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Published

2019

Journal Title

American Journal of Physiology: Endocrinology and Metabolism

Version

Accepted Manuscript (AM)

DOI

10.1152/ajpendo.00265.2019

Pregnancy and diet-related changes in the maternal gut microbiota following exposure to an elevated linoleic acid diet

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Running head: Linoleic acid alters gut microbiome in pregnancy

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Key words: linoleic acid, gut microbiome, pregnancy

ABSTRACT

Dietary intakes of linoleic acid (LA) have increased, including in women of reproductive age. Changes in maternal gut microbiome have been implicated in the metabolic adaptions that occur during pregnancy. We aimed to investigate if consumption of a diet with elevated LA altered fecal microbiome diversity prior to and during pregnancy. Female Wistar Kyoto rats consumed a high LA diet (HLA: 6.21% of energy) or a low LA diet (LLA: 1.44% of energy) for 10 weeks prior to mating and during pregnancy. DNA was isolated from fecal samples prior to pregnancy (embryonic day 0 (E0)), or during pregnancy at E10 and E20. The microbiome composition was assessed with 16S rRNA sequencing. At E0, the beta diversity of LLA and HLA groups differed with HLA rats having significantly lower abundance of the genera *Akkermansia, Peptococcus, Sutterella* and *Xo2d06* but higher abundance of *Butyricimonas* and *Coprococcus*. Over gestation, in LLA but not HLA rats, there was a reduction in alpha diversity and an increase in beta diversity. In the LLA group, the abundance of *Akkermansia, Blautia, rc4.4* and *Streptococcus* decreased over gestation, whereas *Coprococcus* increased. In the HLA group, only the abundance of *Butyricimonas* decreased. At E20, there were no differences in alpha and beta diversity, and the abundance of *Roseburia* was significantly increased in the HLA group. In conclusion, consumption of a HLA diet alters gut microbiota composition, as does pregnancy in rats consuming a LLA diet. In pregnancy, consumption of a HLA diet does not alter gut microbiota composition.

61

62 ## INTRODUCTION

63 The omega 6 (n-6) polyunsaturated fatty acid (PUFA), linoleic acid (LA; 18:2n-6; cis, cis-9,
64 12-octadecadienoic acid), is an essential fatty acid that can only be obtained in the diet. In
65 Western societies, LA consumption has increased to three times the recommended daily
66 intake (28). In Australia, LA availability in the diet has increased by 120% (28) and in the
67 USA by 158% (5) over the past decades, primarily due to the increased use of plant-based
68 oils such as corn, safflower, sunflower and soybean in the food supply (35). The high intake
69 of n-6 FA in the Western diet is reflected in the fatty acid profile of pregnant and lactating
70 women (2). Optimal maternal health during pregnancy is critical for fetal development, and
71 maternal stressors can perturb fetal development leading to an increased risk of disease in
72 later life (13). LA can be metabolised into downstream lipid mediators, including pro-
73 inflammatory eicosanoids and prostaglandins (33). We have recently demonstrated, in a
74 rodent model of low verses high LA intake during pregnancy, that elevated maternal LA
75 increases pro-inflammatory prostaglandin concentrations, and alters the circulating lipid
76 profile of the mother during pregnancy (36). Dietary intake is an important determinant of
77 gut microbiota composition (7), suggesting that an elevated maternal LA diet may alter
78 microbiota diversity. Furthermore, gut microbiota diversity is strongly associated a range of
79 host functions that impact health, including inflammation and lipid levels (23).

80 The specific species composition of the gut microbiota can be a disease risk factor, as the
81 microbiota can regulate energy homeostasis and whole body metabolism (14).
82 Mechanistically, this is via the digestion of polysaccharides to produce essential nutrients (6),
83 so that bacterial diversity is important for the metabolism of a diversity of nutrients. LA is
84 biohydrogenated by microbes into the saturated fatty acid stearic acid (19), with a number of

intermediates, or bioactive metabolites (10). Previous research has demonstrated that diet can influence gut microbial diversity (44) and emerging research has demonstrated that pregnancy can impact the diversity of gut microbiota (15). In pregnancy, hormonal alterations modulate the maternal metabolic environment to ensure appropriate fetal nutrition. This places the mother in a state of metabolic dysfunction that becomes more overt as the pregnancy advances. This state of metabolic dysfunction can be further impacted by diet and contributed to pregnancy disorders that occur when physiological metabolic dysfunction becomes pathological. Current hypotheses suggest that changes to the gut microbiota, under the influence of pregnancy specific hormones, may contribute to the pregnancy associated metabolic changes (16). Further, alterations in the maternal gut microbiota during pregnancy can alter the microbiome and immune system of offspring later in life (29).

At this time, we do not know if elevated maternal LA consumption alters the fecal microbiome. Therefore, the current study aimed to investigate the effects of elevated maternal LA consumption on the composition of the gut microbiota prior to and during pregnancy in a rodent model. We hypothesised that exposure to elevated maternal concentrations of LA would alter gut microbiota composition, and pregnancy would reduce microbiota diversity independent of maternal LA intake.

MATERIALS AND METHODS

Ethical approval, experimental animal model and diet

Wistar Kyoto rats (8 weeks of age, n=6) were purchased from the Australian Resource Centre (ARC, WA, Australia) and housed in accordance to the Australian Code of Practice for Care and Use of Animals for Scientific Purpose after ethical approval being granted by the Griffith University Animal Ethics Committee (NSC/01/17/AEC).

Rats were housed in individually ventilated cages under 12 hours light-dark cycle at a temperature of 20-22°C and provided with standard food pellets during acclimatisation and tap water *ad libitum* throughout the study. After a week for acclimatization, female rats were randomised to either a control low linoleic acid (LLA: 1.44%) diet or a high linoleic acid (HLA: 6.21%) diet for 10 weeks. These diets were matched for carbohydrate, protein, fibre, n-3 PUFA and total fat content (36). The diets were matched for total fat intake by increasing the content of MUFA in the LLA diet (36). After 8 weeks of dietary exposure, vaginal impedance was measured daily for at least two estrous cycles using a rodent vaginal impedance reader (Muromachi Kikai Co. Ltd., Japan). Rats were considered ready for mating after 10 weeks of dietary exposure and when vaginal impedance was greater than 4.5×10^3 Ω and at this time were placed with a Wistar Kyoto male rat overnight. The day after mating was considered embryonic day 1 (E1). The rats were fed the LLA or HLA diet during gestation as well. The female rat was weighed daily and monitored for weight gain during pregnancy.

Fecal sample collection for microbiota analysis

To examine the effect of LLA vs. HLA diet on both the non-pregnant and pregnant female microbiota, fecal samples were collected from female rats at three time points; following 10

126 weeks of nutritional intervention (non-pregnant; identified as E0), and at E10 and E20. During
127 the time of fecal sample collection, rats were house individually. The fecal sample was
128 collected in the morning (10:00-11:00 am).

129 ***Extraction of DNA***

130 At the time of collection, fecal samples were weighed, and immediately frozen at −20°C.
131 DNA was extracted from the thawed fecal sample using a QIAamp DNA Stool Mini Kit
132 (Qiagen). Briefly, ~250mg of frozen stool was lysed and the DNA extracted using the
133 manufacturer's instructions. The eluted DNA was suspended in 200µL of buffer (Buffer ATE
134 provided by company) and stored -20°C.

135 ***Processing and analysis of 16S rRNA gene sequencing data***

136 16S sequencing of the V1-V3 region of the 16S rRNA gene was performed by the Australian
137 Genome Research Facility (AGRF), using the forward primer:
138 AGAGTTTGATCMTGGCTCAG and reverse primer: GWATTACCGCGGCKGCTG to
139 amplify the 27F-519R target. The read length for paired end sequences was 2x300 bp. The
140 sequences with 100% overlap were selected for downstream analysis. Paired-ends reads were
141 assembled by aligning the forward and reverse reads using PEAR1 (version 0.9.5). Primers
142 were identified and trimmed. Trimmed sequences were processed using Quantitative Insights
143 into Microbial Ecology (QIIME 1.8) USEARCH (version 8.0.1623) and UPARSE software.
144 Using usearch tools sequences were quality filtered, full length duplicate sequences were
145 removed and sorted by abundance. Singletons or unique reads in the data set were discarded.
146 Sequences were clustered followed by chimera filtered using "rdp_gold" database as
147 reference. To obtain number of reads in each OTU, reads were mapped back to OTUs with a
148 minimum identity of 97%. Using QIIME taxonomy was assigned using Greengenes database
149 (Version 13_8, Aug 2013).

Statistical analysis

The sequencing data did not adhere to the normal distribution and data analysis was performed using non-parametric statistics with $p<0.05$ as cut-off for statistical significance. Data was not corrected for multiple testing due to the small sample size. Data are presented as median and interquartile range (IQR). Gut microbiota composition at the genus level was compared using the Calypso software tool (43). Alpha diversity was assessed with the Chao1 and Shannon indices and beta diversity with unsupervised (PCoA) based on the Bray-Curtis dissimilarity statistic, PERMANOVA (Adonis) and supervised (RDA) analysis. Group comparisons were conducted with the Wilcoxon Rank test and LEfSe (linear discriminant analysis (LDA) effect size) analysis. LEfSe analysis identifies bacterial genera that predominantly explain the differences between the diet groups and the different gestations. We identified discriminating features that were ranked on their effects size based on a log10 scale.

RESULTS

Effect of a high maternal linoleic acid diet on maternal weight

Maternal consumption of HLA for 10 weeks prior to pregnancy and through gestation did not affect body weight either prior to pregnancy or during gestation (Figure 1) compared to LLA controls, similar to our previous study (36).

Gut microbiota composition in response to a HLA diet.

All results are presented at genus level. Before pregnancy (E0), there was no difference in alpha diversity between dams on LLA or HLA diets with either the Chao1 (Figure 2A) and the Shannon index (Figure 2B). There was a significant difference in beta diversity in both unsupervised PCoA (Figure 2C, $p < 0.05$) and supervised RDA analysis (Figure 2D, $p < 0.05$),

with the diet explaining 21% of the variation between the groups. PERMANOVA analysis showed that these variations were significant (P=0.006). In the group comparisons, HLA diet decreased the abundance of *Akkermansia, Peptococcus, Sutterella* and *02d06* and increased the abundance of *Butyricimonas, Coprococcus, Uncl. Clostridiales, Uncl. Victivallaceae* and *Uncl. YS2* (Figure 2E). The difference in the abundance of *Butyricimonas* and *Uncl. Victivallaceae* was significant after correcting for multiple testing (FDR=0.048 for both) but none of the other differences remained. This was confirmed by the LEfSe analysis, which showed that these bacterial genera were the main determinants of differences in the gut microbiota between the diets (Figure 2F).

Gut microbiota composition over gestation with maternal LLA diet

Alpha diversity decreased sharply at E20 as measured by the Chao1 (Figure 3A, $p < 0.05$) and the Shannon index (Figure 3B, $p < 0.05$). There was a significant difference in beta diversity in both unsupervised PCoA (Figure 3C, $p < 0.05$), supervised RDA analysis (Figure 3D, $p < 0.05$) and with PERMANOVA analysis (P=0.03). Gestational age explained 15% of the variation in beta diversity. In the group comparisons, the abundance of *Akkermansia, Blautia, rc4.4, Streptococcus, Uncl. Bacteroidales, Uncl. Christensenellaceae, Uncl. Mogibacteriaceae* and *Uncl. Ruminococcaceae* decreased over gestation and only the abundance of *Coproccocus* increased over gestation (Figure 3E). In the LEfSe analysis, *Streptococcus, Akkermansia, Uncl. Ruminococcaceae* and *Uncl. Bacteroidales* were associated with the gut microbiota at E0, *Peptococcus* with E10 and *Coprococcus, Ruminococcus* and *Bacteroides* with E20 (Figure 3F).

Gut microbiota composition over gestation with maternal HLA diet

Alpha diversity did not change in dams on the HLA diet as measured by the Chao1 (Figure 4A) and the Shannon index (Figure 4B). There was a trend toward significant difference in

beta diversity in the unsupervised PCoA (Figure 4C, p=0.08), supervised RDA analysis (Figure 4D, p=0.07) and PERMANOVA analysis (P=0.08). Gestational age explained only 9% of the variation in beta diversity. In the group comparisons, the abundance of *Butyricimonas* and *Uncl. Lachnospiraceae* decreased over gestation, the abundance of *Uncl. Victivallaceae* decreased significantly at E10 and the abundance of *Uncl. RF39* increased over gestation (Figure 4E). In animals on the HLA diet, *Butyricimonas, Uncl. Lachnospiraceae, Uncl. Ruminococcaceae* and *Uncl. Mogibacteriaceae* were determinants of the gut microbiota at E0, *Sutterella* at E10 and *Uncl. RF39* at E20 in the LEfSe analysis (Figure 4F).

Differences in gut microbiota composition between the maternal diets at E10 and E20

At E10, there was a decrease in alpha diversity as measured by the Shannon index in the HLA diet group (Figure 5A, P=0.017) but not in the Chao1 index (Figure 5B). There still was clustering of the samples from LLA and the HLA group with both PCoA (Figure 5C) and RDA analysis (Figure 5D) though PERMANOVA analysis showed that this was just borderline significant (P=0.06). When comparing the gut microbiota composition between the groups, there was significantly lower abundance of *Akkermansia* in the HLA diet group but higher abundance of *Bilophila, Roseburia*, *Uncl. Barnesiellaceae* and *Uncl. YS2* (Figure 5E). This was confirmed by LEfSe analysis that also identified higher abundance of *Desulfovibrio, Bacteroides* and *Uncl. Victivallaceae* in the HLA group contributing to the differences between the groups (Figure 5F). At E20, there was no difference in alpha diversity with either the Chao1 or the Shannon index (Data not shown). There were no differences in beta diversity in the PCoA analysis (Data not shown), the RDA analysis (Data not shown) and PERMANOVA analysis (P=0.48, data not shown). Only the abundance of *Roseburia* was

220 significantly increased in the dams on the HLA diet in the group comparison (Data not shown)
221 but no differences were observed in the LEfSe analysis (data not shown).

222

223

224 **DISCUSSION**

225 The level of dietary LA strongly influenced female rat gut microbiota composition. Further,
226 a high LA diet was associated with a suppression of the relative reduction in bacterial species
227 diversity observed in pregnant rats on a low LA diet. Our results demonstrate that HLA intake
228 before conception alters the composition of the gut microbiota in female rats, significantly
229 resulting in lower diversity of the gut microbiota in addition to changes in the abundances of
230 specific bacterial genera as compared with a LLA intake. Pregnancy reduces gut microbiota
231 diversity and alters gut microbiota composition only in dams on a low LA diet. Conversely,
232 in dams consuming a HLA diet prior to and during gestation, there are no changes to gut
233 microbiota diversity and only limited changes to gut microbiota composition, with distinct
234 genera changing abundance over gestation in each diet group. In late pregnancy, there are no
235 differences between dams on LLA or HLA diet with respect to gut microbiota diversity and
236 only one genus that was differentially abundant. A recent study demonstrated that microbiota
237 community taxonomic composition and diversity remain stable during pregnancy (9).

238 High intake of LA had large effects on the diversity and composition of the gut microbiota
239 prior to conception, and these changes may have functional consequences. For example, some
240 of the genera that increased in abundance with the HLA diet are known short chain fatty acid
241 producers including *Akkermansia, Butyricimonas, Coprococcus* and members of the
242 *Clostridiales* order. Abundance of *Akkermansia* and especially the species *Akkermansia*

muciniphila abundance has previously been linked to dietary fat intake, although this relationship is complex, with both increased and decreased abundance has been reported depending on the type of lipid, the overall composition of the diet or the presence of additional treatments (22, 24, 31, 34). In general however, *Akkermansia* abundance is negatively correlated with dietary fat intake (30). Here we observed a decrease in *Akkermansia* abundance both in response to HLA diet and over gestation in the LLA group. *Akkermansia* is a mucus degrader that synthesises short chain fatty acids that are generally considered beneficial for the host (e.g. increasing gut barrier function and stimulating beneficial mucosal microbial networks), and a modulator of the immune system (30). Depletion of this genus may therefore have detrimental effects on the host through the reduction in short chain fatty acids.

In response to the HLA diet, we not only observed decreased abundance of *Akkermansia* but also of *Sutterella,* and increased abundance of *Butyricimonas* and *Coprococcus.* Alterations in the abundances of these bacteria in response to increased dietary lipid intake have been reported previously (3, 26, 32), indicating that all of these genera may be sensitive to the fatty acid content of the diet. Human pregnancy has previously been reported to reduce the individual (alpha) diversity of the gut microbiota in humans (21) and rodents (15). Here we observed a similar decrease in gut microbiota alpha diversity, but only in rats on the LLA diet, suggesting that a HLA diet may perturbed normal changes in microbial composition. In a study of Sprague-Dawley rats on high fat and control diet during gestation and lactation, changes to beta diversity were reported only in animals on a high fat diet, not on a control diet (25). This is in contrast to our results, where we only observed altered beta diversity in the animals on the LLA diet. This may be due to differences in the rat strain, dietary composition and the small number of pregnant rats (four) on the control diet. The contrasting results could also be due to the differences between pre-pregnancy exposure to the diet, which

was present in our study but not in the high fat diet study given that it was stated that the dietary effect increased over time and overcame the pregnancy effect at later time points (25). In addition, overall weight and weight gain were not different between the two diet groups at any time point in this study, whereas weight was altered in the high fat diet study, suggesting that the changes in microbiota diversity in the high fat diet study may be related to weight gain/perturbed metabolism. Indeed, host-microbial interactions can impact the host's metabolism (21). In contrast, the changes in gut microbiota that we observed appear to be directly linked to LA dietary composition, rather than a secondary effect of a shift in metabolism.

We observed in this study, that in LLA rats, there was higher microbiome diversity compared to those consuming the HLA diet. This may be due to the reduced concentration of LA in the diet, but may also be due to the elevated concentration of MUFA in the LLA diet. The increase in diversity observed with an increased MUFA diet contradicts the findings from a recent systematic review (41). Wolters *et al.* determined that a high intake of MUFA in non-pregnant humans was thought to decrease bacterial numbers, with no effect on diversity (41). While the effect of an increase in MUFA independently was not assessed in our study, it should be noted that, at this time, there is a paucity of data concerning the effect of an elevated MUFA diet in pregnancy on microbiome diversity and abundance.

Transplantation of human third trimester gut microbiota samples into germ-free mice rendered them insulin resistant and fat, demonstrating the important link between gut microbiota and metabolic syndrome (21). One bacterium that has been associated with altered metabolism in pregnancy is *Blautia.* In early pregnancy, lower abundance of *Blautia* was reported in women with higher integrity of the gut wall barrier (27). Furthermore, women with excessive weight gain in pregnancy have higher abundance of *Blautia* (37) and in women

292 with gestational diabetes mellitus, an inverse correlation between the change in insulin levels
293 over gestation and *Blautia* abundance was reported (12). Here we have observed a decrease
294 in the abundance of *Blautia* over gestation but only in the LLA diet group. This may indicate
295 that the abundance of *Blautia* was higher in the LLA group at the start of pregnancy, though
296 not significantly so, similar to what has been reported previously in young healthy humans
297 on a low fat diet (39). *Blautia* can produce short chain fatty acids and decreases in its
298 abundance have been associated with insulin resistance (18, 40). Therefore, decreased
299 abundance of *Blautia* over pregnancy may be associated with the pregnancy-induced increase
300 in insulin resistance, indicating that a diet high in LA could perturb metabolism.

301

302 Members of the *Clostridium* cluster and *Roseburia* are known to metabolise linoleic acid
303 through conjugation (8), which is not greatly absorbed (20) but may have local beneficial
304 effects on the gut epithelium (17). *Roseburia* is also a short chain fatty acid producer and
305 immune modulator (11), and is increased in women consuming a vegetarian diet in early
306 pregnancy (4). The vegetarian diet in the study (4) was higher in LA content; therefore, HLA
307 diet may specifically increase *Roseburia* abundance over the course of pregnancy, similar to
308 what we observe in rats fed a HLA diet. *Roseburia* abundance increased in pregnancy in
309 BALB/c (11), suggesting that there may be an interaction between dietary intake and
310 pregnancy.

311 Emerging studies in animal models have shown the strong correlation between liver disease
312 and dysbiosis (1). The gut and liver communicate through biliary tract, portal vein and
313 circulation (38). We recently reported alteration in inflammatory cytokines in liver from the
314 rats fed with HLA at E20 (36). This change in hepatic inflammatory cytokines may be
315 associated with change in abundance of microorganisms. For example, in mouse model of

316 immune mediated liver injury, *Akkermansia muciniphila* had protective role by alleviating
317 inflammation (42). Therefore, the decrease in abundance of *Akkermansia* in rats fed with
318 HLA diet may be associated with liver inflammation previously observed in these rats (36).

319 In summary, our data demonstrate that HLA intake lowers the diversity and alters the
320 composition of the gut microbiota in rats. Pregnancy similarly reduces the diversity and alters
321 the composition of the gut microbiota but only in rats that were consuming a LLA diet. For
322 the rats that consumed a HLA diet prior to conception, there were only small changes to the
323 composition of the gut microbiota in pregnancy. These results suggest that HLA intake prior
324 to conception mimics the changes to the gut microbiota normally observed by the end of
325 pregnancy. These changes, may affect the risk of development of pregnancy complications
326 in women consuming a pre-pregnancy diet that is high in LA.

ACKNOWLEDGEMENTS

The authors would like to thank members of the laboratory and our collaborators for helpful discussions.

GRANTS

This work was supported by the Allen Foundation (DHH, AJM), and through the Australian Government's Collaborative Research Networks (CRN) program (AJM). Scholarship funding is provided by Griffith University International Postgraduate Research Scholarship (GUIPRS-NS), Griffith University Postgraduate Research Scholarship (GUPRS-NS) and Griffith Health Top Up Scholarship (NS).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR NOTES

*M. Dekker Nitert and D. Hryciw contributed equally to this work.

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Figure legends

Figure 1. Effect of HLA diet on maternal body weight. There was no difference between the LLA and HLA groups at different ages. Data expressed as mean ± SEM. n=6 (LLA) and n=6 (HLA) at E0, E10 and E20.

Figure 2: Gut microbiota composition at E0 in the LLA and HLA groups. A-B) There was no difference in alpha diversity between dams on LLA or HLA diets. C-D) There was a significant difference in beta diversity in both unsupervised PCoA and supervised RDA analysis, with the diet explaining 21% of the variation between groups. E) HLA diet decreased the abundance of *Akkermansia, Peptococcus, Sutterella* and *02d06* and increased the abundance of *Butyricimonas, Coprococcus, Uncl. Clostridiales, Uncl. Victivallaceae* and *Uncl. YS2*. F) Representation of bacterial genera driving the differences between LLA and HLA diets at E0 as shown by the LEfSe analysis. n=6 (LLA) and n=6 (HLA). *p<0.05, **p<0.01.

Figure 3: Gut microbiota composition over gestation in the LLA group. A-B) Alpha diversity decreased sharply at E20 as measured by the Chao1 and the Shannon index. C-D) There was a significant difference in beta diversity in both unsupervised PCoA and supervised RDA analysis. E) The abundance of *Akkermansia, Blautia, rc4.4, Streptococcus, Uncl. Bacteroidales, Uncl. Christensenellaceae, Uncl. Mogibacteriaceae* and *Uncl. Ruminococcaceae* decreased over gestation and only the abundance of *Coproccocus* increased over gestation in LLA group. F) Representation of the bacterial genera driving the differences between the gestations in animals on the LLA diet. n=6 (LLA) and n=6 (HLA) at E0, E10 and E20. *p<0.05, **p<0.01.

Figure 4: Gut microbiota composition over gestation in the HLA group. A-B) Alpha diversity did not change in dams on the HLA diet as measured by the Chao1 and the Shannon index. C-

D) There was a trend toward significant difference in beta diversity in the unsupervised PCoA (p=0.08) and supervised RDA analysis (p=0.07). E) In the group comparisons, the abundance of *Butyricimonas* and *Uncl. Lachnospiraceae* decreased over gestation, the abundance of *Uncl. Victivallaceae* decreased significantly at E10 and the abundance of *Uncl. RF39* increased over gestation. F) Representation of the bacterial genera driving the differences between the gestations in animals on the HLA diet. n=6 (LLA) and n=6 (HLA) at E0, E10 and E20. *p<0.05, **p<0.01.

Figure 5: Gut microbiota composition at E10 in the LLA and HLA groups. A-B) there was a decrease in alpha diversity as measured by the Shannon index in the HLA diet group but not in the Chao1 index. C-D) There was clustering of the samples from LLA and the HLA group with both PCoA and RDA analysis. E) There was significantly lower abundance of *Akkermansia* in the HLA diet group but higher abundance of *Bilophila, Roseburia*, *Uncl. Barnesiellaceae* and *Uncl. YS2* F) Representation of the bacterial genera driving the differences between LLA and HLA diets at E10. n=6 (LLA) and n=6 (HLA). *p<0.05.