 To further understand the links between the maternal gut microbiota and the regulation of fetal
320 growth, we assessed placental structure (performed on GD16.5, when placental growth in mice is
321 maximal [24]). When compared to SPF mice, placentas were lighter in GF and BIF mice (Figure 2A).
322 Placental efficiency, defined as the grams of fetus produced per gram of placenta, was significantly
323 improved in the BIF group compared to GF mice (Figure 2B). Analysis of placental compartments
324 showed that lack of maternal gut microbiota significantly hampered growth of the placental labyrinth
325 transport zone (Lz), without compromising the endocrine junctional zone or decidua volumes (Figure
326 2C). It also did not affect placental glycogen storage (Figure 2D) or the volume of the trophoblast
327 (Figure E-F). Analysis of maternal blood spaces revealed that GF and BIF groups had reduced spaces
328 compared to SPF mice, while the volume and the length of fetal capillaries were significantly reduced in
329 the GF compared to SPF (Figure 2F-G). Similarly, surface area for exchange of the Lz was significantly
330 decreased in GF compared to SPF mice (Figure 2H). The barrier between maternal and fetal blood was
331 also determined to be thinner in BIF *versus* GF mice (Figure 2I). Lz apoptosis levels were similar
332 between groups (Figure 2J).
333

334 To define the molecular mechanisms behind the changes in the Lz, we quantified the expression of
335 select genes in micro-dissected Lz. The angiogenic factor *Vegf* was similarly expressed between groups
336 (Figure 3A). However, the expression of signalling pathways involved in cell proliferation and growth,
337 namely the MAPK pathway, was significantly altered by changes in maternal gut microbiota; *Mapk1*
338 was shown to be increased in both GF and BIF, while *Mapk14* (also known as *p38Mapk*) was revealed
339 to be specifically up-regulated in the Lz of BIF mice. In addition, *Dlk1* and *Igf2po*, which are key genes
340 implicated in metabolism and Lz formation, were significantly up-regulated in the BIF group compared
341 to GF mice. The expression of *Akt* did not vary with group (Figure 3A). As informed by western blotting,
342 activation of ERK was reduced in the placental Lz of GF compared to SPF mice, and this effect was
343 reversed by BIF (Figure 3B). p38MAPK protein activity was similar between groups. DLK1 protein level
344 was also lower in GF compared to SPF mice. However, BIF increased DLK1 protein levels when
345 compared to both SPF and GF mice (Figure 3B). Overall, these findings suggest that the maternal gut
346 microbiota, and *B. breve*, regulate the development of the mouse placental Lz via modulation of
347 specific cell growth and metabolic genes/pathways.
348

349 ***Maternal gut microbiota and *B. breve* controls key placental nutrient transporters***

350 To better understand the changes in fetal growth and glycemia between groups, we quantified the
351 expression of selected amino acid, glucose and lipid transporters in the Lz. We found no difference in

352 the expression of system A amino acid transporters (*Slc38a1*, *Slc38a2*, *Slc38a4*) between groups (Figure
353 3C). However, the key glucose transporter *Slc2a1* was up-regulated in the Lz of BIF mice compared to
354 GF mice (*Slc2a3* mRNA levels were similar between groups; Figure 3C). Fatty acid transporters were
355 also altered, with increased levels of *Fatp1* in the GF group compared to SPF and BIF, while *Fatp4* was
356 increased in the BIF group compared to the GF (Figure 3D; *Cd36* and *Fatp3,6* expression levels were
357 unaltered). Collectively, these data suggest that maternal gut microbiota, and *B. breve*, could regulate
358 fetal growth by inducing changes in the expression of key nutrient transporters within the placenta.
359

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

361 To gain further mechanistic understanding of the changes observed in the placental Lz and fetal liver,
362 we analysed >80 metabolites at GD16.5 (Figure 4 and Table S2). We found 5 metabolites significantly
363 altered in the placental Lz (Figure 4). 2-Aminoadipate in the Lz was very low in GF/BIF groups as well as
364 in fetal livers (Figure 4A). Treatment with *B. breve* significantly reduced the concentrations of
365 acetylcarnitine and carnitine in Lz tissue compared to SPF placentas, but not in fetal livers (Figure 4B-
366 C). Levels of formate in placental Lz were significantly elevated in both GF and BIF compared to SPF
367 mice (Figure 4D), with a similar trend (although not significant) in fetal liver samples. Acetate was also
368 altered in the Lz (Figure 4E), with concentrations significantly lower in the SPF compared to the GF
369 group, whilst BIF samples showed intermediate levels (although these levels were much lower than
370 observed in the maternal caecum). Similar to formate, concentrations of acetate in fetal liver followed
371 similar directions to the Lz, yet were not statistically different between groups. These data suggest that
372 maternal gut microbiota, and *B. breve*, regulate the fetal and placental growth via modulation of the
373 placental Lz metabolome.
374

375 **Discussion**

376 In this study, we demonstrate that the maternal gut microbiota and the microbiota member *B. breve*
377 regulate feto-placental growth. To the best of our knowledge, this is the first demonstration of a
378 maternal gut bacterium remotely controlling placental structure and nutrient transporters, with
379 important implications for fetal glycaemia and fetal growth. We observed that the effects of
380 *Bifidobacterium* are partially mediated by altered metabolites in maternal caecum and in placental Lz
381 tissue, with alterations in the expression of key genes in the placental Lz and fetal liver.
382

383 *Bifidobacterium* is the dominant microbiota member in vaginally delivered, breast-fed infants, with
384 certain species and strains known to stimulate and aid the maturation of the immune system [41]. *B. breve*
385 UCC2003 also regulates responses at the gut barrier, inducing homeostatic epithelial cell
386 programming, and protecting against inflammatory insults [28, 42]. Importantly, pregnancy is
387 accompanied by increasing *Bifidobacterium* abundance in the gut of women and mice [5] and
388 alterations in the abundance of *Bifidobacterium* are linked to the development of serious pregnancy
389 complications like preeclampsia [43]. Recently, it has been demonstrated that the maternal gut
390 microbiota regulate embryonic organ growth by promoting fetal neurodevelopment [44]. Our study
391 shows that maternal gut microbiota induces changes in fetal organogenesis and that *B. breve*
392 supplementation restored fetal glycaemia and liver weight. In this regard, fetal brain weight was
393 unaltered in the three experimental groups, whilst liver mass was drastically reduced only in the GF
394 group. Together, these results suggest that untreated GF fetuses prioritize the growth of the brain at
395 expense of the liver. This fetal strategy, known as the ‘brain sparing effect’, is a protective mechanism

396 to preserve oxygenation and nutrient delivery to the brain in situations of placental insufficiency [45–
397 48]. Our RNAseq analysis shows an upregulation of genes involved in oxygen transport and
398 haemoglobin binding, and downregulation of metabolic pathways like steroid hydroxylase activity,
399 carboxylic acid binding or fatty acid metabolism in the BIF group. These data therefore suggest that
400 fetal defenses against growth retardation were better in the BIF group compared to the GF group, and
401 that the downregulation of the metabolic pathways could be due to the fact that BIF fetuses already
402 achieved their hepatocyte maturation or their maximum liver growth potential earlier than the GF
403 group. In fact, *B. breve* supplementation restored fetal glycaemia and weight, achieving similar values
404 to that seen for SPF fetuses.

405
406 Previous *in vivo* studies show different strains of *Bifidobacterium* (including *B. breve*) modulate glucose
407 handling [49], with this genus consistently associated with potential protection against human
408 metabolic disorders e.g. type 2 diabetes [50, 51]. Our observations of reduced maternal gonadal fat
409 mass and maternal liver weight in *B. breve*-treated dams compared to SPF dams, suggest that
410 *Bifidobacterium*, or *B. breve* metabolites, could affect responses of key organs in the mother, and
411 subsequently impact fetal resource allocation. *B. breve* UCC2003 appeared to induce changes in the
412 metabolite milieu, including carnitine and acetate in the maternal caecum and/or placenta, which
413 could be determinant for the effects observed on fetal growth. Carnitine is well-known for mediating
414 the transport of fatty acids into mitochondrial matrix for fatty acid β-oxidation and BIF placentas had
415 lower concentrations of acetylcarnitine and carnitine compared to SPF. These results suggest a
416 potential greater reliance on these compounds for energy production, or enhanced transfer of these
417 fatty acids to the fetus [52]. On the other hand, acetate is a major bifidobacterial fermentation by-
418 product, which directly mediates glucose homeostasis through the free fatty acid receptor 2 [53] and
419 epithelial cell responses. Previous work in adult mice suggests that elevation of gut acetate levels due
420 to *Bifidobacterium* treatment plays a key role in regulating glucose handling systemically and reduces
421 visceral fat accumulation [54]. Acetate also exerts systemic metabolic [55, 56] and immunological
422 effects [57]. More generally, microbial-derived short-chain fatty acids (SCFAs) modulate multiple host
423 physiological systems and during pregnancy are associated with maternal gestational weight, neonatal
424 length and body weight, and protection against allergic airway disease in the developing fetus [58, 59].
425 Acetate crosses the placenta [59], so in our model, the elevated maternal *B. breve*-derived acetate may
426 exert effects on feto-placental growth in three potential ways. Firstly, higher maternal caecum acetate
427 concentrations in SPF and *B. breve* supplemented dams vs. GF dams could indicate maternal effects,
428 through interactions within the maternal gut mucosa and subsequent impact on maternal organs (liver,
429 adipose and spleen). Secondly, effects on the placenta, through the potential use of acetate for cellular
430 metabolism, growth and function. Finally, effects on fetal metabolism following transport of acetate
431 across the placenta to the fetus. Compared to the maternal caecum, levels of acetate were relatively
432 low in the placental Lz and fetal liver (for all 3 groups). This suggests that *B. breve* (and SPF microbiota-
433 derived acetate) may be used to support anabolic processes *in utero* (hence the very low levels
434 detected). Moreover, the observed modulation of immune-associated pathways in the fetal liver,
435 including those associated with G protein-coupled receptor signalling (e.g. *Dusp9*), also indicates a role
436 for direct acetate-associated responses [60]. Further work is required to fully understand the
437 mechanisms behind the differences observed in maternal organs between the SPF and BIF groups
438 (liver, adipose and spleen) and how these changes impact on materno-fetal resource allocation. Thus,
439 future work should assess the ontogeny of these changes and incorporate an additional pregnant SPF

440 group treated with *B. breve* to fully understand the chemical, endocrine and metabolic interactions
441 occurring between *B. breve*, maternal organs (gut, liver, adipose and spleen) and fetal metabolism.
442

443 Administration of *B. breve* significantly reduced the interhaemal membrane barrier thickness of the
444 placenta (compared to GF group), which may facilitate exchange of nutrients and gases. Previous work
445 has shown that the barrier thickness is regulated by *Igf2P0*, as *Igf2P0*/knockout mice have increased
446 thickness of the exchange barrier and reduced passive permeability of the placentas [61]. In our case,
447 *Igf2P0* was significantly elevated in the BIF group compared to the SPF and GF groups and although *in*
448 *vivo* functional assays evaluating the passive and active transport of solutes are required to verify the
449 implications of this effect on fetal nutrient allocation (e.g. performing unidirectional maternal-fetal
450 transfer assays using ⁵¹Cr-EDTA or glucose and amino acid analogues, ³H-MeG or ¹⁴C-MeAIB [23, 61,
451 62]), this result explains, in part, the improvement in fetal weight observed in the BIF group. Moreover,
452 IGFs have been implicated in the regulation of glucose transporters in a variety of organs by utilizing
453 signalling pathways like PI3K/AKT and MAPK [63], and among the different nutrient transporters
454 evaluated, *Slc2a1* mRNA levels were significantly elevated in the BIF group compared to GF group. The
455 other two transporters that were altered, *Fatp1* and *Fatp4*, changed in opposite directions suggesting
456 that *B. breve* could modulate the expression of these two transporters in different ways depending on
457 the direction and magnitude of fatty acid flux at the placental Lz [64, 65]. The divergent expression of
458 *Fatp1* and *Fatp4* in the BIF compared to GF group may also be linked to intracellular carnitine
459 utilization, as *Fatp1* can interact with carnitine palmitoyltransferase 1 to promote fatty acid transport
460 into mitochondria [66].
461

462 Maternal gut microbiota affected placental structure and its vascular bed, which is required for
463 adequate fetal growth [23]. The mechanisms governing these structural changes could be partially
464 mediated by changes in the expression *Igf2P0* and *Dlk1* (two important imprinted genes in placental
465 physiology [67]), and via MAPK upregulation. In addition to the changes above described for barrier
466 thickness, deletion of *Igf2P0* results in feto-placental growth restriction in association with reduced
467 placental surface area for exchange and fetal capillary volume (reviewed by [68]); parameters that
468 were significantly affected in the SPF vs GF groups, and partially restored by *B. breve* administration.
469 *Dlk1*, a non-canonical ligand of the Notch signalling pathway localized to the endothelial cells of fetal
470 capillaries in the placental Lz, regulates placental vascularisation and branching morphogenesis [69]
471 and both, IGF2 and DLK1, can mediate cellular actions via the MAPK pathway [70–72]. We observed no
472 differences in the mRNA levels of *Dlk1* between SPF and GF mice. However, at the protein level, DLK1
473 in the Lz was controlled by the maternal gut microbiota and more specifically by *B. breve*. Similarly,
474 *Mapk1* levels were increased in both GF and BIF groups compared to the SPF, but at the protein level,
475 ERK activity was lower in the GF but not BIF group when compared to the SPF group, suggesting once
476 again that *B. breve* is involved in the regulation of DLK-MAPK signaling. Another important signaling
477 pathway for embryogenesis and placental Lz angiogenesis and vascular remodeling is p38MAPK
478 (encoded by the gene *Mapk14*) [73]. This pathway has been linked to environmental stresses and
479 inflammatory cytokines [74]. However, p38MAPK also regulates many normal cellular processes,
480 including proliferation and cytoskeletal organisation. We observed that exposure to *B. breve* increases
481 the mRNA abundance of *Mapk14* and carnitine, a metabolites that was found to be altered in the Lz of
482 the BIF group compared to the SPF group, can also promote p38MAPK signalling activation in cardiac
483 tissue [75]. Taken together, our findings reveal that (1) maternal gut microbiota promotes fetal and

484 placental growth in mice and (2) *B. breve* UCC2003 treatment may link to the altered
485 metabolites/nutrient milieu in the mother, affecting placental nutrient transporter abundance and
486 placental barrier thickness for exchange, with effects on fetal growth and development (when
487 compared to GF).

488

489 **Limitations of the study**

490 While our study has clear strengths and strong translational implications for pregnancy complication
491 treatments, it has limitations that are important to consider as they impact on the conclusions drawn.
492 First, our study only addresses the effects of *B. breve* UCC2003 in a completely clean and naïve
493 microbiome system (GF model). This is not representative of the human gut environment, and
494 therefore future experiments could include the addition of a SPF group treated with *B. breve* UCC2003
495 and also a similar group treated with another probiotic species (e.g. *Lactobacillus acidophilus*), or a
496 combination of species. This would help to define *Bifidobacterium*-specific effects (driven by key
497 metabolites), including their efficacy, safety and potential use of probiotics during pregnancy.
498 Moreover, it could be argued that the SPF group interferes in the interpretations of the *B. breve*
499 effects. However, there is lack of fundamental knowledge on what is considered “normal or abnormal”
500 in the GF system, as very little research has been done in understanding the role of the maternal gut
501 microbiota on placental development (SPF vs GF). Therefore, the addition of the SPF group is required
502 to define a baseline for adequate feto-placental growth, and it would also be important to understand
503 how reconstitution of GF mice with SPF microbiota also modulates these responses. As previously
504 mentioned, future work should evaluate the response of pregnant SPF mice to *B. breve* UCC2003
505 supplementation (using microbial profiling [e.g. shotgun metagenomics] to follow microbiota changes),
506 as well as the efficiency of *B. breve* UCC2003 using other types of mouse models such as antibiotic-
507 treated mice. These animal models may also reduce issues with the immune naïve physiology of the GF
508 system [8]. Unsurprisingly, we did not see a full ‘rescue’ of placental phenotype in the monocolonised
509 GF *B. breve* (BIF) group, compared to the complex microbiota found in SPF dams. However, structural
510 and functional adaptations of these placentas exposed to *B. breve* were adequate enough to ‘rescue’
511 fetal weight and fetal glycaemia. An array of gut-associated signaling and a diverse metabolite pool are
512 expected to provide more complete placental development. Indeed, other or additional
513 *Bifidobacterium* species and/or strains may be required for placental and fetal development, given
514 strain-specific host physiology responses [42, 76]. Further studies should allow the relative
515 contributions of other microbial- and *Bifidobacterium*-derived factors to be elucidated. Moreover,
516 ideally, future work should analyze fetal and placental growth each day of the supplementation period
517 and use larger cohorts of pregnant mice. It would also be valuable to assess the impact of *B. breve*
518 supplementation from prior to, and/or during the whole pregnancy.

519

520 Exploring three different compartments (i.e. mother, placenta and fetus) with respect to metabolites
521 and elucidating their role is a complex process, and makes interpretations and drawing definitive
522 conclusions difficult. Further studies using e.g. ^{13}C labeled *Bifidobacterium* or specific metabolites for
523 tracking experiments may allow more nuanced interactions to be uncovered in future work.
524 Nonetheless, this study has revealed novel roles for the gut microbiota and specifically *Bifidobacterium*
525 and provides the bases for future therapeutic strategies for treating pregnancy complications. These
526 data suggest an opportunity for *in utero* programming through maternal *Bifidobacterium* and
527 associated metabolites. Overall, although our study was performed in mice and is not representative of

528 a clinical scenario, our study highlights the importance of the maternal gut microbiota during gestation
529 and demonstrates that *B. breve* modulates maternal physiology, placental structure and nutrient
530 transporter capacity with impact on fetal glycaemia and fetal growth (Figure 5). Our findings prompt an
531 in-depth investigation into how additional members of the maternal gut microbiota impact on
532 pregnancy outcomes. These future studies are important for the design of novel therapies to combat
533 fetal growth restriction and other pregnancy complications.

534

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724 the Immune System. *Front Microbiol* 8:2345. <https://doi.org/10.3389/fmicb.2017.02345>

725
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735
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737

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739 T, ZS, RK, MJD contributed analytic tools and performed analysis; DvS contributed reagents; JL-T, ZS,
740 ANS-P, LJH wrote the paper with feedback from all the authors.

741

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749

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753

754 **Data availability**

755 The fetal liver RNA-Seq raw sequencing data are deposited at the National Center for Biotechnology
756 Information (NCBI) under BioProject PRJNA748000. Relevant data are within the manuscript, individual
757 figures and its Supporting Information files. Additionally, data is available from the corresponding
758 authors on reasonable request. Scripts for differential gene expression analysis can be accessed at
759 GitHub, <https://github.com/raymondkiu/Maternal-foetal-microbiota-paper/>

760

761 **Figure legends**

762 **Figure 1. Effects of maternal gut microbiome and *B. breve* supplementation during pregnancy on**
763 **fetal viability, growth and hepatic transcriptome.** (A) Experimental design. (B) Number of viable
764 fetuses per litter (One-way ANOVA with Tukey's multiple comparison). (C) Fetal weight. (D) Circulating
765 glucose concentrations in fetal blood. (E) Fetal organ weights. (F-G) RNA-Seq analysis of fetal liver
766 samples obtained at GD18.5. (F) PCA plot and (G) volcano plots showing up and down-regulated
767 differentially expressed genes (DEGs) in BIF group (compared to GF group). (H) Heat map of the 20
768 most up and down-regulated DEGs (BIF group). (I-J) Functional profiling (g:Profiler) on 602 DEGs. Key
769 enriched GO terms and REACTOME pathways are shown in the figure. Fetal data were obtained on
770 GD16.5 from: SPF (49 fetuses/6 dams), GF (33 fetuses/5 dams), BIF (34 fetuses/6 dams). Dots represent
771 raw data for each variable assessed (individual values). However, the statistical analysis and the
772 mean±SEM reported in the graphs were obtained with a general linear mixed model taking into
773 account viable litter size as a covariate and taking each fetus as a repeated measure followed by Tukey
774 multiple comparisons test (further explanations can be found in the Materials and Methods-statistical
775 analysis section). Identification of outliers was performed with ROUT Method. RNA-seq was performed
776 on fetal livers obtained at GD18.5 from a total of 3 GF and 4 BIF pregnant dams/litters. RNA-Seq data
777 analysis is described in the material and methods section. (NS, not significant; *P<0.05; **P<0.01;
778 ***P<0.001).

779 **Figure 2. Effects of maternal gut microbiome and *B. breve* supplementation during pregnancy on**
780 **placental structure on day 16.5 of gestation. (A)** Placenta weight. **(B)** Placental efficiency determined
781 by dividing fetal by placental mass. Scale bar= 1mm. **(C)** Placental regional analysis. **(D)** Representative
782 staining of placental glycogen with PAS and glycogen abundance. Scale bar= 2.5mm and 250 μ m. **(E)**
783 Representative image of lectin and cytokeratin staining for labyrinth zone structural quantification.
784 Scale bar= 500 μ m and 50 μ m. **(F-I)** Stereological parameters determined in placental labyrinth zone. **(J)**
785 Representative image of TUNEL staining for apoptosis quantification in labyrinth zone. Scale bar=
786 2.5mm and 100 μ m. All data were analyzed by general linear mixed model, taking into account litter
787 size as a covariate and taking each fetus as a repeated measure followed by Tukey multiple
788 comparisons test. ROUT test was used for identification of outlier values. Dots represent raw data
789 (individual values). However, the statistical analysis and the mean \pm SEM reported within the graphs
790 were obtained with the general linear mixed model (further explanations can be found in the Materials
791 and Methods statistical analysis section). Placental weight-efficiency was obtained from: SPF (49
792 fetuses/6 dams), GF (33 fetuses/5 dams), BIF (34 fetuses/6 dams). Laboratorial analysis was performed
793 with: SPF (14-15 placentas from 6 dams), GF (10 placentas from 5 dams) and BIF (9-11 placentas from 6
794 dams). Only placentas collected on day 16.5 of gestation were analysed. One to three placentas per
795 litter were randomly selected and used for assessment. Placentas were analysed blind to the
796 experimental groups. (NS, not significant; *P<0.05; ***P<0.001). Abbreviations: D (decidua), Jz
797 (junctional zone), Lz (labyrinth zone), TB (trophoblasts), FC (fetal capillaries), MBS (maternal blood
798 spaces).

799 **Figure 3. Effects of maternal gut microbiome and *B. breve* supplementation during pregnancy on**
800 **placental gene and protein levels on day 16.5 of gestation. (A)** Gene expression levels in micro-
801 dissected labyrinth zones. **(B)** Immunoblots and protein quantification by Western blot in micro-
802 dissected labyrinth zones. **(C-E)** Gene expression levels in micro-dissected labyrinth zones for amino-
803 acids, glucose and lipid transporters. Western blot data was analysed by one-way ANOVA. qPCR data
804 were analyzed by general linear mixed model, taking into account litter size as a covariate and taking
805 each fetus as a repeated measure followed by Tukey multiple comparisons test. ROUT test was used
806 for identification of outlier values. Dots represent raw data (individual values). However, the statistical
807 analysis and the mean \pm SEM reported within the graphs (for qPCR data) were obtained with the general
808 linear mixed model (further explanations can be found in the Materials and Methods statistical analysis
809 section). Gene expression analysis was performed with: SPF (13 placentas from 6 dams), GF (11
810 placentas from 5 dams) and BIF (14 placentas from 6 dams). Protein quantification was performed
811 with: SPF (4 placentas from 4 dams), GF (5 placentas from 5 dams) and BIF (5 placentas from 5 dams).
812 Only placentas collected on day 16.5 of gestation were analysed. For qPCR, one to three placentas per
813 litter were assessed and selection of the samples was conducted at random. For protein expression
814 analysis, 1 placenta per litter was selected. (NS, not significant; *P<0.05; **P<0.01).

815 **Figure 4. Metabolomic profiling of placental labyrinth zone and fetal liver on day 16.5 of gestation.**
816 Data were analysed by Kruskal-Wallis test followed by multiple comparisons using the Benjamini &
817 Hochberg false discovery rate method and Dunn's test. ROUT test was used for identification of outlier
818 values. Different superscripts indicate significant differences between groups (a versus b, P<0.05). Data
819 presented as mean \pm SEM. Number of litters analysed per group: SPF (8 placentas-livers from 4 dams),
820 GF (8-7 placentas-livers from 4-5 dams), BIF (6-7 placentas-livers from 5 dams). Only tissues collected

821 at GD16.5 were analysed. Selection of the samples was conducted at random. (NS, not significant;
822 *P<0.05; **P<0.01; ***P<0.001).

823 **Figure 5. Summary illustration showing the most relevant results on how the maternal gut**
824 **microbiota and *B. breve* affects mother, placenta and fetus during gestation.** The effects of lacking
825 maternal gut microbiota on maternal, placental and fetal phenotype are shown in red circles (SPF vs GF
826 comparisons). Our results suggest that lacking maternal gut microbiota aside from inducing changes in
827 the maternal digestive tract, pancreas and caecum metabolites, has important implications for the
828 correct growth of the fetus and its placenta. The effects of *B. breve* administration compared to the
829 SPF and GF groups are shown in blue and red arrows, respectively. Overall, *B. breve* induces changes in
830 the maternal compartment that affect the structure, metabolome and function of the placenta in
831 association with alterations in fetal metabolism, growth and hepatic transcriptome. Abbreviations: SPF
832 (Specific-Pathogen-Free mouse); GF (Germ-Free mouse); BIF (Germ-Free mouse treated with *B. breve*
833 UCC2003); Lz (labyrinth zone); MBS (maternal blood spaces); FC (fetal capillaries); SA (surface area for
834 exchange); BT (barrier thickness); DEG (differentially expressed genes)

835 **Supplementary Figure 1. Colonization levels of *B. breve* determined in maternal faecal samples on**
836 **gestational day (GD), 12 and 14.** Analysis performed by two-ways ANOVA (****P<0.0001). Data
837 displayed as mean ± SEM. Number of dams for GF and BIF groups are 5 and 6, respectively. Assessment
838 was performed only on dams sacrificed at GD16.5.

839 **Table 1. Effects of maternal gut microbiome and *B. breve* administration during pregnancy on**
840 **maternal body composition, circulating metabolites and hormones in maternal serum, and**
841 **metabolites in caecum on day 16.5 of gestation.** Body composition and metabolites/hormones in
842 serum were analyzed by one-way ANOVA followed by Tukey multiple comparisons test. Metabolites in
843 maternal caecum were analysed by Kruskal-Wallis test followed by multiple comparisons using the
844 Benjamini & Hochberg false discovery rate method and Dunn's test. ROUT test was used for
845 identification of outlier values. The level of significance was set at P<0.05. NS: not significant. Data
846 presented as mean±SEM. The number of dams used for each group is annotated on the table and only
847 data from dams at day 16.5 of gestation were used.

849 **Supplementary Table 1. List of primers used for placental labyrinth zone qPCR.**

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

853 **Supplementary Table 3. List of differentially expressed genes and pathways detected in the liver**
854 **RNA-Seq on day 18.5 of gestation.**

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Table 1. Body composition and circulating metabolites-hormones

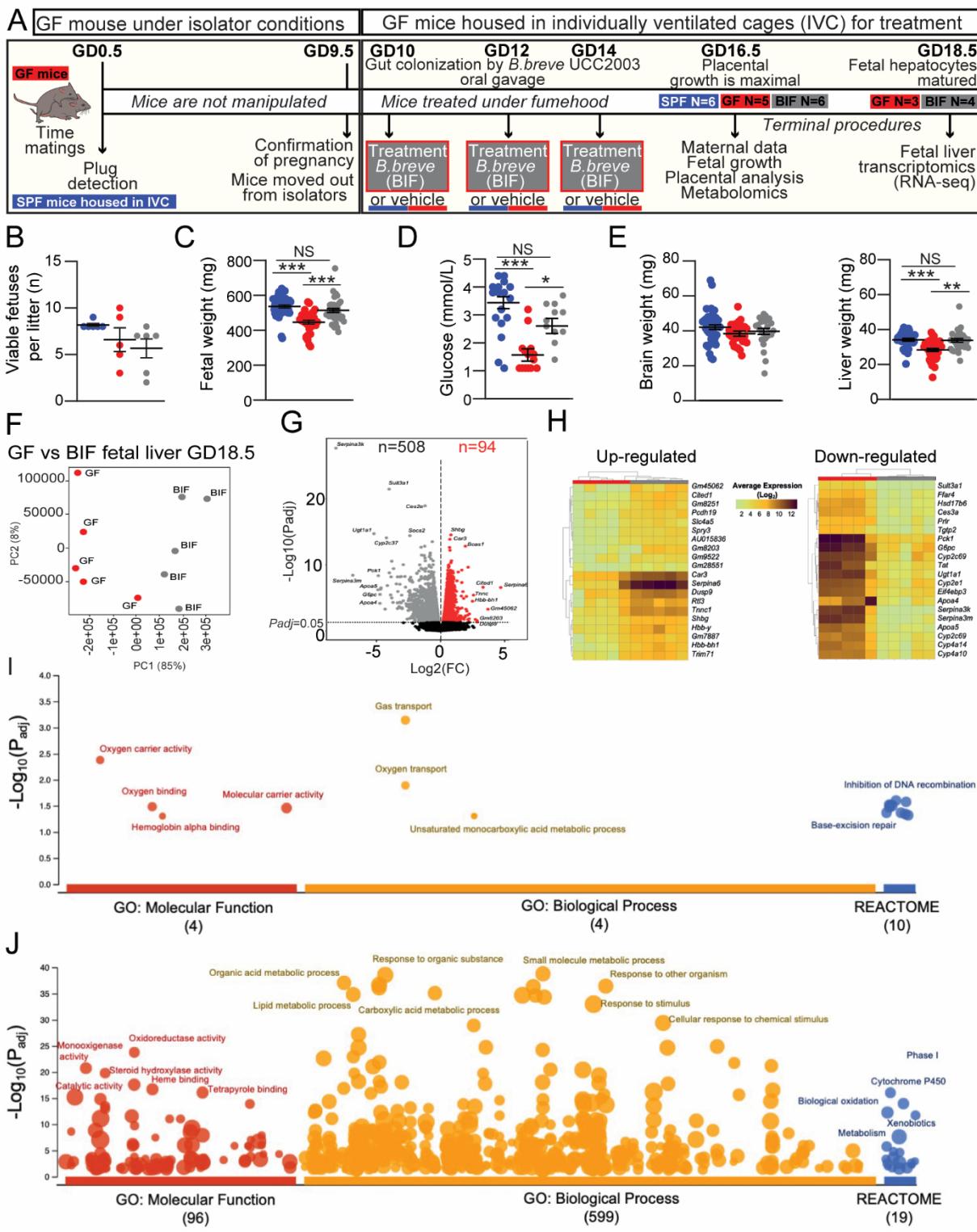
	SPF (n=6)	GF (n=5)	BIF (n=6)	SPF vs GF	SPF vs BIF	GF vs BIF
Hysterectomy weight (g)	26.01±0.91	27.87±0.78	27.17±0.80	NS	NS	NS
Digestive Tract (g)	2.76±0.03	6.83±0.32	7.25±0.63	<0.0001	<0.0001	NS
Caecum (g)	0.66±0.03	3.47±0.25	3.96±0.41	<0.0001	<0.0001	NS
Small intestine (g)	1.66±0.03	2.65±0.11	2.59±0.14	<0.0001	<0.0001	NS
Pancreas (mg)	315.40±30.12	183.40±24.74	190.60±38.71	0.041	0.044	NS
Gonadal fat (mg)	433.10±43.20	297.0±37.02	272.0±27.35	NS	0.016	NS
Liver (g)	2.09±0.10	1.79±0.05	1.55±0.08	NS	0.001	NS
Spleen (mg)	117.90±2.80	91.76±10.60	83.03±6.72	NS	0.0127	NS
Glucose (mmol/L)	8.08±0.78	8.38±1.18	8.88±0.74	NS	NS	NS
Insulin (μg/L)	0.12±0.004	0.19±0.05	0.20±0.06	NS	NS	NS
Leptin (pg/mL)	2465±177.1	2739±486	2425±303	NS	NS	NS
Cholesterol (mmol/L)	1.33±0.03	1.56±0.08	1.41±0.09	NS	NS	NS
Triglycerides (mmol/L)	1.54±0.08	1.79±0.14	1.50±0.11	NS	NS	NS
Free Fatty Acids (μmol/L)	890.6±101.3	1440±362	1092±114.5	NS	NS	NS

Maternal caecum metabolites

	SPF (n=3)	GF (n=4)	BIF (n=5)	SPF vs GF	SPF vs BIF	GF vs BIF
Butyrate (mmol/Kg)	12.47±7.97	0.00±0.00	0.00±0.00	0.006	0.008	NS
Citrulline (mmol/Kg)	0.30±0.06	0.00±0.00	0.00±0.00	0.006	0.008	NS
Fucose (mmol/Kg)	0.08±0.01	0.00±0.00	0.00±0.00	0.006	0.008	NS
Isobutyrate (mmol/Kg)	0.41±0.24	0.00±0.00	0.00±0.00	0.006	0.008	NS
Isovalerate (mmol/Kg)	0.09±0.01	0.00±0.00	0.00±0.00	0.006	0.008	NS
Malonate (mmol/Kg)	0.09±0.02	0.00±0.00	0.00±0.00	0.006	0.008	NS
Methylamine (mmol/Kg)	0.05±0.02	0.00±0.00	0.00±0.00	0.006	0.008	NS
Propionate (mmol/Kg)	4.48±1.99	0.00±0.00	0.00±0.00	0.006	0.008	NS
Trimethylamine (mmol/Kg)	0.06±0.007	0.00±0.00	0.00±0.00	0.006	0.008	NS
Valerate (mmol/Kg)	0.57±0.27	0.00±0.00	0.00±0.00	0.006	0.008	NS
2.methylbutyrate (mmol/Kg)	0.05±0.01	0.00±0.00	0.00±0.00	0.006	0.008	NS
5.Aminopentanoate (mmol/Kg)	0.33±0.14	0.00±0.00	0.00±0.00	0.006	0.008	NS
Acetate (mmol/Kg)	35.49±20.66	0.55±0.06	3.22±1.39	0.006	0.129	0.094

865 Figure 1

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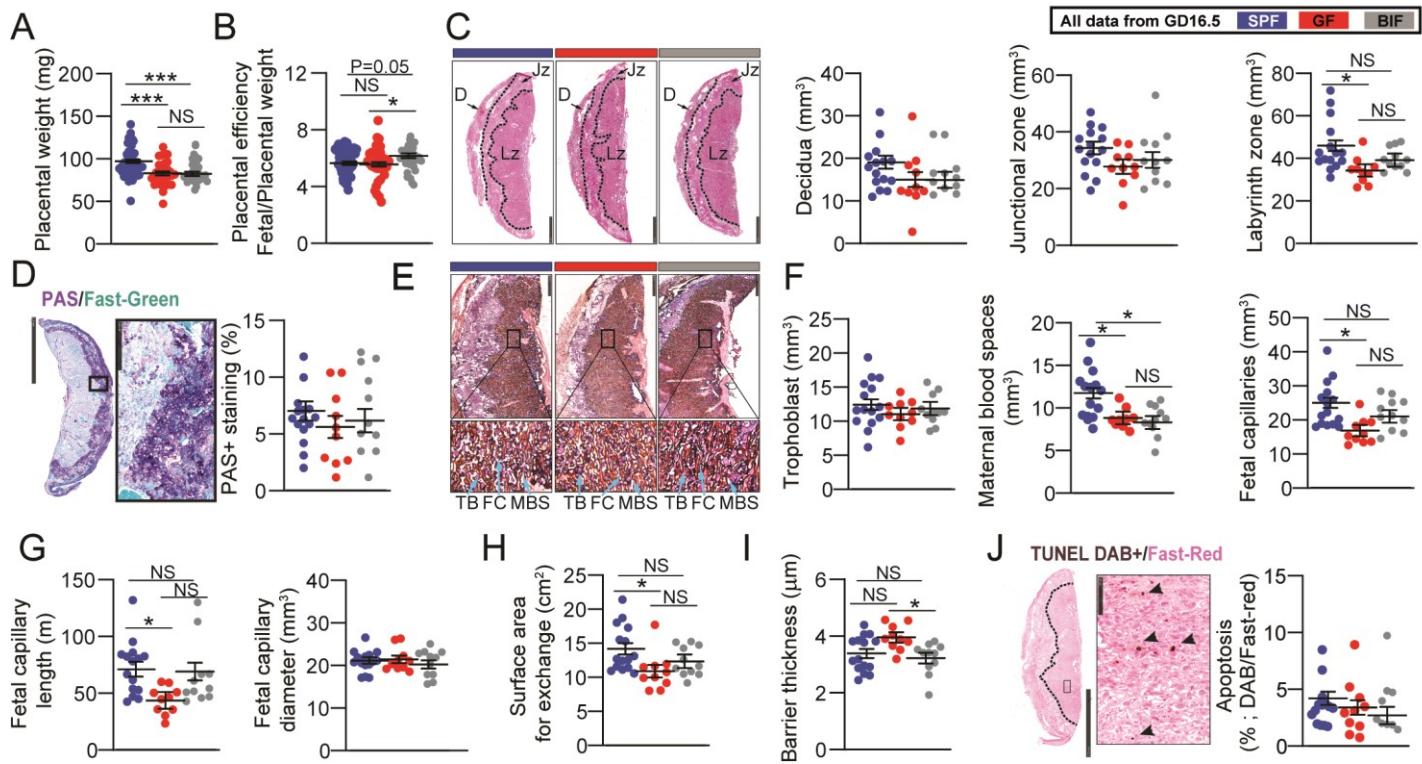
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871 Figure 2

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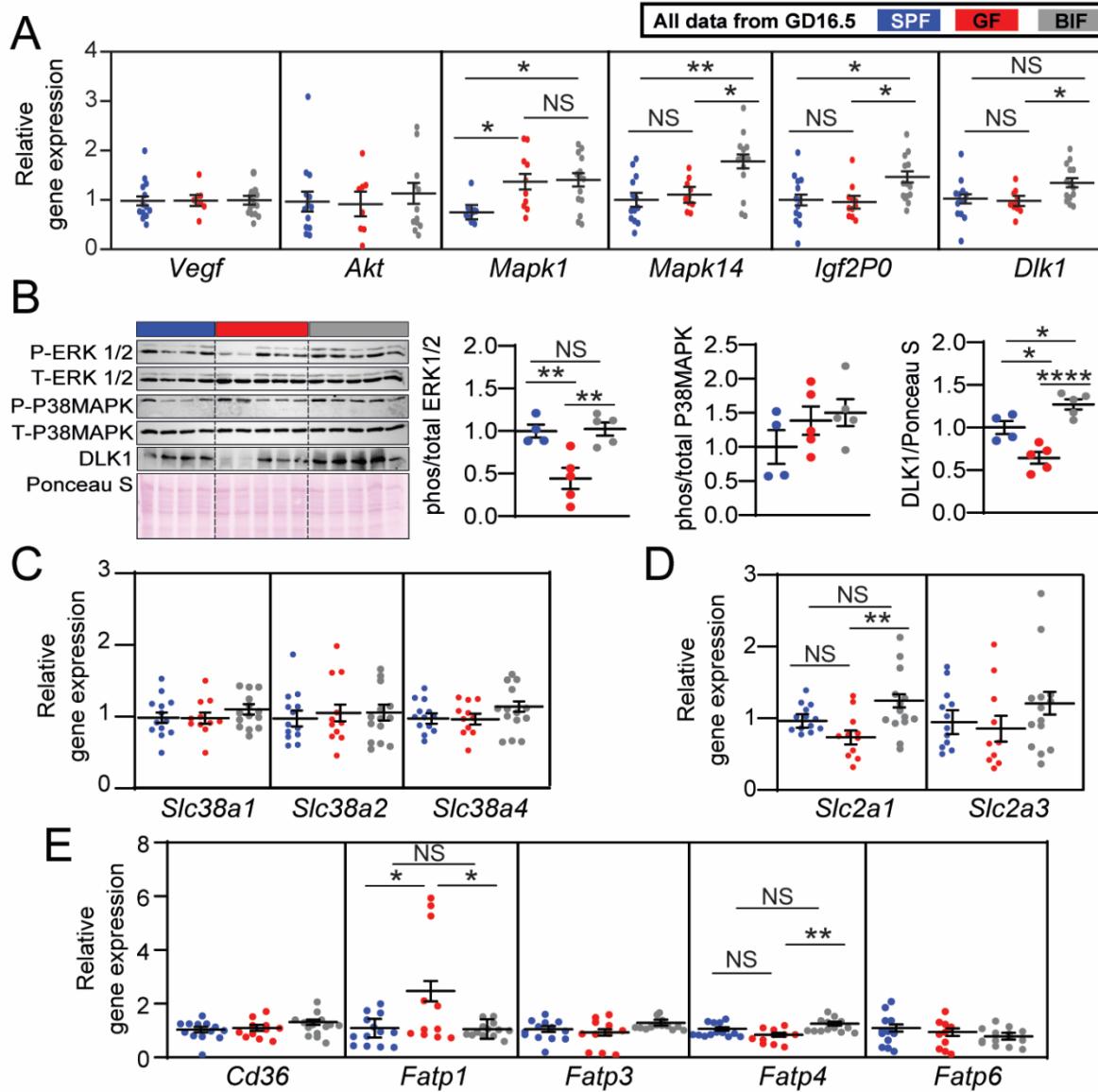
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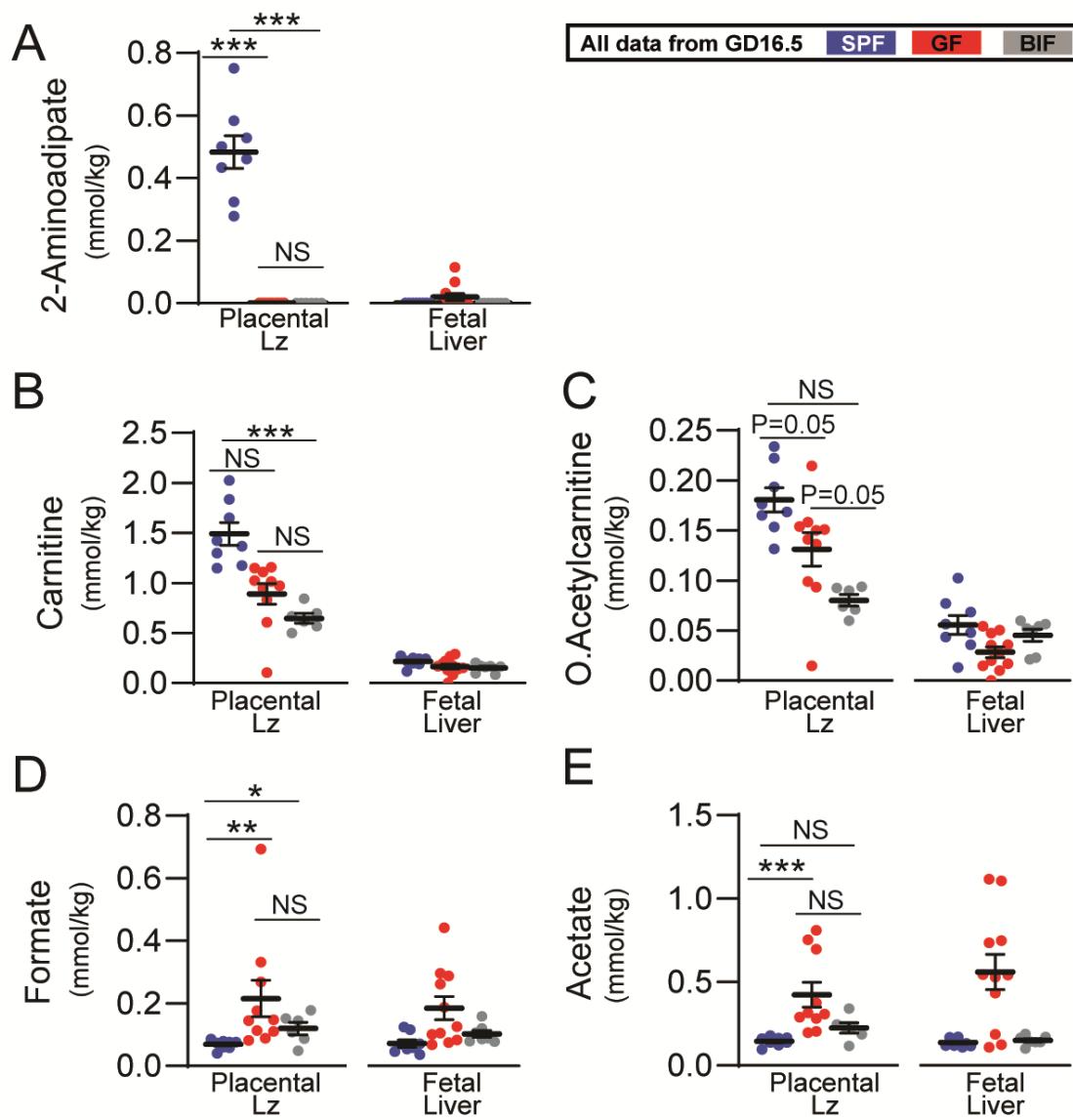
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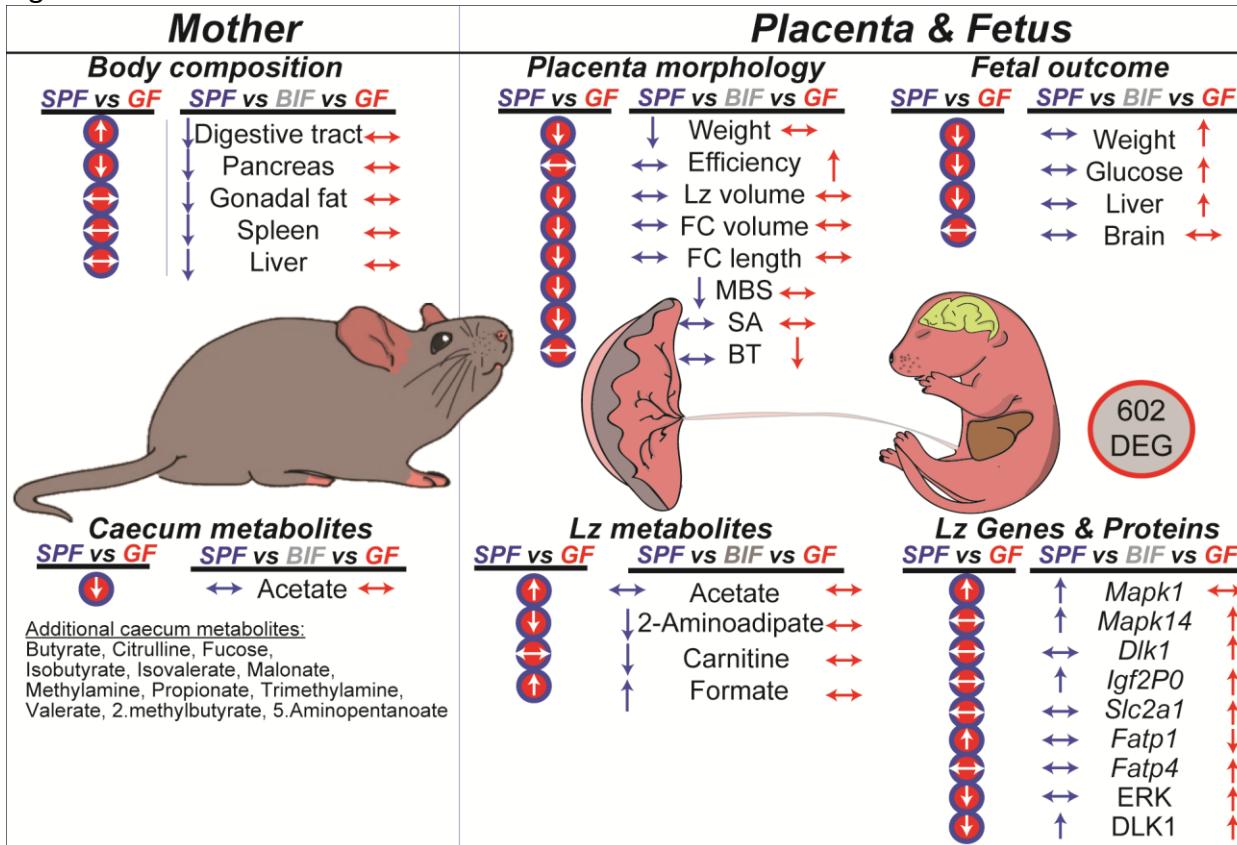
Figure 4



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Figure 5



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Supplementary Table 1	<i>Forward</i>	<i>Reverse</i>
<i>Hprt</i>	<i>CAGGCCAGACTTGTGGAT</i>	<i>TTGCGCTCATCTTAGGCTTT</i>
<i>Ubc</i>	<i>GGAGTCGCCGAGGTCA</i>	<i>AAAGATCTGCATCGTCTCTCAC</i>
<i>Vegf</i>	<i>GAAGCTACTGCCGTCCGATT</i>	<i>CTTCATCGTTACAGCAGCC</i>
<i>Akt</i>	<i>GCCGCCTGATCAAGTTCTCC</i>	<i>TTCAGATGATCCATGCAGGGG</i>
<i>Mapk1</i>	<i>TGCTTCTCTCCGCACAAA</i>	<i>GGCCAGAGCCTGTTCAACTT</i>
<i>Mapk14</i>	<i>AGCTGTCGAGACCGTTTCAG</i>	<i>GATGGGTCAACCAGGTACACG</i>
<i>Dlk1</i>	<i>GAAAGGACTGCCAGCACAAG</i>	<i>CACAGAAGTTGCCTGAGAACG</i>
<i>Igf2P0</i>	<i>GAGGAAGCTCTGCTGTTGG</i>	<i>CAAAGAGATGAGAACGACCAAC</i>
<i>Slc38a1</i>	<i>CGGCGCCTTCCCTTATTTC</i>	<i>CCGTTAACTCGAGGCCACTT</i>
<i>Slc38a2</i>	<i>TTCTGATTGTGGTGATTGCAAGAA</i>	<i>CAGGATGGGCACAGCATACA</i>
<i>Slc38a4</i>	<i>AAGGTAGAGGCGGGAAAGGG</i>	<i>AGGAACTTCTGACTTCCGGCA</i>
<i>Slc2a1</i>	<i>GCTTATGGGCTTCTCCAAACT</i>	<i>GGTGACACCTCTCCCACATAC</i>
<i>Slc2a3</i>	<i>GA TCGGCTCTTCCAGTTG</i>	<i>CAA TCA TGCCACCAACAGAG</i>
<i>Cd36</i>	<i>ATGGGCTGTGATCGGAAC TG</i>	<i>GTCTTCCAATAAGCATGTCTCC</i>
<i>Fatp1</i>	<i>GGCTCCTGGAGCAGGAACA</i>	<i>ACGGAAGTCCCAGAAACCAA</i>
<i>Fatp3</i>	<i>GAGAACTTGCACCGTATGC</i>	<i>GGCCCCTATATCTTGGTCCA</i>
<i>Fatp4</i>	<i>GATTCTCCCTGTTGCTCCTGT</i>	<i>CCATTGAAGCAAACAGCAGG</i>
<i>Fatp6</i>	<i>AACCAAGTGGTGACATCTGC</i>	<i>TCCATAAAGTAAAGCGGGTCAG</i>

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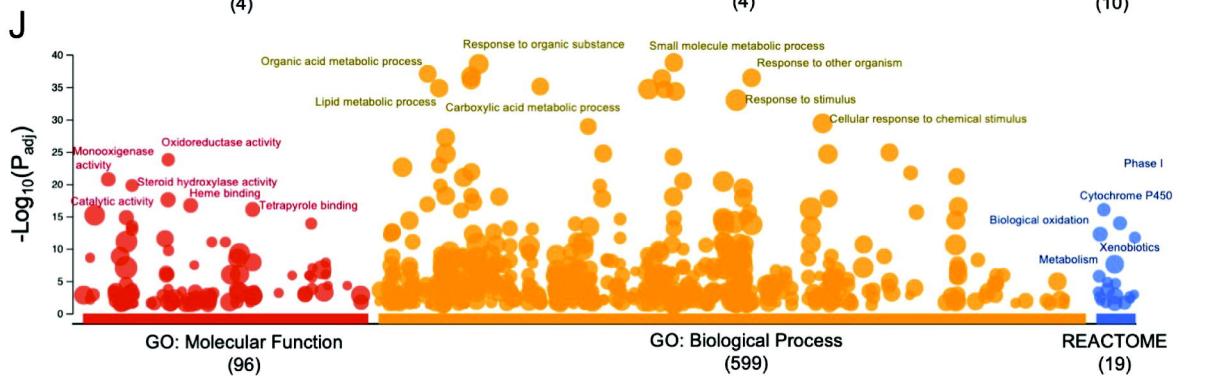
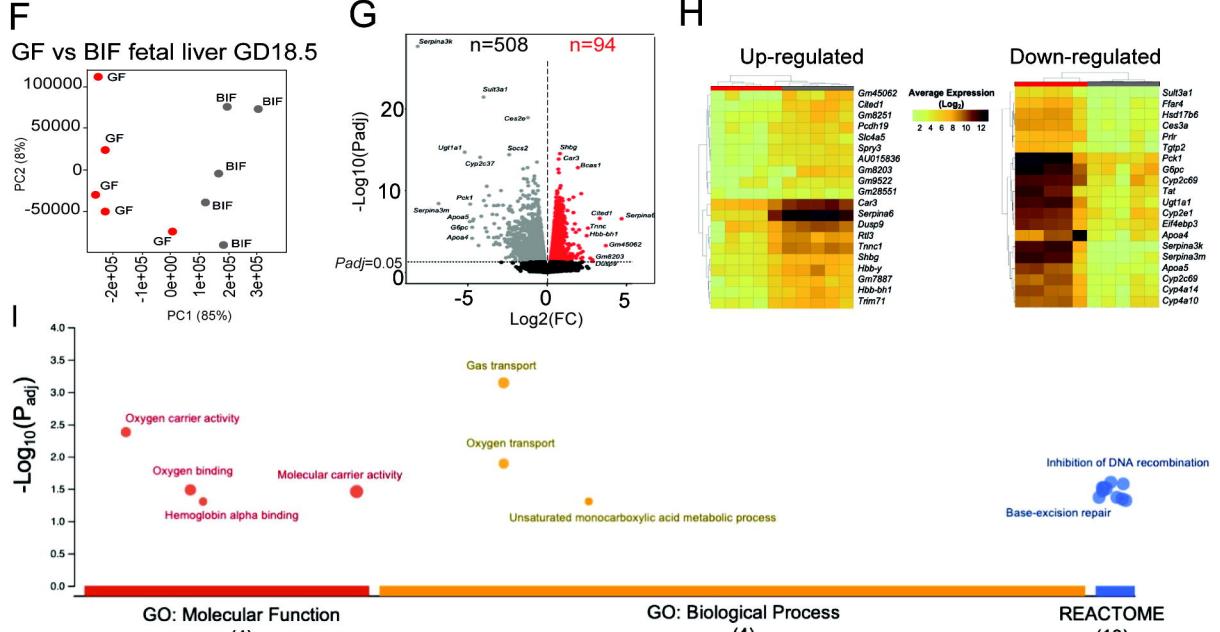
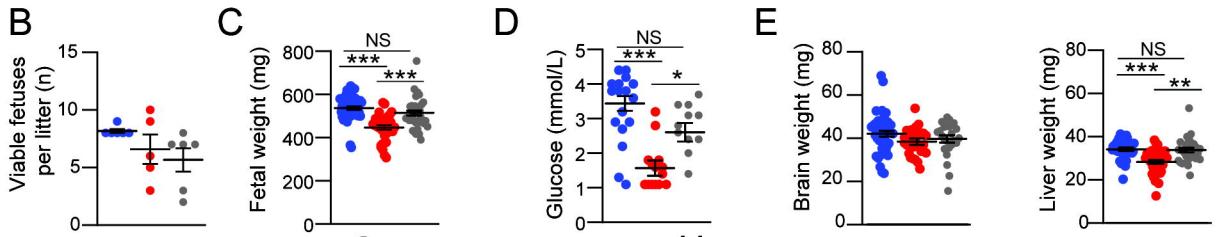
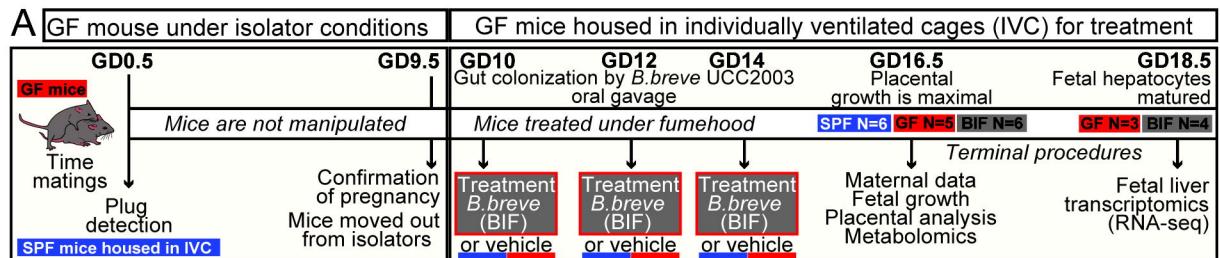
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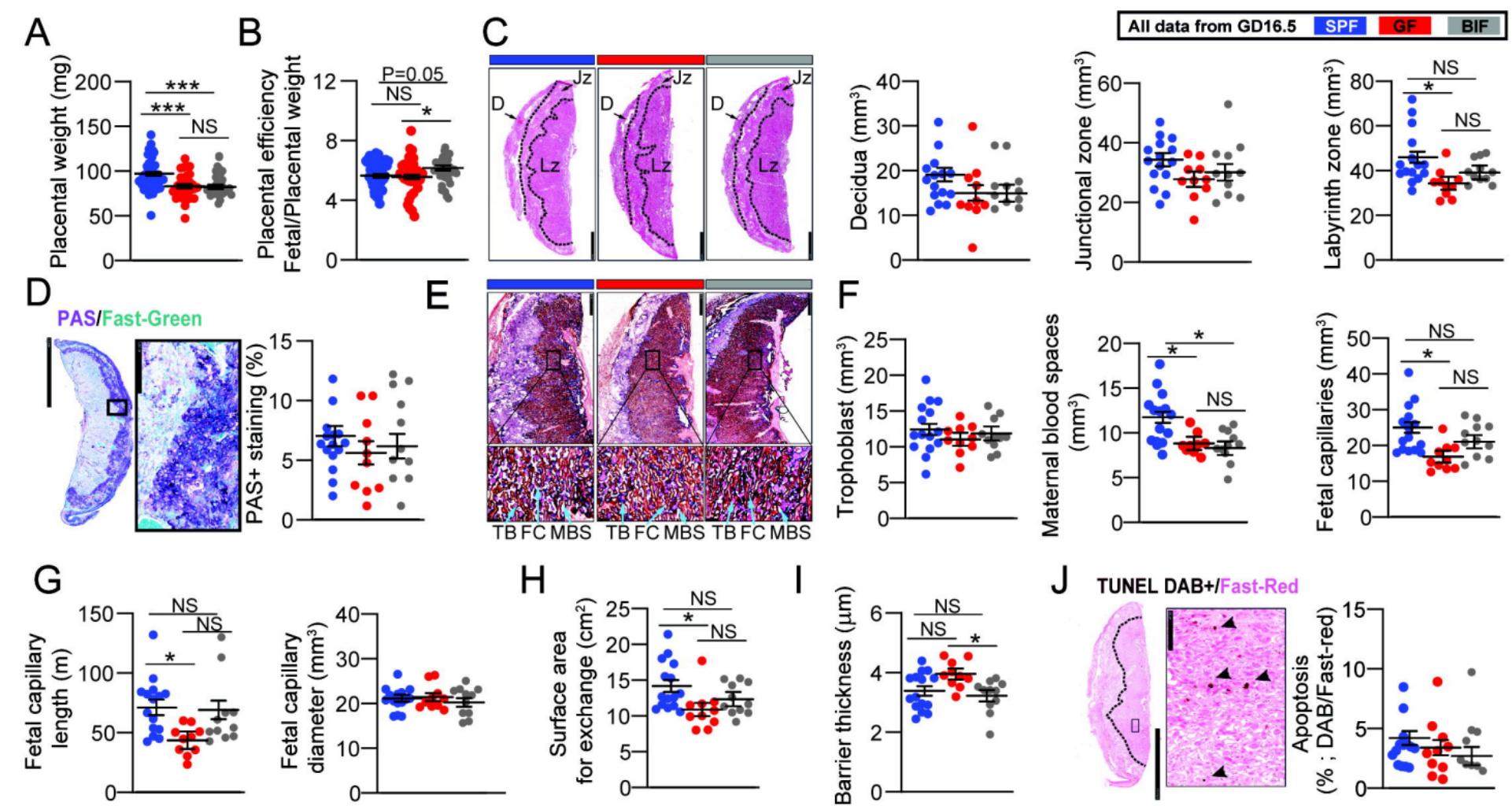
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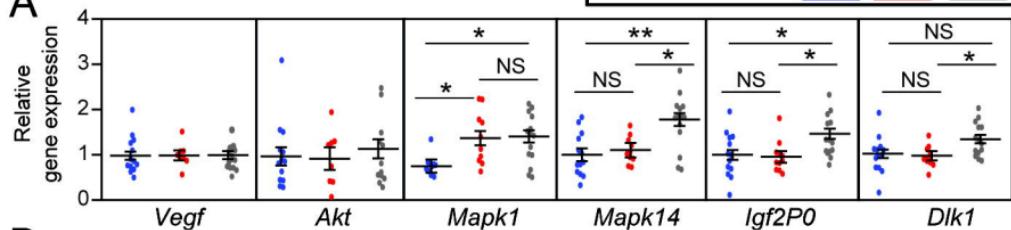
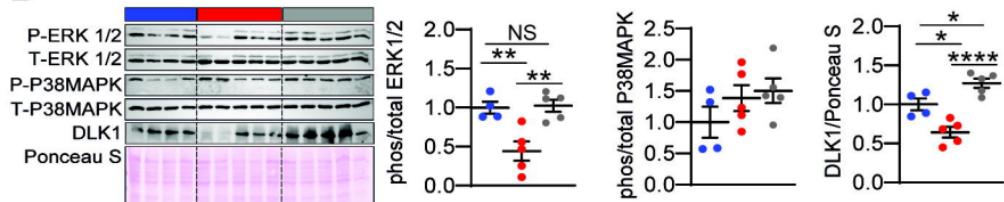
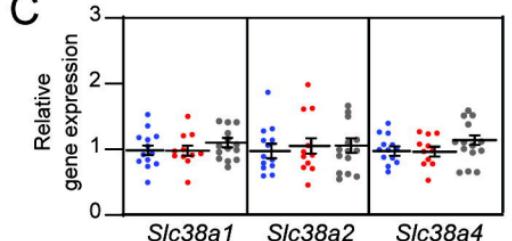
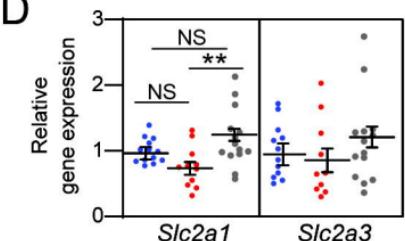
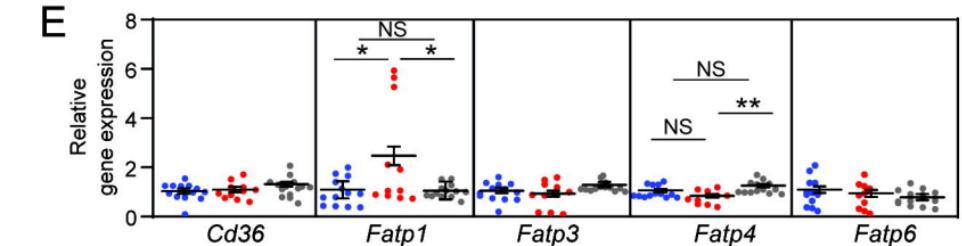
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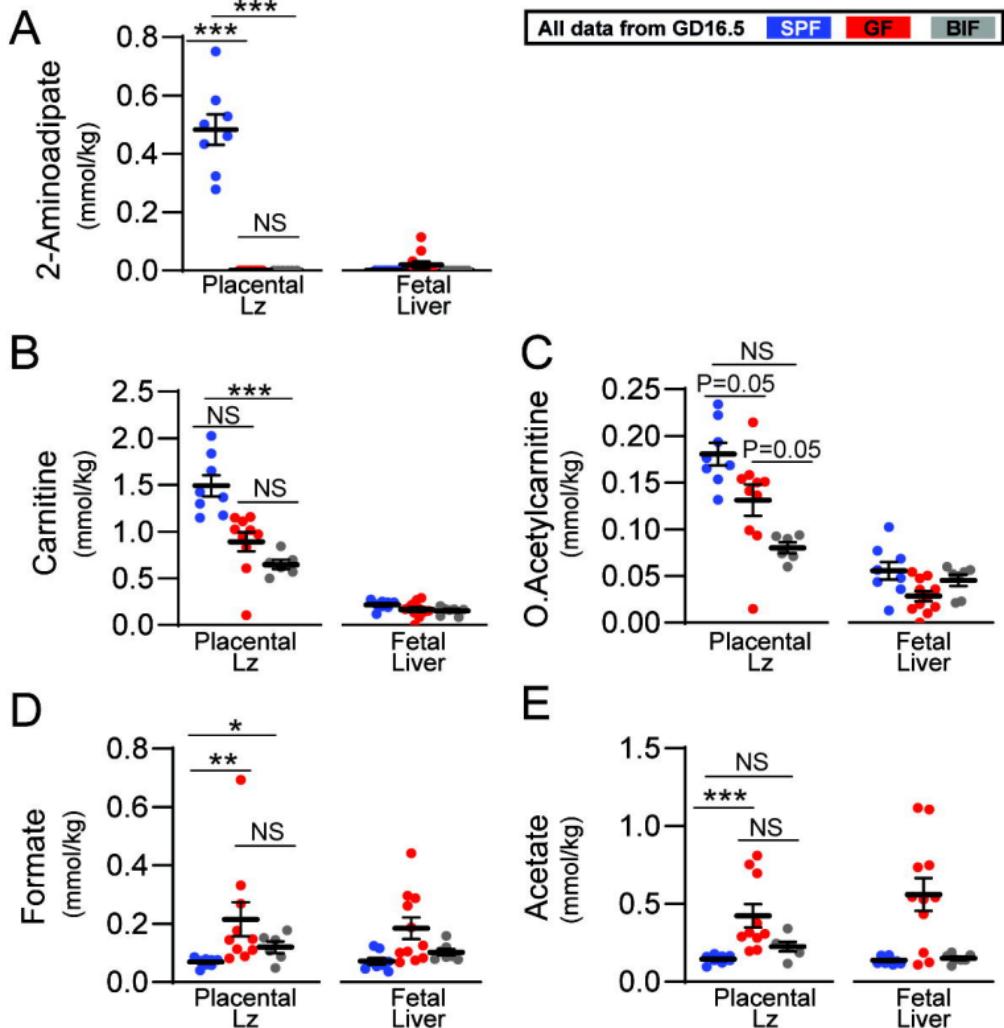
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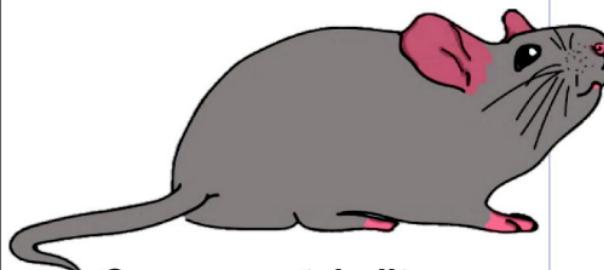


Mother

Body composition

SPF vs GF **SPF vs BIF vs GF**

Digestive tract ↔
 Pancreas ↔
 Gonadal fat ↔
 Spleen ↔
 Liver ↔



Caecum metabolites

SPF vs GF **SPF vs BIF vs GF**

↔ Acetate ↔

Additional caecum metabolites:

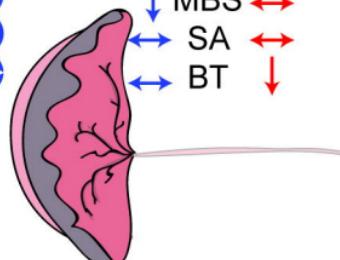
Butyrate, Citrulline, Fucose,
 Isobutyrate, Isovalerate, Malonate,
 Methylamine, Propionate, Trimethylamine,
 Valerate, 2.methylbutyrate, 5.Aminopentanoate

Placenta & Fetus

Placenta morphology

SPF vs GF **SPF vs BIF vs GF**

Weight ↔
 Efficiency ↑
 Lz volume ↔
 FC volume ↔
 FC length ↔
 MBS ↔
 SA ↔
 BT ↓



Fetal outcome

SPF vs GF **SPF vs BIF vs GF**

↔ Weight ↑
 ↔ Glucose ↑
 ↔ Liver ↑
 ↔ Brain ↔

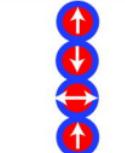


602
DEG

Lz metabolites

SPF vs GF **SPF vs BIF vs GF**

↔ Acetate ↔
 ↓ 2-Aminoadipate ↔
 ↓ Carnitine ↔
 ↓ Formate ↔



Lz Genes & Proteins

SPF vs GF **SPF vs BIF vs GF**

↑ Mapk1 ↔
 ↑ Mapk14 ↔
 ↔ Dlk1 ↑
 ↔ Igf2P0 ↑
 ↔ Slc2a1 ↑
 ↔ Fatp1 ↑
 ↔ Fatp4 ↑
 ↔ ERK ↑
 ↑ DLK1 ↑



