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Effect of Pulse Consumption on Obesity and the Metagenome †

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Abstract: Grain legumes, which are commonly referred to as pulses, are staple foods in many parts of the world, but are infrequently consumed in most economically developed countries where the obesity pandemic is prominent. However, even in low pulse consuming countries such as the United States, there are sub-groups of individuals who consume large amounts of pulses. Systematic reviews of population studies indicate that pulse consumers have a lower risk for developing obesity. To determine whether these population-based findings could be modeled in preclinical systems in which such findings can be deconstructed, we used rat and mouse models of dietary induced obesity and reported that lipid accumulation was inhibited. In this study, we examined the relationship between inhibition of fat accumulation and changes in the gut associated microbiome in male C57BL/6 mice fed either a high fat diet with casein as the protein source or that diet formulation in which one of four pulses (chickpea, common bean, dry pea, or lentil) was substituted to provide 70% dietary protein with the remainder provided by casein. The seeds of each pulse were soaked, cooked, and then freeze-dried and milled; the resulting powder was used for diet formulation. Mice were ad libitum fed over the 17-week duration of the feeding trial. Cecal content was obtained at necropsy and immediately snap frozen in liquid nitrogen. Extracted genomic DNA was processed for 16s rRNA sequencing on an Illumina system. Significant differences were observed between each pulse and the high fat control diet in microbial phylogenetic diversity ($p < 0.001$) and accumulation of lipid in adipose depots ($p < 0.01$). Differences among pulses were also observed in both metrics. Microbiome alpha and beta diversity metrics, differences in abundance for each detected taxon among treatment groups and their relationships to changes in lipid accumulation in adipose storage depots are reported.

Keywords: adiposity; chickpea; common bean; dry pea; gut associated microbiome; intestinal function; lentil; pulses

1. Introduction

The occurrence of obesity, a global pandemic, increases the risk for a number of aging associated chronic diseases including non-alcoholic fatty liver disease, type-2 diabetes, cardiovascular disease, and certain types of cancer [1]. In these disease states, insulin resistance, chronic inflammation, and oxidation mediated cell damage are known to contribute to disease pathogenesis [2–6]. Deregulation of interconnected cell signaling pathways among tissues underlies the metabolic dysfunction that is observed. While the importance of environmental effects on gene expression are well established, there has been a lack of compelling evidence that an individual's diet exerts effects on the signaling

networks that impact disease outcomes including mortality. This has led to increased reliance on prescribed drugs, while limiting the attention given to food-based interventions in chronic disease prevention and control [7]. However, emerging evidence now supports significant impacts of diet that are mediated by its interaction with the gut microbiome that result in reduced risk for chronic diseases [8–12]. As an example of this, recent meta-analyses indicate that consumption of grain legumes, i.e., common bean, chickpea, dry pea, and lentil, also referred to as pulses [13], is associated with improved weight management relative to populations in which the consumption of pulses is low [14,15]. Given the complexity of obesity, we recently sought to determine whether the impact of pulses on body weight observed in prospective clinical studies could be reproduced under the controlled conditions that it is possible to achieve with the use of preclinical rodent models [16]. In polygenic models for obesity in both rats and in mice, pulse consumption was shown to be anti-obesogenic [16–18].

It is widely recognized that food components play a major role in establishing and maintaining the gut microbiome. Of the wide array of bioactive constituents in foods, dietary fiber is a key determinant. While there is no requirement for dietary fiber, the level currently recommended (14 g/1000 kcal) is achieved by less than 25% of the US population and the average short fall, exceeds 50% [19]. The problem can be traced to low food quality relative to dietary fiber content; particularly, a lack of grain legumes, i.e., pulse crops in the diet. Our recent publications document that the most commonly consumed pulses, dry bean (*Phaseolus vulgaris*, L.), chickpea (*Cicer arietinum* L.), dry pea (*Pisum sativum* L.), and lentil (*Lens culinaris* L.) have 2–3 times more fiber per 100 kcal edible portion than other commonly promoted dietary fiber sources, e.g., cereal grains [20,21]. Whether or not these pulses have equivalent effects on the composition and function of the gut microbiome is not known.

The objectives of the work reported herein were: (1) to compare the effects among pulses, i.e., chickpea, common bean, dry pea, and lentil, on the metagenome within the cecum, and (2) to investigate relationships between the effects of these pulses on the microbiome relative to the accumulation of lipid in adipose depots.

2. Materials and Methods

2.1. Experimental Animals and Design

Details of the feeding study have previously been reported [18]. Briefly, NCI C57BL/6NCrl male mice (21–28 days of age) were obtained from Charles River Laboratories NCI (Frederick, MD, USA). Upon arrival, the mice were fed a purified high fat diet. Mice were housed in solid bottomed polycarbonate rodent cages and maintained on a 12 h light/dark cycle at 27.5 ± 2 °C with 30% relative humidity. All mice had *ad libitum* access to diet and distilled water. All animal studies were performed in accordance with the Colorado State University Institutional Animal Care and Use Committee (protocol 18-7746A). At 5 weeks of age mice were randomized to their treatment groups. Mice were either continued on the high fat (HF) formulation (control diet) or were fed the HF diet formulation to which common bean, chickpea, dry pea, or lentil was added. The formulation of the experimental diets and the rationale for the concentration of pulses has been published. The experimental duration was 17 weeks. At necropsy, inguinal subcutaneous and abdominal visceral adipose tissue were harvested and weighed. Content of the cecum was harvested and snap frozen in liquid nitrogen until it was processed for genomic DNA extraction.

2.2. Microbiota Characterization

Using intestinal specimens collected at necropsy, DNA was extracted using the QIAamp PowerFecal DNA Kit (Qiagen, Germantown, MD, USA). 2 × 250 bp paired-end sequencing libraries of the V4 region of the 16s rRNA gene were constructed by using the Schloss MiSeq Wet Lab SOP followed by sequencing on an Illumina MiSeq instrument (Illuminia, San Diego, CA, USA) [22]. All 16S rRNA gene sequences were demultiplexed and processed with the open source bioinformatics tool QIIME 2, version qiime2-2020.2 [23]. Sequence reads were denoised using DADA2 and truncated at 220 bp and 120 bp for forward and reverse reads, respectively, aligned, filtered, checked for

chimeras, and OTUs were classified based on the Greengenes classifier, 13_8 99% OTUs from 515F/806R region of sequences (gg-13-8-99-515-806-nb) [24,25]. Functional predictions were made analyzing sequences by PICRUSt2 to infer functional content and visualized using STAMP v2.1.3 [26,27].

2.3. Statistical Analyses

Data were evaluated ANOVA, PERMANOVA, regression analysis, or multivariate analysis techniques. The Benjamini–Hochberg method was used to adjust *p*-values to control the false discovery rate. Data analyses were conducted using Systat, version 13.0 (Systat Software, Inc., San Jose, CA, USA), CLC Genomics Workbench version 20.0.4 (Qiagen Bioinformatics, Redwood City, CA, USA) and RStudio version 1.1.456 (RStudio, Boston, MA, USA) running R version 3.6.3 (The R Foundation for Statistical Computing, Vienna, Austria) and SIMCA v15 software (Sartorius Stedim Biotech, Umea, Sweden).

3. Results and Discussion

3.1. Effects of Pulses on Adipose Depot Mass

Previously, we have reported that feeding diets containing 70% of dietary protein from chickpea, common bean, dry pea or lentil reduced visceral and subcutaneous fat pad mass of male C57BL/6 mice [18]. In the analysis presented in Figure 1, all individual fat pad masses for an animal across treatment groups were subjected to unsupervised principal components analysis. The relative clustering of pulses in terms of their adipose tissue mass in comparison to the positive control (high fat obesogenic diet) and negative control (low fat non obesogenic diet) is shown in the three-dimensional scores plot.

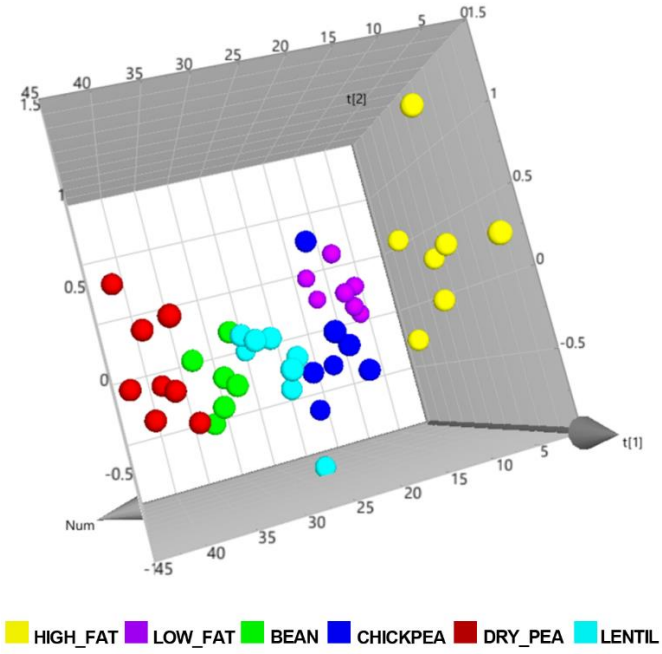

Figure 1. Principal components analysis of the effect of feeding pulse diets on adipose tissue mass.

Given that each principal component assigned to an animal is an unbiased measure of its adipose mass, each animal's first principal component value (PC1) was subjected to ANOVA. The overall *p*-value was highly significant ($p < 0.0009$) and post hoc analysis confirmed that all pulses were significantly different from either control, but not different from one another.

3.2. Effect of Pulses on Cecal Microbiome

It is recognized that the composition of microbiome varies throughout the length of the intestinal tract, particularly as it relates to conditions in the gut lumen relative to the growth of facultative and obligate anaerobes which are important groupings of commensal microorganisms. Therefore, we decided to evaluate the impact of pulse consumption on microbial composition of the luminal content of the cecum. In the mouse, the cecum interfaces with the ileum, i.e., the distal segment of the small intestine that plays a central role it metabolic signaling, and the ascending colon with which it abuts and that plays key roles in water reabsorption and short chain fatty acid metabolism. The focus on the cecum is in marked contrast to other reports of the impact of pulses on the fecal microbiome [28–31], which are translationally important, but may not capture information relevant to metabolic effects of the microbiome in regions of the gut that are anerobic.

Microbial abundance data (level of genus) from the 16s rRNA analyses of DNA extracted from cecal luminal content, were subjected to unsupervised principal components analysis in Simca in the same way the adipose depot mass data were evaluated in order to permit an opportunity to compare the clustering of data by diet group. The three dimensional scores plot is Figure 2.

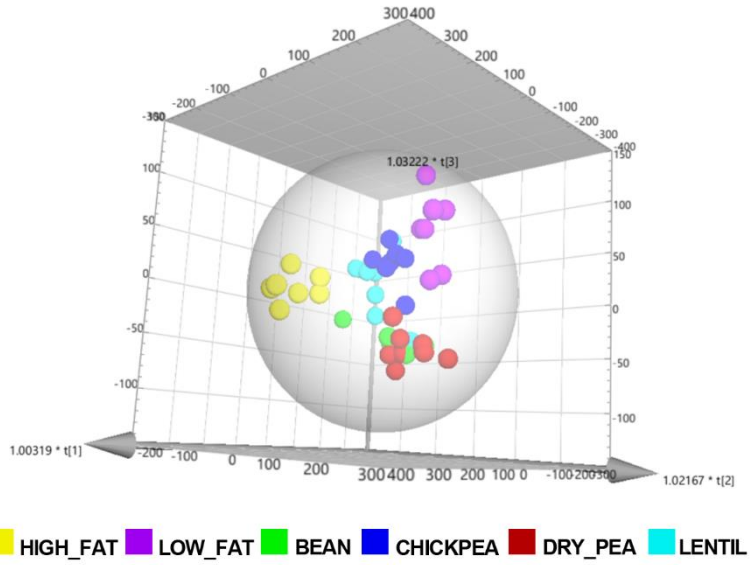

Figure 2. Principal components analysis of the effect of feeding pulse diets on the abundance of microorganism (level of genus).

Using the same strategy as described for adipose depot mass, each animal's first principal component for microbial abundance was evaluated by ANOVA. The model fit had a R^2 of 0.76, overall effect of diet, $p < 0.001$. Post hoc pairwise comparisons among treatment groups revealed that the unbiased principal component for the positive control (high fat, obesogenic) was significantly different from all other diet groups but differences among pulses and between each pulse and the negative control (low fat, non-obesogenic) were not significant.

Since there were overall effects of diet group on the principle components for adipose mass and microbial abundance (genus level), the relationship between these variables was examined via regression analyses (Figure 3). Overall, these analyses support a relationship between cecal microbial composition and adipose tissue mass, using unbiased measures of both variables. This opens the door to using the loading values from the principal components analyses to identify microorganisms (genus level) that contribute to this relationship.

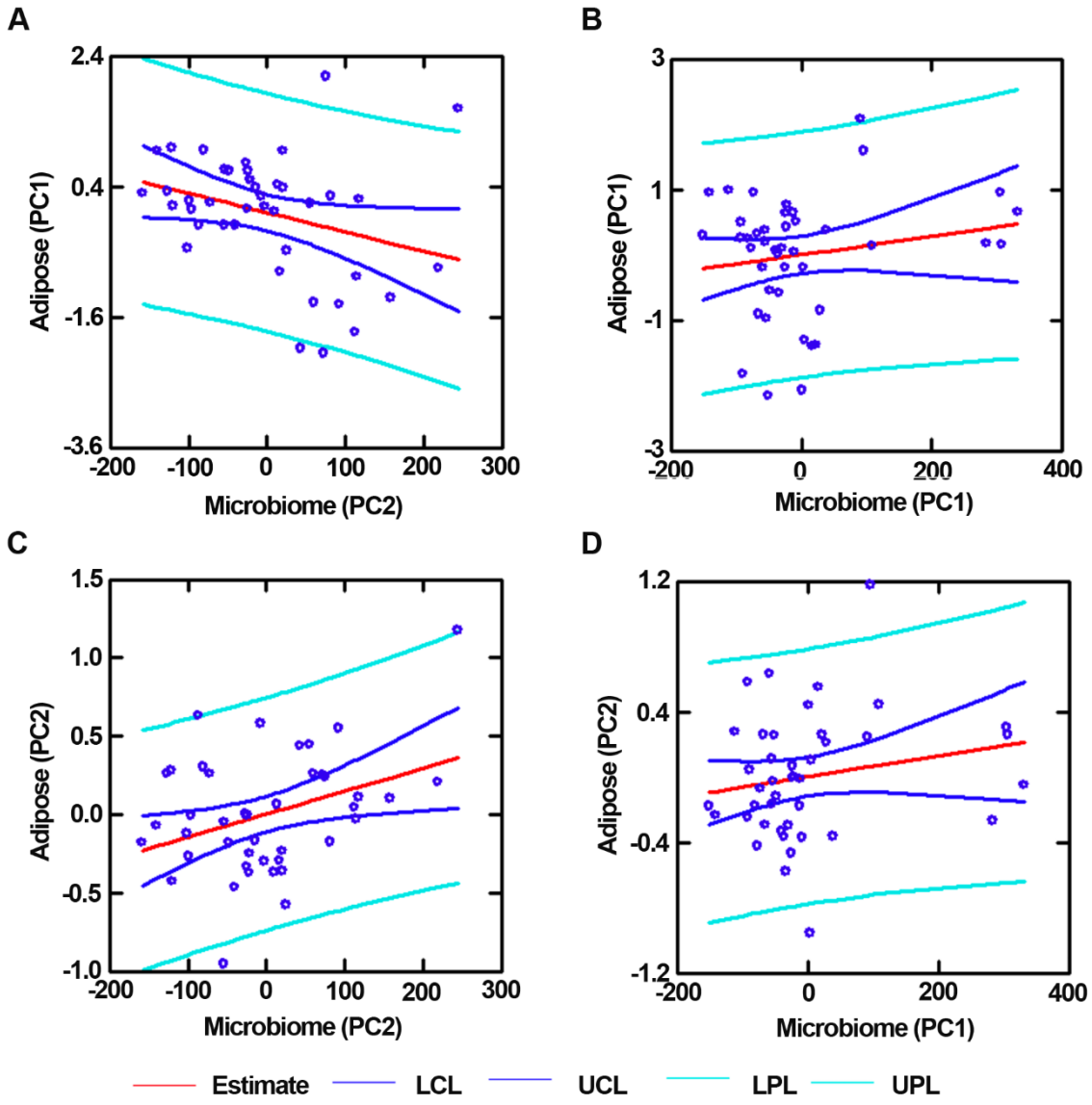

Figure 3. Regression analyses evaluating the relationship among principal components for adipose depot mass and microbial abundance (cecal luminal content). All regressions were statistically significant ($p < 0.01$), except for the regression analysis shown in panel D; lower confidence limit: LCL; upper confidence limit: UPL; lower prediction limit: LPL; upper prediction limit: UPL.

Another focus of our comparative analyses of the effects of pulses on the microbiome in the luminal content of the cecum employed metrics commonly used in the evaluation of metagenomic data.

Using the taxonomic profiling algorithm in the CLC Genomics Workbench, the impact of pulse consumption on species abundance is shown in Figure 4. Major shifts in percent abundance of microbial species were observed when pulses were compared to either the negative or positive dietary control group. Differences among pulses were also apparent. Of particular note, the changes in Akkermansia muciniphila that were reported by us and determined via qPCR using specific primers [17,18], are consistent with the differences observed in Figure 4. The observed pulse induced increase in A. muciniphila are consistent with health promoting effects of pulses. Gut levels of A. muciniphila have been reported to be inversely associated with obesity, diabetes, and inflammation [32–37]. At the level of phylum, pulses, collectively, induced an increase in bacteria in the phylum Bacteroidetes vs. Firmicutes ($p < 0.001$ for all). Given the controversial nature of the literature about whether an increase in the Bacteroidetes to Firmicutes ratio is consistent with health benefits [38–43], the importance of this observed requires further investigation.

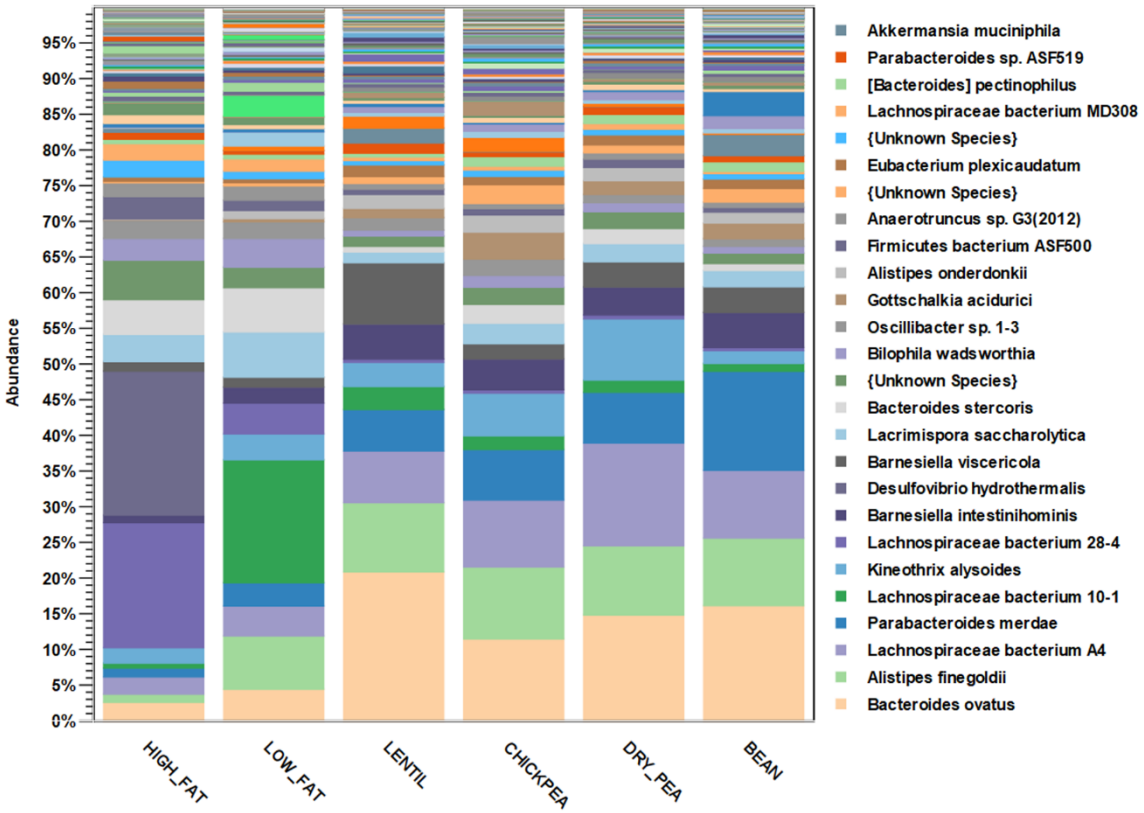

Figure 4. Effect of pulse consumption on microbial abundance (species level) using CLC Genomics Taxonomic Profiler.

We also evaluated the effect of pulse consumption on phylogenetic diversity using CLC Taxonomic Profiler (Figure 5). Unlike other metrics that have been presented thus far, statistically significant differences in phylogenetic diversity were observed. The rank order from lowest to highest was: lentil ≤ dry pea < common bean < chickpea. The lowest phylogenetic diversity was observed for the positive control (high fat, obesogenic). While the commonly held view is that higher phylogenetic diversity is prognostic for a healthy gut, there is no consensus on this point [9]. However, a phylogenetically diverse microbiota, gives rise to an immense metabolic potential. The microbiome consists of the genes that the cells constituting the microbiota harbor. A human microbiome collectively contains on the order of 3 million non-redundant genes; whereas, the human genome is comprised of approximately 20,000 genes [44]. Unsurprisingly, the gut microbiota executes essential functions that the body itself is incapable of performing. These functions include promotion of gut maturation, education of the immune system, protection against viral and bacterial pathogens, influence on brain activities and bodily.

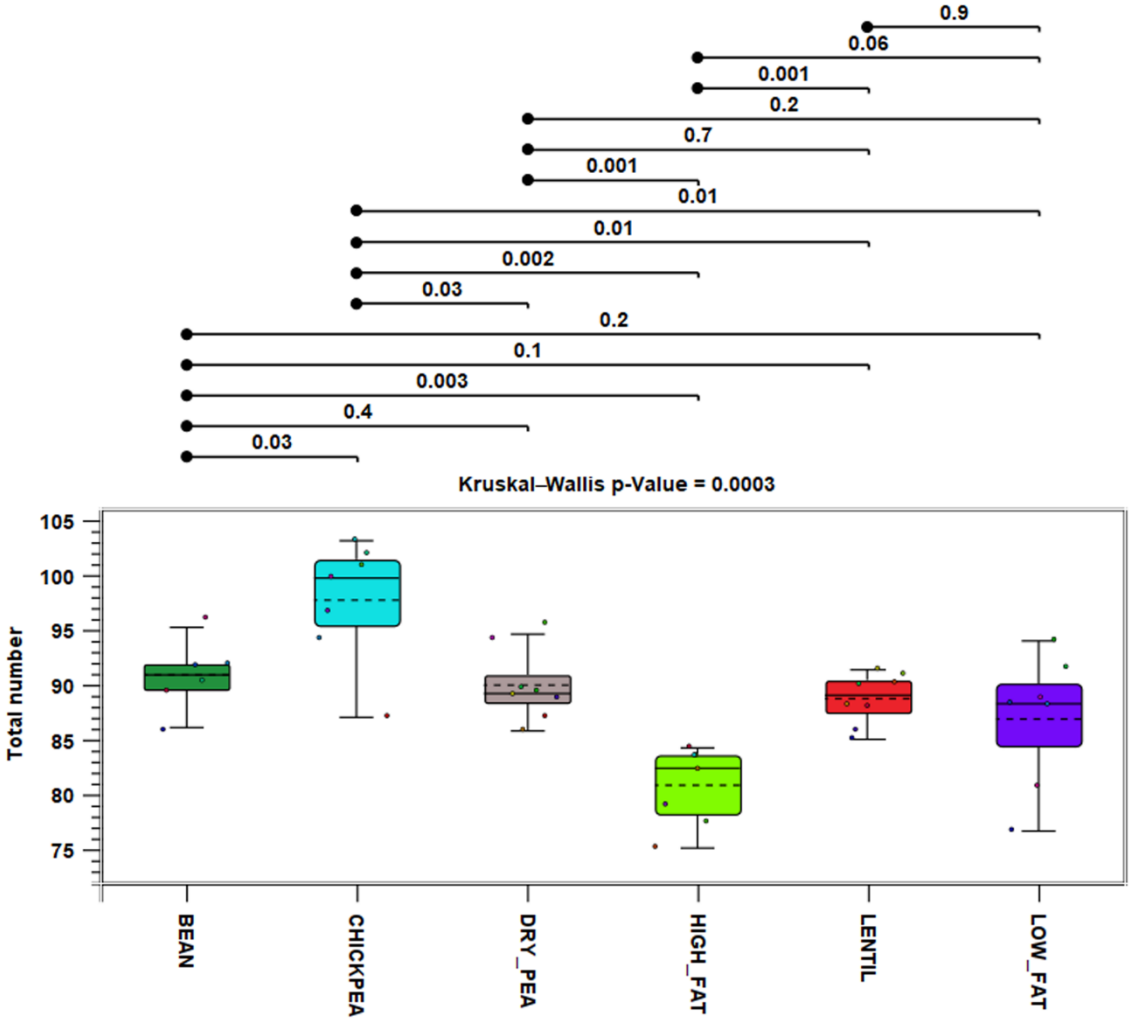

Figure 5. Effect of pulse consumption on phylogenetic diversity.

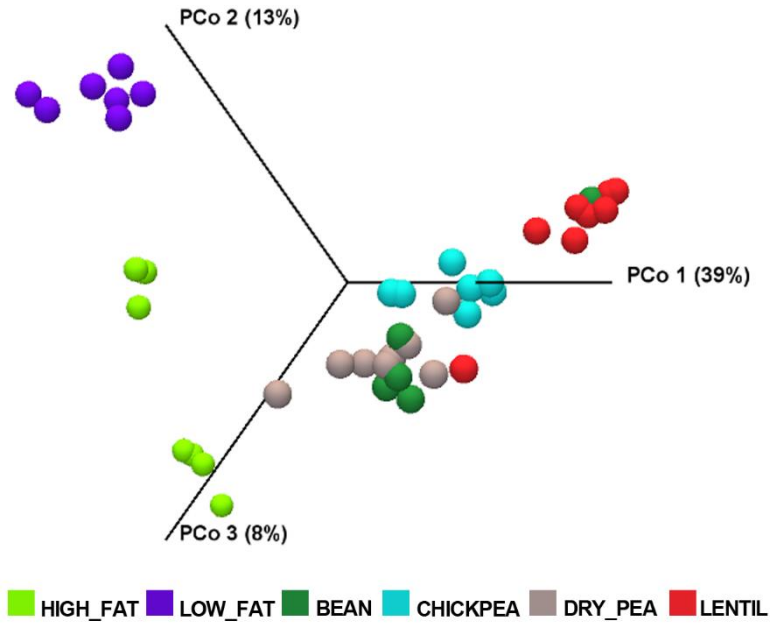

Figure 6. The effect on beta-diversity was also assessed

While the information in Figure 6 is similar to that presented in Figure 2, the statistical approach (Bray-Curtis) used to generate this figure is commonly used in the evaluation of metagenomic data. The hierarchical cluster analysis of these data showed that the positive control (high fat, obesogenic diet) was on a branch distinct from all other diet groups.

The metagenomic data were also evaluated for predictions of function using PICRUSt. That output was subject to unsupervised principal components in Simca using the same strategy described in the presentation of Figures 1 and 2. The scores plot from that analysis is Figure 7.

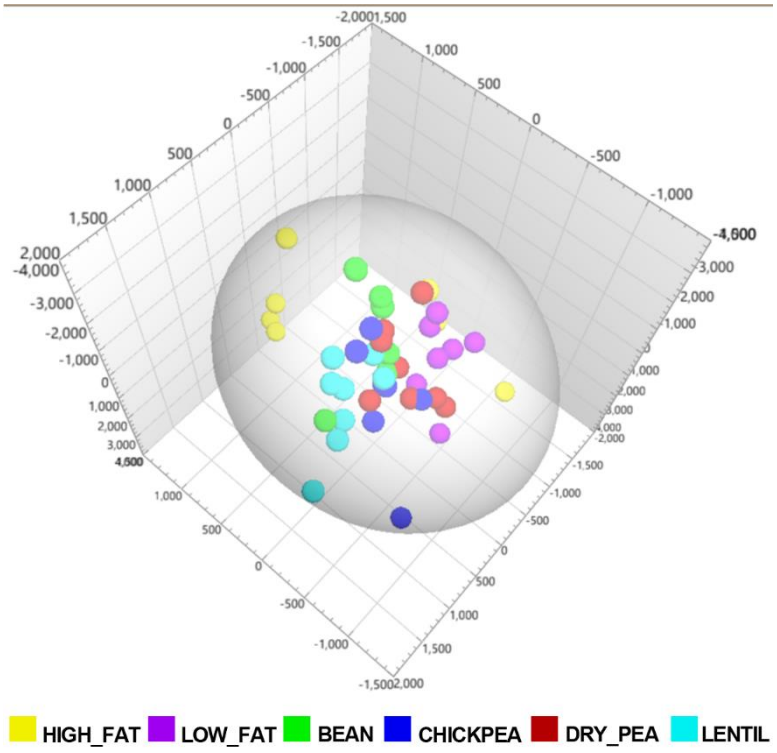

Figure 7. Effect of pulse consumption predicted function of the microbiome.

The ANOVA of the principle components for each animal from the PCA supported the assessment that there was an overall effect of diet group on predicted functional activity categorized via Kegg defined metabolic pathways. The post hoc analysis indicated that pulses differed from the control groups and among one another. This supports the hypothesis that all pulses are not created equal in terms of microbial populations whose cecal colonization they support.

4. Final Comments

There has been an explosive expansion of the literature on food, the gut microbiome, and human health and disease with major reviews being published regularly as illustrated in these citations [45–47]. From this burgeoning literature, the central thesis we advance is that increased granularity in the assessment pulse derived chemical exposures in the gut is required to understand their effects on microbial ecology and function. The ultimate effectors of the microbiota on their host are an array of molecules produced due to the transcription activity of the microbiome. Analysis of the effect of pulses on the meta-transcriptome and meta-metabolome are logical next steps in elucidating the role of the microbiome in mediating pulse health effects.

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