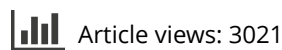
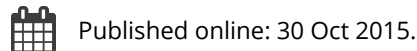
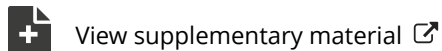


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# Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother's periconceptional diet

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**Keywords:** gut, high fat diet, intestine, microbiome, obesity, pregnancy

Shifts in the maternal gut microbiome have been implicated in metabolic adaptations to pregnancy. We investigated how pregnancy and diet interact to influence the composition of the maternal gut microbiota. Female C57BL/6 mice were fed either a control or a high fat diet for 8 weeks prior to mating. After confirmation of pregnancy, maternal weight gain and food intake were recorded. Fecal pellets were collected at 2 timepoints prior to mating (at the beginning of the experiment, and after 6 weeks of the specified diet) and at 4 timepoints during pregnancy (gestation day 0.5, 5.5, 10.5, and 15.5). The microbial composition and predicted metabolic functionality of the non-pregnant and pregnant gut was determined via sequencing of the variable 3 region of the 16S rRNA gene. Upon conception, differences in gut microbial communities were observed in both control and high fat-fed mice, including an increase in mucin-degrading bacteria. Control versus high fat-fed pregnant mice possessed the most profound changes to their maternal gut microbiota as indicated by statistically significant taxonomic differences. High fat-fed pregnant mice, when compared to control-fed animals, were found to be significantly enriched in microbes involved in metabolic pathways favoring fatty acid, ketone, vitamin, and bile synthesis. We show that pregnancy-induced changes in the female gut microbiota occur immediately at the onset of pregnancy, are vulnerable to modulation by diet, but are not dependent upon increases in maternal weight gain during pregnancy. High fat diet intake before and during pregnancy results in distinctive shifts in the pregnant gut microbiota in a gestational-age dependent manner and these shifts predict significant differences in the abundance of genes that favor lipid metabolism, glycolysis and gluconeogenic metabolic pathways over the course of pregnancy.

## Background

Obesity affects more than 500 million people worldwide.<sup>1</sup> The steady rise in the prevalence of obesity over the past 3 decades cannot be solely attributed to genetic factors and has led researchers to examine other causes that may contribute to disease risk. Epidemiological and experimental data has shown a relationship between the *in utero* environment and the risk of developing chronic diseases, such as obesity, later in life.<sup>2</sup> Prenatal signals, such as nutrition, modulate disease risk by inducing gene-environment interactions in fetal homeostasis leading to persistent changes in key signaling pathways. Maternal obesity, a key predictor of childhood obesity, is associated with abnormal fetoplacental function,<sup>3</sup> offspring obesity risk,<sup>4</sup> and increased disease risk in general.<sup>5</sup>

The gut microbiota is emerging as a disease risk factor due to its role in regulating energy extraction and whole body

metabolism. These microbes are essential for metabolizing indigestible polysaccharides, producing essential nutrients, and regulating fat storage.<sup>6</sup> The presence and abundance of each bacterial taxa is crucial since different populations differentially metabolize nutrients and have varying capacities of energy harvest.<sup>7</sup> The mammalian gut is generally dominated by 2 phyla, *Firmicutes* and *Bacteroidetes*,<sup>8</sup> and shifts in the relative abundances of these groups have been observed in obesogenic states when compared to the gut microbiota of healthy individuals.<sup>9</sup> Both dietary factors<sup>10</sup> and genetics<sup>9</sup> influence gut microbial composition.<sup>11</sup> High fat (HF) intake is associated with an increased *Firmicutes* to *Bacteroidetes* ratio, increased gut inflammation and intestinal permeability.<sup>12,13</sup> Comparisons of the gut microbiota of obese and lean twins has shown that obesity is associated with a decrease in abundance of *Bacteroidetes* and altered expression of genes involved in metabolic pathways that favor increased energy uptake and adipogenesis.<sup>7</sup>

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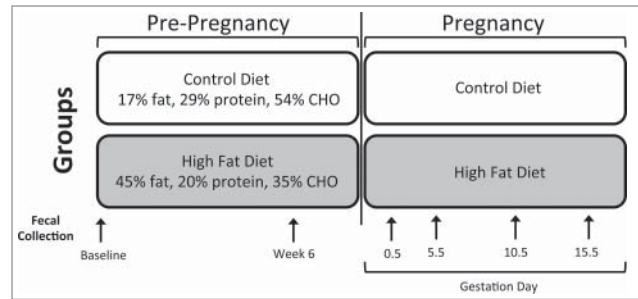
There is evidence, although limited, to suggest that pregnancy is a physiological state that is also associated with shifts in gut microbiota.<sup>14</sup> The biological purpose of these shifts may contribute to the ability of the mother to adapt to pregnancy and facilitates optimal fetal growth and development, but the mechanisms regulating these adaptations are unclear. Over the course of normal pregnancy, key metabolic changes occur in the mother that resemble metabolic dysfunction, including insulin resistance, dyslipidemia and hypertension.<sup>15</sup> Late pregnancy is characterized by increased insulin and leptin resistance and depletion of maternal adipose tissue deposits, facilitating the growth of the placenta and the fetus. Recent studies have suggested that changes to the microbiota may influence these pregnancy-associated metabolic changes, but whether this occurs immediately at the onset of pregnancy or occurs slowly over the course of gestation is unknown. The biomass present in the maternal gut microbiota is known to increase from early to late pregnancy and is accompanied by an increase in diversity.<sup>14,16</sup> Of particular interest, when germ-free mice are colonized with third trimester maternal microbiota, these mice demonstrate heightened inflammatory responses and adipose tissue accumulation.<sup>14</sup> This observation is suggestive of pregnancy-induced changes to the gut microbiota, which appear similar to that observed in obese individuals.

The factors that modulate maternal gut microbial changes are currently unknown; however, body weight may be an associated factor. It has been identified that the maternal gut microbiota may be influenced by pre-pregnancy weight and weight gain over the course of pregnancy.<sup>16</sup> In human studies, overweight pregnant women were found to have significantly higher levels of *Bacteroides* and *Staphylococcus*, where pregnancy weight gain was correlated with *Bacteroides* abundance.<sup>16</sup> However, the relationships between advancing gestation, maternal weight gain, and maternal diet-induced obesity remain unclear. Critically, it is also unknown whether the maternal pre-pregnancy condition modulates pregnancy-associated changes in maternal gut microbial population. Thus, using a mouse model of high fat (HF) diet intake, we investigated pregnancy-associated shifts as well as diet-induced shifts in the maternal gut microbiota and predicted the impacts of these variables on metabolic pathways. This model is novel in that it allows investigations of the interactions between diet and pregnancy and will determine the extent to which microbial shifts in HF pregnancies are due to diet, pregnancy, or an interaction between the 2 conditions. We suggest that since microbiota are responsive to changes in the nutritional environment, changes in the mother's gut niche may impact maternal adaptation to pregnancy, the function of the placenta, and/or the growth of the fetus, and impart an increased risk of obesity and metabolic dysfunction in the offspring.

## Results

### Body weight and caloric intake

Fecal samples were collected from all mice at all timepoints (Fig. 1). Mating of control (n = 5) and high fat (n = 5) fed female mice resulted in samples collected from n = 5 control and



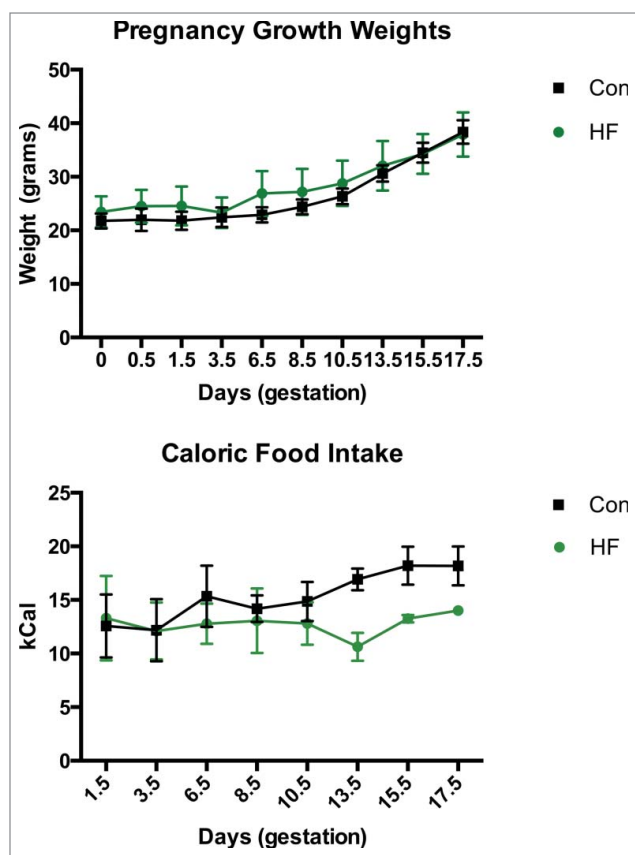
**Figure 1.** Schematic of mouse model. Schematic representation of the experimental design. Six-week-old female C57BL/6J mice (n = 10) were randomly assigned to one of 2 nutritional groups: 1) Control diet (Con; n = 5 17% fat, 29% protein, 54% CHO, 3 kcal/g) or 2) high fat diet (HF; n = 5; 45% fat, 20% protein, 35% CHO, 4.73 kcal/g), fed for 8 weeks and then mated with viable males. Pregnancy was identified when a vaginal plug was visible and designated as gestational day (E) 0.5 in both control (n = 5) and high fat (n = 3) groups. Fecal samples were collected from control mice prior to nutritional randomization (Baseline), after 6 weeks of either control or high fat diet feeding (Week 6) and then at timepoints during pregnancy (0.5, 5.5, 10.5 and 15.5; n = 5 control, n = 3 high fat fed).

n = 3 HF pregnant mice. Females fed a high fat (HF n = 5) diet for 8 weeks were not statistically heavier than low fat control diet-fed females (Con n = 5) at the time of mating. Consistent with previous reports indicating that nutritional excess and obesity reduces fertility in females,<sup>17</sup> 3 out of the 5 HF fed mice became pregnant. Gestational weight gain increased steadily throughout gestation and was not different between treatment groups (Fig. 2). Caloric intake was not statistically different between groups (Fig. 2).

### Pregnancy induces a shift in the gut microbiota of control-fed females

In order to study the impact of pregnancy on the maternal gut microbiota, mice were fed a control diet leading up to and during pregnancy. Fecal samples were collected pre- and post-conception, and the microbial communities were examined at 2 pre-conception and 4 post-conception timepoints (Figs. 1 and 3). Significant differences in the relative abundance of 21 genera were identified between non-pregnant and pregnant animals fed a control diet (Fig. S1 and Table S1). This includes 4 abundant genera (present at greater than 1%), namely *Akkermansia*, *Clostridium*, *Bacteroides* and *Bifidobacterium*, that were significantly increased during pregnancy compared to non-pregnant females (Fig. 3a, c, Table S1). Five taxa were reduced during pregnancy but these were present at low relative abundance (<0.5%), with the exception of *Sarcina* which decreased from 2.94% mean relative abundance to 0.14% with pregnancy (Fig. S1 and Table S1). These taxonomic differences indicate some variation within the non-pregnant and pregnant mice, which were further visualized using a Principal Coordinates Analysis (PCoA) of the Bray Curtis distance between microbial communities (Fig. 3b). Here, we see a distinct separation of the non-pregnant and pregnant animals despite some variation within both groups.

A more detailed investigation of the relative abundance of the most significantly different taxa between non- and pregnant Con



**Figure 2.** Maternal weight gain and caloric intake. Maternal weight gain over the course of pregnancy and caloric intake (expressed as calories consumed / gram body weight) in mothers during pregnancy. Data are mean  $\pm$  SEM; n = 5 in control group, n = 3 in the HF group. Con = control diet, HF = high fat diet.

diet animals shows that 3 taxa are significantly increased in the relative amounts of each bacteria over the course of pregnancy. *Akkermansia* is most abundant during early gestation (E0.5–E5.5) after which it drops to less than half its abundance during mid to late gestation (E10.5–15.5) (Fig. 3c). The relative abundance of *Bifidobacterium* also increases modestly at the onset of pregnancy, peaks dramatically in early gestation (E5.5) after which levels gradually decrease similar to those seen in the non-pregnant mice (Fig. 3c). *Bacteroides* increases with the onset of pregnancy at E0.5 and remains relatively elevated throughout pregnancy compared to non-pregnant females (Fig. S1). Examining these taxa, as well as others, which differ significantly (Fig. S1 and Table S1), we found changes to the gut microbial communities that occur not just at the onset of pregnancy, but also over the course of gestation (Fig. 3c, Fig. S1). In the 5 taxa that were suppressed with pregnancy, and did not display any changes in abundance over the course of gestation, all fell into the Firmicutes and Tenericute phyla, of which the genus *Coprobacillus* and *Clostridium* (phylum Tenericutes, family Erysipelotrichaceae) and *Sarcina* (phylum Firmicutes, family Clostridia) decreased the most upon conception (Fig. S1).

### Pre-conception high fat diet intake modulates pregnancy-induced changes in female gut microbiota: a diet and pregnancy interaction

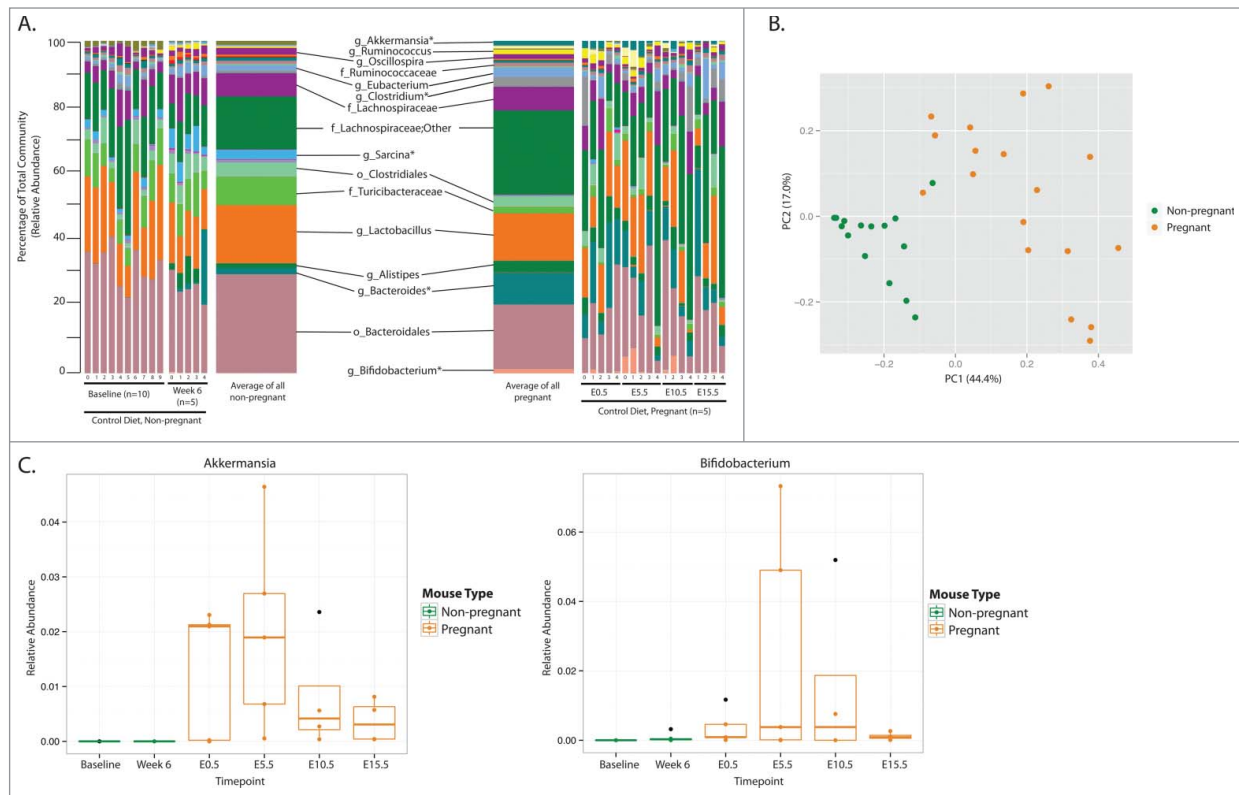
In parallel, we imposed a HF diet challenge in female mice to investigate the interactive effects between diet and pregnancy on the maternal gut microbiota. Eleven genera were identified as being significantly different between the non- and pregnant HF-fed groups most of which were Firmicutes and Bacteroidetes (increased with pregnancy) (Fig. S2 and Table S2) as well as a dramatic increase in *Akkermansia* (16.8 fold increase, phylum Verrucomicrobia) and *Bifidobacterium* (13.9 fold increase, phylum Actinobacteria) with gestation (Fig. 4a). A distinct set of pregnancy associated changes in microbial relative abundance occurs in mice fed a high fat diet compared to those fed a control diet where approximately half of the 11 genera that changed in high fat fed mice did not change in control mice (Table S1 and Table S2). Where pregnancy, independent of diet, induced an increased abundance of *Allobaculum* and *Clostridium* (family Lachnospiraceae), and decreased 5 genera in control mice, HF-exposed mice did not (Table S1 and Table S2). Rather, HF exposed mice display no significant decrease in any genera with the onset of pregnancy.

Consistent with Con fed mice, examination via PCoA indicates a distinct separation between non-pregnant and pregnant HF fed animals along the first axis despite some variation between the mice with each group (Fig. 4b).

When the relative abundances of the 2 genera with the greatest increase in relative abundance are visualized, similar patterns of abundance in *Akkermansia* over the course of gestation were observed in HF-fed pregnancies as in the Con-fed pregnancies (Figs. 3c and 4c). This indicates that the presence and abundance trends of this microbe may be universal in pregnancy. However, it appears that maternal diet may impact the fold increase of this microbe at the onset of pregnancy (Fig. 4b), as the relative abundance of *Akkermansia* is  $\sim$ 10 fold greater in HF-fed pregnancies (Table S1 and Table S2). Similarly, over the course of pregnancy in HF-fed mice, the relative abundance of *Bifidobacterium* generally increases from E0.5–5.5, after which it remains elevated; this trend is unique to HF-fed mice and this genera (Figs. S1, S2, and 4c). Detailed abundances of other significantly different taxa are displayed in Figure S2.

### Pregnancy-induced shifts in maternal gut microbiota: control vs. high fat nutrition

We have previously shown that imbalances in maternal macronutrient intake modifies both maternal and offspring metabolic function.<sup>18–20</sup> Given the established relationship between diet-induced shifts in gut microbiota and metabolic function in non-pregnant animals,<sup>10</sup> we set out to investigate whether there exists an interaction between diet and pregnancy on the gut microbiota. We observed the greatest differences in the gut microbiota, in terms of significantly altered taxa, between Con pregnant and HF pregnant mice (Fig. 5, Fig. S3 and Table S3), where 26 genera were identified as significantly different between Con and HF pregnancies. Taxonomic summaries of these mice over the course of pregnancy (Fig. 5a) and tests of significance across taxonomic groups (Table



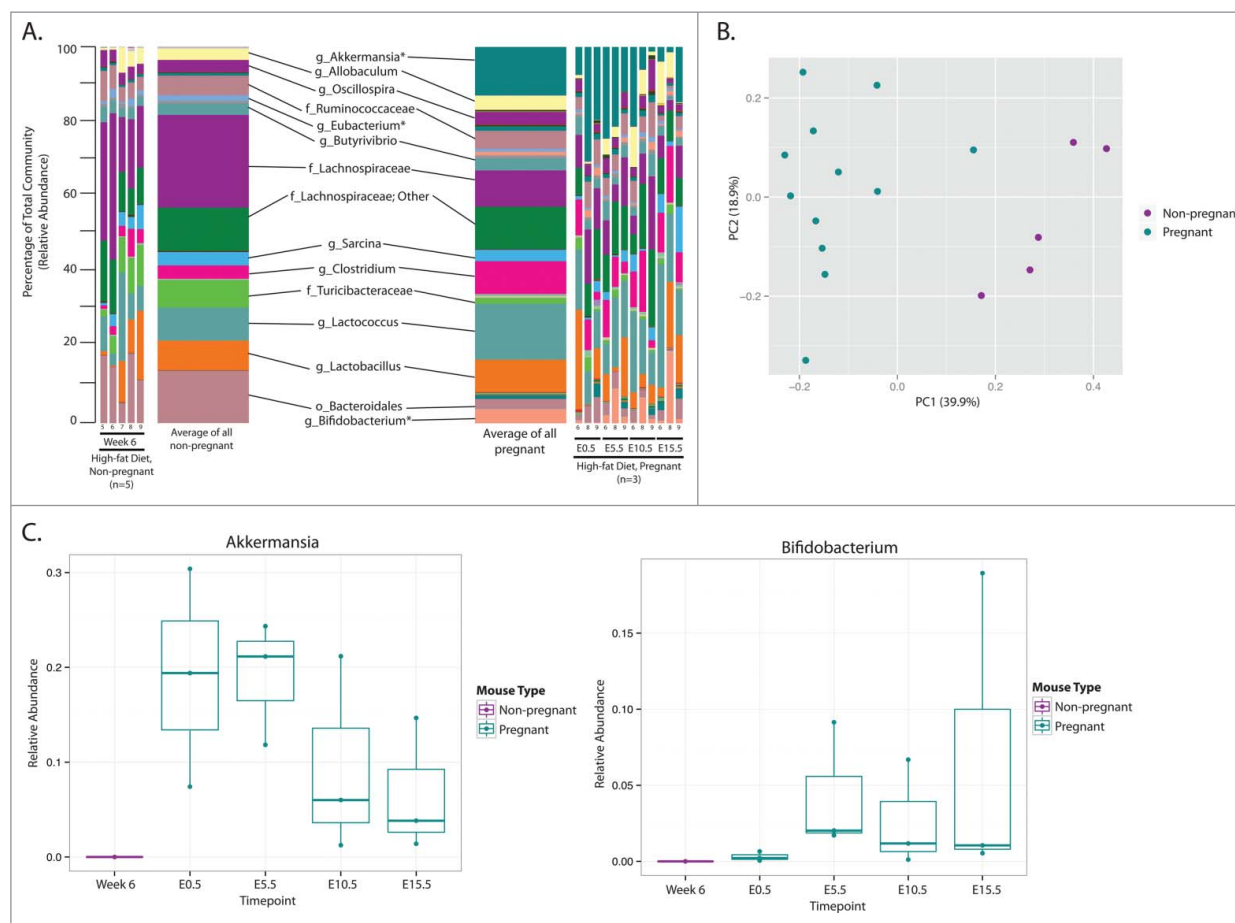
**Figure 3.** Pregnancy alters the maternal gut microbial community of Control-fed mice. Mice fed a control diet were sampled twice during an 8-week period prior to impregnation (Baseline (BL), Week 6 (Wk6)), at 0.5 days of pregnancy (E0.5) and then every 5 days during pregnancy (E5.5, E10.5, E15.5). (A) Taxonomic summaries of microbial relative abundance for each mouse sampled at each timepoint are displayed; the microbial communities of non-pregnant and pregnant mice are summarized and taxonomic classifications, resolved to the order (o), family (f), or genus (g) level, are displayed and those with relative abundance > 1.0% are labeled. Asterisks identify genera that were significantly different between non-pregnant and pregnant mice; additional low abundance genera identified as significantly different are listed in Additional File 2. Further, an average taxonomic summary of each group displays the mean relative abundance of each genus. (B) A Principal Coordinates Analysis (PcoA) using the Bray Curtis distance metric displays separation of non-pregnant and pregnant animals (PERMANOVA,  $P = 0.001$ ). (C) The relative abundance of the genera with the largest change in relative abundance between non-pregnant and pregnant animals are displayed. The x-axis indicates the sampling timepoints and samples are colored by pregnancy information. All other significant genera are displayed in **Figure S1**.

S3), demonstrate that pregnancy-induced shifts in the maternal gut microbiota are dependent upon the mother's diet before and during pregnancy. Indeed, when all 4 groups are visualized together, the first axis separates the mice groups based on diet, and the second axis by pregnancy (Fig. S4). Similarly, these differences are reflected when the 2 pregnant mouse groups are examined; pregnant mice fed a HF diet show distinctive changes in gut bacterial populations compared to pregnant controls (Fig. 5b). These data are robust, given that we observe distinct and significant differences despite relatively small numbers of mice that were successfully fertilized under the high fat diet regimen.

In total, 26 genera were found to be significantly different between Con and HF fed pregnant mice. This is greater than the 21 and 11 taxonomic groups that were altered with pregnancy alone in either Con or HF fed females respectively (Tables S1, S2 and S3). In order to further elucidate whether some of these changes were due more to diet than to pregnancy, we compared the non-pregnant Con to non-pregnant HF gut microbial composition in which we found 11 significantly different genera, all

of which were also different during pregnancy. Thus, 15 genera are specifically related to pregnancy effects and changes in these genera are not due to an underlying diet-associated difference in their gut microbiota before pregnancy (Table S3). In contrast to the diet x pregnancy-induced changes in *Akkermansia*, other bacterial taxa are elevated during pregnancy only when mothers are fed a specific diet. For example, *Allobaculum* steadily increases in relative abundance during pregnancy in HF fed mothers, but has a significantly lower and unchanged abundance in Con mice (Fig 5c). Overall, of the 26 taxa that are different between Con and HF-fed pregnant mice, the majority of them fall into the phylum Firmicutes, many of the order Clostridiales. Of those genera identified as significant, 14 are increased with the HF diet and 12 are increased with the Con diet (Fig. S3 and Table S3). Together, these observations suggest that there is an interactive effect of the mother's periconceptional diet on pregnancy-induced changes in the maternal gut microbiota.

Since previous reports have demonstrated that high fat intake is associated with an increased Firmicutes to Bacteroidetes ratio

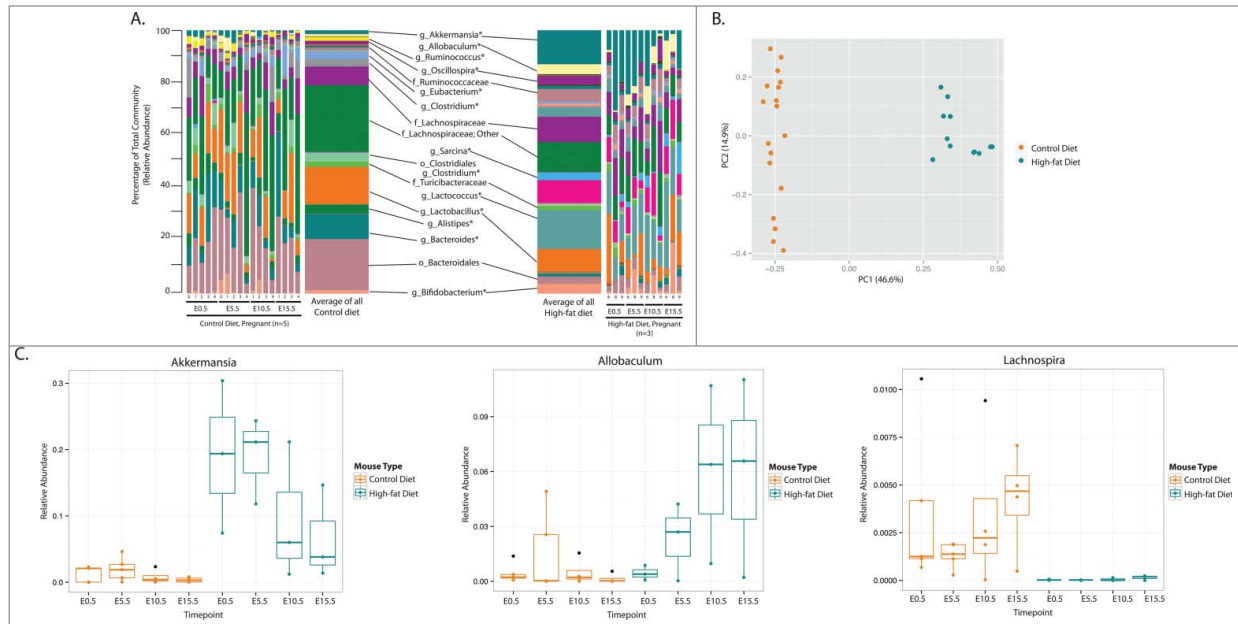


**Figure 4.** The maternal gut microbiota of animals fed a High fat diet is altered upon pregnancy. High fat-fed mice were sampled following 6 weeks of high fat feeding (Wk 6) prior to impregnation at 0.5 days of pregnancy (E0.5) and then every 5 days during pregnancy (E5.5, E10.5, E15.5). **(A)** Taxonomic summaries of microbial relative abundance for each mouse sampled at each timepoint are displayed; the microbial communities of non-pregnant and pregnant mice are summarized and taxonomic classifications, resolved to the order (o), family (f), or genus (g) level, are displayed and all genera present at a relative abundance of >1.0% are labeled. Asterisks identify genera that were significantly different between non-pregnant and pregnant mice on this diet; significantly different low abundance genera are listed in Additional File 4. Also, the mean relative abundance of each genus per group is displayed as an average taxonomic summary. **(B)** A Principal Coordinates Analysis (PcoA) using the Bray Curtis distance metric displays separation of non-pregnant and pregnant animals (PERMANOVA,  $P = 0.001$ ). **(C)** The relative abundances of 2 genera identified as significantly different between non-pregnant and pregnant HF-fed animals are visualized. The x-axis indicates the sampling timepoints and samples are colored by pregnancy information. All other significant genera are displayed in **Figure S2**.

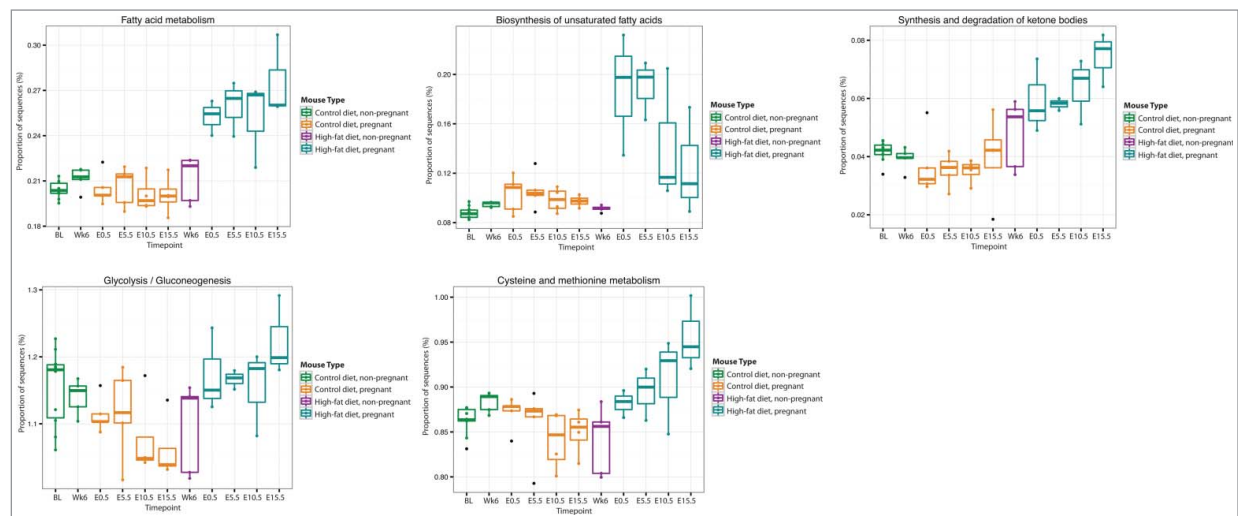
which is suggested to contribute to increased gut inflammation and intestinal permeability,<sup>12,13</sup> the ratio of these 2 phyla was evaluated in non-pregnant and pregnant states with the addition of a dietary challenge. At the onset of pregnancy, control mice show little change in the Firmicutes to Bacteroidetes ratio, but this ratio increases with advancing gestation (**Fig. S5**). In high fat fed mice, this ratio is significantly increased in non-pregnant mice compared to controls and is not further altered with the onset of pregnancy. Our data support previous reports that high fat dietary intake increases the Firmicutes to Bacteroidetes ratio, but pregnancy had no further impact, and the ratio remained relatively constant in HF-fed pregnant mice. This suggested that changes in the ratio of these 2 phyla in pregnant obesogenic states may be primarily due to diet, not due to pregnancy.

#### Maternal high fat diet alters the abundance of microbes containing genes predicted to regulate metabolic pathways related to fuel and vitamin metabolism

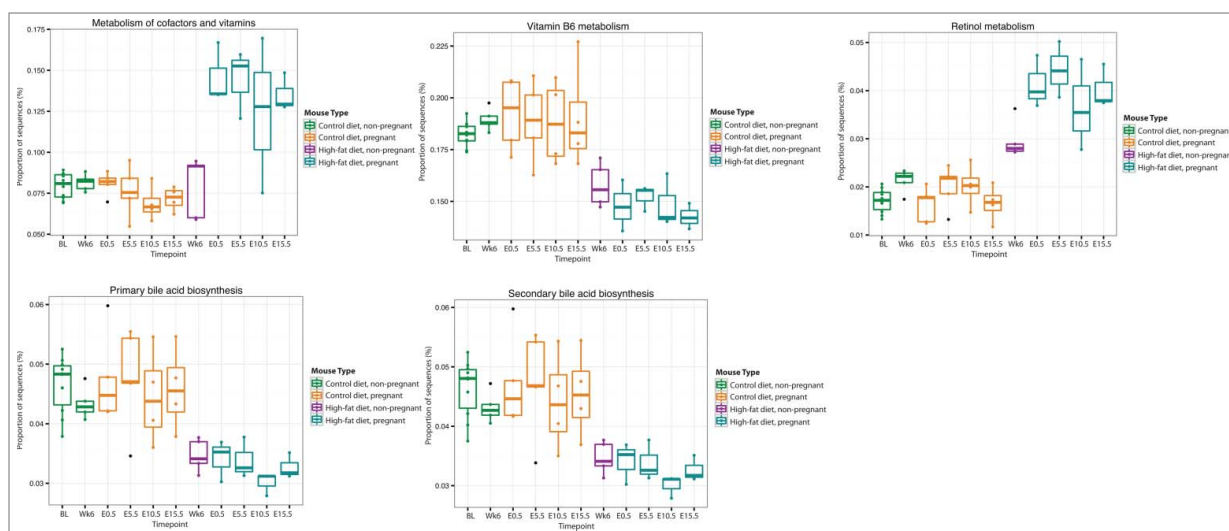
It has been shown that the gut microbiota of obese male mice is enriched in genes encoding for enzymes involved in energy extraction, where these bacterial populations facilitate obese mice to harvest more energy from their diet.<sup>4</sup> We used PICRUSt<sup>21</sup> in combination with our 16S rRNA gene sequencing to estimate changes in microbial metabolic pathways involved in fuel and vitamin metabolism. We demonstrate that both maternal diet and gestational age significantly affect pathways involved in fuel metabolism, (**Fig. 6, Table S4**). While numerous pathways were identified as significantly different between the dietary groups, pathways particularly relevant to maternal metabolism during pregnancy are highlighted in **Figure 6**. The gut microbiota of HF



**Figure 5.** The maternal gut microbiota during pregnancy is dependent on maternal diet. Pregnant mice in the Con and HF groups are compared to each other. (A) Taxonomic summaries of microbial relative abundance reveal more significant differences in taxa than in any other comparisons made. Each mouse sampled at each timepoint are displayed; the microbial communities of Con and HF pregnant mice are summarized and taxonomic classifications, resolved to the order (o), family (f), or genus (g) level, are displayed and all genera present at a relative abundance of >1.0% are labeled. Asterisks identify genera that were significantly different between Con and HF pregnant mice on this diet; significantly different low abundance genera are listed in Additional File 6. Further, an average taxonomic summary displays the mean relative abundance of each genus per grouping. (B) A Principal Coordinates Analysis (PcoA) using the Bray Curtis distance metric displays distinct separation of pregnant animals on differing diets, and distinct clustering of mice from each diet type (PERMANOVA,  $P = 0.001$ ). (C) The relative abundances of 3 genera identified as significantly different between Con-fed and HF-fed pregnant animals are visualized. The x-axis indicates the sampling timepoints and samples are colored by pregnancy information. All other significant genera are displayed in **Figure S3**.



**Figure 6.** Maternal diet- and pregnancy-induced changes in the abundance of microbe containing genes predicted to regulate metabolic pathways related to fuel metabolism. Mice fed a control diet were sampled twice during an 8 week period prior to impregnation (BL, Wk6), at 0.5 days of pregnancy (E0.5) and then every 5 days during pregnancy (E5.5, E10.5, E15.5). HF fed mice were sampled following 6 weeks of high fat feeding (Wk 6) prior to impregnation, and at 0.5 days of pregnancy (E0.5) and then every 5 days during pregnancy (E5.5, E10.5, E15.5). Here, estimations of changes in metabolic pathways between Con pregnant and HF pregnant groups were calculated using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)<sup>21</sup> and the proportion of genes predicted to be present for significantly different pathways of interest are visualized. The corresponding abundances for non-pregnant groups are shown for the sake of comparison. The x-axis indicates the sampling timepoints and samples are colored by diet and pregnancy information. All other significant pathways are listed in **Table S4**.



**Figure 7.** Maternal diet- and pregnancy-induced changes in the abundance of microbe containing genes predicted to regulate metabolic pathways related to vitamin and bile acid metabolism. Mice fed a control diet were sampled twice during an 8 week period prior to impregnation (BL, Wk6), at 0.5 days of pregnancy (E0.5) and then every 5 days during pregnancy (E5.5, E10.5, E15.5). HF fed mice were sampled following 6 weeks of high fat feeding (Wk 6) prior to impregnation, and at 0.5 days of pregnancy (E0.5) and then every 5 days during pregnancy (E5.5, E10.5, E15.5). Here, estimations of changes in metabolic pathways between Con pregnant and HF pregnant groups were calculated using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)<sup>21</sup> and the proportion of genes predicted to be present for significantly different pathways of interest are visualized. The corresponding abundances for non-pregnant groups are shown for the sake of comparison. The x-axis indicates the sampling timepoints and samples are colored by diet and pregnancy information. All other significant pathways are listed in **Table S4**.

pregnant mice is predicted by PICRUSt to be significantly enriched in genes involved in fatty acid and sulfur-containing amino acid metabolism, and glycolysis and gluconeogenesis compared to Con pregnant mice, and these increases are maintained throughout gestation. Interestingly, although there is little effect of a HF diet on unsaturated fatty acid synthesis in non-pregnant animals (Week 6 (Wk6), **Fig. 6**) pregnancy onset in HF-fed mice resulted in a significant rise in the abundance of these genes. Genes involved in the biosynthesis of unsaturated fatty acids are twice as high in HF mothers during early gestation, but these levels decline by mid-gestation (E10.5) (**Fig. 6**). Similarly, there appears to be a diet x gestational age interaction in genes associated with cysteine and methionine metabolism, and in the synthesis and degradation of ketone bodies in HF-fed mice compared to Con mice; where abundance of these sequences is elevated in HF mice and increase further with advancing gestation (**Fig. 6**).

Microbial genes involved in vitamin metabolism and bile metabolism are also predicted to differ between Con- and HF-fed pregnant mice. The microbial communities of Con-fed pregnant mice are enriched in genes involved in vitamin B6 metabolism compared to the microbiome of HF mothers and in contrast, the microbiome of mice fed a HF diet during pregnancy is enriched in genes involved in cofactor and retinol metabolism (**Fig. 7**). Genes involved in primary and secondary bile acid biosynthesis are predicted to be significantly decreased in the microbiome of HF mothers. All other significant metabolic pathways are listed in **Table S4**.

Similar to our investigations into the microbial composition, we performed similar analyses to predict metabolic pathways between

non-pregnant Con and HF fed mice (**Table S5**). In parallel to changes in taxonomic composition, we found a subset of the pathways increased in pregnancy x diet to also be increased with diet, indicating that these effects are multi-factorial.

## Discussion

We present novel data in mice to suggest that diet and pregnancy have interactive effects on the composition of the female gut microbiota. We show that pregnancy-induced changes in the gut microbiota occur immediately at the onset of pregnancy, and are vulnerable to modulation by diet. This is entirely diet x pregnancy related and does not appear to be dependent upon increases in maternal weight gain during pregnancy, consistent with our previous study where a HF diet containing 45% kcal from fat did not increase weight gain during pregnancy but did increase adipose depots.<sup>22</sup> HF diet intake before and during pregnancy resulted in distinctive shifts in the maternal gut microbiota in a gestational-age dependent manner and these shifts resulted in significant differences in the abundance of genes that favor lipid metabolism, glycolysis and gluconeogenesis metabolic pathways over the course of pregnancy.

### Pregnancy is a state that induces shifts in the gut microbiota of control females: shifts that change over the course of gestation

Previous work demonstrates that the pregnant gut microbiota changes from the first to the third trimester of pregnancy in humans.<sup>14,16</sup> We now show in mice that these shifts occur as early as 0.5 days of pregnancy. Most notably, *Akkermansia*



abundance is significantly elevated at the onset of pregnancy (at 0.5 days) and, consistent with other reports,<sup>14</sup> remains elevated throughout pregnancy. It is possible that this immediate shift is linked to reproductive cycle changes in the non-pregnant female, although we did not test this. Previous studies have shown shifts in the vaginal microbiome with reproductive cycles<sup>23</sup> but to our knowledge similar changes in gut microbiota have not been tested.

We hypothesize that gut microbes could participate in or be facilitated by pregnancy-induced changes in maternal endocrine and metabolic function. Early pregnancy is characterized by a maternal anabolic state; where maternal metabolic adaptations, mediated by pregnancy hormones, facilitate lipogenesis, glycogenesis, and adipocyte hypertrophy.<sup>24</sup> By late pregnancy, the mother is catabolic, where increasing production of progesterone, estradiol and placental lactogen participate in mediating insulin and leptin resistance thus ensuring high levels of maternal-fetal glucose transfer for normal growth and development.<sup>24</sup> Gut microbiota can affect host metabolism by modulating energy extraction,<sup>7</sup> immunity,<sup>12,13</sup> and lipid metabolism.<sup>25</sup> Whether maternal endocrine related changes in metabolic function may be mediated by gut microbiota is unknown.

The intestinal epithelium is the interface between the host and the gut microbiota. The gut mucosal layer serves as both a barrier to pathogenic bacteria and a source of oligosaccharides for mucin-degrading bacteria including *Ruminococcus*, *Bacteroides*, *Bifidobacterium*, *Clostridium*<sup>26</sup> and *Akkermansia*.<sup>27</sup> The relative abundance of *Akkermansia* has recently been shown to be inversely proportional to body weight and is decreased in genetically and diet induced obese mice.<sup>28</sup> Our observed increase in the abundance of *Akkermansia* during pregnancy does not correlate with increased maternal body weight. It may be that increased mucin-degrading bacteria serve to provide substrates for other commensal bacteria (*Allobaculum*) and facilitate metabolic pathways that serve as barriers to pathogens and permeability. Estrogen receptors (ERs) have been localized in all segments of the gut epithelium<sup>29</sup> and are known to mediate epithelial function.<sup>30</sup> Whether estrogen participates in changing the composition of the mucus layer during pregnancy is unknown, although it seems likely that an interactive effect between pregnancy-related hormonal changes and microbial population shifts would impact the gut microbial niche environment. Indeed, data in hamsters has shown a positive correlation between circulating leptin levels (an adipokine) and the presence of *Akkermansia* and *Allobaculum*<sup>31</sup> and it is known that leptin levels rise with pregnancy.<sup>32</sup>

Generally, high caloric intake has been shown to favor Firmicutes over Bacteroidetes where in cases of obesity, the ratio of Firmicutes to Bacteroidetes increases,<sup>33</sup> although data are conflicting.<sup>34</sup> Despite similarities in the metabolic constitution of pregnancy and obesity, including insulin and leptin resistance, our data show no significant shift in the Firmicutes to Bacteroidetes phyla ratio either with the onset of pregnancy or throughout advancing gestation in control fed mice, whereas *Akkermansia* increased with advancing gestation. This is consistent with human studies investigating early versus late pregnancy states<sup>14</sup> although maternal gut bacterial counts increase,<sup>16</sup> and

an expansion of  $\beta$  diversity occurs with advancing gestational age.<sup>14</sup>

### Pregnancy induced microbial shifts are dependent upon maternal dietary intake before and during pregnancy

We show that pregnancy-associated shifts in the maternal gut microbiota are dependent upon the mother's diet prior to and during pregnancy. *Akkermansia* (phylum Firmicutes) and *Bifidobacterium* abundance are increased in mothers fed a HF diet before and during pregnancy. Notably, only half of the genera that increased with pregnancy in control animals were also increased in high fat pregnancies; where in high fat pregnancies distinct genera were increased. We recognize the fact that our data are based upon 3 HF pregnancies, but despite this relatively low sample, our data are robust enough to show distinct differences due to pregnancy and to diet. Taken together, these data suggest that a high fat diet before pregnancy significantly alters the type of microbial changes that occur at the onset of pregnancy.

Despite our lack of knowledge regarding the exact role of these pregnancy-associated microbial shifts on maternal metabolism, the predicted metabolic pathways suggest that high fat diet before and during pregnancy increases numerous metabolic signaling pathways. In particular, those of fatty acid, ketone, cysteine, methionine, and vitamin metabolism are significantly elevated in HF pregnancies compared to control pregnancies. Of note, there appears to be a diet x pregnancy interaction in these predicated pathways, where the pregnancy-induced changes in these pathways are dependent upon the preconception diet. It is presently unknown whether these pathways provide substrates for the bacterial niche environment or whether these are substrates that could be utilized by the mother. The uptake of fatty acid, ketone, and sulfur containing amino acids by the mother would be consistent with the metabolic characteristics of late pregnancy where lipolysis results in increased maternal circulating free fatty acids, triglycerides and ketones.<sup>24</sup>

It is interesting to note that predicted primary and secondary bile acid metabolism pathways are decreased in HF pregnancies. Primary bile acid synthesis occurs in the liver where products are transported to the gut within which bacterial dehydroxylation occurs to form secondary bile acids that are integral for cholesterol, glucose and lipid metabolism, as well as fat soluble vitamin absorption<sup>35</sup> and more recently bile acids are thought to play a role as signaling molecules.<sup>36</sup> Bile biosynthesis during pregnancy is thought to remain similar to control values.<sup>37</sup> In cases of obesity, taurine-conjugated bile acids have been shown to be increased in the cecum of high fat-fed mice and may act as a marker of energy utilization behavior between the bacterial community and the host.<sup>38</sup> Whether this occurs during pregnancies complicated by obesity, however, is unknown, but would be consistent with our previously observed changes in maternal metabolic function.<sup>19</sup>

We have predicted changes in metabolic capabilities due to the shift in microbial populations due to periconceptional HF diet using PiCRUST.<sup>21</sup> Further metabolomics studies will be required in order to establish these inferred microbial relationships to metabolic functionality. Nevertheless, these predicated

pathways can be used to probe further into the impact of maternal HF diet on adaptation to pregnancy.

Human data have suggested that pregnancy imposes changes in the maternal gut microbiome<sup>14,16</sup> and our data in mice are consistent with these observations, although the functional consequences are still unclear. No doubt there are significant species differences in gut microbial populations and their influences on host metabolic function both in the pregnant and non pregnant state, but the use of animal models are an essential starting point for studying the gut microbiota especially in circumstances that do not permit invasive investigations in humans—particularly during pregnancy.

In conclusion, we show in mice that diet and pregnancy have interactive effects on the composition of the female gut microbiota. We show that pregnancy-induced changes in the female microbiota occur immediately at the onset of pregnancy, and are vulnerable to modulation by maternal diet. HF diet intake before and during pregnancy results in distinctive shifts in the maternal gut microbial community in a gestational-age dependent manner and that these shifts result in significant differences in the abundance of genes that favor lipid metabolism, glycolysis and gluconeogenic metabolic pathways over the course of pregnancy. Future studies are set to determine whether these shifts result in changes in maternal nutrient absorption and how maternal dietary induced microbiome shifts impact on fetal growth and placental function.

## Methods

### Animal model

All experiments were approved by the Animal Ethics Committee at McMaster University (AUP 12-38-10). Ten six-week-old female C57BL/6J mice were obtained from Jackson Laboratories. All mice were fed a control diet and after one week in our facility, baseline fecal samples were collected ( $n = 10$ , identified as Baseline), after which, mice were randomly assigned to one of 2 nutritional groups: 1) Control diet (Con;  $n = 5$  17% fat, 29% protein, 54% CHO, 3 kcal/g; Harlan 8640 Teklad 22/5 Rodent Diet) or 2) high fat diet (HF;  $n = 5$ ; 45% fat, 20% protein, 35% CHO, 4.73 kcal/g, Research Diets Inc.. D12451) (Fig. 1). All mice were housed individually in standard mouse cages with free access to water and 150 g of food, in the same room with a constant temperature of 25°C, a 12-h light, 12-h dark cycle and maintained on their respective diets for 8 weeks. Body weight and food intake were recorded. Following 6 weeks of nutritional intervention, fecal samples were collected (identified as Week 6) to examine the effect of diet on non-pregnant female microbiota.

After 8 weeks of dietary intervention all mice were housed with a C57BL/6J male and mating was confirmed by the appearance of a vaginal plug. If no plug was sighted after 7 days of consecutive mating, the mice were excluded from the study. Of the  $n = 5$  Con and  $n = 5$  HF females, we were able to confirm pregnancy in  $n = 5$  and  $n = 3$ , respectively. This is not inconsistent with high fat diet-induced difficulties in fertility in mice.<sup>17</sup> The

morning of vaginal plug identification is described as day (embryonic day) E0.5 of gestation. Pregnant females were housed individually and provided with free access to water. Throughout pregnancy maternal gestational weight and food intake were recorded, and maternal fecal samples were collected (in the same manner described above) at embryonic days (E) 0.5, E5.5, E10.5, and E15.5 and stored at  $-80^{\circ}\text{C}$  for future analysis. Fecal samples were collected from all mice throughout pregnancy.

### Genomic DNA extraction and 16S rRNA gene sequencing

Fecal pellets collected from these mice were used to infer gut microbial communities. Genomic DNA was extracted from fecal pellets as previously described<sup>39</sup> with a few modifications. Briefly, 0.2 g of fecal material was mechanically lysed using 800  $\mu\text{l}$  200 mM  $\text{NaPO}_4$ , 100  $\mu\text{l}$  guanidine thiocyanate-ethylenediaminetetraacetic-Sarkosyl, and 0.2 g of 2.8 mm ceramic beads (Mo Bio). A further mechanical and enzymatic lysis was performed using 0.2 g of 0.1-mm glass beads (Mo Bio), 50  $\mu\text{l}$  lysozyme (100 mg/mL), 50  $\mu\text{l}$  mutanolysin (10 U/ $\mu\text{l}$ ), and 10  $\mu\text{l}$  of RNase A (10 mg/mL) and incubated for 1 hour at 37°C. A second enzymatic lysis using 25  $\mu\text{l}$  25% sodium dodecyl sulfate, 25  $\mu\text{l}$  proteinase K, and 62.5  $\mu\text{l}$  5 M NaCl was further incubated for 1 hour at 65°C. The resulting solution was centrifuged (12,000  $\times$  g), supernatant removed, and combined with an equal volume of phenol-chloroform-isoamyl alcohol in a new microcentrifuge tube. This was centrifuged (12,000  $\times$  g) and the solution of the lowest density removed and combined with 200  $\mu\text{l}$  DNA binding buffer (Zymo), which was transferred to a DNA column (Zymo), washed, and eluted into sterile  $\text{H}_2\text{O}$ .

PCR amplification of the variable 3 (V3) region of the 16S rRNA gene was subsequently performed on the extracted DNA from each sample independently using methods described previously.<sup>39,40</sup> 341F and 518R rRNA primers were used, modified to include adapter sequences specific to the Illumina technology, and 6-base pair barcodes to allow multiplexing of samples. DNA products of this PCR amplification were subsequently sequenced using the Illumina MiSeq platform, resulting in 150bp paired-end reads.

### Processing and analysis of 16S rRNA gene sequencing data

Resultant FASTQ files were processed using a custom in-house developed pipeline for the processing of 16S rRNA gene sequencing data used previously.<sup>39</sup> In this pipeline, Cutadapt<sup>41</sup> is used to trim any reads exceeding the length of the 16S rRNA V3 region, PANDASeq<sup>42</sup> to align paired-end reads, AbundantOTU+<sup>43</sup> to group reads into Operational Taxonomic Units (OTUs) based on 97% similarity, and the RDP Classifier<sup>44</sup> as implemented in Quantitative Insights Into Microbial Ecology (QIIME)<sup>45</sup> against the Feb 4 2011 release of the Greengenes reference database<sup>47</sup> to assign a taxonomy to each OTU. Any OTU not assigned to the Bacterial domain were culled, as was any OTU to which only 1 sequence was assigned. This processing resulted in a total of 7985925 reads (mean 120999 reads per sample; range: 16524–237026) and 4982 OTUs (mean 631 OTUs per sample; range: 200–1242).

Analyses of these data were completed using various open source software. Taxonomic summaries were computed using QIIME.<sup>45</sup> Measures of  $\beta$  diversity were computed using Phyloseq's implementation of the Bray Curtis distance metric, and tested for whole community differences across groups using vegan's implementation of permutational multivariate analysis of variance (PERMANOVA) in the adonis command.<sup>48</sup> These results were visualized via Principal Coordinates Analysis (PCoA) ordination.<sup>46</sup> Calculations of genera which differed significantly between mouse groups were computed using DESeq2<sup>50</sup> (considered significant if the p-value was <0.01 after adjustment for multiple testing via DESeq2's implementation of the Benjamini-Hochberg multiple testing adjustment procedure) and visualized using R's ggplot2 package.<sup>49</sup> Estimations of changes in metabolic pathways were calculated using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)<sup>21</sup> and significantly different pathways of interest (considered significant if the p-value was <0.01 after adjustment for multiple testing) were visualized using R's ggplot2.<sup>49</sup>

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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#### Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

#### Authors' Contributions

WG assisted in the animal study, performed genomic DNA extraction, prepared samples for sequencing, interpreted data, and drafted the manuscript. FJW processed and analyzed sequencing data, performed statistical analysis, and drafted the manuscript. WG and FJW contributed equally to the preparation of this manuscript. MGS interpreted data and assisted in drafting the manuscript. CM carried out the animal work and collected samples. JDS interpreted data and helped to draft the manuscript. DSM designed the study, participated in animal work and data interpretation, and drafted the manuscript.

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