The influence of maternal malnutrition on folate and inositol production and transport in the placenta and gut – a mechanism for fetal growth restriction and fetal disorders?

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Abstract

Maternal malnutrition and micronutrient deficiencies can alter fetal development. However, the mechanisms underlying these relationships are poorly understood. A systems-approach was used to investigate the effects of malnutrition on maternal gut microbes and folate/inositol transport in the maternal/fetal gut and placenta. Female mice were fed a control diet (CON) diet, undernourished (UN, restricted by 30% of CON intake) or a high fat diet (HF, 60% kcals fat) during pregnancy. At gestational day 18.5 we assessed circulating folate levels by microbiological assay, relative abundance of gut lactobacilli by G3PhyloChip™, and folate/inositol transporters in placenta and maternal/fetal gut by qPCR/immunohistochemistry. UN and HF-fed mothers had lower plasma folate concentrations vs. CON. Relative abundance of three lactobacilli taxa were higher in HF vs. UN and CON. HF-fed mothers had higher gut *Pcft* and *Rfc1,* and lower *Smit2,* mRNA expression vs. UN and CON. HF placentae had increased *Fr*β expression vs. UN. mRNA expression of *Pcft, Frα* and *Smit2* was higher in gut of HF fetuses vs. UN and CON. Transporter protein expression was not different between groups. Maternal malnutrition alters abundance of select gut microbes and folate/inositol transporters, which may influence maternal micronutrient status and delivery to the fetus, impacting pregnancy/fetal outcomes.

Keywords: folate, inositol, nutrition, gut, placenta

Introduction

Suboptimal maternal nutrition, characterised by both underweight and obesity, are associated with reduced serum folate concentrations, even if the diet is sufficient in folate.[1–5] Low folate status in turn can increase the risk of adverse pregnancy outcomes including fetal growth restriction and neural tube defects (NTDs).[6–8] Additionally, myo-inositol (inositol), a pseudovitamin of the vitamin B family, is important for fetal development. Inositol by-products are necessary for neurulation and transport to the fetus via the placenta, suggesting they are essential for brain development.[9–11] Although these micronutrients are important for optimal embryonic/fetal development, and changes in maternal micronutrient status during pregnancy can adversely affect developmental trajectories, the extent to which malnutrition impacts pregnancy phenotypes that influence the transfer and production of these micronutrients from mother to fetus is unclear.

Folate is a naturally occurring essential B vitamin that is required for DNA synthesis and as such, is important during periods of rapid tissue growth, such as embryogenesis and fetoplacental development.[12–14] Folic acid is a synthetic form of folate found in dietary supplements.[12] Folate transfer to the fetus is mediated by the placental transporters folate receptor alpha and beta (*Frα*/β), proton coupled folate transporter (*Pcft*) and reduced folate carrier 1 (*Rfc1*).[15, 16] *Frα* is the main folate transporter responsible for maternal-to-fetal transfer across the placenta (in both mice and humans) and works together with *Pcft* and *Rfc1* to control the uptake and release of folate.[17–19] *Pcft* is a high-affinity folate transporter and *Rfc1* mediates folate transport to the fetus via bi-directional transport.[15, 18, 20–22] Furthermore, inositol is transferred to the fetus by placental transporters sodium (Na+) myo-inositol co-transporter 1 and 2 (*Smit1/2*) and tonicity-responsive enhancer binding protein (*TonEBP*).[23, 24] *Smit1* is a high affinity transporter that is highly expressed prenatally in the fetal central nervous system and placenta.[25] Similarly, *TonEBP* is a main signal transcription factor of *Smit1* and the absence of *TonEBP* results in reduced *Smit1* transcription.[24] The SI also acts as a main regulator of folate/inositol uptake and transport via *Pcft, Rfc1, Smit2, TonEBP*. *Pcft* and *Rfc1* are expressed in the duodenum and jejunum, which are the preferred sites of folate absorption, and *Pcft* is responsible for the majority of folate intestinal absorption.[15, 26–28] Moreover, *Smit2* is highly expressed in the intestinal epithelium of the gut, playing a greater role than *Smit1* in mediating gut inositol uptake/transport, opposite to their roles in the placenta.[29] Maternal gut folate and inositol absorption therefore is likely critical to the early supply of these micronutrients to the developing embryo/fetus, and throughout gestation.[15, 27, 28, 30] To our knowledge, the presence of these transporters has not been investigated in the fetal gut. As such, their roles and importance in the fetus remain unclear. Further, it remains unclear how adverse conditions common during pregnancy, including maternal malnutrition, may compromise the uptake and transfer of these nutrients to the fetus.

In addition to dietary intake of nutrients, specific gut microbes play key roles in nutrient metabolism and vitamin production.[31, 32] Lactobacilli and bifidobacteria produce folate and increase production of these vitamins when paired with prebiotic fibres, therefore potentially compensating for the lack of dietary intake of folate in the host.[26, 31, 33–39] The small intestine (SI), particularly the duodenum, jejunum, and colon, are sites for bacterial folate production and absorption.[26, 40] Although there is limited information on bacterial production of inositol, it is absorbed in the SI.[41] Once absorbed from the maternal gut into the blood, these micronutrients are then transferred transplacentally to the fetus.[14, 17, 42, 43]

While maternal malnutrition has been associated with reduced folate status and adverse pregnancy outcomes, the mechanisms through which it leads to poor fetal development are not fully understood.[1–3, 5–7] Most studies have focused on the effects of either UN or HF/obesity separately. Given the importance of both the maternal gut and placenta to micronutrient status and transport in pregnancy, a systems physiology approach, considering the holobiont (the host and their collection of microbes), the placenta, and the fetal gut, is needed to go beyond investigating, in isolation, single elements along this nutritional pipeline in pregnancy that influence micronutrient delivery to the fetus.[44] The present study aimed to investigate how global nutritional challenges that differentially affect pregnancy phenotypes impact circulating folate levels and folate/inositol pathways the placenta and maternal/fetal guts. As a first step, we sought to answer three questions: does maternal malnutrition alter microbes in the maternal gut that could be important for folate production; does malnutrition impact maternal micronutrient levels and folate and inositol transport in the maternal gut and placenta; does malnutrition affect gut folate and inositol pathways in the developing fetus, which may contribute to the programming of gut dysfunction long-term? We hypothesised that maternal undernutrition and high fat diet would decrease circulating folate levels, and result in pregnancy adaptations including increased abundance of lactobacilli in the maternal gut and altered expression of genes and proteins in the maternal gut and placenta related to folate/inositol production and transport. We also hypothesised that the developing fetus would try to adapt to this maternal malnutrition by increasing the expression of folate and inositol pathways in the gut.

Methods

Animal model and diet

All experiments were approved by the Animal Ethics Committee at Mount Sinai Hospital, Toronto, Canada. C57BL/6J female mice (Jackson Laboratories, Bar Harbour, ME, USA) were randomised to three nutritional groups: control (CON), undernourished (UN) and high fat (HF). Female mice were fed a control diet (23.4% saturated fat by weight; Dustless Precision Pellets S0173, BioServe, Frenchtown, NJ, USA) *ad libitum* before and throughout pregnancy (n=7), or mice were moderately undernourished restricting food by 30% of control intake from gestational day (GD) 5.5-17.5 of pregnancy (n=7) or mice were fed a 60% high fat diet (60% kcals as fat, 37.1% saturated fat by weight; D12492, Research Diets, New Brunswick, NJ, USA) from 8 weeks before mating and during pregnancy (n=8).[45] There were differences in macronutrient and micronutrient content between diets (Table 1). Fathers (Jackson Laboratories, Bar Harbour, ME, USA) were fed a control diet *ad libitum* and were mated with females at approximately 10 weeks of age. The UN diet was applied from GD 5.5-17.5 to allow us to model reduced fetal growth, but without embryonic lethality or preterm birth, whilst the high fat diet was applied before and during pregnancy to model increased maternal adiposity without resulting in fetal growth restriction.[45–47]

RNA extraction and expression in placenta and gut

At GD18.5, maternal, placental, and fetal tissues were collected and snap frozen in liquid nitrogen and stored at -80 until analysed. RNA was extracted from whole jejunum homogenates from each mother (CON n=7, UN n=7, HF n=8), from whole placental homogenates from one male and one female placenta from each litter, and from whole fetal gut homogenates from one male and one female fetus from each litter (females: CON n=7, UN n=4, HF n=8 HF; males: CON n=6, UN n=6, HF n=8) using the Qiagen RNeasy Plus Mini Kit (Qiagen, Toronto ON). RNA was quantified by spectrophotometry (DeNovix, Wilmington, DE, USA) and 1 µg of RNA was reverse transcribed by using 5X iScript Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions.

Maternal jejununal SI mRNA expression of *Pcft* and *Rfc1*(folate transporters), *Smit2* and *TonEBP* (inositol transporters), and placental and fetal gut expression of *Frα, Frβ, Pcft, Rfc1, Smit1/2* and *TonEBP* were measured by real time qPCR using SYBR Green I (Bio-Rad, Mississauga ON) and 5 µM of forward and reverse primer mix (Bio-Rad CFX384, Mississauga ON, Canada). Primer sequences for genes of interest and three stably expressed reference genes were designed using Primer BLAST software (NCBI) or from the literature (Supporting Information Table S1). A standard curve was run for each gene. Standards, samples, and controls were run in triplicate under the following cycling conditions: 95°C for 30 seconds, 60°C for 20 seconds, 39 successive cycles at 95°C for 5 seconds and 60°C for 20 seconds, and melt curve at 65°C with 0.5°C increments for 5 seconds/cycle. Target gene expression was normalised to the geometric mean of the following reference genes: Beta-actin (*Actb*), Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and Hypoxanthine Phosphoribosyltransferase 1 (*Hprt1*) (placental samples); *Actb*, Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta (*Ywhaz*) and TATA Box Binding Protein (*TBP*) (maternal SI and fetal gut samples). Data were analysed using the Pfaffl method.[48]

Localisation of FRβ, PCFT and SMIT2 protein

Protein expression and localisation of FRβ in placentae and PCFT and SMIT2 in maternal jejunum were assessed by immunohistochemistry (IHC). Placentae and SI samples were fixed in paraformaldehyde, paraffin embedded, and sections cut at 5-µm. To visualise FRβ protein expression, placental sections from 1 male and 1 female placenta per litter were stained using a rabbit polyclonal anti-FOLR2 antibody (Abcam, ab228643, Cambridge, United Kingdom) at a 1:50 dilution. To visualise PCFT and SMIT2 protein expression, SI sections were stained with rabbit polyclonal anti-HCP1/PCFT antibody (Abcam, ab25134, Cambridge, United Kingdom) and rabbit polyclonal anti-SLC5A11/SMIT2 antibody (LifeSpan BioSciences, LS-C403570-120, Seattle, WA, USA). Slides were deparaffinised in 100% xylene, rehydrated in decreasing concentrations of ethanol, and quenched using 3.0% hydrogen peroxide in methanol at room temperature. Antigen retrieval was performed by microwave treatment of 10mM solution of citric acid and sodium citrate (pH 6.0). Slides were washed with 1X phosphate-buffered saline (PBS) and incubated in a humidified chamber for 1 hour at room temperature with protein-blocking solution (Agilent Dako, Santa Clara, ON, Canada) to block nonspecific sites. Slides were incubated overnight at 4°C with the specific primary antibody. A negative control was included in each run for each primary antibody (primary antibody omitted in replace of antibody diluent). Slides were incubated with 1:200 goat biotinylated anti-rabbit antibody (BA-1000, Vector Labs, Burlingame, CA, USA) in a humidified chamber for 1 hour at room temperature, followed by incubation with streptavidin-horse radish peroxidase (HRP, 1:2000 dilution in 1X PBS, Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. To visualise HRP enzymatic activity, the reaction was initiated by 3,3' diaminobenzidine (DAB; Vectastain Elite ABC HRP Kit, Vector Laboratories, Brockville, ON, Canada) for 3 minutes and 30 seconds. Slides were counterstained with Gill's #1 haematoxylin (Sigma-Aldrich, Oakville, ON, Canada).

To evaluate the localisation and expression of immunoreactive (ir) FRβ, PCFT and SMIT2 stained sections, sections were semi-quantitatively analysed as previously described.[49] Images were taken on the EVOS FL Auto 2 (Thermo Fisher Scientific, Waltham, MA, USA) at 20X magnification by one researcher blinded to the animal IDs and groups. Entire placentae and SI sections were scanned to note staining patterns. Placental images were taken in the decidua, junctional and labyrinth layers and chorionic plate. Across all samples using the automate function (EVOS FL Auto 2, Thermo Fished Scientific, Waltham, MA, USA), six images were randomly captured within each layer of the placenta. Using the same function, six images were randomly captured along the length of the maternal small intestine. Intensity of staining was scored based on the scoring system described previously by a single observer blinded to the experimental groups. [49] Staining intensity was classified on a 5-point scale: 0 = absent, 1 = weak, 2 = moderate, 3 = strong and 4 = very strong.[49] For all sections, average staining intensity across the six images was calculated to determine the mean intensity for each animal. Semi-quantitative staining intensity data are presented as mean intensity staining for the specified protein and diet group calculated from the average staining intensity across all six images.

16S rRNA gene sequencing and analysis

DNA was extracted from maternal caecal contents at GD18.5, as previously described.[45] Diversity and relative abundance of maternal gut microbes were measured using the G3 PhyloChip™.[45]

Circulating folate measures

To determine maternal folate status, circulating folate levels were measured using the *Lactobacillus casei* microbiological assay on maternal plasma samples, as previously described.[50, 51]

Statistical analysis

Analyses were performed using JMP 14 (SAS Institute Inc., Cary, NC, USA). Outcome measures were analysed for normality and equal variances (Levene's test). Data that were normal and had equal variances were analysed by parametric tests. If data were non-normal, a log transformation was applied to normalise. Data that could not be normalised were analysed by non-parametric tests. Differences between dietary groups for outcome measures were compared by ANOVA (Tukey's post hoc), or Welch ANOVA (Games-Howell post hoc), or Kruskal-Wallis (Steel-Dwass post hoc). Relationships between variables were determined by Pearson or Spearman correlation analysis. $P = <0.05$ was deemed statistically significant. Data are presented as means ± standard deviation (SD) or median interquartile range (IQR), with 95% confidence interval diamonds in figures.

Results

Malnutrition is associated with reduced maternal plasma folate levels and fetal weight

Maternal plasma folate levels were lower in both UN and HF fed mothers compared to CON mothers ($p<0.0001$, Figure 1A), with HF fed females having the lowest circulating folate levels. Maternal plasma folate levels were positively associated with fetal weight ($r = 0.712$, $p<0.01$, Figure 1B). The mean daily diet and vitamins consumption can be found in Table 1. Based on diet composition, HF fed females had the lowest folate consumption.

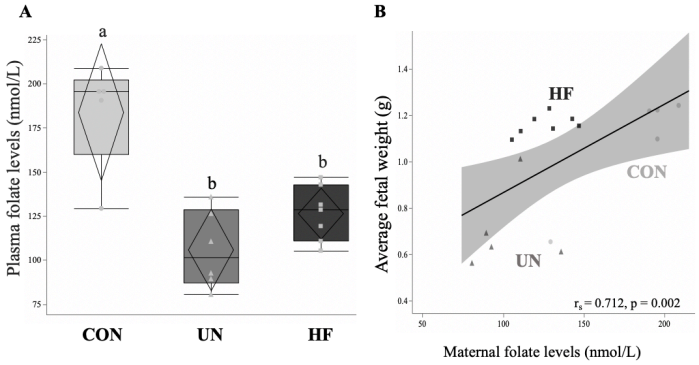

Figure 1. Plasma folate levels in CON, UN and HF mothers and association with fetal weight at GD18.5. Maternal plasma folate levels were lower in UN and HF mothers compared to CON ($p<0.0001$) (A). Data are quantile box plots with 95% confidence diamonds. Groups with different letters are significantly different (Tukey's post hoc). Mean fetal weight was positively associated with maternal plasma folate levels (B). Data are linear regression with 95% confidence intervals ($p<0.01$). rs = Spearman correlation coefficient. (Circle = CON, Triangle = UN, Square = HF).

Table 1. Composition of CON, UN and HF diets.

	CON	**UN**	**HF**
Average amount of diet (g) consumed per day during pregnancy	3.79 ± 0.48	2.97 ± 0.42	3.32 ± 0.58
Average daily B vitamin consumption during pregnancy			
B6 (mg/g)	0.122 ± 0.015	0.096 ± 0.013	0.030 ± 0.0052
B9 (mg/g)	0.043 ± 0.0054	0.034 ± 0.0047	0.009 ± 0.0015
B12 (μg/g)	0.189 ± 0.024	0.148 ± 0.021	0.043 ± 0.0075

a) Data are mean (± standard deviation) amount of diet (g) consumed per day and mean daily B vitamin consumption (mg/g).
b) The form of vitamin B9 in the control diet was folate (at 11.4 mg/kg of diet) while the form in the high fat diet was folic acid (at 2.0 mg/kg of diet).

The maternal gut harbours an array of lactobacilli and is altered by HF diet

To determine the impact of maternal malnutrition on gut lactobacilli we measured the relative abundance of lactobacilli in maternal caecal contents. We first identified 15 maternal gut bacterial taxa (empirical operational taxonomic units (eOTU)) within the lactobacilli genera in pregnant mice at GD18.5 (Figure 2). Of these, HF fed mothers had a higher relative abundance of three specific lactobacilli eOTU compared to UN mothers ($p<0.05$, Figure 3).

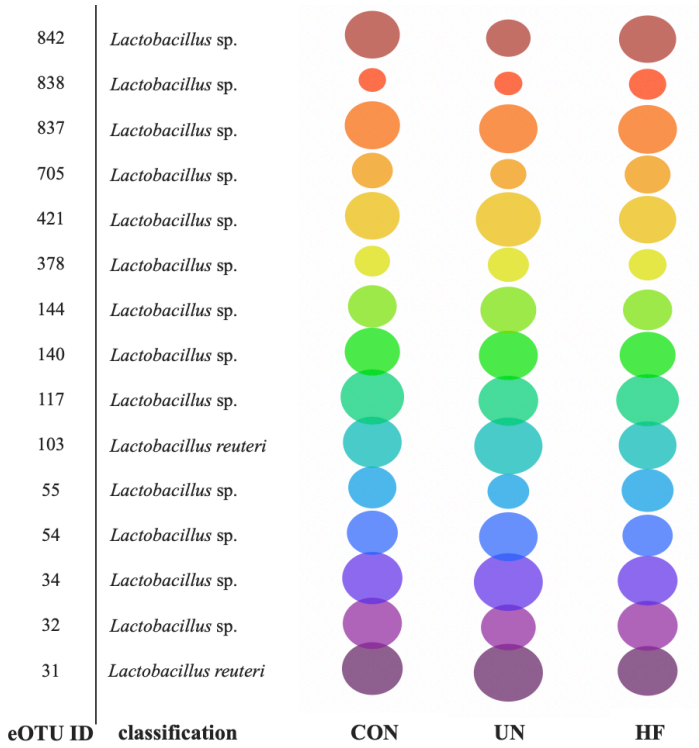

Figure 2. Array of lactobacilli in CON, UN, and HF maternal gut. Bubble plot of relative abundance of 15 gut bacterial taxa (eOTU) within the Lactobacillus genera in pregnant mice at GD18.5. Relative abundance of each eOTU is indicated by the size of the bubble.

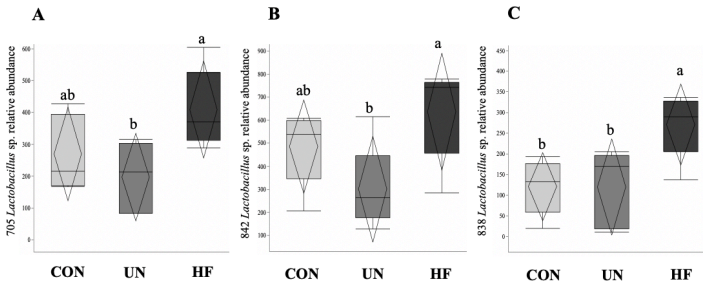

Figure 3. Abundance of lactobacilli in CON, UN and HF maternal gut. HF fed mothers had a higher relative abundance of three specific lactobacilli eOTU compared to mice fed an UN diet (A & B) ($p<0.05$) and compared to mice fed a CON and UN diet (C) ($p<0.05$). Data are quantile box plots with 95% confidence diamonds. Groups with different letters are significantly different (Tukey's *post hoc*).

Maternal malnutrition is associated with altered expression of folate and inositol transporters in maternal SI

Since folate and inositol uptake and transport in the gut is also important for determining maternal micronutrient status, we measured mRNA expression of genes involved in folate and inositol transport/uptake in the SI. Maternal HF diet was associated with higher mRNA expression of the folate transporters *Pcft* compared to UN mothers ($p<0.001$, Figure 4A) and *Rfc1* compared to UN and CON mothers ($p<0.01$, Figure 4B). At the protein level, ir-PCFT staining was localised to the SI muscularis mucosae, mucosa, and the simple columnar epithelium surrounding the villi (Figure 5). Semi-quantification of ir-PCFT staining intensity did not differ between the diet groups (Table 2). Inositol transporter *Smit2* mRNA expression levels were lower in the SI of HF fed compared to UN mothers ($p<0.05$, Figure 4C). There was no difference in *TonEBP* mRNA expression levels between the diet groups (Figure 4D). Ir-SMIT2 staining was localised to the muscularis mucosae and simple columnar epithelium (Figure 6). There was no difference in ir-SMIT2 staining intensity between diet groups (Table 3).

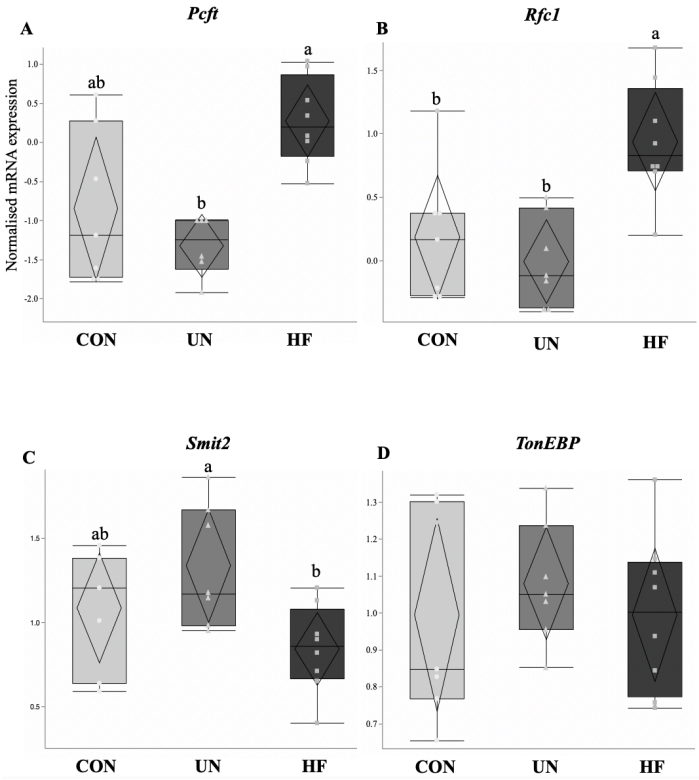

Figure 4. **Maternal HF diet alters folate and inositol gene expression in the maternal SI.** HF fed diet was associated with higher mRNA expression levels of the folate transporters *Pcft* compared to UN diet ($p<0.001$) (A) and *Rfc1* compared to UN and CON diet ($p<0.01$) (B). HF diet was associated with lower expression levels of inositol transporter *Smit2* compared to UN mothers ($p<0.05$) (C). There was no difference in expression levels of *TonEBP* between diet groups (D). Data are quantile box plots with 95% confidence diamonds. Groups with different letters are significantly different (Welch/Games-Howell or Tukey's *post hoc*). (Circle = CON, Triangle = UN, Square = HF).

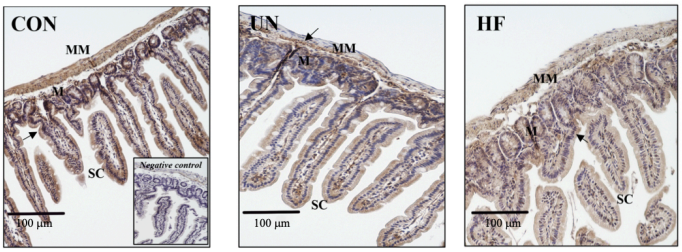

Figure 5. Folate transporter PCFT protein expression in CON, UN and HF maternal gut. PCFT protein expression was not significantly different among CON, UN and HF maternal SI samples. Staining was localised to the muscularis mucosae (MM), mucosa (M), and simple columnar epithelium (SC). Arrows indicate areas where ir-PCFT staining is localized.

Table 2. PCFT maternal SI staining intensity.

	CON	UN	HF	p-value
ir-PCFT in maternal SI				
Muscularis mucosae	2.0 ± 1.45	2.1 ± 1.08	2.0 ± 1.45	NS
Mucosa	3.9 ± 0.10	2.4 ± 1.26	1.9 ± 1.85	NS
Villi	3.8 ± 0.25	2.6 ± 0.69	2.0 ± 1.46	NS

a) Data are mean (± standard deviation) intensity of ir-PCFT staining for the specified diet group determined from the average staining intensity across all six images for each SI tissue layer and each dam. Intensity was score at 0 = absent, 1 = weak, 2 = moderate, 3 = strong, 4 = very strong.

b) Difference between diet groups determined at 5% level of significance. NS signifies no statistical difference between CON, UN, and HF within the specified tissue layer.

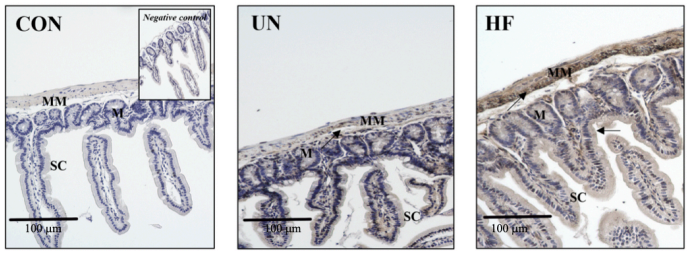

Figure 6. Inositol transporter SMIT2 protein expression in CON, UN and HF maternal gut. SMIT2 protein expression was not significantly different among CON, UN and HF maternal SI samples. Staining was localized to the muscularis mucosae (MM) and simple columnar epithelium (SC). Arrows indicate areas where ir-SMIT2 staining is localized.

Table 3. SMIT2 maternal SI staining intensity.

	CON	UN	HF	p-value
ir-SMIT2 in maternal SI				
Muscularis mucosae	2.1 ± 1.71	1.9 ± 1.84	1.6 ± 1.54	NS
Mucosa	1.1 ± 1.92	1.3 ± 2.31	1.3 ± 1.63	NS
Villi	1.3 ± 2.31	1.7 ± 2.08	1.1 ± 1.20	NS

a) Data are mean (± standard deviation) intensity of ir-SMIT2 staining for the specified diet group determined from the average staining intensity across all six images for each SI tissue layer and each dam. Intensity was score at 0 = absent, 1 = weak, 2 = moderate, 3 = strong, 4 = very strong.

b) Difference between diet groups determined at 5% level of significance. NS signifies no statistical difference between CON, UN, and HF within the specified tissue layer.

Maternal undernutrition is associated with decreased placental Frβ gene expression

Maternal UN was associated with reduced placental mRNA expression of *Fr*β, but not *Frα*, compared to CON and HF placentae ($p<0.05$, Figure 7D). There were no differences in mRNA expression levels of placental *Pcft*, *Rfc1*, *Frα, TonEBP*, and *Smit1* between the diet groups (Figure 7). Fetal weight was negatively associated with placental *Frα* mRNA expression ($r=0.337$, $p<0.05$, Figure 8A) and positively associated with *Fr*β mRNA expression ($r=0.596$, $p<0.001$, Figure 8B). When stratifying by sex, we found that HF male placentae had lower *Rfc1* mRNA expression compared to CON and that HF and CON male placentae had higher *Fr*β mRNA expression compared to UN (Supporting Information Figure S1 B & D). Female placenta had higher *Fr*β mRNA expression compared to CON and UN (Supporting Information Figure S1D). At the protein level, ir-FRβ staining intensity did not differ between the diet groups (Table 4). ir-FRβ staining was localized to the decidua, junctional and labyrinth zones, with most intense staining appearing in the labyrinth zone (Figure 9).

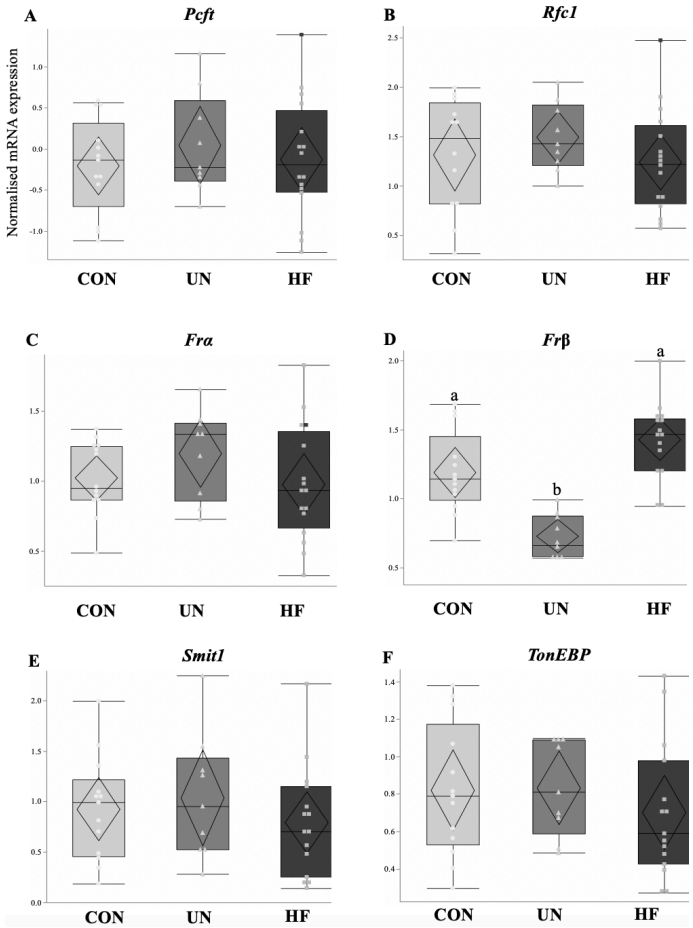

Figure 7. Folate and inositol transporter mRNA expression in CON, UN and HF placentae. *Fr*β mRNA expression was reduced in UN placentae compared to CON and HF placentae ($p<0.05$) (D). Data are quantile box plots with 95% confidence diamonds. Groups with different letters are significantly different (Tukey's *post hoc*). (Circle = CON, Triangle = UN, Square = HF).

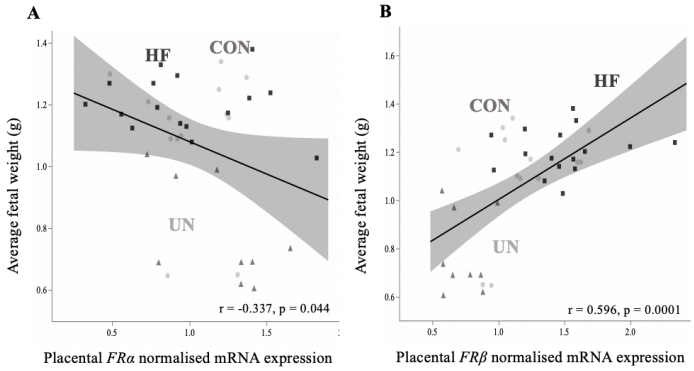

Figure 8. Association between fetal weight and folate receptor *α* and β mRNA expression at GD18.5. Fetal weight was negatively associated with placental *Frα* expression (A) and positively associated with *Fr*β expression (B). Data are linear regression with 95% confidence intervals, $p<0.05$. r = Pearson correlation coefficient. (Circle = CON, Triangle = UN, Square = HF).

Table 4. FRβ placenta staining intensity.

	CON	UN	HF	p-value
ir-FRβ in placenta				
Decidua	2.1 ± 1.35	3.0 ± 1.00	3.0 ± 1.04	NS
Junctional zone	2.1 ± 1.30	3.2 ± 0.96	3.2 ± 0.92	NS
Labyrinth zone	1.8 ± 1.68	2.4 ± 1.39	3.1 ± 1.03	NS
Chronic plate	1.9 ± 1.56	2.3 ± 1.59	2.5 ± 1.40	NS

a) Data are mean (± standard deviation) intensity of ir-FRβ staining for the specified diet group determined from the average staining intensity across all six images for each placental tissue layer and each animal. Intensity was score at 0 = absent, 1 = weak, 2 = moderate, 3 = strong, 4 = very strong.

b) Difference between diet groups determined at 5% level of significance. NS signifies no statistical difference between CON, UN, and HF within the specified tissue layer.

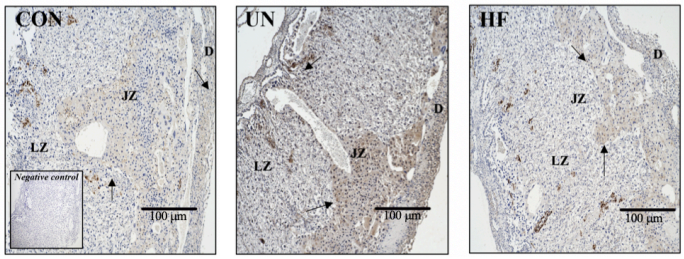

Figure 9. FRβ protein expression in CON, UN and HF placentae. FRβ protein expression was not significantly different among CON, UN and HF placentae. Staining was localized to the decidua (D), junctional (JZ), and labyrinth zones (LZ). Arrows indicate areas where ir-FRβ staining is localized.

Maternal malnutrition is associated with altered folate and inositol gene expression in the fetal gut

To better understand the effects of malnutrition on folate and inositol pathways in the developing fetal gut, we measured mRNA expression levels of folate and inositol transporters. Consistent with gene expression changes in the maternal gut, fetuses from HF fed mothers had higher gut mRNA levels of the folate transporter *Pcft* compared to fetuses from CON and UN mothers ($p<0.0001$, Figure 10A) and higher *Frα* compared to fetuses from UN mothers ($p<0.01$, Figure 10B). In contrast to the maternal SI, fetuses from HF fed mothers had higher gut inositol transporter *Smit2* mRNA expression compared to fetuses from CON and UN mothers ($p<0.01$, Figure 10C). There were no differences in mRNA expression levels of fetal gut *TonEBP* between the diet groups (Figure 10D). When stratifying by sex, we found that both HF female and male fetuses had higher gut mRNA expression of *Pcft* compared to CON and UN (Supporting Information Figure S2A). *Frα* mRNA expression was higher in HF male fetal gut compared to CON and UN male fetal gut (Supporting Information Figure S2B) and *Smit2* mRNA expression was higher in HF female fetal gut compared to CON female fetal gut (Supporting Information Figure S2C).

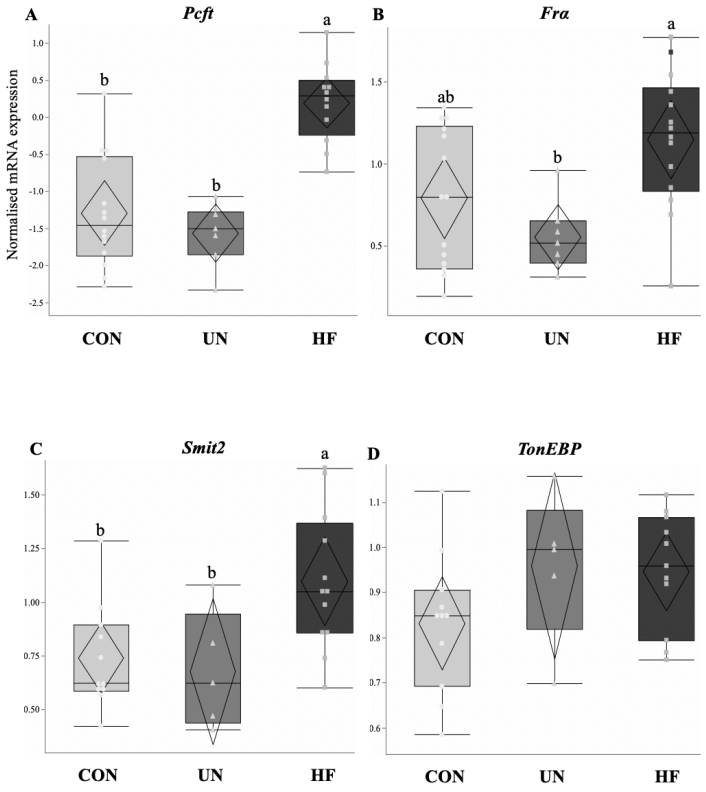

Figure 10. Maternal malnutrition alters folate/inositol gene expression in the fetal gut. Fetuses from HF fed mothers had higher mRNA expression levels of the folate transporters *Pcft* compared to CON and UN ($p<0.0001$) (A), and higher *Frα* compared to UN ($p<0.01$) (B). Fetuses from HF fed mothers had higher expression levels of the inositol transporter *Smit2* compared to UN ($p<0.01$) (C). There was no difference in expression levels of the inositol transporter *TonEBP* between diet groups (D). Data are quantile box plots with 95% confidence diamonds. Groups with different letters are significantly different (Tukey's *post hoc*). (Circle = CON, Triangle = UN, Square = HF).

Principal component analysis of maternal and fetal variables

To visualise our maternal and fetal outcome measures and determine whether we could discriminate between dietary groups based on these measures, we performed principal component analysis (PCA). First, a PCA of maternal plasma folate levels, relative abundance of significant maternal gut lactobacilli (eOTU) and mRNA levels of folate and inositol genes revealed that HF mothers clustered separately from CON and UN (Figure 11A). The variables with the greatest eigenvalues (EV) were represented by *Pcft* and *Rfc1* mRNA expression and the three significantly different lactobacilli (EV 3.10, explaining 44.3% of the variance in the data), and by *Pcft* and *Smit2* mRNA expression (EV 1.35, explaining 19.3% of the variance in the data) (Supporting Information Figure S3A). Second, a PCA of fetal variables, including placental and gut folate/inositol genes and fetal and placental weights, revealed fetuses from HF mothers clustered separately from UN whereas fetuses from CON mothers were intermingled (Figure 11B). The variables with the highest eigenvalues were represented by fetal weight, *Pcft, Frα,* and *Smit2* gut mRNA expression and *Frβ* placental mRNA expression (EV 2.74, explaining 45.9% of the variance in the data) and by *Pcft, Smit2* gut mRNA expression and *Frβ* placental mRNA expression (EV <1, explaining 19.8% of the variation (Supporting Information Figure S3B).

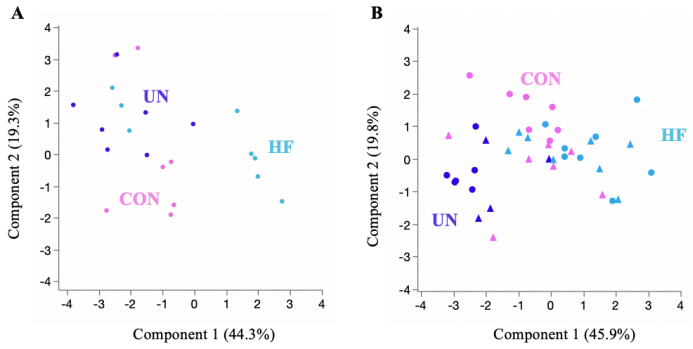

Figure 11. Principal component analysis (PCA) of maternal (A) and fetal (B) variables. (A) HF fed mothers are separated from CON and UN mothers. The first component explains 44.3% of the variation and the second component 19.3%. (B) Fetuses from HF fed mothers are separated from UN; fetuses from CON are mixed. The first component explains 45.9% of the variation and the second component 19.8%. (Triangle = Female, Circle = Male).

Discussion

Folate is recognised as an essential micronutrient for optimal fetal growth and development, however few studies have examined the effects of malnutrition on folate transport in the mother and fetus, with most studies considering placental pathways.[6,7] Here, we used a systems-physiology approach, considering the maternal holobiont, placenta and fetus, to comprehensively examine the effects of adverse nutritional environments on pregnancy phenotypes, specifically pathways related to folate and inositol production, transport and uptake along the nutritional pipeline in pregnancy. We found the greatest differences in gut lactobacilli relative abundance and gene expression were between mothers and/or fetuses fed HF diets compared to those that were UN.

Importantly, to our knowledge, this is the first study to measure mRNA levels of folate and inositol transporters in the fetal gut and show early life programming of offspring gut micronutrient pathways by exposure to malnutrition *in utero*, which may have consequences for long-term offspring gut function.

The maternal gut is an underappreciated player in micronutrient status and the programming of fetoplacental development. To our knowledge, no studies have looked at a direct role of maternal gut microbes in placental development/function and associations with offspring growth and development. Previous research has investigated the gut-placenta-fetus pipeline with respect to tryptophan absorption and serotonin production.[56] However, an evidence gap remains as the mechanisms by which the maternal gut and the gut microbiome affect placental development are unclear. Thus, a novel aspect of our study was to interrogate maternal gut function in response to malnutrition. The gut microbiome is a dynamic and complex network of microorganisms that interact with the environment and host, and is strongly influenced by diet and nutrition.[52, 53] Some of these microbes, including lactobacilli and bifidobacteria, are known to produce folate and can alter host micronutrient levels.[26, 31, 35–37, 54, 55] Lactobacilli are a dominant genera of microbes in the mouse gut.[57] We found that HF fed mothers had a higher relative abundance of three specific lactobacilli eOTU compared to UN mothers, results that are in part consistent with our hypothesis that malnutrition would alter levels of maternal gut lactobacilli. Despite these findings, both HF and UN mothers had lower circulating folate levels, consistent with our observation that UN and HF fed mice consumed less, albeit still adequate, folate from their diets and consistent with reports of lower circulating folate levels with maternal underweight and obesity. [1–5] In the current study, we do not have direct evidence for reduced maternal gut bacterial production of folate impacting host micronutrient levels, therefore, the specific links between host folate status and gut bacterial folate production have yet to be disentangled. Whilst we could not detect bifidobacteria, it is known that species in this genus also contribute to folate production in the host and are a major component of the mouse microbiome.[31, 33]

We also found that that folate transporter expression is responsive to dietary intakes even at nutrient intake levels that are considered adequate or better. Maternal HF diet was associated with higher mRNA expression of folate transporters Pcft and Rfc1 in the maternal gut, which is consistent with studies that show low folate intake is associated with upregulation of folate transporters.[58] Whilst Rfc1 contributes to intestinal folate absorption, Pcft plays a dominant role in the uptake and transport of folates in the intestine.[15, 59] An additional novel finding of our study is that maternal UN was associated with lower maternal SI mRNA expression of Pcft and Rfc1, since we would expect the expression to be higher given that low folate intake is usually observed with higher folate transporter expression.[58] Maternal HF diet was also associated with lower Smit2 mRNA expression in maternal SI. We were not able to measure circulating inositol levels in mothers and do not know levels in the diets and it may be that, as with folate content, inositol content varied between the diets. However, at this time we cannot speculate on the cause of the reduced Smit2 expression or its physiological relevance.

Furthermore, our study highlights an important relationship between maternal malnutrition and placental folate/inositol transporters. We found that UN placentae had lower Frβ mRNA expression levels compared to CON and HF placentae, whilst there was no difference in Frα, Pcft, and Rfc1 placental mRNA expression between the diet groups. In our study, there was no difference in Smit1 and TonEBP placental mRNA expression between the diet groups suggesting that inositol transport from mother to fetus is not altered as a result of maternal malnutrition. Whether this indicates that inositol levels have less of an impact on overall fetal development than other micronutrients remains to be determined.

Less is known about the presence and function of folate and inositol transporters in the fetal gut, and how *in utero* exposures may programme their development and function. To our knowledge, our findings are the first to show that maternal malnutrition affects folate and inositol pathways in the developing fetus and are consistent with our findings in the maternal SI. We found that fetuses from HF fed mothers had higher gut mRNA expression levels of folate transporters *Pcft* and *Frα* and inositol transporter *Smit2*. Whilst it has yet to be conclusively determined if bacteria colonisation of the fetal gut occurs *in utero*, lactobacilli species are among the first microbes to colonise the infant gut after vaginal birth.[60] Therefore, the presence of folate and inositol transporters in the fetal gut may be a preparatory step for the micronutrient production that is likely to occur after bacterial colonisation in the early newborn gut. However, if malnutrition alters the expression of these key transporters *in utero*, it may have long-term implications for their function and ultimately, host micronutrient status. Overall, in our study maternal HF diet had the greatest effect on folate and inositol pathways in the developing fetus at GD18.5 and fetuses can be discriminated based on maternal diet when considering fetal weight and transporter expression.

A limitation to our study is its cross-sectional design. It remains to be determined whether maternal malnutrition impacts gut bacterial folate production and intestinal and placental folate and inositol gene/protein expression in very early pregnancy. That said, our findings are still relevant since suboptimal micronutrient status, production and transport of folate and inositol at the end of pregnancy could be a marker of in utero events earlier in pregnancy, and a window to future maternal and offspring health. Additionally, the CON/UN and HF diets used were produced by different manufacturers and had differences in their nutritional composition (neither diet indicated the amount of inositol). While not the purpose of our study, future studies should explore how differences in micro/macronutrient levels impact the maternal holobiont, placenta, and fetus, with respect to micronutrient production and transport pathways, by controlling for specific micronutrients. Here, our aim was to use established diets that

elicit specific pregnancy phenotypes to examine the subsequent effect on circulating folate levels and pregnancy physiology across the gut-placental-fetal pipeline. For example, while our HF model had a high proportion of calories from fat, it did not result in fetal growth restriction as seen in many other animal models which is not entirely reflective of the human situation.[61] That said, our HF model does parallel diets from populations who do consume higher amounts of fat, such as the Alaskan Inland Inuit populations who have undergone nutritional adaptations (such as high carbohydrate, low protein diets that are regularly over 40% fat under normal conditions) due to less than favourable environmental changes, and populations residing in the Pacific Islands, who have high levels of daily absolute fat intake and saturated fat intake as a result of various social determinants (e.g. availability of processed foods and access to fresh local food). [62, 63] Another limitation is that the inositol content of the diets was not defined by the manufacturers, and thus we do not know whether changes in inositol transporters reflect differences in micronutrient availability from the diet. Further, maternal plasma inositol concentrations could not be measured due to limited sample volume, which was prioritised for measuring folate. Similarly, fetal plasma folate concentrations could not be measured due to limited sample volume, therefore we do not know the effects of these adverse exposures on circulating micronutrient levels in the fetus at term. However, others have shown that maternal diets deficient in folate, resulting in low maternal serum folate levels, impacts fetal growth at the end of gestation (GD 18.5).[64]

Our systems-physiology approach, considering the maternal holobiont, placenta and fetus, provided us with the unique opportunity to examine the effects of two common nutritional adversities with known effects on pregnancy phenotypes, on pathways important for folate and inositol production and transport across the pregnancy nutritional pipeline. In our model a high fat diet with lower (but adequate) folate content before and during pregnancy has the greatest effect on these pathways in the mother and the fetus at the end of pregnancy. Our findings provide a first step to more complete insight into microbial and micronutrient physiology in pregnancy and, once carefully characterised across pregnancy and if translated to human cohorts, may aid in efforts to identify new biomarkers and therapeutic targets for women and pregnancies at high-risk of micronutrient-associated fetal disorders or poor fetal growth and development.

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Author Contributions and Notes

Conceptualisation: KLC; Methodology: KLC; Investigation: EP, KLC; Analysed data: EP, KLC; Wrote paper: EP, KLC; Reviewed and revised paper: TVM, KLC, EP.
The authors have no conflicts of interest and nothing to disclose.
Supporting information is appended at the end of this pre-print.

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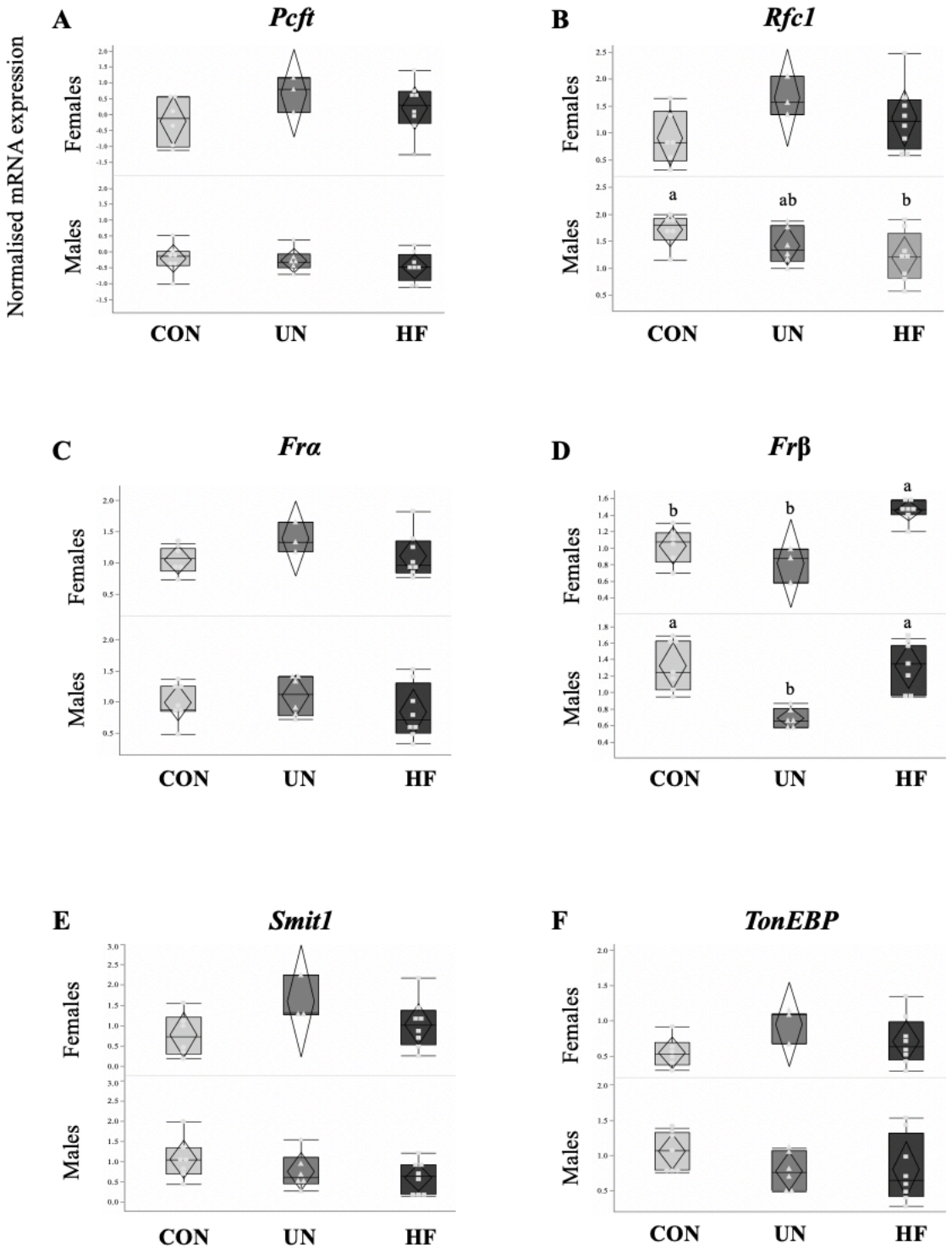
Supporting Information Table S1. Primer sequences for quantitative PCR (qPCR).

Gene Name	Sequence of Primers	Gene Bank Accession ID or PMID
Frα	F: 5'-GGCCCTGAGGACAATTTACA-3' R: 5'-TCGGGGAACACTCATAGAGG-3'	23904512
Frβ	F: 5'-CCAGCAAGTGGACCAGAGTT-3' R: 5'-AAGGAGGTACGACAGGCTTC-3'	XM_006507371.3
Rfc1	F: 5'-TGGGTGTTGTAGTCTGCGT-3' R: 5'-CACTCCACCTTGCACTACCC-3'	XM_006513420.3
Pcft[P]	F: 5'-TGCCCGGCTATATGTGGTTC-3' R: 5'-TGCACAGTAACCACCACGAA-3'	NM_026740.2
Pcft[SI]	F: 5'-TGCTAGCCCCTCCGTGTTTGC-3' R: 5'-CCATCCGGAAGGTGCGACTGT-3'	20053979
TonEBP	F: 5'-AGCCAAAAGGGAACTGGAG-3' R: 5'-GAAAGCCTTGCTGTGTTCTG-3'	19381288
Smit1	F: 5'-CTCCACTCTAATGGCTGGCTTCTT-3' R: 5'-GCCACAATATCCTGCCCACAATC-3'	16644257
Smit2	F: 5'-CACAGTGCCAGCACCATCT-3' R: 5'-GCACCAGGGCAAACACAAAT-3'	XR_001785540.1

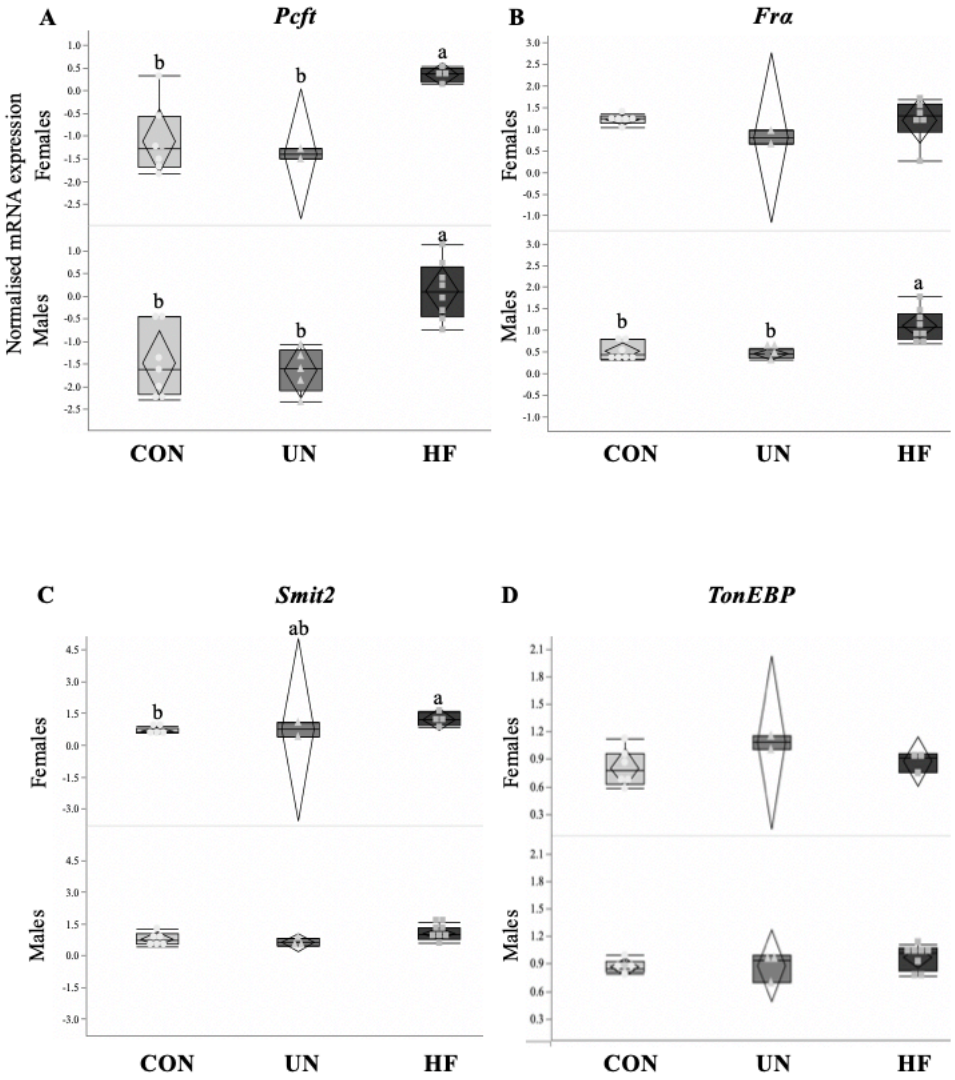
a) F: forward, R: reverse

b) [P] Primer sequence specific for placenta

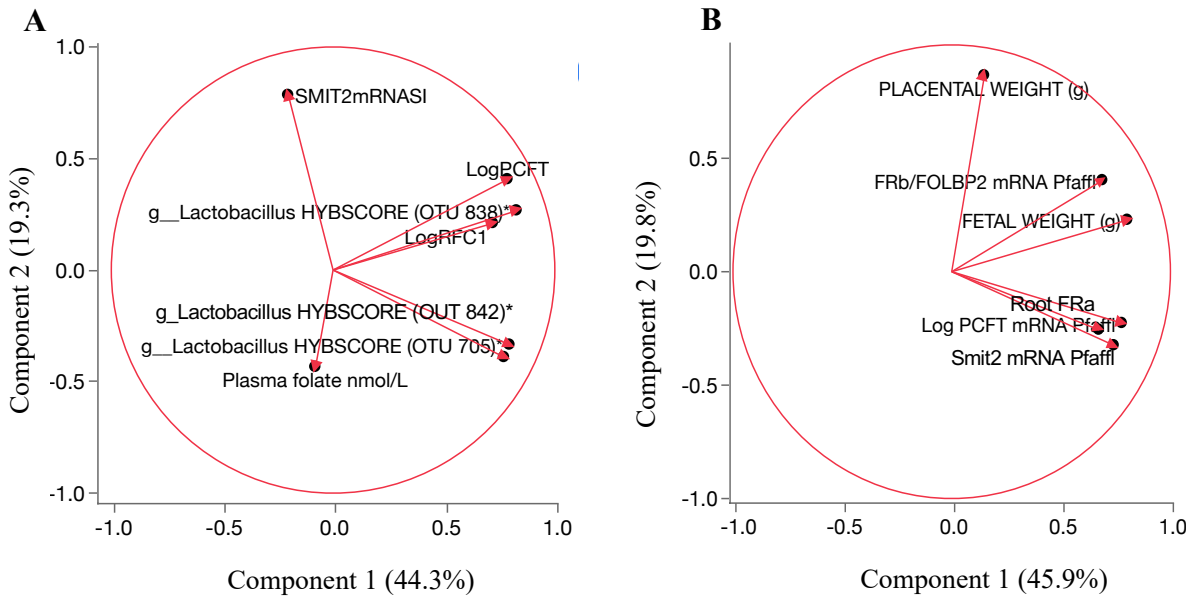
c) [SI] Primer sequence specific for small intestine



Supporting Information Figure S1. Folate and Inositol transporter mRNA expression in CON, UN, and HF placentae stratified by fetal sex. *Rfc1* mRNA expression was reduced in HF male placentae compared to CON male placentae ($p<0.0755$) (B). *Frβ* mRNA expression was higher in HF female placentae compared to CON and UN female placentae ($p<0.001$) and higher in CON and HF male placentae compared to UN ($p<0.001$) (D). Data are quantile box plots with 95% confidence diamonds. Groups with different letters are significantly different (Tukey's *post hoc*). (Circle = CON, Triangle = UN, Square = HF).



Supporting Information Figure S2. Folate and Inositol transporter mRNA expression in CON, UN, and HF fetal gut stratified by fetal sex. *Pcft* mRNA expression was higher in HF female and male fetal gut compared to CON and UN fetal gut ($p<0.05$ for female; $p<0.001$ for male) (A). *Frα* mRNA expression was higher in HF male fetal gut compared to CON and UN male fetal gut ($p<0.001$) (B). *Smit2* mRNA expression was higher in HF female fetal gut compared to CON female fetal gut ($p<0.05$) (C). Data are quantile box plots with 95% confidence diamonds. Groups with different letters are significantly different (Tukey's *post hoc*). (Circle = CON, Triangle = UN, Square = HF).



Supporting Information Figure S3. Correlations determined by PCA of maternal (A) and fetal (B) variables. (A) *Pcft* and *Rfc1* mRNA expression and eOTU 838 are correlated. Maternal plasma folate levels and *Smit2* mRNA expression were negatively correlated. (B) *Pcft, Frα, Smit2* mRNA expression were correlated and *Fr*β and fetal weight (g) were positively correlated.