Determining the true digestibility of plant-based proteins and their effect on obesity, NAFLD, and the gut microbiome.

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Abstract

Introduction

The Protein Digestibility Amino Acid Corrected Score (PDCAAS) is the current FDA standard for determining protein quality. As more isolated plant proteins come onto the marketplace, the database of current PDCAAS values for these plant proteins needs to be expanded. There is also a need to understand more the health effects of isolated plant proteins. A serious and growing health issue is non-alcoholic fatty liver disease (NAFLD), for which no pharmacological intervention exists to treat it. Here, we determined PDCAAS values for wild rice, brown rice, pea flour, and two different pea flour protein isolates. In addition, we report the effect of feeding wild rice, brown rice, and pea flour on obesity, liver lipids, an early stage of NAFLD, and the gut microbiome.

Methods

Male Wistar rats (n=8 per group) were fed a diet of either casein (control diet, CC), wild rice (WR), brown rice (BR), pea flour (PF), pH precipitated pea protein isolate (PPI pH), salt solubilized pea protein isolate (PPI salt), or protein-free for 2-10 weeks. For the first 2 weeks of feeding the diets, which was for PDCAAS determination, each diet except the protein-free contained either 10% protein or as close as possible to it. After 2 weeks the PPI pH, PPI salt, and protein-free rats were euthanized, and the remaining rats were switched to a high fat background diet (High Fat Diet; HFD) to induce NAFLD. The experimental diets contained 40% by weight of either wild rice, brown rice, or pea flour and fed for 8 more weeks. Cecal contents, livers, and epididymal fat pads were harvested. Microbiome analysis of cecal contents was conducted by 16S sequencing.

Results

The PDCAAS values for the plant proteins tested are 0.61 for BR, 0.60 for WR, 0.73 for PF, 0.61 for PPI pH, and 0.70 for PPI salt. In the HFD trial, both WR and BR significantly reduced total liver lipids compared to CC ($p < 0.0001$). However, epididymal fat pad weight was not reduced in either of the rice groups compared to CC ($p > 0.05$). Serum cholesterol was also reduced in WR compared to CC ($p < 0.0188$). Additionally, p-AMPK/AMPK and ACC/pACC activation was not seen in the HFD trial ($p > 0.05$). In the HFD trial, microbiome analysis showed that the beta-diversity measure of community dissimilarity indicated that WR had a significantly different microbial profile compared to the animals fed the CC diet ($p < 0.003$) while BR had an increased abundance of lactobacillus ($p < 0.03$) compared to animals fed the CC diet.

Conclusions

Pea flour had the highest PDCAAS values among the plant protein sources. Wild rice feeding, but not pea flour feeding, reduced fatty liver and altered the gut microbiome. Wild rice should be further explored as a dietary approach to prevent development of non-alcoholic fatty liver disease (NAFLD), and to investigate whether hepatic lipid reductions are mediated by changes in the gut microbiome.

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Chapter I: Introduction

Plant based proteins are becoming increasingly popular in the US due to their perceived positive benefits for the environment as well as potential health benefits. However, the overall nutritional quality of these proteins has often been debated. One such issue is that plant proteins are known to be of lower protein quality than animal proteins for two key reasons: plant proteins are limiting in at least one essential amino acid, indicated as a lower amino acid score (AAS), and plant proteins are less digestible than animal proteins [1, 2]. Protein Digestibility Amino Acid Corrected Score (PDCAAS) is the current standard for determining protein quality [3]. With an increase in plant-based proteins being produced for use as a food ingredient, there is a need for the expansion of the database for PDCAAS values of plant proteins. Additionally, plant-based diets have been suggested to have health benefits relative to omnivorous diets. For example, plant-based diets are associated with less weight gain when compared to omnivorous or more animal-based diets [4-7]. This is important due to the rising prevalence of obesity in the United States [8-10]. An increased prevalence non-alcoholic fatty liver disease (NAFLD) has accompanied the rise in obesity. No pharmacological intervention exists to treat NAFLD [11]. Currently, the only recommended treatment is weight loss of 7-10% of an individual's body weight [12]. Considering the difficulty and sustainability for some people to achieve this body weight loss, if plant-based diets can reduce weight gain, they may also assist to reduce the incidence of obesity and NAFLD. Further research is needed to understand the potential health benefits of plant proteins in order to determine the overall quality of plant-based diets.

In the next chapter, this thesis will review protein quality and the history of protein quality methods leading to the PDCAAS methodology as well as the current literature and values for PDCAAS. Additionally, literature covering the characteristics and prevalence of obesity and its co-morbidity, NAFLD, will be reviewed with a focus on how plant-based diets and plant proteins may influence these adverse health conditions. Finally, important aspects of the gut microbiome will be covered and how it is linked to obesity, NAFLD, plant-based diets, and large intestinal fermentation.

Chapter 3 reports on the animal study involving feeding various plant protein sources. Specifically, wild rice, brown rice, pea flour, and multiple pea protein isolates were investigated for their protein quality and effects on obesity, NAFLD, and the gut microbiome in rats fed a high fat background diet. Lastly, chapter 4 contains appendices that provide additional information and detailed method descriptions used in the study described in chapter 3.

Chapter II: Literature Review

Protein Quality

History of Protein Quality Measures

Protein quality (PQ) is determined by two key aspects of a food protein: protein digestibility and the amino acid score which is defined as a protein's most limiting amino acid [3]. It is commonly regarded that plant proteins are of lower quality than animal proteins because of these two factors. Plant proteins either do not meet the requirements for one or more essential amino acid or are less digestible than animal-based proteins [1, 2]. However, due to environmental concerns and philosophical views against consuming meat, the use of plant-based proteins as a food ingredient has increased dramatically in the market over the last few years [13]. Because of this, defining and understanding plant protein quality has become more important than ever.

Various approaches have been used in the past to define PQ in foods. One of the earliest methods of measuring PQ was the protein efficiency ratio (PER). PER uses a single level of protein (10%) in the diet of rats and measures the ratio of weight gain to protein consumed over a 28 day period [14]. This was the official method for determining protein quality by the U.S. and Canada from 1919 until 1993. The equation used to calculate PER is as follows:

$$PER = (Weight\ gained\ of\ test\ animal)/(Protein\ consumed)$$

PER, however, was a highly variable assay because it did not consider the strain of rats used or their level of food consumption [15, 16]. Other limitations of PER include that it only measures growth and does not take into account maintenance requirements for the animal, that PER values are not proportional to one another (i.e. a PER value of 4 does

not mean the protein is twice as efficient as a protein with a PER value of 2), and the assay only considers one protein level which is problematic for plant protein sources of less than 10% protein [14]. Subsequently, Bender and Doell et al. proposed a modified version of the PER called the net protein ratio assay (NPR) [16]. This method considers the protein maintenance requirement by introducing a protein-free group to determine the level of protein needed to prevent weight loss. NPR also only takes 14 days instead of 28 days. The equation used to calculate NPR is shown below:

$$NPR = \{(weight\ gained\ of\ test\ animal) + (weight\ loss\ of\ protein\ free\ animal)\} / (Protein\ consumed\ by\ test\ animal)$$

The net protein utilization (NPU) assay takes the NPR one step further by measuring the nitrogen content of the test protein instead of using the body weight of an animal, as first described in Bender and Miller et al [17]. NPU can be calculated using the following formula:

$$NPU = \{(Body\ nitrogen\ of\ test\ animal) - (Body\ nitrogen\ of\ protein\ free\ animal) + (Nitrogen\ consumed\ by\ protein\ free\ animal)\} / (Nitrogen\ consumed\ by\ test\ animal)$$

This is a more accurate measure than NPR because it considers the biological value (BV) of the test protein. BV was first calculated using the Thomas-Mitchell method in 1923 [18] and is used in several PQ methods. The Thomas-Mitchell method is a 1 weeklong animal feeding trial where urine and fecal samples are collected daily, body weight

measurements are taken on the first and last days of the trial, nitrogen content of the test proteins are calculated, and daily nitrogen intakes are recorded. The BV of a food is the proportion of absorbed protein from a food that is incorporated into tissue proteins. It is a measure of how readily the consumed protein can be used by the animal. The equation for BV is as follows:

$$BV = \{(N_i - N_{e(f)} - N_{e(u)})/(N_i - N_{e(f)})\} \times 100$$

Where N_i = nitrogen intake from test protein, $N_{e(f)}$ = ((nitrogen excreted in feces from test protein fed animals) – (nitrogen excreted in feces from protein free animals)), and $N_{e(u)}$ = ((nitrogen excreted in urine from test protein fed animals) – (nitrogen excreted in urine from protein free animals)).

A limitation with BV is that this measure does not account for protein digestibility or amino acid concentrations. Differences in digestibility of different proteins does not affect their BVs [18].

The most modern and accurate measures of protein quality are the Protein Digestibility Corrected Amino Acid Score (PDCAAS) and the Digestible Indispensable Amino Acid Score (DIAAS). PDCAAS replaced PER as the preferred method of determining protein quality by the FDA and FAO/WHO in 1993 [19]. However, Rutherford et al. found that DIAAS and PDCAAS values for the same protein were significantly different. These differences were mainly due to the DIAAS method correcting digestibility for each amino acid of the test protein and not the entire protein and PDCAAS truncating the results of values >1 down to 1.0 [20]. However, when the same proteins were compared and not truncated, the digestibility differences still caused a

significant difference between the 2 methods [20]. Notably, the PDCAAS values were all higher than the DIAAS values. This difference was even more noticeable with lower quality plant proteins having an elevated value in the PDCAAS assay versus the DIAAS assay [20]. Consequently, the FDA pushed to use DIAAS as the preferred method for defining protein quality in 2013, but it was unsuccessful due to the greater technical challenges and expense of the method relative to the PDCAAS method [20, 21]. A new or modified method for measuring PQ that had the accuracy of DIAAS but the relative ease and lower cost of the PDCAAS would be highly desirable.

The Protein Digestibility Corrected Amino Acid Score (PDCAAS)

The 1989 Joint FAO/WHO Expert Consultation describes the currently accepted procedure for determining PDCAAS values as an *in vivo* model using rats [19]. PDCAAS is calculated as the product of the true digestibility of a protein and its amino acid score. Values range between 0 and 1 [3, 22]. A value of 1 represents a complete and well-digestible protein, while a value at or close to 0 represents an incomplete protein that is poorly digested and/or has an extremely low amino acid score. True digestibility (TD) is the amount of nitrogen intake versus the amount of nitrogen excreted in the feces, corrected for the metabolic fecal nitrogen not from dietary intake [19]. The calculation for TD is as follows:

$$TD = \frac{I - (F - F_k)}{I} \text{ X } 100$$

Where I is dietary nitrogen intake, F is fecal nitrogen, and F_k is the metabolic fecal nitrogen. F_k is determined by feeding of a protein-free diet and is calculated by the following equation:

$$F_k = total\ diet\ consumed\ by\ test\ protein\ group\ (g) \times \frac{mg\ of\ fecal\ nitrogen\ for\ the\ protein-free\ group}{g\ of\ diet\ consumed\ by\ the\ protein-free\ group}$$

The other component of the PDCAAS calculation is the amino acid score of a protein. The amino acid score is defined as the most dietarily limiting essential amino acid, using either the 1991 reference values for preschool aged children between 2-5 years old [23], or 2005 reference values based on preschool aged children between 1-3 years old [24]. Despite the updated reference values and contention from others in the field [25, 26], most literature still uses the 1991 reference values [3, 20].

The PDCAAS method being widely used by the nutrition community. However, it is a time-consuming and labor-intensive assay. As described by Hughes et al., the method requires feeding male Sprague-Dawley rats diets containing either a test protein, casein (as a positive control), or no protein (for determination of metabolic nitrogen excretion) [3]. Rats are fed their respective diets for 9 days, with food intake and feces collected daily for the final 5 days. The feces must then be weighed, dried, and analyzed for nitrogen content. Nitrogen content in the diets and feces may be determined using either the Kjeldahl or Dumas methods. The Dumas method has now become the preferred method due to its optimization by Saint-Denis et al. in 2004, using an automated nitrogen analyzer, manufactured by the LECO Corporation [27]. The Dumas method was first

described by Jean-Baptiste Dumas in 1883 and utilizes combustion of the test sample to burn and oxidize the organic elements into combustion gases. All other gases are eliminated except for nitrogen and nitrogen oxides. These gases are carried through a thermal-conductivity cell which generates an electrical signal proportional to the amount of nitrogen present [27]. The nitrogen value is then multiplied by 6.25 (Jones' Factor) to calculate the amount of protein [28]. Although Jones' factor is universally used, more specific values for unique animal and plant proteins have also been developed and result in more accurate measures of protein content [29]. Sosulski et al. claims that a more accurate nitrogen-to-protein-conversion factor is 5.70 for any blended diet of both animal and plant proteins. The issue with Jones' Factor is that it assumes the nitrogen content of proteins to be 16% [28], which may not be true for certain plant proteins. Therefore, a lower nitrogen-to-protein conversion factor should be used for those plant proteins to give a more accurate measure of their protein content. Once fecal nitrogen, dietary nitrogen intake, and metabolic fecal nitrogen have all been correctly calculated, these values can be used to determine the TD of a protein. TD multiplied by the most limiting amino acid ratio, determined from the amino acid concentration compared to the amino acid reference values, equals a proteins' PDCAAS value.

Definition, characteristics, and labeling across food industry

As of 1993, PDCAAS is the accepted method for determining PQ for foods in the U.S. and the FAO/WHO [19]. Consequently, if a company wants to make a protein claim on their product in the United States, they need a PDCAAS value to substantiate their claim. According to 21CFR101.54 from the FDA, a protein source can be called a "good"

source of protein if it has 10-19% of the Daily Value (DV) per reference amount customarily consumed (RACC) [30]. A protein source is considered "high" if the protein amount is above 20% DV per RACC [30]. The protein amount is determined by its PDCAAS value, and the protein content that is included on the nutrition label. A company must also provide the DV percentage of protein on the nutrition label if they wish to make a protein claim on the product. As more companies wish to make protein claims for their products, a greater need for known PDCAAS values will also be needed.

Many common foods already have PDCAAS values published for them and are available to consumers. Some known PDCAAS values are shown in **Table 1** below.

Table 1: List of PDCAAS Values

Protein Source	PDCAAS Values
Casein	1.00 [3, 31]
Soy Protein Isolate	1.00 [32]
Soy Protein Concentrate	1.00 [3]
Whey Protein Isolate	1.00 [32]
Whey Protein Concentrate	1.00 [32]
Pea Protein Concentrate	0.89 [3]
Cooked Peas	0.59 [20]
Kidney Beans	0.64 [20]
Rice	0.61 [20]
Oats	0.67 [20]
Peanuts	0.50 [20]

Rice Protein Concentrate	0.41 [20]
Beef	1.00 [33]
Almonds	0.23 [3]
Gluten	0.25 [22]
Whole Wheat	0.40 [32]
Pork	1.00 [33]
Chicken	1.00 [33]
Rye	0.59 [33]
Barley	0.59 [33]
Chickpeas	0.52 [34]
Pinto Beans	0.59 [3]
Black Beans	0.53 [35]
Navy Beans	0.67 [35]
Eggs	1.00 [33]
Corn	0.43 [33]

As mentioned above, PDCAAS values above 1.0 are truncated to 1.0. This list shows that soy and animal proteins are considered complete and well-digestible proteins because they all have a PDCAAS value of 1.00, the highest possible score. On the contrary, most plant-proteins are of lower quality than animal proteins because they have a lower PDCAAS value. Except for soy, pea protein concentrate is the next highest quality plant protein among those that have been tested so far. Based on the current

literature, most legumes and lentils have moderate PDCAAS values ranging between 0.5 and 0.6, while most grains and seeds have very low PDCAAS values and are low quality protein sources.

Despite plant proteins being of lower dietary quality relative to animal proteins, plant proteins can complement each other to improve their overall protein quality. This concept is known as protein complementation. Two proteins complement each other when each has a low amino acid score due to deficiencies in a different essential amino acid, but when combined, each protein makes up for (complements) the lower amount of essential amino acid in the other protein, and thus the amino acid score is greatly improved. An example of this is rice and beans, where rice is high in the sulfur containing amino acids cysteine and methionine, but low in lysine. Beans are usually low in methionine but are high in lysine. Therefore, these proteins make up for each other and together form a complete protein. Consuming meals in which proteins complement each other are commonly eaten and thus help alleviate the problem of plant proteins being of lower quality to animal proteins. However, few studies determining the PDCAAS values of protein combinations have been published. This will be important to better understand protein quality for plant proteins and for labeling purposes of new plant protein sources.

Plant-Based Diets and Obesity

Characteristics and Prevalence of Obesity

Obesity in the United States continues to be a major health issue for many individuals. Overweight and obesity are the first markers of metabolic syndrome which can lead to more adverse health effects. According to the World Health Organization (WHO), obesity is defined as a condition of abnormal and excessive fat accumulation in the adipose tissue to the degree where normal health status may be impaired [9]. Commonly, the Body Mass Index (BMI) is used to assess a person's degree of adiposity. It is calculated by dividing an individual's body weight in kilograms by their height in meters squared. This measure works very well for estimating adiposity in most people but is inaccurate in individuals who are either extremely muscular or extremely obese. As stated in the WHO technical report series #894, an optimal BMI is between 18.5 and 24.9, while someone is considered overweight who has a BMI between 25.0 and 29.9, and an obese person has a BMI >30.0 [9]. However, other measures of adiposity may be as important or more so in assessing the risk of excess body fat, including body fat percentage, waist-to-hip ratio, waist circumference, and body fat distribution [36]. Waist circumference and waist-to-hip ratio have been used as measures of central obesity and abdominal fat, which are known to increase the risk of co-morbidities compared to subcutaneous fat [36]. Thus, by itself, BMI is an imperfect measure for assessing the health risks associated with obesity.

Obesity rates over the last couple of decades have been skyrocketing worldwide. According to the CDC, every state in the U.S. in 2020 reported at least 20% obesity

prevalence with several states reporting over 40% obesity prevalence [37]. These values are also significantly higher in the Latino and Black communities [38]. Additionally, in the U.K., obesity rates have increased by 30% in women, 40% in men, and 50% in children within the last decade, resulting in 23% of adults being obese in 2007 and a prediction of 50% for 2050 [39]. Additionally, the World Health Organization's 2018 Obesity and Overweight Report estimates that obesity occurs in 39% of the global population [40]. The cause of this massive increase in obesity incidence is most likely due to an increase in the consumption of high fat diets. The consumption of a high fat diet has been directly linked to obesity and other co-morbidities, such as cardiovascular disease, type 2 diabetes mellitus, and certain cancers [41]. This excess consumption of dietary fats causes an increase in blood lipid levels which, in turn, deposit in excess into the adipose tissue [41]. This excess deposit of lipids in the adipose tissue causes obesity as we know it today and through further mechanisms causes the development of other non-communicable diseases.

The main function of adipose tissue is to store energy in the form of lipids. Another important function of adipose tissue is, therefore, to store excess lipids to prevent their accumulation in other tissues and blood. The adipocytes that make up the adipose tissue grow larger and increase in number to accommodate the excess dietary lipids consumed from a high fat diet. Once dietary lipids exceed the storage capacity of adipose tissue the adipocytes are no longer able to control the breakdown of lipids (lipolysis), which leads to an increase in fatty acids (FA) in the circulation and accumulation in non-adipose tissues such as the liver and skeletal muscle [42, 43]. This

can lead to the development of other diseases such as Type 2 diabetes (T2D), cardiovascular disease (CVD), and non-alcoholic fatty liver disease (NAFLD) [42, 44].

Plant Food's Impact on Obesity

One of the major reasons for the alarmingly high prevalence in obesity is due to shortcomings in the Traditional/Total Western Diet (TWD). The TWD is defined as a diet high in the consumption of fatty and processed meats, saturated fats, refined grains, salt, and sugars but lacking in the consumption of fresh fruits and vegetables [45]. A high consumption of processed meats and grains is known to increase the risk of chronic non-communicable diseases (NCDs), like obesity [45]. Meanwhile, whole grains, which the TWD is lacking in, are known to reduce risk of NCDs [46-48]. Fresh fruits and vegetables that are high in dietary fiber and phytochemicals can increase the feeling of satiety, reducing food intake and helping prevent obesity and other NCDs [49]. Phytochemicals are broad and varied in nature and function but may aid in fatty acid metabolism and mediate inflammation [50].

Many studies have examined whether consumption of plant-based diets results in weight or body fat loss. For example, Mishra et al. in 2013 conducted a multicenter study assessing weight loss of American adults switching to a plant-based diet. At 10 different job sites of the same U.S. Company, male and female employee volunteers were randomly divided into a diet intervention group and a control group. The control group was not given any dietary guidelines to follow, whereas the diet intervention group was asked to follow a low-fat, vegan diet for 18 weeks. They were also given vegan options in the cafeteria to make better diet choices while working. The diet intervention group saw

statistically significant reductions in body weight, BMI, and plasma lipids compared to the control group [5]. Kahleova et al. found similar results feeding a vegetarian diet to 74 male and female adults with T2D. Again, those consuming the plant-based diet had reductions in body weight, BMI, and LDL cholesterol [6]. Kahleova et al. further showed that a vegetarian diet shifted fatty acid metabolism, promoting β-oxidation and improving cellular response to insulin [6]. In humans, the fatty acid composition in serum phospholipids reflects both the effects of dietary fatty acid intake and endogenous fatty acid metabolism including: synthesis, β-oxidation, de-saturation, elongation and lipoperoxidation [51]. The key association from this study is that the reported metabolic shift was due to an increase in linoleic acid in serum phospholipids that caused significant changes in fatty acid composition, along with a reduction in visceral fat volume, and increased insulin sensitivity [6]. This same finding was seen in a 2001 study by Pelikanova et al [52]. If vegetarian diets can increase serum levels of linoleic acid which past research shows may cause a shift in fatty acid composition and metabolism, then it is possible that vegetarian diets would be able to reduce the risk of metabolic syndrome and obesity. Finally, Barnard et al. conducted a 74-week randomized clinical trial where individuals were randomly assigned to follow either a low-fat vegan diet, or the 2003 American Diabetes Association (ADA) dietary guidelines [7]. In contrast with other studies, patients on the vegan diet did not lose more weight than those on the conventional ADA diet plan. [7]. However, a recent meta-analysis of randomized controlled trials found that, overall, studies in which subjects consumed a vegetarian or vegan diet lost more weight than those on a non-vegetarian diet [4]. Therefore, vegetarian

diets should be further investigated for their ability to prevent and reduce obesity prevalence.

Inflammation during Obesity

It is now recognized that obesity results in a low-grade chronic inflammation. This chronic inflammation may be responsible for some of the deleterious effects of obesity. In 2010, Dowd et al. found that increased adiposity is associated with high serum levels of C-reactive protein (CRP) [53]. Discovered in 1930, CRP's main function in normal circumstances is an acute phase reactant in response to infection, trauma, and inflammation [54]. Specifically, CRP is secreted by the liver due to stimulation from the pro-inflammatory cytokine, IL-6 [54]. Chronically elevated levels of CRP have been associated with increased risk of cardiovascular disease (CVD). Specifically, CRP facilitates the uptake of lipids and monocytes that differentiate into macrophages into vessel walls [55-57]. CRP acts as an opsonin by binding to pathogenic microorganisms and damaged cells and mediating their phagocytosis [54]. This is an important role for helping host defense against infection, thus boosting the immune system.

Chronic inflammation can lead to liver damage. Alanine transaminase (ALT), along with alkaline phosphatase (ALP) and aspartate aminotransferase (AST), are sensitive indicators of both acute and chronic hepatocellular injury caused by inflammation of the liver [58, 59]. Other pro-inflammatory cytokines, such as Tumor Necrosis Factor-α (TNF-α) and Monocyte Chemoattractant Protein-1 (MCP-1), activate NF-κB and other inflammatory mediators, leading to chronic inflammation. Lipopolysaccharide (LPS) is a bacterial toxin from the gut microbiome that activates

Toll-like receptors and mediates signaling through NF-κB that also promotes increased expression of pro-inflammatory cytokines which can exacerbate the chronic low-grade inflammation associated with obesity [60].

As previously stated, vegetarian and vegan diets have been shown to prevent the development of obesity and other co-morbidities. These beneficial effects include the reduction of inflammation caused by obesity. Specifically, vegetarian and vegan diets have been associated with lower levels of CRP compared to omnivorous diets [61]. In a study by Franco-de-Moraes et al., 268 individuals were divided into 3 diet groups, a strict vegetarian diet, a lacto-ovo-vegetarian diet, or an omnivorous diet based on their dietary choices over the last year [62]. Results showed that CRP, LPS, and TNF-α/IL-10 levels were all significantly higher in the omnivorous group compared to the strict vegetarian group [62]. The noticeable increase in LPS by the omnivorous group possibly shows a relationship between animal products increasing LPS producing gram-negative bacteria in the gut microbiome that would further contribute to the chronic low-grade inflammation caused by obesity, as previously stated. Another trial comparing the anti-inflammatory effects between a vegan diet and the American Heart Association (AHA) diet supports the findings of the previous study as the vegan diet significantly reduced CRP levels compared to the omnivorous AHA diet [63]. However, a cross-sectional study comparing 36 omnivores and 36 vegans found no significant differences between any of the examined inflammatory biomarkers [64]. Therefore, specific plant foods might be a contributing factor to the reduction of inflammation caused by obesity seen in certain vegetarian and vegan diets compared to animal-based diets.

Uniquely, some studies have compared the differences in anti-inflammatory effects between animal and plant proteins. Markova et al. found that high protein diets of both animal protein and plant protein were able to reduce AST levels over the course of 6 weeks of high protein feeding [65]. However, this significant reduction in AST did not differ between the 2 different protein diets [65]. In a randomized, clinical trial by Holmer-Jensen et al., 11 obese patients consumed 4 different high fat diets consisting of either cod protein, whey protein, casein, or gluten [66]. The researchers found that all 4 proteins significantly reduced MCP-1 levels, with whey protein reducing levels significantly less than the other protein sources [66]. However, like the previous study discussed, the plant protein did not significantly reduce inflammation levels compared to other animal proteins. Finally, Lopez-Legarrea et al. conducted an 8-week randomized trial where 96 participants consumed either a high protein diet consisting of animal, meat, fish, or plant protein, or a normal protein control diet [67]. The results of this study showed that the animal and meat protein diets raised CRP, IL-6, and TNF-α levels after 8 weeks of feeding, while the plant and fish protein diets had no impact on inflammatory status [67]. Based on the current literature, it seems that plant proteins can reduce chronic inflammation caused by obesity but may not offer any additional anti-inflammatory effects compared to other animal proteins.

Some plant-related products have shown signs of reducing inflammation. Callcott et al. demonstrated in 2018 that polyphenols from rice can alleviate obesity-related oxidative stress and inflammation [68]. This study followed an *ex vivo* model where blood was drawn from 22 fasted, obese patients. Varying levels of purple, red, and brown

rice-derived polyphenols were incubated in human plasma before being analyzed for pro-inflammatory cytokine activity. Although the colored rice polyphenols did not impact plasma IL-6 levels, they all reduced TNF-α levels significantly [68]. In an intervention study by Callcott at al., they found that pigmented rice consumption was able to reduce inflammatory biomarkers in an obese cohort [69]. Red rice was able to significantly reduce plasma IL-8 concentrations 1 hour after eating, while purple rice was able to reduce plasma IL-6 and TNF-α concentrations 30 minutes after consumption [69]. Brown rice did not show any protection against inflammation in this epidemiological study [69]. This study, along with the previous *ex vivo* study, show that polyphenols from pigmented rice have significant ability to reduce inflammation due to obesity. Finally, this same group was able to show anti-inflammatory effects of colored rice in healthy individuals, not just in obese patients. Callcott et al. fed 24 healthy individuals red, purple, and brown rice and measured plasma levels of pro-inflammatory cytokines [70]. This method is very similar to what was done with the obese cohort study. The results using healthy human subjects were even more astounding than in the obese patients as purple and red rice were able to significantly reduce IL-6, IL-8, IL-10, and TNF-α plasma concentrations for 30 minutes to 4 hours after consumption [70]. Again, brown rice had no effect on reducing pro-inflammatory cytokine concentrations in the plasma [70]. These three rice studies along with the other studies looking at vegetarian diets and plant proteins show that plant foods and plant-based diets have anti-inflammatory properties that could help protect against obesity and its co-morbidities like NAFLD by reducing chronic inflammation in the adipose tissue and liver.

Non-Alcoholic Fatty Liver Disease (NAFLD)

Characteristics as a Risk Factor for Obesity

Non-alcoholic fatty liver disease (NAFLD) is defined as an excess accumulation of lipids in the liver and is considered a largely asymptomatic disease [12, 71]. NAFLD is highly associated with obesity and affects 25% of people worldwide [12]. NAFLD affects 80% of overweight and obese adults in North America and 57% worldwide [71].

There are two forms of NAFLD. One is simple fatty liver (non-alcoholic fatty liver, NAFL) which is the most common form of NAFLD and does not include much inflammation or scarring. The other is non-alcoholic steatohepatitis (NASH), which is the more severe form of NAFLD that results in irreversible scarring, inflammation, cirrhosis, and fibrosis of the liver, which may lead to liver cancer and liver failure [72, 73]. Unfortunately, there are no pharmacological treatments for NAFLD at this time, and the only known treatments are dietary modification and weight loss [11, 74], which are difficult for many individuals to adopt. Understanding how NAFLD develops is an important step in finding a better treatment or prevention for the disease.

Development of Fatty Liver Disease

NAFLD is the most common type of liver disease, characterized by aberrant fat accumulation in the liver that is not due to excessive alcohol consumption [75]. Development of NAFLD has been suggested to follow a “two-hit” or “multi-hit” model [12]. The “two-hit” hypothesis states that lipid accumulation in the liver from consumption of a high fat diet, a sedentary lifestyle, and other metabolic diseases, acts as

a "first hit." The excess fat in the cells of the liver allows for inflammation and other damage, a.k.a., the “second hit”, to initiate a cascade that leads to fibrosis [76, 77]. However, Buzzetti et al. in 2016 published a more robust “multi-hit” hypothesis that has begun to replace the “two hit” hypothesis [78]. This is due to many individuals with other NCDs developing NAFLD and NASH without it being caused by the issues stated in the “two hit” hypothesis. The “multi-hit” hypothesis states that there are several more factors that impact the development of NAFLD that are not included in the “two hit” hypothesis. These factors include dietary habits, environmental factors, genetics, hepatic insulin resistance, obesity, adipocyte proliferation, and the gut microbiome [78]. Insulin resistance can increase liver lipid accumulation by increasing *de novo* hepatic lipogenesis, inhibiting lipolysis in the adipose tissue, and increasing adipokine secretion [79]. The inhibition of lipolysis in the adipose tissue leads to a decrease in lipid oxidation and mobilization. Additionally, the gut microbiome can impact the development of NAFLD and NASH. Certain gram-negative bacteria can increase intestinal permeability which allows lipopolysaccharide (LPS) to enter the portal circulation. [76]. LPS is a potent inflammatory agent known to cause metabolic endotoxemia and increase liver inflammation [76]. The influence of the gut microbiome on NAFLD will be discussed in further detail in a section below. Given our current knowledge, it is likely that development of NAFLD and NASH is the result of multiple metabolic insults to the liver.

Fatty Acid and Cholesterol Metabolism in the Liver

Many of the metabolic pathways that maintain proper liver homeostasis become dysregulated during obesity and T2D. For example, the hyperinsulinemia that

accompanies T2D can cause the liver to become insulin resistant. This is due in part to the upregulation of lipogenic transcription factors like sterol regulatory element binding protein-1c (SREBP1c) [80]. This upregulation of SREBP1c results in increased lipid synthesis in the liver. However, the type of lipid that accumulates in the liver seems to be a factor In the development of NAFLD and NASH. In 2010, Koliwad et al. used a DIO diet using mice overexpressing diacylglycerol acyltransferase 1 (DGAT1). DGAT1 and DGAT2 catalyze the final step in triglyceride synthesis [80]. Mice that overexpressed DGAT1 in macrophages were protected from macrophage activation, systemic inflammation, and hepatic insulin resistance caused by diet induced obesity [81]. Yamaguchi et al. conducted a similar study in 2007 where they inhibited triglyceride synthesis by knocking out DGAT2 using a DIO mouse model [82]. This study found that DGAT2 KO mice had decreased levels of obesity and fatty liver, and showed major improvements in serum free fatty acids, insulin, and glucose levels. However, there was a significant increase in hepatic free fatty acids, liver damage, inflammation, and fibrosis [82]. The study by Koliwad et al. tried to accelerate triglyceride synthesis while the study by Yamaguchi et al. hindered triglyceride synthesis. The knockout study found an increase in liver damage due to an excess of hepatic free fatty acids, but less overall fat in the liver. Meanwhile the overexpression study did not find any liver damage due to low amounts of free hepatic fatty acids. These two studies together show that high amounts of hepatic free fatty acids lead to greater severity of NAFLD and NASH compared to a high concentration of triglycerides. This is interesting because it shows that the buildup of free fatty acids in the liver is what causes liver damage, not necessarily the accumulation of

all types of lipids. When adiposity increases, secretion of the anti-inflammatory adipokine adiponectin is reduced [83, 84]. Adiponectin activates AMP-activated protein kinase (AMPK) to its phosphorylated form (pAMPK) which promotes β-oxidation of fatty acids and inhibits lipid synthesis [83, 84]. With adiponectin reduced and less activation of AMPK, β-oxidation is reduced and the amount of lipid in the liver increases.

Another type of lipid with emerging implications in NAFLD and NASH development is cholesterol [85-87]. The cholesterol pool in the body comes from either *de novo* cholesterol synthesis from cells or dietary cholesterol consumption. The rate-limiting enzyme in cholesterol synthesis is 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) [88]. Cholesterol homeostasis is maintained in the liver by a family of transcription factors known as SREBPs [88]. Low-density lipoprotein receptors (LDLr) are also vital to maintaining cholesterol homeostasis by regulating plasma cholesterol levels in a negative feedback loop. LDLr in hepatocytes bind LDL cholesterol from the plasma, lowering plasma cholesterol concentrations and increasing cellular levels of cholesterol [89]. Elevated levels of LDL cholesterol have become a well-known risk factor for CVD and other metabolic diseases like NAFLD [90, 91]. The cholesterol that is formed by *de novo* synthesis in hepatocytes can be esterified by acyl-CoA-cholesterol acyl transferase and packaged into apolipoprotein B-100 (apoB-100) [92]. ApoB-100 forms a shell around the triglyceride rich very low-density lipoprotein (VLDL) which moves triglycerides and cholesterol esters into the bloodstream and to peripheral tissues away from the liver [92, 93]. Cholesterol from the peripheral tissues can accumulate in the liver by a process known as reverse cholesterol transport that is mediated by high

density lipoproteins (HDL) [92]. HDL is considered a cholesterol rich lipoprotein, however, unlike LDL cholesterol, high HDL cholesterol levels have been known to reduce risk of CVD [94, 95]. Interestingly, raising HDL cholesterol has not been shown to improve CVD risk, and HDL cholesterol has not shown benefits with other metabolic diseases [94-96]. HDL cholesterol is also in charge of moving dietary cholesterol from the intestines to the liver, where it is internalized by the hepatic scavenger receptor, SR-B1 [93]. Once cholesterol has been taken up by hepatocytes from HDL, it can be converted into bile acids or excreted into bile and eliminated in the feces [97]. This utilization and elimination of cholesterol by the liver is why HDL cholesterol is aptly named “good cholesterol” because it moves cholesterol into the liver for total body cholesterol maintenance and homeostasis. Meanwhile, LDL cholesterol is considered “bad cholesterol” because elevated levels of LDL cholesterol correlate with elevated plasma cholesterol levels as LDLr are not able to remove sufficient cholesterol from the plasma. This is another risk factor for metabolic disease, as previously stated [90, 91, 98]. Plasma triglyceride, HDL cholesterol, and LDL cholesterol levels are important measures associated with NAFLD development.

Gut Microbiome

The gut microbiome is defined as the population of microorganisms that live in the gut of a host. The gut microbiome can be found all along the gastrointestinal tract, but in humans this is mostly in the large intestines, while in rats and many other animals it is mostly in the cecum. The gut microbiome consists of many different species of microorganisms, which make up trillions of cells that together form the gut microbiome [83-85]. The gut microbiome consists of bacteria, fungi, viruses, and other eukaryotes, and is influenced by many factors and has become an important field of interest for understanding disease in its host. Factors that can change the gut microbiome are host genetics, disease, physical activity, diet, and environment [84].

Characteristics Relating to Obesity and NAFLD

The gut microbiome is now being targeted as an NAFLD therapeutic strategy, as it is clearly connected to diet, obesity, and insulin resistance [99]. The "multi-hit" hypothesis used for understanding the development of NAFLD considers the gut microbiome as one of the "multiple hits" for development of NAFLD and NASH [78]. Specifically, certain microbiome profiles have been associated with obesity and NAFLD [100, 101]. Most notably, obesity has been associated with an increase in the *Firmicutes* to *Bacteroidetes* ratio [102, 103]. Additionally, human studies have shown correlations between lean subjects and greater abundance of *Bacteroidetes, Bifidobacterium,* and *Lactobacillus,* and lower abundance of *Firmicutes* and *Actinobacteria* [104-108]. The associations between the gut microbiome and NAFLD are slightly more complicated and controversial.

In 2016, Wang et al. reported a greater abundance of gram-negative bacteria in nonobese patients suffering from NAFLD relative to nonobese subjects without NAFLD [109]. This greater abundance in gram-negative bacteria was correlated with a greater abundance of *Bacteroidetes* and lower abundance of *Firmicutes* [109]. However, this is the opposite trend of what has been shown in obese patients, as Wang et al.'s findings indicate that patients suffering from NAFLD, but not obesity, have a lower *Firmicutes* to *Bacteroidetes* ratio, not a higher ratio [102, 103, 109]. A decrease in short chain fatty acid (SCFA) producing bacteria, an increase in LPS producing bacteria, an increase in serum ALT levels, and a decrease in *Ruminococcus* was also seen in NAFLD patients [109]. However, Jiang et al. and Del Chierico et al. found an increase in *Ruminococcus* and *Dorea* in both of their respective studies using an obese animal model and an adolescent human model suffering from NAFLD, NASH, or obesity compared to healthy controls not suffering from obesity or NAFLD [110, 111]. Additionally, Raman et al. found an increase in the phylum *Firmicutes* in patients suffering from NAFLD, contrary to the decrease in *Firmicutes* that was shown in Wang et al [109, 112]. Finally, Michail et al. showed that children with NAFLD had higher levels of *Prevotella* compared to healthy children [113]. Again, the opposite was found in a 2016 study where human patients suffering from liver fibrosis and NASH had significantly decreased levels of *Prevotella* [114]. Clearly, there are conflicting results as it pertains to the gut microbiome and fatty liver. These microbiome markers associated with NAFLD are population and study-specific and is something that should be considered when using results related to the gut microbiome to make health claims. Understanding how the different genera in the gut

microbiome communicate with the liver and host tissues for positive health outcomes, and how to elevate those beneficial bacteria is important for proper recommendations to achieve better health outcomes.

Influences of Whole Grain Rice Components on the Microbiome

Past research has shown that wild rice can induce beneficial changes to the gut microbiome. Moghadasian et al. reported that LDLr KO mice fed an atherogenic diet with 60% wild rice reduced levels of inflammation compared to mice fed a control atherogenic diet without wild rice, and that the gut microbiota profile also differed significantly between the groups [115]. Most notably, mice fed wild rice had greater abundances of *Anaeroplasma, Acetatifactor muris, Lactobacillus, Oscillospira,* and *Dubosiella newyorkensis* compared to the mice on the atherogenic control diet [115]. Also, wild rice fed mice had decreased abundance of *Barnesiella, Butyrivibrio*, and *Oscillibacter* compared to the atherogenic control fed mice [115]. In 2020, Hou et al. fed mice either a normal chow diet, a high fat diet, or a high fat diet with wild rice for 11 weeks [116]. Investigators performed 16S sequencing and found that mice fed the high fat diet significantly reduced the richness and diversity of the gut microbiome compared to the normal chow diet, but wild rice feeding reversed these changes so that the taxonomic profile was significantly similar to the normal chow diet [116]. Specifically, the high fat diet decreased the abundance of *Bacteroidetes* and increased the abundance of *Firmicutes*, but wild rice feeding recovered these levels and reduced the *Firmicutes* to *Bacteroidetes* ratio compared to the high fat diet [116]. Mice fed a high fat diet with wild rice also significantly increased the abundance of *Lactobacillus* and reduced the

abundance of *Prevotella* and *Staphylococcus* compared to both the normal chow and high fat diets [116]. These shifts in the gut microbiome by wild rice feeding are also associated with reductions in liver steatosis, oxidative stress, and systematic low-grade inflammation that development in the high fat diet. There is no defined mechanism so far on how wild rice feeding can induce these changes to the gut microbiome.

Dietary fiber is a common nutrient consumed to improve gut health. Some soluble dietary fibers are also known as "prebiotics" as they can provide fuel for beneficial bacteria. These beneficial bacteria are often called "probiotics", as they are known to produce fermentation byproducts that can be used positively by the host [117]. Unfortunately, almost all of the dietary fiber in rice is insoluble and non-fermentable, and therefore, cannot be used by the gut microbiome [118]. Instead, rice is high in polyphenolic compounds which are reported to have prebiotic effects and contribute certain health benefits [115, 116, 119-124].

The main polyphenolic compounds found in wild rice are phenolic acids (mainly ferulic acid), phytic acids, phytosterols, sinapic acid, anthocyanins, and proanthocyanidins [125, 126]. Specifically, proanthocyanidins have been shown to shift the gut microbiome to a predominance of *Bacteroidetes, Lactobacillus,* and *Bifidobacterium* [123, 124]. As previously mentioned, *Lactobacillus* and *Bifidobacterium* are two well-known probiotic gena in the human gut microbiota that have been documented with certain health benefits. These polyphenolic compounds are mainly found in the bran of the rice grain. Nealon et al. used an animal model to investigate the effects of rice bran supplementation on the gut microbiome [122]. Mice were fed either

the AIN-93G control diet, the control diet with 10% rice bran, or the control diet with 10% fermented rice bran for 15 weeks [122]. The results of this study showed that the fermented rice bran diet caused an increase in abundance of *Roseburia* while the non-fermented rice bran caused an increase in abundance of *Clostridiales* [122]. There was also no distinct difference between the rice bran fed diet groups, but they were both significantly different from the microbiome of the AIN-93G control diet fed mice [122]. This study shows that rice bran, which is rich in polyphenols, can alter the gut microbiome and promote the growth of certain beneficial bacteria. Zhang et al. and Pham et al. both conducted *in vitro* digestion studies on these rice polyphenols to better understand how these phenolic compounds affect the gut microbiome [119, 120]. Although these results are not the most relevant because they were done in an *in vitro* model instead of an *in vivo* model, the researchers were still able to report that the polyphenolic compounds in whole grain rice stimulated the growth of *Lactobacillus, Bifidobacterium, A. muciniphila,* and *F. prausnitzii* [119, 120]. As discussed in the previous section, the growth of these specific bacteria has been associated with positive health outcomes in humans.

Proteolytic versus Saccharolytic Fermentation

Both unabsorbed carbohydrates and proteins can be fermented in the large intestine. Saccharolytic fermentation refers to fermentation of dietary fibers, unabsorbed sugars, and other unabsorbed carbohydrates by the bacteria in the gut microbiome. Proteolytic fermentation refers to fermentation of undigested proteins. Saccharolytic fermentation is considered beneficial for the host, as most of the metabolic products are

Short Chain Fatty Acids (SCFAs). SCFA production by the gut microbiome is thought to be a key determinant in regulating host health, including adiposity and fatty liver. An increased abundance of saccharolytic fermenters in the large intestine and increased SCFA production have been correlated with several health benefits, such as reduced adiposity and improved glycemic control [127-132]. However, proteolytic fermentation produces metabolic products which are considered toxic to the host [133]. Some of these toxic protein fermentation end products include amines, indoles, thiols, H_2S, and cresols [133, 134]. Most notably, Chaves et al. found that p-cresol activates macrophage micropinocytosis which leads to increased LDL cholesterol uptake [135]. As discussed in earlier sections, increased LDL cholesterol can lead to increased risk of obesity and NAFLD. Many of the other toxic protein fermentation products have been linked to increased risk of colon cancer, irritable bowel disease (IBD), hypercholesteremia, and other NCDs [133-136]. The link between protein fermentation and colon cancer has been most commonly seen in people that consume a diet high in red meat or a Traditional Western Diet that involves excess protein consumption that is malabsorbed [134, 137]. The distinction between bacteria that carry out proteolytic fermentation and those that carry out saccharolytic bacteria can be complex, as a particular genus can ferment both undigested protein and dietary fiber.

Two examples of bacteria that ferment both proteins and carbohydrates are *Prevotella* and *Alistipes.* Specifically, *Prevotella* has been characterized as showing both protease and saccharolytic activity in the oral and gut microbiomes [138, 139]. Deschner et al. found that protease activity by *Prevotella intermedia* modulates the virulence of

LPS [138]. As discussed previously, LPS is a potent inflammatory agent known to cause metabolic endotoxemia and increase inflammation [76]. On the other hand, Kovatcheva-Datchary et al. conducted a clinical trial where healthy subjects consumed barley-kernel bread, high in dietary fiber, or white bread for 3 days [140]. After the feeding, researchers conducted 16S rRNA sequencing on the patients' fecal samples and found a significant increase in *Prevotella* abundance in the patients that consumed the high fiber bread compared to the patients who consumed the white bread [140]. These results showing the ability of *Prevotella* to ferment dietary fiber have been well-supported by other literature [141-143]. Meanwhile, Parker et al. noted that *Alistipes* has the highest number of proteolytic fermentation pathways among commensal bacteria [136]. Known metabolites of *Alistipes* proteolytic fermentation include ammonia, H_2S, cresol, indole, and phenol [134]. Kaur et al. found that *Alistipes* fermentation contributed to histidine degradation/THF production, indole production, and phenol production [144]. Histidine degradation/THF production has been found to release excess ammonia, which when absorbed, damages colonic cells and can lead to the development of colon cancer [145]. *Alistipes* has also been shown to ferment dietary fibers and other complex carbohydrates [146-148]. Specifically, Tian et al. fed mice either a control chow diet or a chow diet with barley leaf dietary fiber [147]. Researchers found that barley leaf dietary fiber feeding caused an increase in *Alistipes* abundance, which also correlated with a reduction in inflammation biomarkers [147]. Kiewiet et al. conducted a double-blind, placebo control trial where healthy adults consumed an inulin supplement once a day for 2 months [148]. Patients consuming inulin developed a more diverse gut microbiome and had an increase

in *Alistipes shahii* [148]. The ability for certain bacteria gena to perform multiple forms of fermentation leads to the idea that determining the species of bacteria present in the gut microbiome gives a more definitive conclusion on how the gut microbiome interacts with its host. Looking at associations at just the genus level is not sufficient to draw conclusions between the gut microbiome and certain diseases.

Chapter III: Determining the Protein Digestibility of Plant Based Proteins and Their Effect on Obesity, NAFLD, and the Gut Microbiome

Introduction

Plant based food proteins are becoming increasingly popular in the U.S. due to their perceived benefits for the environment, certain health benefits [4, 13], and the desire by some to avoid consuming animal products for personal reasons. However, the overall nutritional quality of plant-based proteins has been debated. One issue is that plant proteins are usually of lower quality than animal proteins for two key reasons: plant proteins are limiting in at least one essential amino acid, and plant proteins are less digestible than animal proteins [1, 2]. With an increase in the types of plant-based proteins being produced as food ingredients, there is a need expand our knowledge of how they differ in protein quality, as plant proteins vary considerably in their protein digestibility and their amino acid profile. The Protein Digestibility Amino Acid Corrected Score (PDCAAS) is the current standard for determining protein quality [3]. Consequently, whenever a new plant protein comes onto the market, a PDCAAS value needs to be measured for that specific product. It is important for consumers to know the quality of the proteins in the plant protein products they are purchasing. In the U.S., a PDCAAS value and the crude protein content for that food is required to make a protein claim on a product. Therefore, the objective of the present study was to determine the protein quality of wild rice, brown rice, pea flour, and 2 different pea flour protein isolates, as determined by the PDCAAS procedure.

Plant-based diets have been reported to lower weight gain when compared to omnivorous or animal-based diets [4-7]. This is of major importance due to the rise in the prevalence of obesity in the United States [8-10]. As obesity prevalence has risen, so has

non-alcoholic fatty liver disease (NAFLD). NAFLD affects 25% of people worldwide [12], and 80% of overweight and obese adults in North America [71]. Unfortunately, no pharmacological intervention exists to treat NAFLD [11] and the most common treatment option is weight loss by diet and exercise. Currently, the widely accepted hypothesis for the development of NAFLD is the "multi-hit" hypothesis [78]. This hypothesis states that there are several metabolic insults that lead to development of NAFLD [76, 77]. One of these insults is a dysregulated gut microbiome, or dysbiosis. Specifically, certain microbiome profiles have been associated with obesity and NAFLD [100, 101]. Most notably, obesity and NAFLD have been associated with an increase in the *Firmicutes* to *Bacteroidetes* ratio [102, 103]. There are many other postulated associations between NAFLD and the gut microbiome, but the findings are inconsistent [109-114]. Further research is needed to better understand the link between NAFLD, the gut microbiome, and diet, including protein quality. Therefore, another objective of this study was to determine the effect that brown rice, wild rice, and pea flour consumption have on the development of fatty liver disease, the earliest manifestation of NAFLD, as well as obesity and changes to the gut microbiome, using a diet-induced obesity (DIO) animal model.

Materials and Methods

Animals and Experimental Design

For this study 52 male Wistar rats (initial body weight 73-114 g, 4-6 weeks of age) were purchased from Envigo Laboratories (Indianapolis, IN). Animals were singly housed in hanging wire cages and kept on a 12-h light/dark cycle. All animal use

procedures were approved by the University of Minnesota Animal Care and Use Committee (protocol 2005-38167A).

All animals were fed an adaptation diet (AIN-93G Diet) for 5 days after arrival to acclimatize the rats to a powdered diet. After the 5 day adaptation period, rats were divided into 7 diet groups and fed diets formulated according to the 1989 FAO/WHO Joint Expert Consultation on Protein Quality [19]. Briefly, the diets consisted of 10% protein and the animals were offered 15 g/day of diet for 9 days. For the final 5 days, food intake was recorded, and fecal collections were made daily. The fecal collections were composited, and nitrogen content determined by the Dumas method using a Leco (model # FP828) nitrogen analyzer. The 7 diets consisted of brown rice (BR), wild rice (WR), casein control (CC), pH precipitated pea flour protein isolate (PPI pH), salt solubilized pea flour protein isolate (PPI salt), pea flour (PF), and protein-free (PFR). There were 8 rats/group except for the protein-free group, which consisted of only 4 rats.

After the 9 days of experimental feeding, the rats on the protein-free, salt solubilized pea protein isolate, and pH precipitated pea protein isolate diets were euthanized. The rats on the other 4 experimental diets were switched to a high fat, diet induced obesity (DIO) model and fed *ad libitum* for 8 more weeks. During those 8 weeks, weekly body weight and food intake measurements were recorded. After 8 weeks on the DIO experimental diets, animals were anesthetized with isoflurane and blood was collected by cardiac puncture. Blood was centrifuged at 3,000 *g* for 20 minutes and serum aliquots were stored at -80°C until use. Ceca were harvested and their contents were stored in 15 mL polypropylene conical tubes, frozen on dry ice, and stored at -80°C until

further use. Empty ceca were rinsed, weighed, and discarded. One epididymal fat pad from each animal was harvested, rinsed, weighed, frozen on dry ice, and stored at -80°C. Livers were excised, rinsed, weighed, frozen on dry ice, and stored at -80°C until further analysis. To preserve tissue RNA for future analysis, approximately 1.0 g of liver and 0.3 g epididymal fat pad were stored in 2 mL of RNA*later* solution and stored at 4°C.

Diet composition

As previously stated, the 7 diets consisted of brown rice, wild rice, protein-free, casein control, pH precipitated pea flour protein isolate, salt solubilized pea flour protein isolate, and pea flour. The pea flour and the two pea protein isolates were provided by the laboratory of Dr. Pam Ismail. The procedure for developing the pea flour and the two isolation methods for the pea protein isolates are described by Hansen et al. [149]. The brown rice was the Essential Everyday® brand purchased from Cub Foods in Roseville, MN. The wild rice was cultivated wild rice from various locations across Minnesota and was provided by the Minnesota Cultivated Wild Rice Council. Both types of rice were cooked in an AROMA® (model ARC-9045B) rice cooker, dried at 60°C in a low temperature oven for 24 hours, and then milled using an Udy Mill (model 3010-0310). Amino acid analysis of the experimental proteins was performed by Eurofins Scientific, Inc. (Des Moines, IA) and the proximate analysis was performed by Medallion Labs (Golden Valley, MN; Appendices 1 and 2). Diet compositions for the 7 experimental diets used for the first 9 days of experimental feeding are provided in **Table 2**. Note that in **Table 2**, 200 g of the protein-free diet was added to the casein, pea flour, and both pea protein isolate diets to dilute the diets to 10% protein by weight. For the protein quality

feeding trial, the dietary protein concentration is set to 10% by convention, to properly calculate the protein-digestibility amino acid corrected score (PDCAAS).

In the DIO model, where brown rice, wild rice, and pea flour were the experimental diets fed to rats, a high fat version of the AIN-93G control diet was used as a casein control diet. The DIO experimental diets contained 40% of the experimental plant protein source and 25% fat by weight. Animals were kept on the same protein source as they were fed in the previous portion of the study. The macronutrient content of the diets was matched as closely as possible. Due to the low protein concentration in wild rice, brown rice, and pea flour, casein was included in these diets to ensure each diet contained 20% protein. Diet compositions for the high fat diets are shown in **Table 3**.

Table 2: Diet Compositions for Protein Quality Trial

Diet Composition

Nutrient (g/kg)	Casein Control (AIN-93G)	Wild Rice	Brown Rice	Protein Free	PPI (scaled-up salt)	PPI (scaled-up pH)	Pea Flour
Sucrose	119.8	94.1	89	99	119.8	119.8	119.8
Corn Starch	529.55	0	0	465.3	593.06	586.31	291.86
Maltodextrin	224.84	0	0	264.2	152.84	152.84	52.84
Soybean Oil	84	62.3	45.4	70	84	84	78.1
Casein	120	0	0	0	0	0	0
Cellulose	60	23.1	25.4	50	60	60	60
Mineral Mix	42	35	35	35	42	42	42
Vitamin Mix	12	10	10	10	12	12	12
L-Cystine	3.6	3	3	3	3.6	3.6	3.6
Choline bitartrate	3	2.5	2.5	2.5	3	3	3
BHT	0.017	0.014	0.014	0.014	0.017	0.017	0.017
Cholesterol	1.2	1	1	1	1.2	1.2	1.2
Wild Rice	0	769	0	0	0	0	0
Brown Rice	0	0	788.7	0	0	0	0
Pea Protein Isolate (salt)	0	0	0	0	128.49	0	0
Pea Protein Isolate (pH)	0	0	0	0	0	135.25	0
Pea Flour	0	0	0	0	0	0	535.6
Total Weight	1200.0028	1000.014	1000.014	1000.014	1200.0028	1200.017	1200.017
Nutrient Composition							
% Carbohydrates	73%	67.31%	68.45%	83%	72%	71.58%	64.28%
% Lipids	7%	7.00%	6.75%	7%	7%	7.00%	7.00%
% Protein	10%	10.00%	6.28%	0%	10%	10.00%	10.00%
% Fiber	5.00%	5.00%	4.75%	5.00%	5.00%	5.00%	5.00%
% Sum	95%	89.31%	86.22%	94.85%	94.035%	93.58%	86.28%
kcal/g	4.019	3.80	3.67	4.019	3.99	3.97	3.68

Table 3: Diet Compositions for High Fat DIO Trial

Diet Composition

Nutrient (g/kg)	Casein Control	Wild Rice	Brown Rice	Pea Flour
Sucrose	100	100	100	100
Corn Starch	176.49	18.5	2.7	42.5
Maltodextrin	172	0	0	0
Lard	187.5	184.5	179.1	184.2
Soybean Oil	62.5	61.5	59.7	61.4
Casein	200	148	168.2	110.4
Cellulose	50	36	38.8	50
Mineral Mix	35	35	35	35
Vitamin Mix	10	10	10	10
L-Cystine	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5
BHT	0.014	0.014	0.014	0.014
Cholesterol	1	1	1	1
Wild Rice	0	400	0	0
Brown Rice	0	0	400	0
Pea Flour	0	0	0	400
Total Weight	1000	1000.014	1000.014	1000.014

Nutrient Composition

% Carbohydrates	45%	41.97%	40.47%	37.17%
% Lipids	25%	25.00%	25.00%	25.00%
% Protein	20%	20.00%	20.00%	20.00%
% Fiber	5.00%	5.00%	5.00%	5.00%
% Sum	95%	91.97%	90.47%	87.17%
kcal/g	4.92	4.80	4.74	4.61

Nitrogen Determination and PDCAAS Calculations

Nitrogen content of the diets and composited fecal samples was determined using the Dumas method via the Leco nitrogen analyzer. Feces was cleaned of any spilled or caked-on diet, ground, and stored at -20°C until further use. Nitrogen content of the experimental diets was measured to confirm that the diets contained 10% protein. The concentration of nitrogen in the feces was used to determine True Digestibility (TD) of the experimental proteins. The Jones factor of 6.25 was used to convert dietary and fecal nitrogen to protein for all the diet groups except for the WR and BR groups, where a more accurate factor of 5.61 was used. According to Sosulski et al., a lower nitrogen-to-protein conversion value should be used for specific plant proteins [29]. The calculation for TD is as follows:

$$\mathrm{TD} = \frac{\mathrm{I} - (\mathrm{F} - \mathrm{F_k})}{\mathrm{I}} \ \mathrm{X}\ 100$$

Where I is dietary nitrogen intake, F is fecal nitrogen, and F_k is metabolic fecal nitrogen. F_k is determined using the protein-free rat group and is calculated by using the equation as follows:

$$F_k = total\ diet\ consumed\ by\ test\ protein\ group\ (g) \times \frac{mg\ of\ fecal\ nitrogen\ for\ the\ protein-free\ group}{g\ of\ diet\ consumed\ by\ the\ protein-free\ group}$$

Finally, the calculation for PDCAAS is:

$$PDCAAS = TD \times AAS$$

where AAS is the amino acid score. The amino acid score is the most limiting essential amino acid, based on the 1991 reference values for preschool aged children between 2-5 years old [23].

Additionally, an *in vitro* PDCAAS assay was performed on the 7 experimental proteins to compare to the in vivo PDCAAS results, using a commercial kit (Megazyme, Dublin, Ireland, cat# K-PDCAAS) and used according to the manufacturer's instructions (**Appendix 3)**. Briefly, samples were sequentially incubated in pepsin and trypsin plus chymotrypsin for 1 hour and 4 hours, respectively. The samples were then placed in boiling water for 10 minutes and incubated at 4°C overnight. Finally, the samples were centrifuged at 15,000 *g* and the supernatant was assayed colorimetrically for amino acid content using ninhydrin at a wavelength of 570 nm.

Liver Lipids and Cholesterol

Approximately 1 g of liver from each animal was homogenized and lipids were extracted using chloroform:methanol (2:1) [150]. Extracted lipids were dried under N_2 gas and quantified gravimetrically. Lipid samples were reconstituted with 10 mL chloroform:methanol (2:1) and cholesterol was measured enzymatically using a previously described method [151].

DNA extractions and microbiome sequencing

DNA was extracted from approximately 180-220 mg of cecal contents using the QiAmp® DNA Stool Mini kit (cat# 51604) and quantified spectrophotometrically with a Nanodrop spectrophotometer using a baseline correction wavelength of 340 nm. DNA eluates were then submitted to the University of Minnesota Genomics Center (UMGC)

for sequencing of the 16S ribosomal subunit with the V5/V6 region amplicon on an Illumina MiSeq, using 2x300 bp paired end reads. The primer sequence used by UMGC was GTGCCAGCMGCCGCGGTAA. Data was preprocessed using DADA2 and analyzed using phyloSeq and microeco in R.

Leukocyte cell derived chemotaxin 2 (LECT2)

Serum LECT2 concentrations were determined by ELISA from a kit purchased from Biohippo (cat# GA-E1048RT). Briefly, samples, standards, and blanks were loaded into the provided 96 well plate with LECT2 antibody and streptavidin-HRP. The plate was incubated on a plate rocker at 37℃ for 60 minutes. The plate was rinsed with washing solution 5 times before chromogen A was added and again incubated at 37℃ for 10 minutes. Finally, the stop solution was added to the plate and absorbance was measured at 450 nm.

Serum triacylglycerols and cholesterol

Serum triacylglycerols and serum cholesterol were measured spectrophotometrically using commercial kits purchased from Pointe Scientific, Inc. (cat# 23666410 and 23666200 respectively).

AMPK and ACC quantification by Western blot

AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) were quantified via Western Blot. Both the phosphorylated and unphosphorylated form of both enzymes was quantified using a LI-COR Biosciences imager (model# Odyssey Fc) and a ratio for pAMPK/AMPK and ACC/pACC was calculated. The LI-COR imager uses near-infrared detection to measure the concentration of a compound based on its near-infrared

fluorescence. Primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Further details are presented in **Appendix 4**.

Statistical analysis

All data except microbiome data was analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test and LSMEANS using SAS version 9.4. A p-value less than 0.05 denotes statistical significance while a p-value between 0.05 and 0.1 denotes a trend.

Bioinformatics

Microbiome data was analyzed in R using DADA2 and phyloSeq/microeco packages for determination of alpha diversity by Shannon-Weaver Index, beta diversity by Bray-Curtis PCoA, taxonomical abundance, and dissimilarity by PERMANOVA Statistical significance of taxonomical abundance was determined using SAS version 9.4 by Duncan's multiple range test. A p-value less than 0.05 denotes statistical significance while a p-value between 0.05 and 0.1 denotes a trend.

Results

Pea flour had the best protein quality among plant proteins tested

Among the 5 plant proteins tested in this animal experiment, pea flour had the highest PDCAAS value (**Table 4),** differences that were statistically significant ($p < 0.0001$). However, the pea flour PDCAAS was significantly lower than that of casein ($p < 0.0001$). Both pea protein isolates had a greater protein digestibility compared to pea flour, however, they both had a lower amino acid score than the pea flour, thus lowering

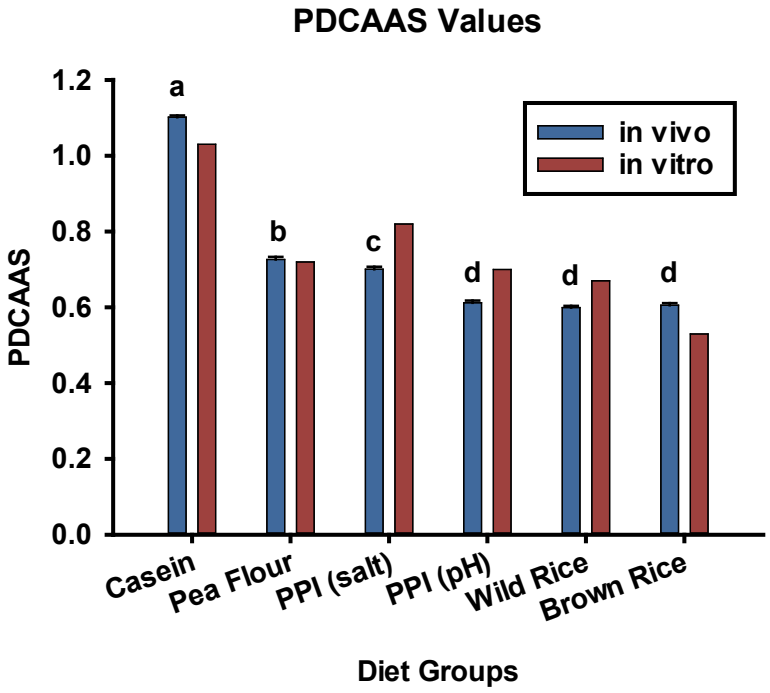
their PDCAAS value. The *in vitro* PDCAAS assay correlated well with the *in vivo* results ($R^2 = 0.810$), but some of the experimental plant proteins had noticeable differences between the two assays (**Figure 1)**. Most notably, BR had the least agreement between the *in vivo* and *in vitro* determinations. When BR is removed from the *in vivo* and *in vitro* correlation, there appears to be a strong correlation between the two assays (**Figure 1B**) ($R^2 = 0.915$).

Table 4: Amino Acid Score, True Digestibility, and PDCAAS values for Brown Rice, Wild Rice, Pea Flour, Pea Protein Isolates, and Casein

Diet Group	Amino Acid Score	Limiting Amino Acid	True Protein Digestibility*	In vivo PDCAAS*
Casein (control)	Lowest Amino Acid is Tryptophan @ 1.16	No limiting AA	0.95 ± 0.0039[a]	1.11 ± 0.0045[a]
Pea Flour	0.81	Tryptophan	0.89 ± 0.0084[c]	0.73 ± 0.0068[b]
PPI (salt)	0.75	Cysteine/Methionine	0.93 ± 0.0088[b]	0.70 ± 0.0066[c]
PPI (pH)	0.65	Cysteine/Methionine	0.94 ± 0.0094[a, b]	0.61 ± 0.0061[d]
Wild Rice	0.72	Lysine	0.84 ± 0.0063[d]	0.60 ± 0.0045[d]
Brown Rice	0.78	Lysine	0.78 ± 0.0070[e]	0.61 ± 0.0054[d]

*Values for True Protein Digestibility and *in vivo* PDCAAS represent mean ± SEM, n=8. Values not sharing a common letter are statistically different, $p < 0.05$.

A



B

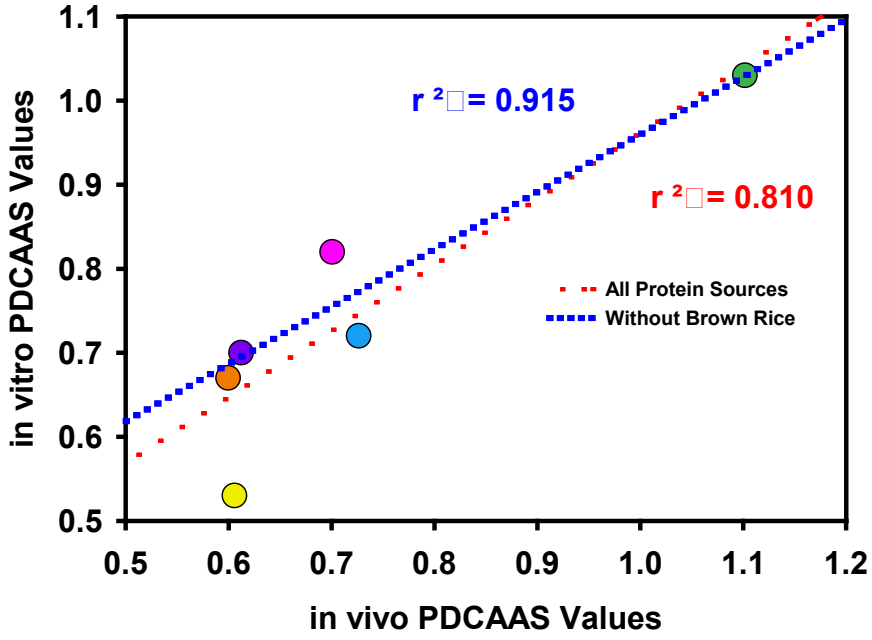
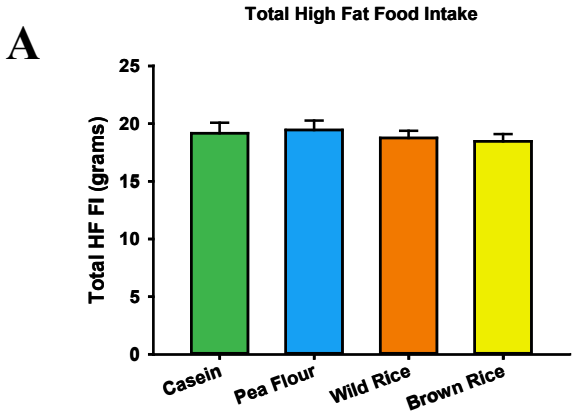


Figure 1: PDCAAS value comparison between in vivo and in vitro methods
PDCAAS values for each diet group in vivo and in vitro. (B) Correlation plot between *in vivo* and *in vitro* assays. Values for *in vivo* PDCAAS represent mean ± SEM, n=8. Values not sharing a common letter are statistically different, $p < 0.05$.

Weight gain in the pea flour diet was not influenced by food intake

In the DIO phase, food intakes did not differ among the diet groups, although there were significant differences in body weight during the 8 weeks of feeding (**Figure 2A and C**) ($p < 0.0001$). Rats fed PF had a significantly greater change in body weight compared to rats fed the control diet (**Figure 2B**) ($p = 0.0124$). There was no change in body weight between either rice diet and the control diet ($p > 0.05$).



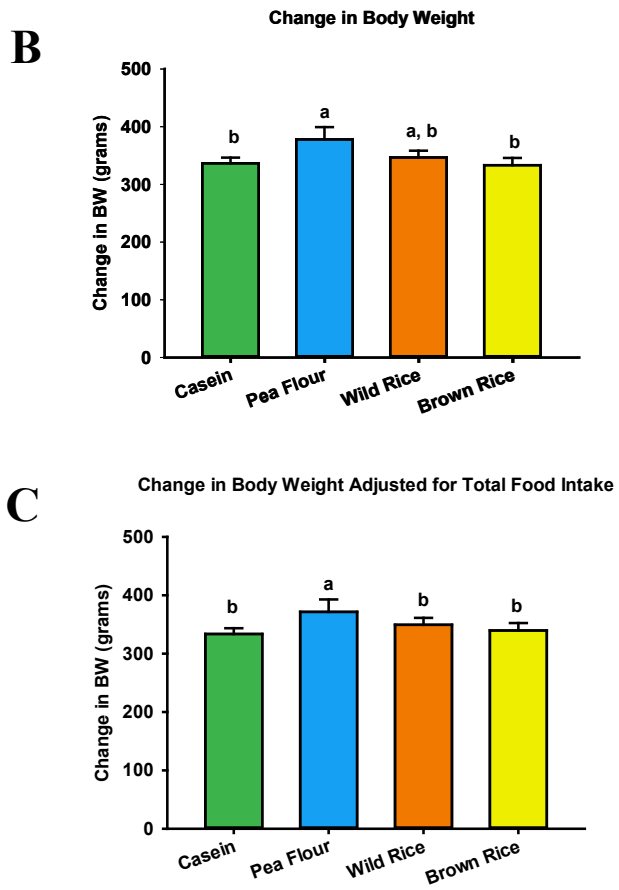


Figure 2: Food intake and body weight in rats fed brown rice, wild rice, pea flour, and a high fat casein control.
(A) Food Intake. (B) Change in Body Weight from end of Protein Quality feeding trial to end of high fat feeding trial. (C) Change in Body Weight using Food Intake as a covariant. Values represent mean ± SEM, n=8. Values not sharing a common letter are statistically different, $p < 0.05$.

Epididymal fat pad weight did not differ among the diet groups

Epididymal fat pad weight correlates highly with total body fat [11]. Using body weight as a covariant to normalize tissue weight relative to body weight, there were no differences in epididymal fat pad weight among any of the diet groups (**Figure 3**) ($p > 0.05$).

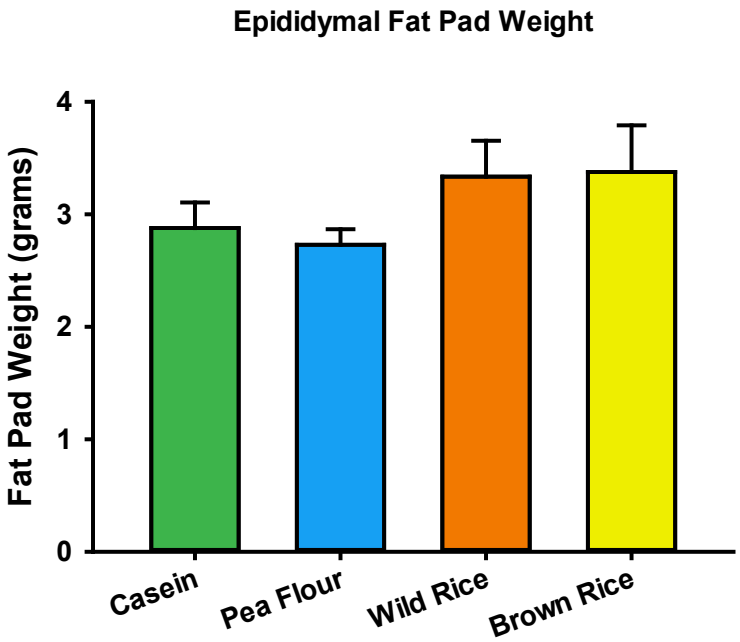
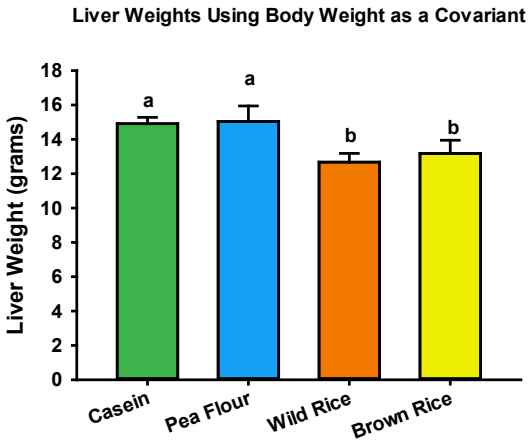


Figure 3: Epididymal fat pad weight by diet group
Values represent mean ± SEM, n=8. No letters mean no significant difference among any of the groups, $p > 0.05$.

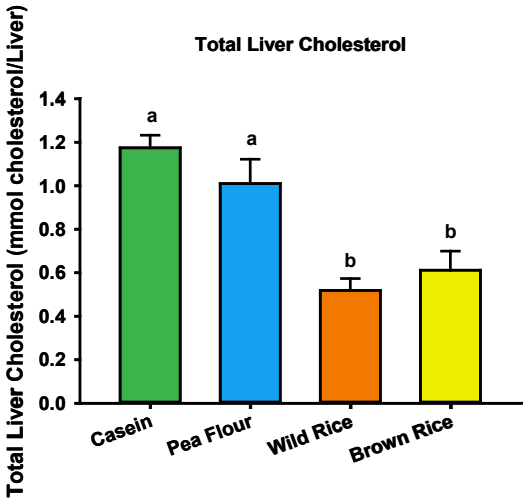
Liver weight, liver lipids, and liver cholesterol were reduced in wild rice and brown rice groups

Both WR and BR diets reduced liver weight ($p < 0.0219$), total liver lipids ($p < 0.0026$), and total liver cholesterol ($p < 0.0001$) compared to the high fat control diet (**Figure 4**). Liver weight was also reported as a function of body weight. PF fed rats did not see any significant changes in liver weight, liver lipids, or liver cholesterol ($p > 0.05$).

A


Liver Weights Using Body Weight as a Covariant
Liver Weight (grams)
18
16
14
12
10
8
6
4
2
0
a
a
b
b
Casein
Pea Flour
Wild Rice
Brown Rice

B


Total Liver Cholesterol
Total Liver Cholesterol (mmol cholesterol/Liver)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
b
Casein
Pea Flour
Wild Rice
Brown Rice

C

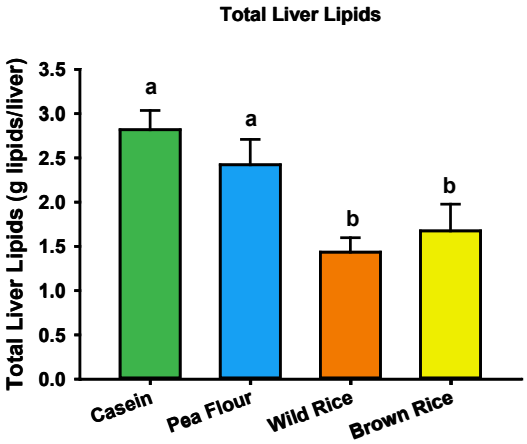

Total Liver Lipids
Total Liver Lipids (g lipids/liver)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
b
Casein
Pea Flour
Wild Rice
Brown Rice

Figure 4: Average liver weight, liver lipids, and liver cholesterol by diet group
Values represent mean ± SEM, n=8. Values not sharing a common letter are statistically different, $p < 0.05$.

Serum LECT2 concentrations did not differ among diet groups

Serum LECT2, a putative serum biomarker for NAFLD [152-154], did not differ among any of the diet groups (**Figure 5**). However, when just comparing the rice diets to the high fat control, BR serum LECT2 levels were significantly reduced compared to the control. WR serum LECT2 levels showed a trend towards a difference from the high fat casein control group ($p = 0.08$). Overall, serum LECT2 did not correlate significantly with total liver lipids ($r = 0.25$, $p=0.18$).

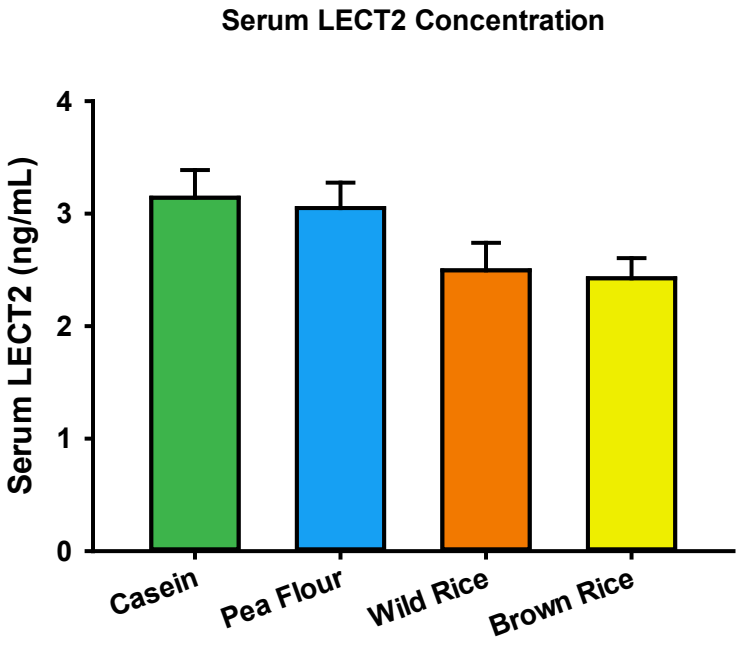
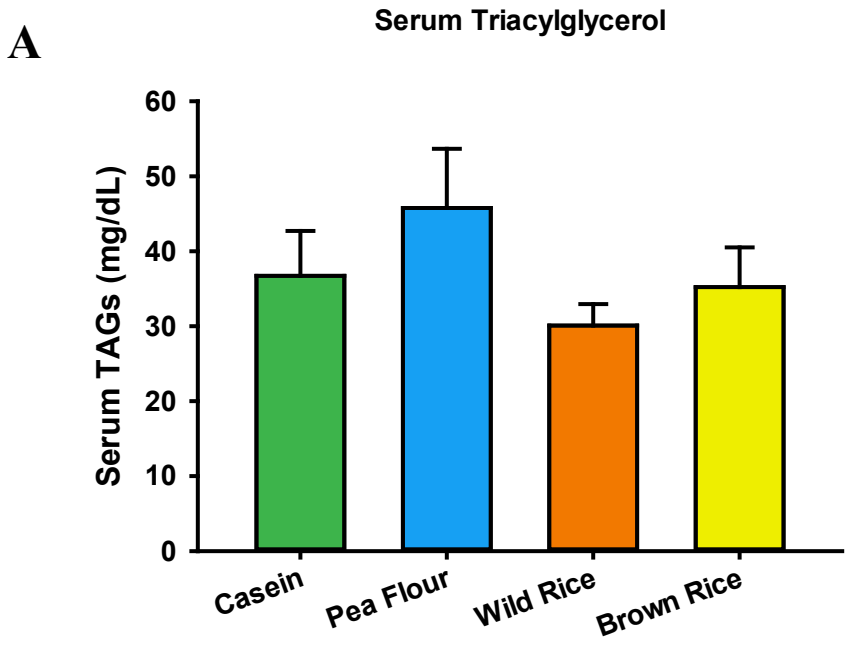


Figure 5: Serum LECT2 concentrations by diet group
Values represent mean ± SEM, n=8 (BR group n = 7), $p > 0.05$.

Wild rice feeding reduced serum cholesterol levels

Serum triacylglycerols were not significantly different by diet group (**Figure 6A**). However, serum cholesterol levels were significantly reduced by WR feeding compared to the casein control group ($p < 0.0188$). Additionally, PF showed a trend to reduce serum cholesterol compared to the casein control group ($p < 0.0596$) (**Figure 6B**). This suggests that WR offers health benefits beyond reducing liver total lipids and cholesterol.



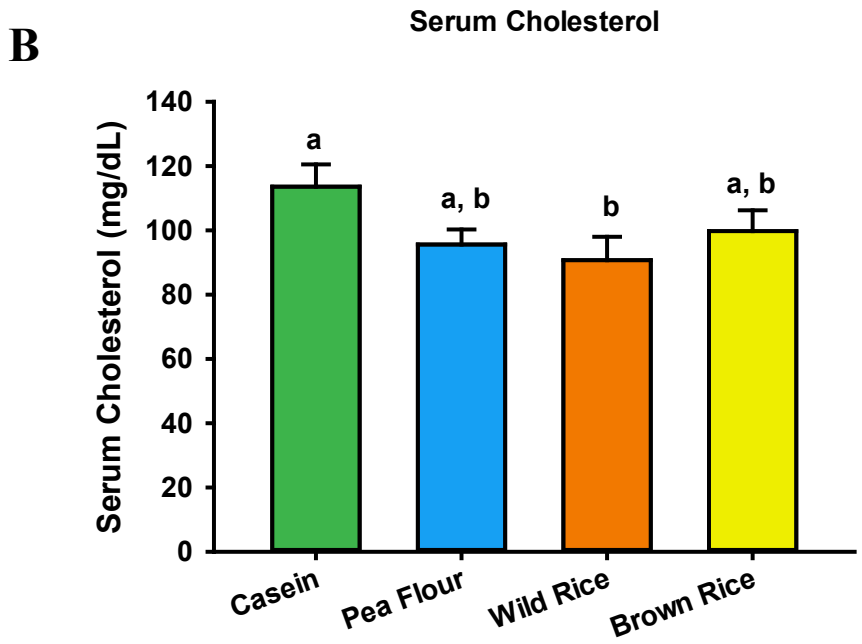
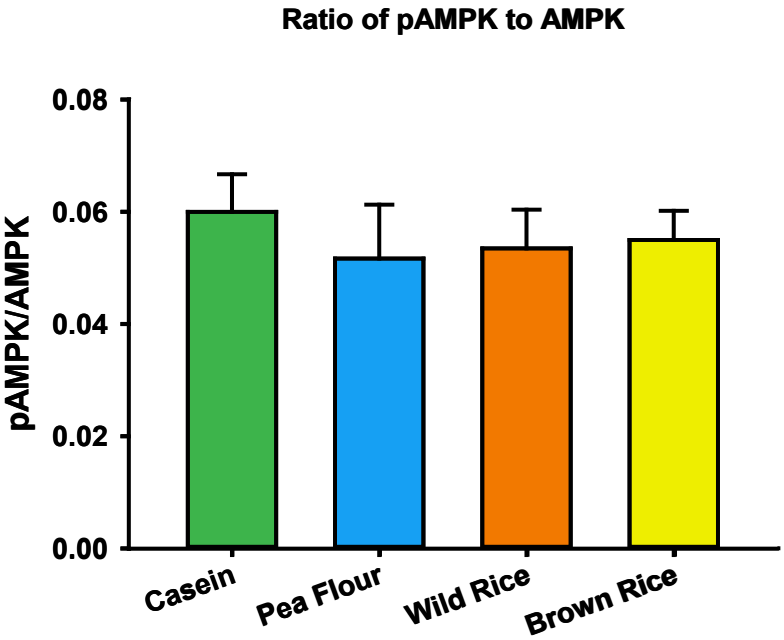


Figure 6: Average Serum Triacylglycerol and Cholesterol Levels by Diet Group
(A) Serum Triacylglycerol. (B) Serum Cholesterol. Values represent mean ± SEM, n=8 (BR group n=7). Values not sharing a common letter are statistically different, $p < 0.05$.

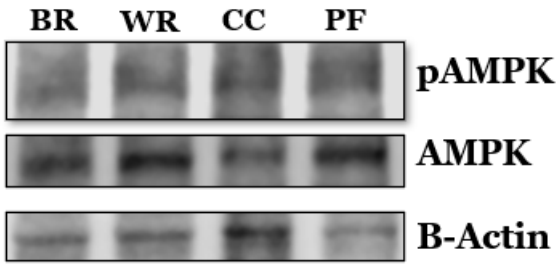
Plant proteins did not cause shifts in enzymes regulating lipid metabolism

AMP-activated protein kinase (AMPK) is the central regulator for lipid metabolism in the liver [84]. Acetyl-CoA carboxylase (ACC) is the rate-limiting step in fatty acid synthesis [72]. AMPK is activated by phosphorylated leading to increased β-oxidation. ACC is active in the unphosphorylated state, which promotes fatty acid synthesis. There were no significant changes in activation of AMPK or ACC by any of the experimental diet groups compared to the casein control diet (**Figure 7**).

A


Ratio of pAMPK to AMPK
pAMPK/AMPK
0.08
0.06
0.04
0.02
0.00
Casein
Pea Flour
Wild Rice
Brown Rice

B


BR
WR
CC
PF
pAMPK
AMPK
B-Actin

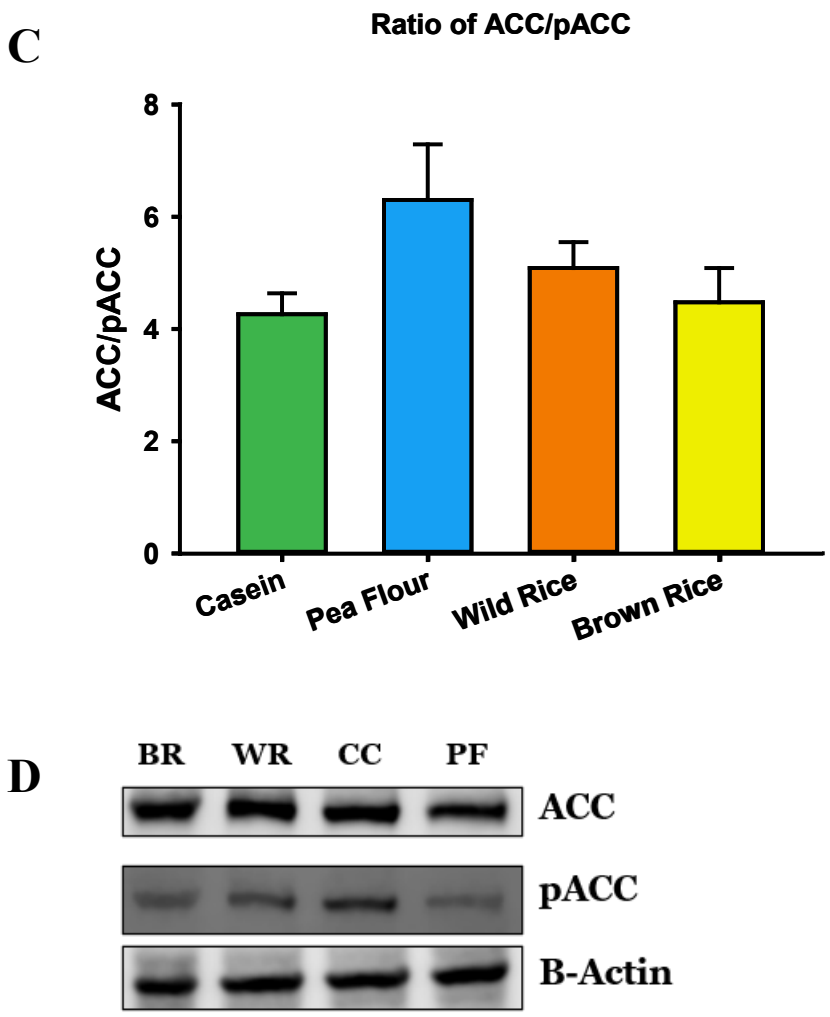


Figure 7: Ratio of pAMPK:AMPK and ACC:pACC by Diet Group
(A) pAMPK:AMPK (B) Images from AMPK Western Blot (C) ACC:pACC (D) Images from ACC Western Blot. Values represent mean ± SEM, n=8, $p > 0.05$.

Rats fed pea flour had the largest empty ceca

Most fermentation by the gut microbiome in the rat occurs in the first part of the large intestine, the cecum. Greater fermentation is indicated by an increase in empty cecal tissue weight [155]. Rats fed the pea flour diet had the heaviest empty cecum weight of all the diet groups (**Figure 8**). Although PF empty cecal weight did not significantly

differ from the control, this numerical increase suggests that PF had the most fermentation in the gut microbiome of all the diet groups. Meanwhile, WR fed rats had the smallest empty ceca weight of all the diet groups (**Figure 8**), suggesting that wild rice feeding does not promote fermentation in the large intestine.

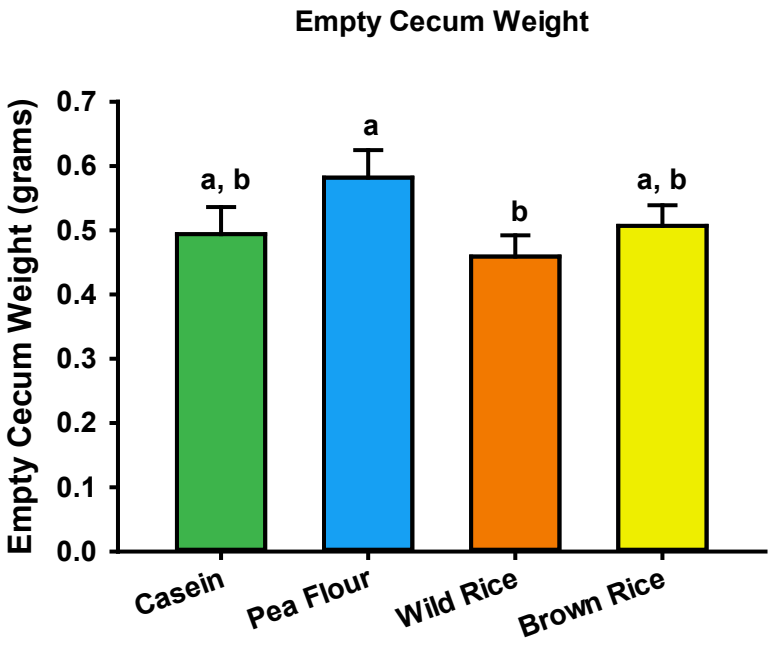


Figure 8: Empty Cecum Weight by Diet Group
Values represent mean ± SEM, n=8. Values not sharing a common letter are statistically different, $p < 0.05$.

Wild rice and pea flour feeding caused significant changes to the gut microbiome

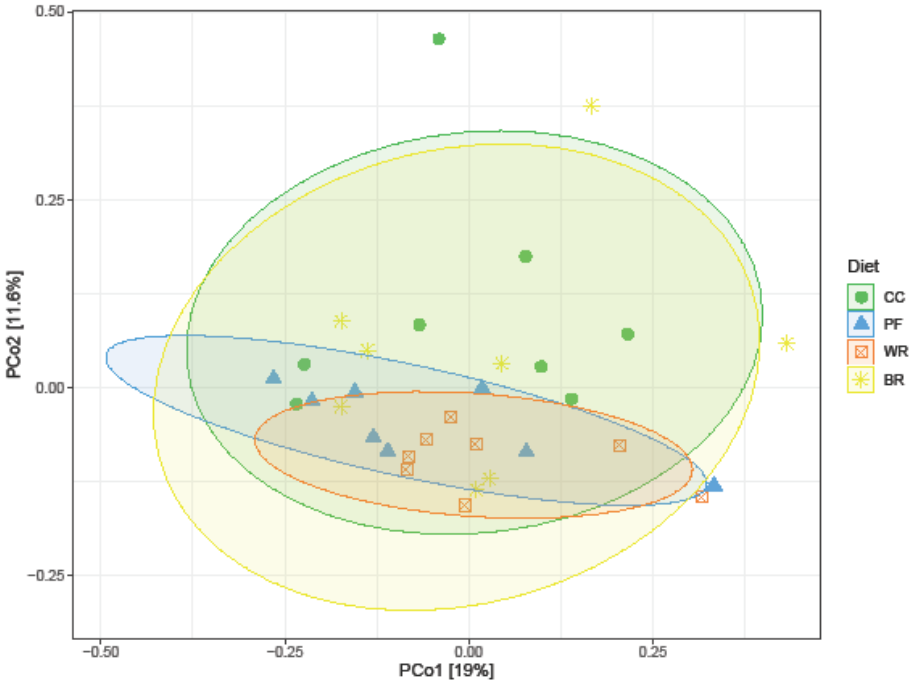
Beta-diversity analysis of the gut microbiome, as shown by a Bray-Curtis distance PCoA plot, revealed that rats fed the WR and PF diets had a significantly different gut microbiome than the casein control diet (**Figure 9A**). The WR group was the most

dissimilar to the casein control group of the three experimental groups ($p = 0.003$). This indicates that WR had more influence on the microbiome than either the PF or BR diets. The heat map in **Figure 9B** shows how every animal in each diet group compares to one another for the top 40 most abundant genera in the rats' gut microbiomes. In the figure, each column represents a different animal, and each row represents a different bacteria genus. This further illustrates how similar each animal was in the wild rice group specifically compared to other rats in other diet groups. The Shannon-Weaver index is a measure of alpha-diversity used to quantify specific biodiversity in each animal. There were no significant differences between any of the diet groups in the Shannon-Weaver Index ($p = 0.3673$, **Figure 9C**).

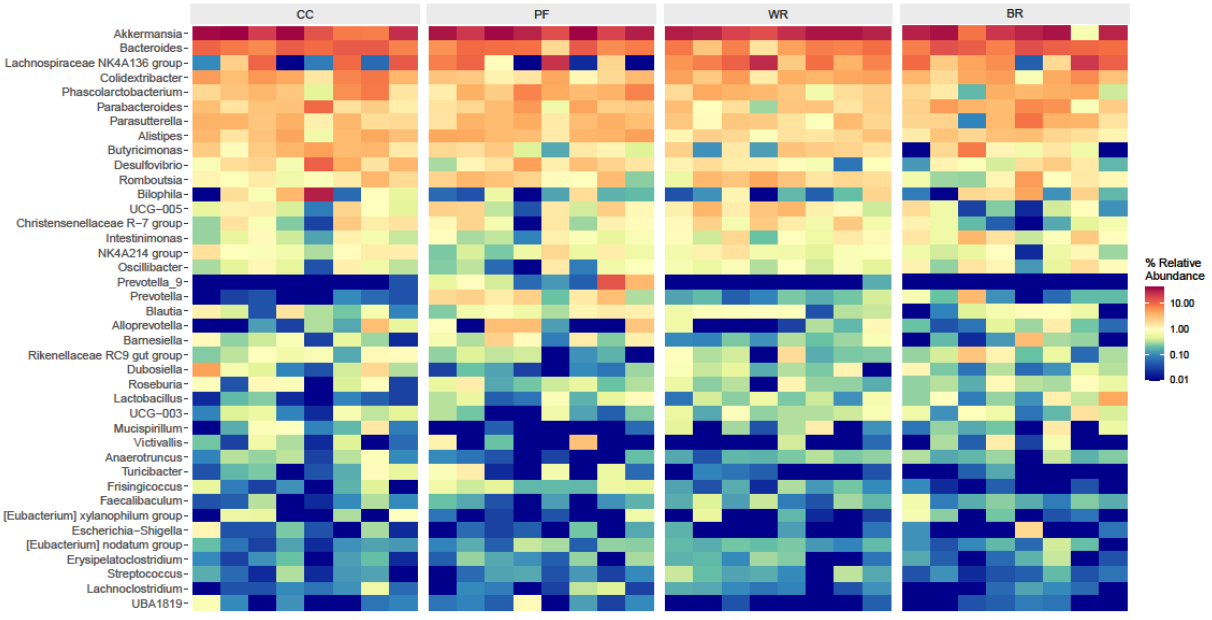
Taxonomic analysis revealed no significant difference between any of the diet groups in the *Firmicutes:Bacteroidetes* ratio ($p = 0.2842$, **Figure 9D**). An increase in the ratio is associated with increased prevalence in obesity [102, 103]. Further taxonomic analysis showed that BR fed rats had significantly greater abundance of *Lactobacillus spp.* than the control diet ($p = 0.0288$, **Figure 9E**). As previously stated, *Lactobacillus* is a popular probiotic genus that has been found to be in greater abundance in healthy individuals [107, 108, 119]. Abundance of *Bifidobacterium* was significantly reduced in WR fed rats compared to the casein control diet ($p = 0.0124$, **Figure 9F**). This is consistent with the report by Moghadasian et al., who found that mice fed wild rice had a low abundance of *Bifidobacterium* in their gut microbiota [115]. One of the greatest differences in taxonomic profiles among the diets was in the abundance of *Prevotella spp.*, which was much greater in rats fed the PF diet ($p = 0.0017$, **Figure 9G**). Increases

in *Prevotella* abundance have been shown to be highly correlated with NAFLD development [113, 114]. *Oscillibacter* abundance tended to be greater in the BR group compared to all other groups (**Figure 9H**), however the differences did not achieve statistical significance ($p = 0.159$). This is different from what was found in a previous wild rice feeding trial using mice in which wild rice feeding significantly decreased *Oscillibacter* abundance compared to the high fat control diet group [115]. Finally, the genus *Alistipes* was significantly less abundant in rats fed the WR diet compared to the casein control ($p = 0.0217$, **Figure 9I**). *Alistipes* abundance was also significantly greater in the PF fed group compared to the BR fed group, however neither of these shifts were significantly different from the casein control diet. The reduction in *Alistipes* abundance in the BR group showed a trend compared to the casein control ($p = 0.094$). Like *Prevotella*, increases in *Alistipes* abundance have been linked to NAFLD and liver fibrosis [114].

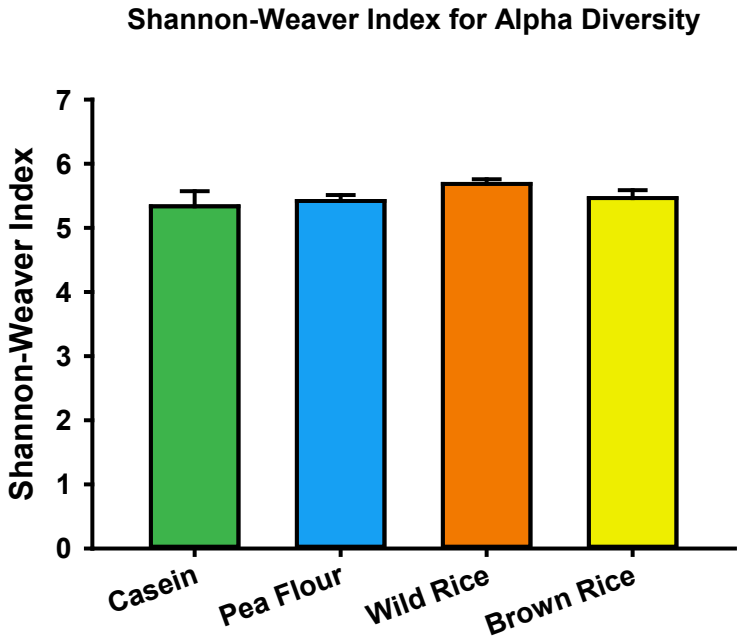
A


0.50
0.25
0.00
−0.25
PCo2 [11.6%]
−0.50
−0.25
0.00
0.25
PCo1 [19%]
Diet
CC
PF
WR
BR

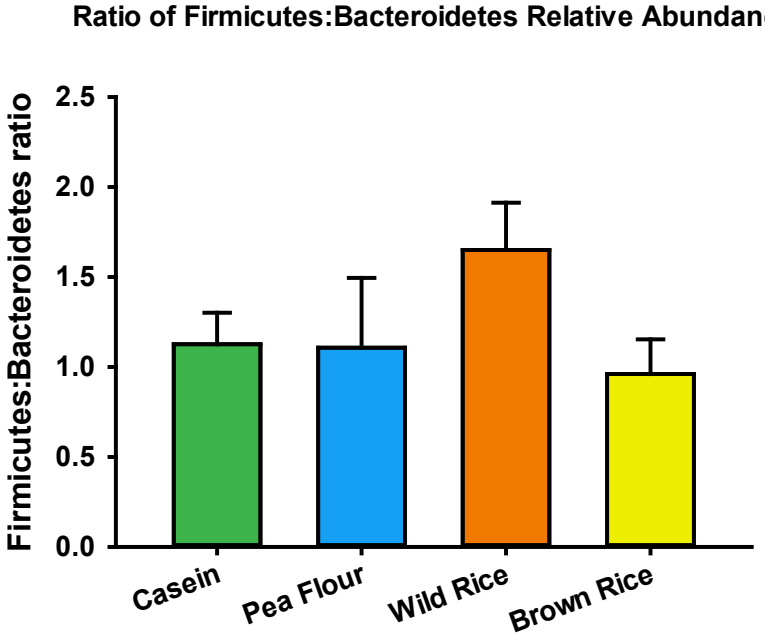
B


CC
PF
WR
BR
Akkermansia
Bacteroides
Lachnospiraceae NK4A136 group
Colidextribacter
Phascolarctobacterium
Parabacteroides
Parasutterella
Alistipes
Butyricimonas
Desulfovibrio
Romboutsia
Bilophila
UCG−005
Christensenellaceae R−7 group
Intestinimonas
NK4A214 group
Oscillibacter
Prevotella_9
Prevotella
Blautia
Alloprevotella
Barnesiella
Rikenellaceae RC9 gut group
Dubosiella
Roseburia
Lactobacillus
UCG−003
Mucispirillum
Victivallis
Anaerotruncus
Turicibacter
Frisingicoccus
Faecalibaculum
[Eubacterium] xylanophilum group
Escherichia−Shigella
[Eubacterium] nodatum group
Erysipelatoclostridium
Streptococcus
Lachnoclostridium
UBA1819
% Relative Abundance
10.00
1.00
0.10
0.01

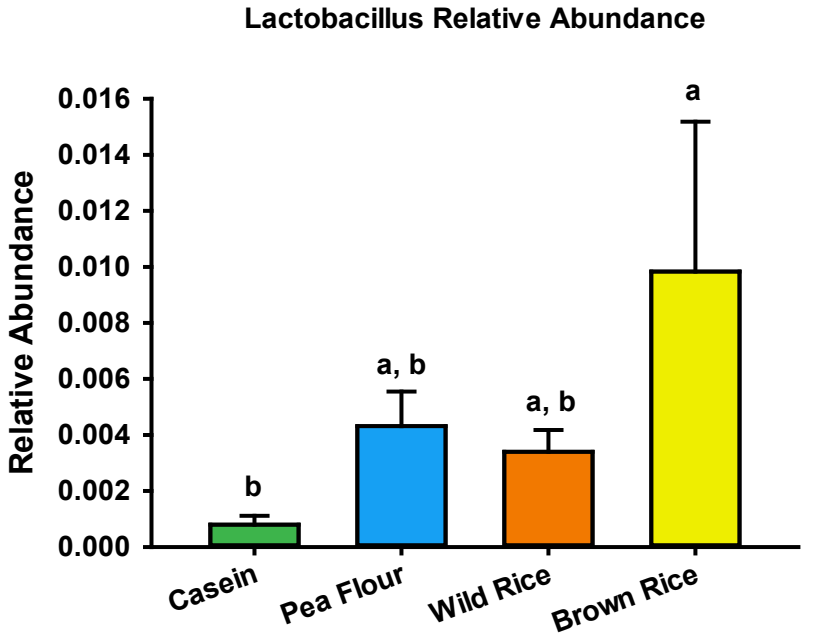
C


Shannon-Weaver Index for Alpha Diversity
Shannon-Weaver Index
7
6
5
4
3
2
1
0
Casein
Pea Flour
Wild Rice
Brown Rice

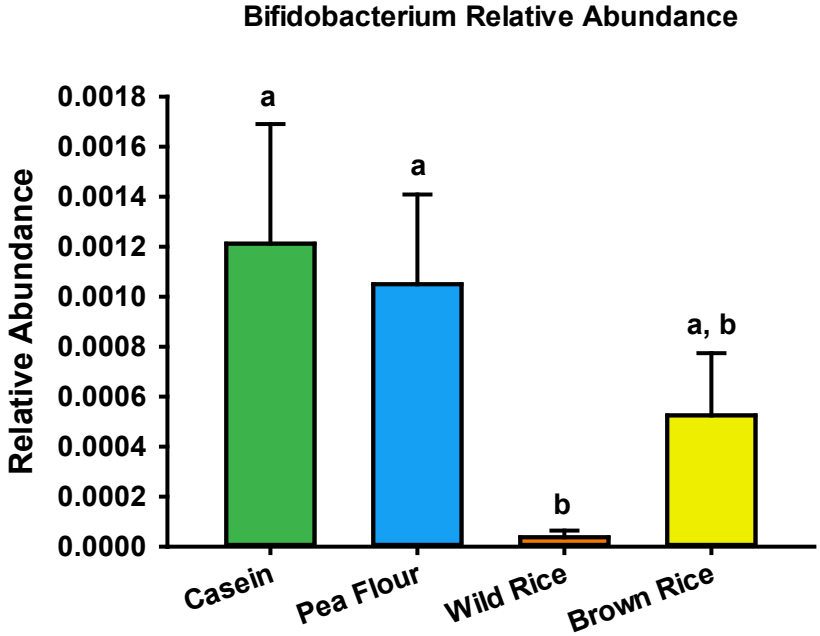
D


Ratio of Firmicutes:Bacteroidetes Relative Abundance
Firmicutes:Bacteroidetes ratio
2.5
2.0
1.5
1.0
0.5
0.0
Casein
Pea Flour
Wild Rice
Brown Rice

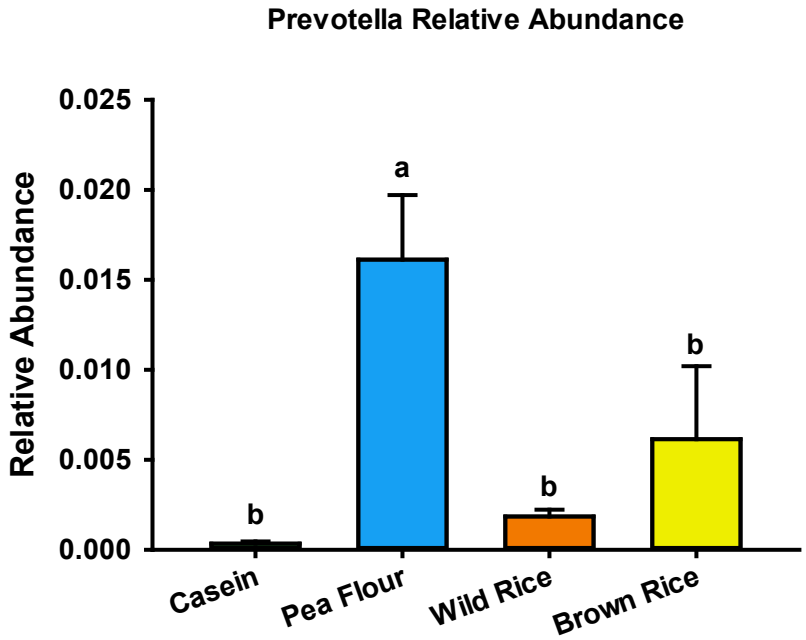
E


Lactobacillus Relative Abundance
Relative Abundance
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
b
a, b
a, b
a
Casein
Pea Flour
Wild Rice
Brown Rice

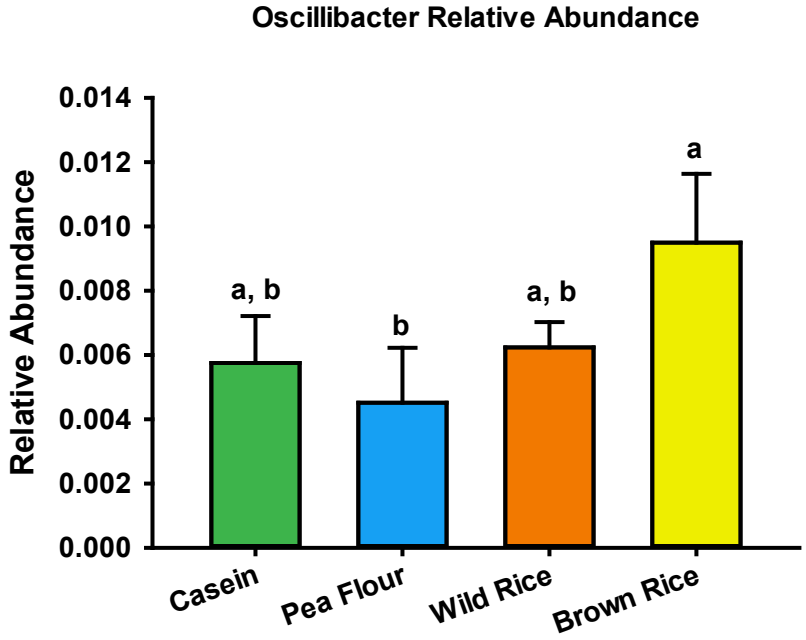
F


Bifidobacterium Relative Abundance
Relative Abundance
0.0018
0.0016
0.0014
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
a
a
b
a, b
Casein
Pea Flour
Wild Rice
Brown Rice

G


Prevotella Relative Abundance
Relative Abundance
0.025
0.020
0.015
0.010
0.005
0.000
a
b
b
b
Casein
Pea Flour
Wild Rice
Brown Rice

H


Oscillibacter Relative Abundance
Relative Abundance
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
a, b
b
a, b
a
Casein
Pea Flour
Wild Rice
Brown Rice

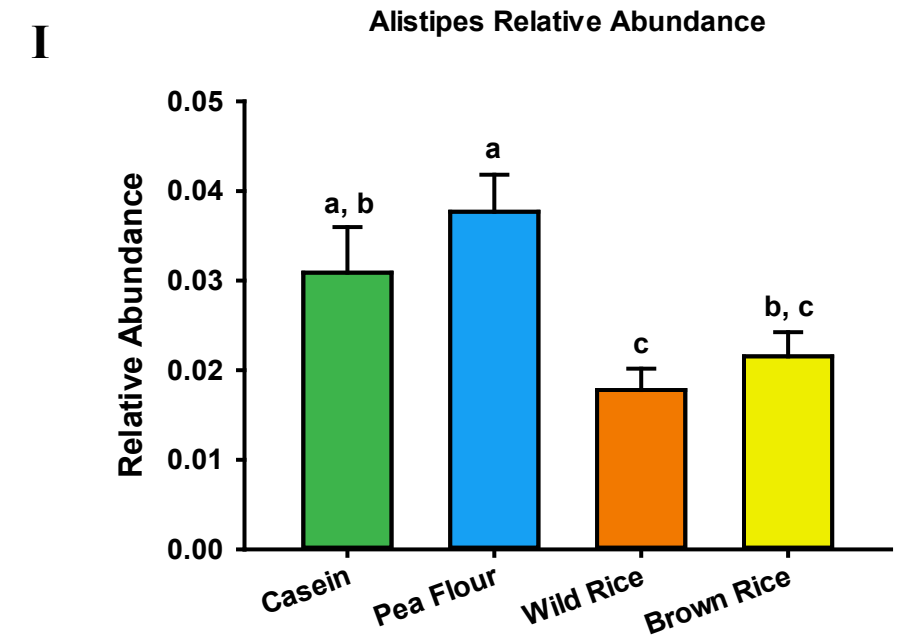


Figure 9: Changes in the Gut Microbiome by Diet Group
(A) Bray Curtis principles coordinate analysis for beta-diversity. Each point represents an animal. Data was statistically analyzed by Adonis (BR vs WR, $p = 0.079$; BR vs CC, $p = 0.067$; BR vs PF, $p = 0.021$; WR vs CC, $p = 0.003$; WR vs PF, $p = 0.011$; CC vs PF, $p = 0.016$). (B) Heat map of each animal's relative abundance of top 40 bacteria gena. Each column represents a different animal. (C) Shannon-Weaver Index as a measure of alpha-diversity. (D) Ratio of the relative abundance of *Firmicutes:Bacteroidetes*. (E) *Lactobacillus* relative abundance. (F) *Bifidobacterium* relative abundance. (G) *Prevotella* relative abundance. (H) *Oscillibacter* relative abundance. (I) *Alistipes* relative abundance. Values represent mean ± SEM, n=8. Values not sharing a common letter are statistically different, $p < 0.05$.

Discussion

This study examined the protein quality of a high protein pea flour, of two pea protein extracts, and of brown and wild rice. Further, the effect of pea flour and the two rices were further studied for several health benefits and their effect on the gut microbiome. Due to health, environmental, and personal concerns, the use of plant-based

proteins has increased drastically in the market over the last few years [4, 13]. Unfortunately, plant proteins are known to be of lesser quality than animal proteins for two main reasons: they are harder to digest, and they are usually limiting in at least one essential amino acid [1, 2]. The protein quality for many plant protein products is not known, therefore, studies like this are important for expanding the knowledge of protein quality of plant proteins being considered for human consumption. The most widely accepted measure of protein quality is the protein digestibility amino acid corrected score (PDCAAS). A standardized PDCAAS assay was first described by the WHO and FAO in 1989 [19] and has been increasingly used since its modernization by the FDA and the U.N. in 1993 [3, 20, 31, 32]. The PDCAAS assay is now the only accepted protein quality assay for food labeling purposes in the United States, and for that reason PDCAAS was used in the present study.

This study determined PDCAAS values for five plant-based proteins. The highest PDCAAS value of the plant proteins was pea flour (PF). The PDCAAS value for PF was 0.73. The most limiting amino acid in PF is tryptophan with an amino acid score of 0.81. The true protein digestibility of PF is 0.89. Both values are close to 1, making PF a higher quality protein than most other plant-proteins [3, 20, 22, 32-34]. Both pea protein isolates (PPIs) had lower PDCAAS values than PF. The salt solubilized and pH precipitated PPIs had true protein digestibility values of 0.93 and 0.94, respectfully. However, their PDCAAS values were lower because the amino acid score was significantly lower in both PPIs compared to the PF (0.70 and 0.61, respectfully). Additionally, the limiting amino acid for the PPIs were cysteine and methionine, not tryptophan (although both

PPIs did not meet the 1991 reference values for tryptophan either). Both cysteine and methionine are sulfur containing amino acids, so it would appear that in the protein extraction method of Hansen et al. [149] a majority of the protein lost during processing were proteins that contained relatively higher amounts of these two amino acids. Finally, brown rice and wild rice had PDCAAS values of 0.61 and 0.60, respectively. These values are both considerably higher than previously reported PDCAAS values for rice [3, 19, 156]. However, the *in vitro* PDCAAS value for BR was similar to the previously reported value and was lower than the one determined using the *in vivo* assay. This is most likely due to the low concentration of protein in BR, which at 7.96% makes it impossible to formulate a diet containing the 10% protein required for the PDCAAS assay. However, since the *in vivo* PDCAAS uses nitrogen intake in the calculation, it is likely that using a dietary protein concentration of less than 10% did not invalidate the results. It is possible that the *in vitro* PDCAAS results for BR were impacted by the lack of crude protein in BR. The research that compares *in vivo* and *in vitro* PDCAAS assays all cover protein sources with crude protein content of at least 10%, so it is difficult to confirm if this suspicion is warranted [35, 157-159]. This should be something to consider going forward when using an *in vitro* PDCAAS assay instead of an *in vivo* model for low protein foods. The *in vitro* model correlated only moderately well with the *in vivo* model when all the experimental proteins were considered. However, when BR was removed, the correlation between the two methods increased (R^2 = 0.810 vs. 0.915).

In the second phase of the animal study, we examined the effect of pea flour and brown and wild rice on fat pad weight and liver lipids to understand the potential for

these plant protein sources to reduce obesity and the development of fatty liver. Given that the prevalence in obesity and non-alcoholic fatty liver disease (NAFLD) are rising rapidly [12], and no pharmacological treatments exist to treat NAFLD [11], finding dietary interventions for these two conditions is highly desirable. Since recent studies are finding associations between the large intestinal bacterial profiles and NAFLD [100-103, 109-114], we also examined the gut microbiome. Plant-based diets have also been known to lower weight gain when compared to omnivorous or more animal based diets [4-7]. Therefore, in this phase of the study, the health benefits of plant-based proteins and how they shift the gut microbiome was examined.

Both WR and BR were able to reduce liver lipids and cholesterol in these rats, with WR showing further benefits by reducing serum cholesterol levels. However, feeding PF did not show any significant reduction in liver or serum lipids and cholesterol. Epididymal fat pad weight, a marker for total body fat [11], was not impacted by any of the experimental diets. Lipids and cholesterol are either absorbed from the small intestines or synthesized metabolically. The key hepatic enzymatic regulators of lipid metabolism and fatty acid synthesis are AMPK and ACC, respectively. None of the diet groups caused any shifts in AMPK or ACC activation, suggesting no change in hepatic lipid oxidation or synthesis pathways.

Both WR and PF caused significant shifts in the gut microbiome compared to the high fat casein control diet. As previously stated, there is evidence of an association between NAFLD and the gut microbiome [109, 110, 112, 113, 160, 161], although for certain bacteria, the findings are inconsistent. Our findings provide mixed support for

such an association. Most notably, obesity and NAFLD have been significantly associated with an increase in *Firmicutes*:*Bacteroidetes* [102, 103], but there were no changes to this ratio among any of the diet groups. *Lactobacillus* and *Bifidobacterium* are two bacterial genera that have been associated with increased abundance in subjects with reduced liver lipids [162-164]. This study showed that BR was able to increase abundance of *Lactobacillus* compared to control, a possible contributing factor to the reduction in liver lipids in the BR diet group. However, *Bifidobacterium* abundance was significantly reduced by the WR fed group. Similar to our finding, Moghadasian et at. reported a greatly reduced *Bifidobacterium choerinum* abundance during WR feeding [115]. The reason for this reduction by wild rice is uncertain.

The group fed PF also saw significant changes in the gut microbiome. The PF group had the largest empty cecum weight of all the diet groups, suggesting the most fermentation [155, 165]. This was confirmed by the significant increase in *Prevotella spp.* in the PF group compared to all the other diet groups. This is consistent with previous reports indicating that *Prevotella* abundance increases in plant-based diets [166]. Interestingly, this increase in fermentation and *Prevotella* abundance did not associate with any physiological benefits. The literature surrounding the impact of *Prevotella* is also very perplexing. Increases in *Prevotella* abundance have had positive correlations with NAFLD development [113, 114]. However, *Prevotella* is very high in individuals living in non-industrialized societies compared to more western, industrialized societies, whose gut microbiomes tend to be largely dominated by *Bacteroidetes* [167-170]. The greater fermentation in the PF group may represent an

increase in proteolytic fermentation, as *Prevotella* has been shown to express proteolytic activity [138, 139]. This is also consistent with the lower protein digestibility of pea flour protein, relative to casein, which would result in more protein passing into the large intestine. As previously stated, proteolytic fermentation has been associated with greater risk of colon cancer and irritable bowel disease (IBD) [133, 135, 136]. This association is mostly seen in diets high in red meats, not plant-based proteins, however [137]. Further research should be done to look for toxic metabolites of protein fermentation in the cecal contents of the PF group. An example of this would be p-cresol, a bacterial metabolite of tyrosine, which has been shown to activate macrophage micropinocytosis, which leads to LDL cholesterol uptake, and mediates pro-inflammatory responses in NAFLD development [135, 171]. This reuptake of LDL cholesterol may be the contributing factor to why PF did not show the same NAFLD preventing effects as the other plant products. Ideally, the PPIs that were tested in the protein quality feeding trial should also be examined to see whether they impart health benefits, or if the increase in true protein digestibility that was shown in the PPIs would yield different results.

Conclusion

Based on the studies reported here, pea flour should be considered a high-quality plant protein due to its high amino acid score and protein digestibility. Wild rice, brown rice, salt solubilized pea protein isolates, and pH precipitated pea protein isolates should all be considered moderate-quality plant proteins. Both pea protein isolation methods can improve total protein digestibility compared to pea flour, but they both lower the PDCAAS scores overall due to loss of sulfur containing amino acids during protein

isolation. This lowers their amino acid score and subsequently their PDCAAS score. Additionally, the *in vitro* PDCAAS assay can be considered a suitable alternative to the *in vivo* PDCAAS assay when testing a protein source greater than 10% crude protein content.

Wild rice and brown rice both show the ability to prevent the development of fatty liver, an early stage of NAFLD, when consuming a high fat diet. However, pea flour does not appear to have this same protective effect. Additionally, it is unclear if serum LECT2 is an appropriate biomarker for NAFLD development. LECT2 serum levels have long been considered a serum biomarker for NAFLD development [152-154], but this experiment showed that total hepatic lipids did not correlate well with serum LECT2 levels. The mechanism by which brown and wild rice reduce liver total lipids and cholesterol is not yet clear, but it may be linked to changes in the gut microbiome.

Finally, wild rice and pea flour feeding both caused significant shifts in the gut microbiome. The mechanism by which wild rice leads to potentially beneficial shifts, and whether these shifts in the gut microbiome are responsible for reducing in liver lipids is unclear. The most notable shift in the gut microbiome among the different diets was pea flour feeding significantly increasing *Prevotella* relative abundance compared to all other diet groups. *Prevotella* is a bacterium that can ferment undigested proteins [138, 139], potentially leading to an increased risk in colon cancer and IBD, and increases in *Prevotella* abundance have been associated with NAFLD development [113, 114]. However, *Prevotella* has commonly been associated with increased abundance in non-industrialized societies compared to western, industrialized societies [167-170]. Thus, the

physiological implications of this increase in *Prevotella* in the present study are uncertain, based on the lack of changes in liver lipids or epididymal fat pad weight and the contrasting findings in the literature. Finally, determining the mechanism of how wild rice feeding can reduce the development of fatty liver is an opportunity for future studies.

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Chapter IV: Appendices

Appendix 1: Amino Acid Analysis by Eurofins

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ANALYTICAL REPORT

AR-20-QD-183015-01

Client Code: QD0009543

Received On: 06Oct2020
Reported On: 14Oct2020

Eurofins Sample Code:	464-2020-10060530	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Brown rice	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Cooked and ground brown rice	**Sample Reference:**	

QQ141 - Tryptophan (AOAC, Most Matrices)	**Reference** AOAC 988.15 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 09Oct2020

Parameter	**Result**
Tryptophan	0.13 %

QQ177 - Cystine & Methionine (AOAC, Most Matrices)	**Reference** AOAC 994.12 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 12Oct2020

Parameter	**Result**
Cystine	0.16 %
Methionine	0.22 %

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference** AOAC 982.30 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 14Oct2020

Parameter	**Result**
Alanine	0.50 %
Arginine	0.72 %
Aspartic Acid	0.79 %
Glutamic Acid	1.37 %
Glycine	0.42 %
Histidine	0.21 %
Isoleucine	0.32 %
Leucine	0.65 %
Phenylalanine	0.43 %
Proline	0.34 %
Serine	0.41 %
Threonine	0.31 %
Total Lysine	0.36 %

10/14/20 6:53 pm

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Client Code: QD0009543

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Client Sample Code:	Brown rice	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Cooked and ground brown rice	**Sample Reference:**	

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference**	**Accreditation**	**Completed**
	AOAC 982.30 mod.	ISO/IEC 17025:2017 A2LA 2927.01	14Oct2020

Parameter	**Result**
Tyrosine	0.33 %
Valine	0.51 %

Respectfully Submitted,

Loreen Jones
Support Services Coordinator

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ANALYTICAL REPORT

AR-20-QD-183035-01

Client Code: QD0009543

Received On: 06Oct2020
Reported On: 14Oct2020

Eurofins Sample Code:	464-2020-10060534	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Casein	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Casein used for diets	**Sample Reference:**	

QQ141 - Tryptophan (AOAC, Most Matrices)	**Reference** AOAC 988.15 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 09Oct2020

Parameter	**Result**
Tryptophan	1.27 %

QQ177 - Cystine & Methionine (AOAC, Most Matrices)	**Reference** AOAC 994.12 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 12Oct2020

Parameter	**Result**
Cystine	0.38 %
Methionine	2.51 %

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference** AOAC 982.30 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 14Oct2020

Parameter	**Result**
Alanine	2.91 %
Arginine	3.52 %
Aspartic Acid	6.77 %
Glutamic Acid	21.11 %
Glycine	1.77 %
Histidine	2.83 %
Isoleucine	4.84 %
Leucine	9.23 %
Phenylalanine	4.91 %
Proline	10.64 %
Serine	5.51 %
Threonine	4.19 %
Total Lysine	7.59 %

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ANALYTICAL REPORT

AR-20-QD-183035-01

Received On: 06Oct2020
Reported On: 14Oct2020

Eurofins Sample Code:	464-2020-10060534	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Casein	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Casein used for diets	**Sample Reference:**	

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference**	**Accreditation**	**Completed**
	AOAC 982.30 mod.	ISO/IEC 17025:2017 A2LA 2927.01	14Oct2020

Parameter	**Result**
Tyrosine	5.19 %
Valine	6.21 %

Respectfully Submitted,

Sean Howard
Evening Manager

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ANALYTICAL REPORT

AR-20-QD-183016-01

Client Code: QD0009543

Received On: 06Oct2020
Reported On: 14Oct2020

Eurofins Sample Code:	464-2020-10060531	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Pea protein meal	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Pea protein meal ground	**Sample Reference:**	

QQ141 - Tryptophan (AOAC, Most Matrices)	**Reference** AOAC 988.15 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 09Oct2020

Parameter	**Result**
Tryptophan	0.20 %

QQ177 - Cystine & Methionine (AOAC, Most Matrices)	**Reference** AOAC 994.12 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 12Oct2020

Parameter	**Result**
Cystine	0.27 %
Methionine	0.21 %

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference** AOAC 982.30 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 14Oct2020

Parameter	**Result**
Alanine	0.88 %
Arginine	1.58 %
Aspartic Acid	2.30 %
Glutamic Acid	3.35 %
Glycine	0.90 %
Histidine	0.50 %
Isoleucine	0.83 %
Leucine	1.43 %
Phenylalanine	0.96 %
Proline	0.72 %
Serine	0.95 %
Threonine	0.77 %
Total Lysine	1.51 %

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ANALYTICAL REPORT

AR-20-QD-183016-01

Received On: 06Oct2020
Reported On: 14Oct2020

Eurofins Sample Code:	464-2020-10060531	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Pea protein meal	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Pea protein meal ground	**Sample Reference:**	

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference**	**Accreditation**	**Completed**
	AOAC 982.30 mod.	ISO/IEC 17025:2017 A2LA 2927.01	14Oct2020

Parameter	**Result**
Tyrosine	0.56 %
Valine	0.97 %

Respectfully Submitted,

Loreen Jones
Support Services Coordinator

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ANALYTICAL REPORT

AR-20-QD-183017-01

Client Code: QD0009543

Received On: 06Oct2020
Reported On: 14Oct2020

Eurofins Sample Code:	464-2020-10060532	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Pea protein isolate - pH	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Pea protein isolated at high pH	**Sample Reference:**	

QQ141 - Tryptophan (AOAC, Most Matrices)	**Reference** AOAC 988.15 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 09Oct2020

Parameter	**Result**
Tryptophan	0.81 %

QQ177 - Cystine & Methionine (AOAC, Most Matrices)	**Reference** AOAC 994.12 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 12Oct2020

Parameter	**Result**
Cystine	0.60 %
Methionine	0.84 %

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference** AOAC 982.30 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 14Oct2020

Parameter	**Result**
Alanine	3.64 %
Arginine	7.89 %
Aspartic Acid	10.32 %
Glutamic Acid	15.91 %
Glycine	3.54 %
Histidine	2.25 %
Isoleucine	4.02 %
Leucine	7.54 %
Phenylalanine	4.66 %
Proline	3.60 %
Serine	4.51 %
Threonine	3.12 %
Total Lysine	6.70 %

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Received On: 06Oct2020
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Eurofins Sample Code:	464-2020-10060532	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Pea protein isolate - pH	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Pea protein isolated at high pH	**Sample Reference:**	

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference**	**Accreditation**	**Completed**
	AOAC 982.30 mod.	ISO/IEC 17025:2017 A2LA 2927.01	14Oct2020

Parameter	**Result**
Tyrosine	3.20 %
Valine	4.38 %

Respectfully Submitted,

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ANALYTICAL REPORT

AR-20-QD-183018-01

Client Code: QD0009543

Received On: 06Oct2020
Reported On: 14Oct2020

Eurofins Sample Code:	464-2020-10060533	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Pea protein isolate - salt2	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Pea protein isolated with high salt	**Sample Reference:**	

QQ141 - Tryptophan (AOAC, Most Matrices)	**Reference** AOAC 988.15 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 09Oct2020

Parameter	**Result**
Tryptophan	0.91 %

QQ177 - Cystine & Methionine (AOAC, Most Matrices)	**Reference** AOAC 994.12 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 12Oct2020

Parameter	**Result**
Cystine	0.87 %
Methionine	0.87 %

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference** AOAC 982.30 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 14Oct2020

Parameter	**Result**
Alanine	4.30 %
Arginine	7.69 %
Aspartic Acid	11.10 %
Glutamic Acid	16.06 %
Glycine	4.09 %
Histidine	2.39 %
Isoleucine	4.17 %
Leucine	7.35 %
Phenylalanine	4.87 %
Proline	4.05 %
Serine	4.75 %
Threonine	3.84 %
Total Lysine	7.38 %

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ANALYTICAL REPORT

AR-20-QD-183018-01

Received On: 06Oct2020
Reported On: 14Oct2020

Eurofins Sample Code:	464-2020-10060533	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Pea protein isolate - salt2	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Pea protein isolated with high salt	**Sample Reference:**	

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference**	**Accreditation**	**Completed**
	AOAC 982.30 mod.	ISO/IEC 17025:2017 A2LA 2927.01	14Oct2020

Parameter	**Result**
Tyrosine	3.67 %
Valine	4.61 %

Respectfully Submitted,

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ANALYTICAL REPORT

AR-20-QD-183014-01

Client Code: QD0009543

Received On: 06Oct2020
Reported On: 14Oct2020

Eurofins Sample Code:	464-2020-10060529	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Wild rice	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Cooked and ground wild rice	**Sample Reference:**	

QQ141 - Tryptophan (AOAC, Most Matrices)	**Reference** AOAC 988.15 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 09Oct2020

Parameter	**Result**
Tryptophan	0.20 %

QQ177 - Cystine & Methionine (AOAC, Most Matrices)	**Reference** AOAC 994.12 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 12Oct2020

Parameter	**Result**
Cystine	0.19 %
Methionine	0.35 %

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference** AOAC 982.30 mod.	**Accreditation** ISO/IEC 17025:2017 A2LA 2927.01	**Completed** 14Oct2020

Parameter	**Result**
Alanine	0.70 %
Arginine	0.96 %
Aspartic Acid	1.26 %
Glutamic Acid	2.26 %
Glycine	0.59 %
Histidine	0.34 %
Isoleucine	0.53 %
Leucine	0.86 %
Phenylalanine	0.63 %
Proline	0.52 %
Serine	0.70 %
Threonine	0.44 %
Total Lysine	0.54 %

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Client Code: QD0009543

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ANALYTICAL REPORT

AR-20-QD-183014-01

Received On: 06Oct2020
Reported On: 14Oct2020

Eurofins Sample Code:	464-2020-10060529	**Sample Registration Date:**	06Oct2020
Client Sample Code:	Wild rice	**Condition Upon Receipt:**	acceptable, non-perishable
Sample Description:	Cooked and ground wild rice	**Sample Reference:**	

QQ176 - Amino Acids by AH (AOAC, Most Matrices)	**Reference**	**Accreditation**	**Completed**
	AOAC 982.30 mod.	ISO/IEC 17025:2017 A2LA 2927.01	14Oct2020

Parameter	**Result**
Tyrosine	0.40 %
Valine	0.73 %

Respectfully Submitted,

Loreen Jones
Support Services Coordinator

Appendix 2: Proxy Analysis by Medallion Labs

Medallion Labs
www.medallionlabs.com 800-245-5615 info@medlabs.com

Order Number:	**2020-001886**	**Completed Date:**	06-Mar-2020
		Submitted Date:	27-Feb-2020
Submitter:	Daniel Gallaher		
Company:	U of MN - Twin Cities		
Company Address:	1334 Eckles Ave		
	Dept of Food Science and Nutrition		
	St Paul, MN 55108		
Results Email:	dgallahe@umn.edu		
Invoice Email:	dgallahe@umn.edu		
Purchase Order:	CC		

Order # Sample ID: 2020-001886-01 **Company:** U of MN - Twin Cities

Customer Sample ID: Wild Rice University of Minnesota

Sample Description:

round Cultivated Wild Rice G

Analytical Testing

Method:	Component:	Result:	Test Date:
Ash	Ash	1.303 %	05-Mar-2020
[2] Calories	Calories	362 Calories/100 g	06-Mar-2020
	Calories, 2020	362 Calories/100 g	06-Mar-2020
	Calories from Fat	9 Calories/100 g	06-Mar-2020
[2] Carbohydrates	Carbohydrates	75.3 %	06-Mar-2020
Fat (Gravimetric)	Total Fat	1.0 %	04-Mar-2020
Fiber (AOAC 991.43)	Total Dietary Fiber	3.5 %	04-Mar-2020
Moisture by Forced Air Oven	Moisture	9.406 %	03-Mar-2020
Protein	Protein (6.25)	13.0 %	04-Mar-2020
[2] Total Starch	Total Starch	74.6 %	06-Mar-2020

Order # Sample ID: 2020-001886-02 **Company:** U of MN - Twin Cities

Customer Sample ID: Brown Rice University of Minnesota

Sample Description: Long Grain Brown Rice

Analytical Testing

Method:	Component:	Result:	Test Date:
Ash	Ash	1.091 %	05-Mar-2020
[2] Calories	Calories	359 Calories/100 g	06-Mar-2020
	Calories, 2020	359 Calories/100 g	06-Mar-2020
	Calories from Fat	25 Calories/100 g	06-Mar-2020
	Calories (Insoluble Fiber Subtracted)	359 Calories/100 g	06-Mar-2020
[2] Carbohydrates	Carbohydrates	75.5 %	06-Mar-2020
Fat (Gravimetric)	Total Fat	2.8 %	04-Mar-2020
Fiber (AOAC 991.43)	Total Dietary Fiber	2.8 %	04-Mar-2020
Moisture by Vacuum Oven	Moisture	12.691 %	06-Mar-2020
Protein	Protein (6.25)	7.96 %	04-Mar-2020
[2] Total Starch	Total Starch	75.9 %	06-Mar-2020

Results Approved By: Steven Murray
(Authorized Reviewer)

Analytical Method References:

Method Name	Method Reference
Ash	AOAC: 923.03*
Calories	Please contact for Method Details
Carbohydrates	Please contact for Method Details
Fat (Gravimetric)	AOAC: 948.15, 922.06, 925.32, 950.54, 922.09*
Fiber (AOAC 991.43)	AOAC: 991.43*
Moisture by Forced Air Oven	AOAC: 926.07, 925.10*, AACC: 44.15A, 44.31*
Moisture by Vacuum Oven	AOAC: 945.43*, 934.01*
Protein	AACC 46-30*; AOAC 992.15*
Total Starch	AOAC 979.10*, AACC 76-11*

* This method has been modified.

Appendix 3: Megazyme in vitro Protein Digestibility Assay

INTRODUCTION:

Protein is an important nutritional component for all mammals. Unlike most plants and microorganisms that can biosynthesise all twenty standard amino acids needed for health and reproduction, typical mammals cannot synthesise all amino acids for survival. These amino acids which cannot be synthesised are referred to as the essential amino acids and are required to be obtained through the diet by ingesting protein-containing foods comprised of these essential amino acids. Digestive enzymes can hydrolyse peptide bonds in the ingested proteins to release individual amino acids and small peptides which can be absorbed into the body, providing nutritional benefits.

International and local governmental regulatory bodies throughout the world have introduced standards by which the quality of protein in a food product may be judged for its completeness in delivery of the essential amino acids needed for human growth and sustenance. These regulatory requirements not only assess the compositional presence of the essential amino acids but the digestibility of the proteins and relative efficiency of the release of the amino acids present. The quality of a protein is judged from a regulatory standpoint as a function of the efficiency of protein digestion multiplied by the presence of essential amino acids. This is in essence the foundation of the most widely accepted protein quality test, known as the Protein Digestibility Corrected Amino Acid Score (PDCAAS) test, required by most regulatory bodies when asserting a protein content claim on a commercial food product.

Traditionally, protein digestibility has been evaluated *in vivo* using rat subjects to measure the amount of protein digestion that occurs when fed a protein containing product. While yielding suitable results this *in vivo* measurement process is costly and time consuming.

Additionally, this type of testing is prohibitive to food companies and individuals which uphold animal testing bans for compassionate reasons.

The Animal-Safe Accurate Protein Quality Score (ASAP-Quality Score) provided by this kit (**K-PDCAAS**), is an *in vitro* enzyme digestion method that has a very high correlation to the rat digestion model and uses the same casein standard as a completely digestible control.

NOTE: For this method each sample must also be analysed for amino acid profile, including cysteine and methionine as well as tryptophan. Analysis of protein by Dumas and moisture are required for reporting purposes.

PRINCIPLE:

Protein samples are digested by pepsin in dilute HCl (pH 2) followed by digestion with trypsin and chymotrypsin in a neutral buffer to simulate the physiological conditions of gastric and intestinal digestion, respectively (1, 2).

(1) Proteins $\xrightarrow{\text{(pepsin; pH 2.0, 37°C)}}$ proteins + peptides + amino acids

(2) Proteins $\xrightarrow{\text{(trypsin + chymotrypsin; pH 7.4, 37°C)}}$ proteins + peptides + amino acids

Undigested proteins are removed by precipitation with trichloroacetic acid. Amine groups of amino acids made available for reaction by the digestion are quantified by the reaction with ninhydrin to form ruhemann's purple (3).

(3) Ninhydrin + α-amino acids $\xrightarrow{\text{(pH 5.5, 70°C)}}$ Ruhemann's purple + $RCHO + H_2O + CO_2$

The amount of ruhemann's purple formed in this reaction is proportional to the amount of reactive α-amino acids present in the sample and is measured by the increase in absorbance at 570 nm. When corrected for the relative reactivity of certain α-amino acids, an *in vitro* digestibility score can be calculated. This digestibility score, in conjunction with the essential amino acid analysis of the sample, is used to calculate the PDCAAS result.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for the measurement of α-amino acids in all types of food products and protein concentrates.
The range of this method is from 0 to 1 for *in vitro* digestibility. The range of this method is from 0 to 1 for PDCAAS.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.
For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 50 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Pepsin (125 mg)

Stable for > 2 years below -10°C.

Bottle 3: Chymotrypsin (125 mg)
Stable for > 2 years below -10°C.

Bottle 4: L-Glycine (500 mg)
Stable for > 5 years at room temperature.

Bottle 5: **Control A** - control powder (~ 5 g)
Stable for > 5 years; store sealed at below -10°C.

Bottle 6: **Control B** - control powder (~ 5 g)
Stable for > 5 years; store sealed at below -10°C.

Bottle 7: **Control C** - control powder (~ 5 g)
Stable for > 5 years; store sealed at below -10°C.

Bottle 8: **Control D** - control powder (~ 5 g)
Stable for > 5 years; store sealed at below -10°C.

Bottle 9: **Control E** - control powder (~ 5 g)
Stable for > 5 years; store sealed at below -10°C.

Bottle 10: **Control F** - control powder (~ 5 g)
Stable for > 5 years; store sealed at below -10°C.

Bottle 11: Casein control powder (~ 5 g)
Stable for > 5 years; store sealed at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Pepsin Solution (1 mg/mL) – Weigh 1 mg of Pepsin (bottle 1) per sample into a suitably sized centrifuge tube. Add 2 additional mg so that there is extra for pipetting. Add 1 mL of the 0.06 N HCl per mg of Pepsin to the centrifuge tube. Lightly vortex to mix. Example: 15 samples in batch = (17 x 1 mg) 17 mg. Add 17 mL (17 x 1 mL) of 0.06 N HCl.
Note: Make fresh daily, use within 30 min.

2 & 3. Trypsin/Chymotrypsin Solution (5 mg/mL) – Weigh 1 mg of Trypsin (bottle 2) and 1 mg of Chymotrypsin (bottle 3) per sample into a suitably sized centrifuge tube. Add 2 additional mg of each enzyme so that there is extra for pipetting. Add 200 µL of the 0.001 N HCl per sample (plus an additional 400 µL) to the centrifuge tube. Lightly vortex to mix. Example: 15 samples in batch = (17 x 1 mg) 17 mg of Trypsin and Chymotrypsin. Add 3400 µL (17 x 200 µL) of 0.001 N HCl.
Note: Make fresh daily, use within 30 min.

4. To prepare the 10 mM L-glycine stock, weigh 37.5 mg of L-glycine into a 50 mL volumetric flask. Add approx. 25 mL sodium acetate buffer (50 mM, pH 5.5) and stir until dissolved. Bring to volume (50 mL) with sodium acetate buffer (50 mM, pH 5.5), cap and invert several times to mix.

months at 4°C.

5-11. Use the contents of bottles 6-11 as supplied. Stable for > 5 years; store sealed at below -10°C (bottles 5-10) or at room temperature (bottle 11).

PREPARATION OF REAGENT SOLUTIONS (NOT SUPPLIED):

1. Ninhydrin Reagent (2% solution)

NOTE: After using ninhydrin, purge the stock bottle with nitrogen and seal with parafilm before returning to the refrigerator. If ninhydrin is not capped with nitrogen, oxidation will occur and the reagent will no longer work sufficiently.

(Sigma cat. no. N7285 or equivalent). Use as supplied.

2. Hydrochloric acid (0.06 N, pH 2.0): 1 L

Place approx. 900 mL of distilled water in a 1 L beaker. Add 5 mL of 12 N HCl while stirring. Adjust the pH to 2.0 with 2 N NaOH. Transfer to a 1 L volumetric flask and bring to volume (1 L) with distilled water. Transfer to a suitable sealed container. Store for up to 1 year at room temperature.

3. Trichloroacetic acid (40% w/v): 100 mL

Add 40 g of trichloroacetic acid (Sigma cat. no. T6399) to approx. 80 mL of distilled water and dissolve by stirring. Make to volume (100 mL) with distilled water. Store for up to 1 year at room temperature.

4. Hydrochloric acid (0.001 N, pH 3.0): 500 mL

Place approx. 450 mL of distilled water in a 500 mL beaker. Add 5 mL of 0.1 N HCl while stirring. Adjust the pH to 3.0 with 0.1 N HCl/NaOH. Transfer to a 500 mL volumetric flask and bring to volume (500 mL) with distilled water. Transfer to a suitable sealed container. Store for up to 1 year at room temperature.

5. Tris Buffer (1.0 M, pH 7.4): 250 mL

Place 150 mL of distilled water in a beaker. Add 30.29 g of Tris base while stirring. Slowly add 15 mL 12 N HCl. Adjust the pH to 7.4 with 1 N HCl and transfer to a 250 mL volumetric flask. Bring to volume (250 mL) with distilled water and mix. Sterile filter buffer and transfer to a sealed container. Store for up to 4 months at room temperature.

6. Sodium acetate buffer (50 mM, pH 5.5): 1 L

hydroxide solution. Transfer to a 1 L volumetric flask and make to volume with distilled water. Store for up 6 months at room temperature.

7. **Reagent Alcohol (50% v/v): 1 L**

   Add reagent alcohol (VWR Scientific Products cat. no. BDH-1156-4LP) to an equal volume of distilled water and mix thoroughly. Store for up 1 year at room temperature.

EQUIPMENT (RECOMMENDED):

1. 96 well microplate (e.g. clear flat-bottomed, glass or plastic).
2. Centrifuge Tubes (50 mL, 15 mL and 2 mL), e.g. cat. no. 21008-240, cat. no. 89039-670 and cat. no. 22179-008 (VWR Scientific Products).
3. Centrifuge Tubes 50 mL, round bottom, e.g. cat. no. P20504MPI (Beckman Coulter).
4. Disposable funnels, e.g. cat. no. 30246-021 (VWR Scientific Products).
5. Culture Tubes, 16 x 100 mm and 13 x 100 mm, e.g. cat. no. 47729-576 and cat. no. 47729-572 (VWR Scientific Products).
6. Culture Tube Caps, 16 mm and 13 mm, e.g. cat. no. 60828-766 and cat. no. 60828-746 (VWR Scientific Products).
7. Media bottles 1 L, 500 mL and 250 mL, e.g. cat. no. 16157-191, cat. no. 16157-169 and cat. no. 6157-136 (VWR Scientific Products).
8. Water bottles, e.g. cat. no. 10111-950 (VWR Scientific Products).
9. Disposable Pipets, e.g. cat. no. 414004-001 (VWR Scientific Products).
10. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 200 µL and 1 mL).
11. Positive displacement pipettor, e.g. Eppendorf Multipette® with 5.0 mL and 50 mL Combitip®.
12. Stop clock.
13. pH Meter.
14. Analytical balance (capable of weighing to +/- 0.0001 g).
15. Microplate reader set at 570 nm.
16. Heated water bath (capable of 95°C).
17. Shaking incubator (capable of 37°C and 70°C).
18. Centrifuge with rotor capable of 15,000 x *g*, e.g. Avanti J-26XPI cat. no. J326XPI-IM-4AB (Beckman Coulter).
19. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).

STANDARD ASSAY PROCEDURE:

NOTES:

1. Calibration standards are only needed if generating a calibration curve for calculating *in vitro* protein digestibility. A previously generated curve can be used but only if a casein control sample is tested as a cross reference to the curve.
2. For each batch of samples that is applied to the PDCAAS procedure a blank sample (i.e. an empty tube to which all reagents are added) must also be included.

A. SAMPLE EXTRACTION:

1. Mill cereal, plant or food product to pass a 0.5 mm screen.
2. Accurately weigh approx. 500 mg of milled, calibration or casein control sample into a 50 mL Beckman centrifuge tube. Tap the tube to ensure that all of the sample drops to the bottom of the tube.

3. Gastric and Intestinal Digestion:

   a. Add 19 mL of HCl (0.06 N) and cap the tube. Mix thoroughly by vortex and incubate for 30 min at 37°C in a hot air, shaking incubator set at 300 rpm.
   b. Add 1 mL of pepsin solution (bottle 1) to each sample and cap each tube. Mix thoroughly by vortex and incubate for 60 min at 37°C in a hot air shaking incubator set at 300 rpm.
   c. After the pepsin incubation is complete, remove samples and adjust the pH to 7.4 by the addition of 2 mL of 1.0 M Tris buffer, pH 7.4. Cap the tubes and mix each sample thoroughly by vortex.

   d. Add 200 µL of Trypsin/Chymotrypsin mixture to each sample, mix thoroughly by vortex and incubate for 4 h at 37°C in a hot air, shaking incubator set at 300 rpm.
   e. At the end of the Trypsin/Chymotrypsin incubation place the samples in a boiling water bath for 10 min.
   f. Remove all samples from the boiling water bath and mix thoroughly by vortex.

4. Allow the samples to cool to room temperature for at least 20 min and transfer 4 mL of each to a 16 x 100 culture tube. **Note: Remaining sample can be frozen for future analysis. Store at -80°C.**
5. Add 1 mL of 40% TCA solution, cap and mix thoroughly by vortex. Incubate the samples at 4°C overnight (at least 16 h). **Note: Analysis can be held at this point.**

at room temperature.

7. Make a 10-fold and 20-fold dilution in acetate buffer (50 mM, pH 5.5) into a culture tube for all samples. The blank and controls (A-F) only require a 10-fold dilution, while the casein requires a 20-fold dilution.
   Note: All samples require a minimum of a 10-fold dilution to bring the TCA concentration down to a level that will not affect the Colourimetric Determination of Amines (Section C).
8. All diluted supernatants of the sample solutions, including the sample blanks, calibration samples and the casein control samples, are applied to the Colourimetric Determination of Amines (see section C).

B. PREPARATION OF THE L-GLYCINE CALIBRATION STANDARDS:

Prepare the standard L-glycine standard solutions in 15 mL culture tubes as described in the **Table 1** and treat as samples in the Colourimetric Determination of Amines (see section C). Stable for up to 1 month at 4°C.

Table 1. Preparation of the L-gLycine calibration standards.

L-Glycine Standards (mM)		Sodium Acetate (50 mM, pH 5.5)	L-Glycine standard
ST 11	1	9 mL	1 mL of 10 mM L-glycine
ST 10	0.75	9.25 mL	0.75 mL of 10 mM L-glycine
ST 9	0.5	9.5 mL	0.5 mL of 10 mM L-glycine
ST 8	0.25	9.75 mL	0.25 mL of 10 mM L-glycine
ST 7	0.1	9.9 mL	0.1 mL of 10 mM L-glycine
ST 6	0.075	9 mL	1 mL of STD 10
ST 5	0.05	9 mL	1 mL STD 9
ST 4	0.025	9 mL	1 mL STD 8
ST 3	0.01	9 mL	1 mL STD 7
ST 2	0.007	9 mL	1 mL STD 6
ST 1	0.005	9 mL	1 mL STD 5
ST 0	0	10 mL	0

C. COLOURIMETRIC DETERMINATION OF AMINES:

NOTE: For each batch of samples that is applied to the Colourimetric Determination of Amines, **a calibration curve of L-glycine must be performed concurrently using the same batch of reagents (see section B).**

1. Set up the reactions for the Colourimetric Determination of Amines as shown in **Table 2** using the suggested format in the microplate (**Table 3**).

Table 2. Reaction set up for the colourimetric determination of amines.

Wavelength:	570 nm	
Cuvette:	96-well (e.g. clear flat-bottomed, glass or plastic)	
Temperature:	70°C	
Final volume:	0.300 mL	
Sample solution:	0-1 mM of L-glycine (in a 0.100 mL sample volume)	
Pipette into wells	Sample	Standard
sample solution (incl. blank)	0.100 mL	-
standard solutions (glycine)	-	0.100 mL
ninhydrin reagent* (2%)	0.050 mL	0.050 mL
Place the lid on the plate and cover with foil and place on a pre-heated tray in a hot air incubator*** at 70°C for 35 min at 100 rpm. Remove the plate from the incubator. Keep covered with foil while allowing to cool for 10 min. **Then add:**		
reagent alcohol (50% v/v)	0.150 mL	0.150 mL
Mix** and read the absorbances of the solutions at 570 nm against the L-glycine standard (ST 0).		

* Ensure that the ninhydrin has warmed to room temperature before use.

** For example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

*** Alternatively, the microplate can be incubated in a plate reader that has a heating facility. In this instance, there is no requirement to cover with foil.

NOTE: If the absorbance of the samples is above the range of the glycine standards it is necessary to further dilute the samples and perform the Colourimetric Determination of Amines again. Calculated amine levels will need to be corrected by this dilution factor.

Table 3. Plate layout for the colourimetric determination of amines reaction.

	1	2	3	4	5	6	7	8	9	10	11	12
A	ST11	ST10	ST 9	ST 8	ST 7	ST 6	ST 5	ST 4	ST 3	ST 2	ST 1	ST
B	ST11	ST10	ST 9	ST 8	ST 7	ST 6	ST 5	ST 4	ST 3	ST 2	ST 1	ST
C	Blank	A	B	C	D	E	F	Casein	S1	S2	S3	S4
D	Blank	A	B	C	D	E	F	Casein	S1	S2	S3	S4
E	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16
F	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16
G	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
H	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24

ST 0 - ST 11 = L-Glycine standards 0-11 **A - F** = Control samples A-F **S1 - S24** = Test samples 1-24

SAMPLE PREPARATION:

1. Ground, frozen samples should be stored below -10°C and thoroughly homogenised prior to weighing.
2. Refrigerated samples should be weighed cold and returned to the fridge or freezer as soon as possible.
3. Do not allow refrigerated or frozen samples to warm to room temperature before weighing. Weigh "as is" to ensure integrity of the matrix.
4. Liquid samples should be thawed under a stream of nitrogen prior to weighing.

CALCULATION:

1. L-Glycine standard curve

Using the absorbance values generated for the L-glycine standards (0-1 mM) plot a linear standard curve of absorbance (y-axis) versus concentration (mM) (x-axis). Include the zero point (ST 0) in the curve. See **Figure 1** (page 13) as an example. Determine the primary amine concentration (C1) of unknown samples using the following equation:

primary amine concentration (mM):

Y=A*C1+B where:

C1 = unknown concentration of primary amines (mM)
Y = absorbance
B = y-intercept
A = slope of the line

2. Primary amine concentration corrected for dilution and weight

Calculate the primary amine concentration in the original sample solution (C2) by multiplying the value obtained above by the dilution factor and also adjusting for any deviation from nominal sample size.

C2 = C1*D*1.25*(0.5)/W

where:
C1 = concentration of primary amines in the diluted sample
D = dilution factor of the sample prior to amine determination
1.25 = dilution with TCA (all samples equal)
W = sample weight (g)
0.5 = nominal sample size (g)

3. Primary amine concentration corrected for amino acids present

Using constants for various amino acids calculate a corrected primary amine concentration (CN) for amino acids present:

CN = C2+((Prol*2*10)/(Lys*0.5*10))+(Hist*0.2*10)+(Arg*0.2*10)

where:
C2 = corrected primary amine concentration (mM)
Prol, Lys, Hist and Arg = concentration of L-proline, L-lysine, L-histidine and L-arginine respectively in the original sample
2, 0.5, 0.2 and 0.2 = constants for the various amino acids

4. Data fit comparison to literature values

Using the literature values for the rat model in **Table 4** below, fit the corrected primary amine concentration (CN) for the standard samples to the corresponding literature values using a linear regression.

Table 4. Literature values of primary amine concentrations.

Matrix	Literature Value (mM)
Control A	83
Control B	86
Control C	91
Control D	84
Control E	84
Control F	81
Casein	100

NOTE: If a previously generated correlation equation is used, adjust for the relative variability of the casein control sample.

5. *In vitro* digestibility

Using the equation from the Data Fit calculate the *in vitro* digestibility of the sample:

***In Vitro* Digestibility = (M*X+B)/100**

where:

X = corrected primary amine concentration (CN) for each sample
M = slope of the line (1.1135 in the example above)
B = y-intercept (74.125 in the example above)
100 = conversion from percentage to g

> **NOTE:** These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

6. Determine the amino acid ratio and limiting amino acid

Determine the amount of amino acid for each of the samples on a g/100 g protein basis using the total Dumas protein result generated separately:

AA (g/100 g) = (AA g/100 g Sample)/(Dumas protein (%))

Calculate the ratio of amino acid (mg/g protein) in the sample with the recommended amount. **Table 5** shows the recommended amount.

Ratio = (mg/protein in sample)/(mg/g protein in reference sample) **Table 5.** FAO recommended values for essential amino acids.

ESSENTIAL AMINO ACIDS for Human Nutrition	FAO Recommended Values (2011) – mg/g protein	FAO Recommended Values (1991) – mg/g protein*
Histidine	20	19
Isoleucine	32	28
Leucine	66	66
Lysine	57	58
Methionine + Cysteine	27	25
Phenlyalanine + Tyrosine	52	63
Threonine	31	34
Tryptophan	8.5	11
Valine	43	35

*1991 referenced values are required to be used for compliance with the U.S. Code of Federal Regulations.

The essential amino acid with the lowest ratio is the limiting amino acid. See **Table 6** below for example.

Table 6. Example of essential amino acid with limiting ratio.

Amino Acid	Sample Name (g/100g sample)	Sample Name (g/100 g protein)	Sample Name (mg/g protein)	1991 Reference Protein (mg/g protein)	Ratio	
L-Cysteine + L-Methionine*	1.38	2.94	29.44	25.00	1.178	
L-Tryptophan*	0.85	1.82	18.20	11.00	1.655	
L-HydroxyProline	ND					
L-Aspartic acid	3.20					
L-Threonine*	1.01	2.16	21.63	34.00	0.636	
L-Serine	1.56					
L-Glutamic Acid	6.66					
L-Proline	1.19					
L-Glycine	1.82					
L-Alanine	1.51					
L-Valine*	1.81	3.87	38.74	35.00	1.107	
L-Isoleucine*	1.45	3.09	30.94	28.00	1.105	
L-Leucine*	2.57	5.51	55.10	66.00	0.835	
L-Tyrosine + L-Phenylalanine*	3.26	6.98	69.83	63.00	1.108	
L-Lysine*	1.49	3.18	31.82	58.00	0.549	1
L-Histidine*	0.85	1.81	18.14	19.00	0.955	
L-Arginine	5.51					
Total Protein =	36.08					

*essential amino acid for nutrition

[1] limiting amino acid for sample

7. *In Vitro* PDCAAS Score

Calculate the *in vitro* PDCAAS score by multiplying the *in vitro* digestibility (Step 5) with the limiting amino acid ratio (lowest value).

PDCAAS = Digestibility x Ratio

where:

PDCAAS = *in vitro* PDCAAS score
Digestibility = *in vitro* digestibility from step 5
Ratio = ratio of the limiting essential amino acid from step 6

Appendix 4: Western Blot Method

Western Blot Method
AMPK and p-AMPK

Reagents

Extraction buffer RIPA lysis buffer with phosphatase inhibitors

- 2 mL 5X RIPA + 8 mL ddH2O + 100 uL protease & phosphatase inhibitor
- From protein isolation method, can use leftovers

BME (2-mercaptoethanol)/Laemmli buffer

- 4X Laemmli Buffer from Bio-Rad (#161-0747)
- Add 2-mercaptoethanol (BME) according to spreadsheet at 10% of Laemmli Buffer for 2.5% BME/tube

1X Tris/glycine/SDS buffer

- Purchased in concentrate from Bio-Rad (#1610772)

Transfer Buffer

- From Bio-Rad "Trans-Blot Turbo RTA Transfer Kit, Nitrocellulose" (#170-4270), 5X
- 600 mL ddH2O + 200 mL 5X transfer buffer + 200 mL 200 proof ethanol

1X TBS, pH 7.6

- 10X (dissolve 24 g Tris and 88 g NaCl in 900 mL of water and then adjust the pH to 7.6 and final volume to 1L); adjust with 6N HCI to bring the PH close to 7.6.
- 1X TBS(100ml TBS-10X +900ml DD H2O)
- Will need TBS-T, too (add 1% Tween)

Other Materials

- boil-safe microcentrifuge tubes
- gel-loading tips, 200 µL
- Bio-Rad Mini-Protean Precast TGX Gels, 4-15% (#4561086)
- Bio-Rad "Trans-Blot Turbo RTA Transfer Kit, Nitrocellulose" 170-4270
    - 1 L 5X transfer buffer
    - 80 transfer stacks
    - 40 nitrocellulose membranes
- LI-COR Chameleon Duo Pre-Stained Protein Ladder 928-60000
- Blocking solution (5% BSA powder in TBS)
- Primary antibodies (dilute in TBS-T + 5% BSA)
    - AMPK Mouse: 1:1000 dilution (CellSignaling, 2793s)
    - pAMPK Rabbit: 1:250 dilution (CellSignaling, 2535S) (dilution adjusted based on the result)
    - B-actin Rabbit: 1:5,000 dilution (for 3h RT) (CellSignaling, 4967S)
- Secondary antibodies (near-IR from LI-COR, dilute in TBS-T)
    - Goat-anti-Mouse, LI-COR 925-68070
    - Goat-anti-Rabbit, LI-COR 926-32211

Method

1. Thaw whole-protein isolates on ice.
2. Make BME/Laemmli Buffer, add to each tube
3. Add lysis buffer and protein sample to the tubes as designated in the spreadsheet. Centrifuge (quick spin)
    a. Can freeze sample for 1 month.
4. Prepare the cassette and box for electrophoresis.
    a. Rinse all components with DI water.
    b. Remove tape from the bottom of the cassette
    c. Assemble the chamber with clamps, dam, and cassette (no tape). 1 goes on red side. Words face inside. Red matches to red.
    d. Fill the chamber with 1X Tris/glycine/SDS buffer.
    e. Make sure the red and black electrodes/connections line up.
    f. Remove comb from the gel cassette and fix the lanes if needed.
5. Load the samples and molecular weight markers with loading tips onto the gel in the appropriate lanes. Avoid bubbles
6. Run gels on a fixed voltage of 200V for 30 minutes (30-35).
7. Remove the chamber and place the gel cassette in the buffer in the box. Rinse the other components.
8. Prepare the nitrocellulose membrane with transfer stacks.
    a. Place the nitrocellulose membrane in a plastic tray and the transfer stacks in another, using forceps
    b. Add 30 mL of transfer buffer to the nitrocellulose membrane and 50 mL to the transfer stacks.
    c. Place on rocker for 5 minutes.
9. Prepare the transfer assembly box.
    a. Rinse the transfer assembly box with DI water and dry the electrodes with a paper towel.
    b. Pipette transfer buffer from trays onto the bottom of the transfer box (2-3 mL).
    c. Place first transfer stack in the center.
    d. Place membrane on top of transfer stack.
    e. Pipette 1 mL of transfer buffer on top of the membrane; avoid bubbles.
    f. Open the gel cassette and gently place on top of the membrane (wear clean gloves, use hands gently grab the bottom of the gel).
        i. Do not move once in place.
    g. Pipette 1-2 mL of transfer buffer to ‘rinse.’
    h. Place 2nd transfer stack on top and roll to remove any bubbles.
    i. Place the top of the transfer box on top and lock into place.
10. Turn on the BIO-RAD Trans Turbo Blot system and select “Turbo” on the home screen and select 1 TGX gel.
    a. Settings should read: 1 MINI TGX or MIXED MW, 2.5 A, 25 V, 3 minutes
11. Place in slot A and hit RUN for slot A.
12. Rinse the membrane with DI water once.
13. Add Ponceau Stain and quickly pour back into container.

14. Wrap in plastic wrap and scan for confirmation of a successful transfer.
15. Cut the membrane to fit in black box
16. Rinse membrane with TBS
    a. 5–7-minute rinses, 3 times
    b. During these TBS rinses, prepare the 5% BSA blocking buffer
        i. 1 g of BSA powder in 20 mL TBS
17. Cover with blocking buffer. Incubate for 1 h, room temp, on rocker.
    a. Quick rinse with TBS-T after incubation with blocking buffer.
18. Dilute primary antibody in 0.25 g of BSA in 5 mL of TBS-T
    a. 5 µL of 1° AMPK and 20 µL of 1° p-AMPK in 5 mL of primary dilution buffer for 1:1000 AMPK 1° & 1:250 p-AMPK 1°
19. Incubate membranes with 5 mL of the 1° antibody solution on a rocker overnight at 4° C (cold room).
20. Discard the primary solution, wash the membrane with TBS-T at room temp on rocker
    a. 2 brief rinses
    b. 4x 5 min
21. Dilute the 2° antibodies in TBS-T (no BSA). All 2° antibodies are light sensitive- cover during incubation.
    a. 1:10,000 dilution (1 µL of 2° in 10 mL of TBS-T).
        i. For AMPK 2°, 1:2000 dilution (5 µL in 10 mL TBS-T).
        ii. For PAMPK 2°, 1:2500 dilution (4 µL in 10 mL TBS-T).
22. Discard final wash and add the required amount of 2°. Incubate the membrane for 1 h at room temperature, in dark, on rocker.
23. Wash the membrane with TBS-T at room temp on rocker
    a. 2 brief rinses
    b. 2x 5 min TBS-T
    c. 2x 5 minutes in TBS (to remove Tween)
24. Use LI-COR to scan membrane, scan 700 and 800 channels for 10 minutes each
25. For loading control, use striping buffer for 8-minute wash.
26. For loading control, rewash membrane (quick rinse with TBS followed by 1x 5 min rinse)
27. Next incubate membrane with blocking buffer (5% BSA) for 60 minutes.
28. Incubate with loading control 1° antibody (b-actin, 1:1000 in 5% BSA + TBS-T) for overnight at 4°C on rocker.
    a. 0.5 grams BSA in 10 mL TBS-T with 10 µL B-actin 1°.
29. Wash, 4x, 5 min each, TBS-T
30. Incubate with 2° for 1h at RT on rocker (in dark)
    a. 1:10,000 dilution factor (1 µL of 2° in 10 mL of TBS-T).
31. Wash the membrane with TBS-T at room temp on rocker
    a. 2 brief rinses
    b. 2x 5 min TBS-T
    c. 2x 5 minutes in TBS (to remove Tween)
32. Use LI-COR to scan membrane, scan 800 channel for 10 minutes

Appendix 5: SAS Input Code

```
* Wild rice and pea protein isolate protein quality Study    ;
*                           ;
*   Joey Eggers                              ;
* Created March 14 12 2021           ;
* Last Edited May 11 2022 by JEE    ;
*                            ;
*******************************;


data all_data (label= 'data from all groups') pea_protein (label='Pea
Protein only') rice (label='Brown and Wild Rice only');
infile datalines;
input Animal Diet Block PDCAAS_BW_1 PDCAAS_BW_2 PDCAAS_BW_3 HF_BW_0
HF_BW_1 HF_BW_2 HF_BW_3 HF_BW_4 HF_BW_5 HF_BW_6
HF_BW_7 HF_BW_8 Fecal_Weight PDCAAS_FI_1 PDCAAS_FI_2 PDCAAS_FI_3
PDCAAS_FI_4 PDCAAS_FI_5 HF_FI_1 HF_FI_2 HF_FI_3
HF_FI_4 HF_FI_5 HF_FI_6 Liver_Weight Fat_Pad_Weight Empty_Cecum_Weight
PDCAAS Liver_lipid_conc Liver_chol_conc LECT2_serum_conc
pAMPK_AMPK_ratio ACC_pACC_ratio
serum_TAG serum_cholesterol lacto_abd bifido_abd firm_bact_ratio
prev_abd akk_abd oscilli_abd rose_abd faeca_abd ali_abd blautia_abd
dorea_abd proteo_abd actino_abd rumino_abd


;
label
PDCAAS_BW_1='Initial PDCAAS Rat Body Weight(g)'
PDCAAS_BW_2='Week 2 PDCAAS Rat Body Weight (g)'
PDCAAS_BW_3='Final PDCAAS Rat Body Weight (g)'
HF_BW_0='Initial High Fat Diet Rat Body Weight (same as PDCAAS_BW_3)
(g)'
HF_BW_1='Week 1 High Fat Diet Rat Body Weights (g)'
HF_BW_2='Week 2 High Fat Diet Rat Body Weights (g)'
HF_BW_3='Week 3 High Fat Diet Rat Body Weights (g)'
HF_BW_4='Week 4 High Fat Diet Rat Body Weights (g)'
HF_BW_5='Week 5 High Fat Diet Rat Body Weights (g)'
HF_BW_6='Week 6 High Fat Diet Rat Body Weights (g)'
HF_BW_7='Week 7 High Fat Diet Rat Body Weights (g)'
HF_BW_8='Week 8 High Fat Diet Rat Body Weights (g)'
Fecal_Weight='PDCAAS Rat Fecal Contents (g)'
PDCAAS_FI_1='Day 1 PDCAAS Food Intake (g)'
PDCAAS_FI_2='Day 2 PDCAAS Food Intake (g)'
PDCAAS_FI_3='Day 3 PDCAAS Food Intake (g)'
PDCAAS_FI_4='Day 4 PDCAAS Food Intake (g)'
PDCAAS_FI_5='Day 5 PDCAAS Food Intake (g)'
HF_FI_1='Week 1 High Fat Diet Food Intake (g)'
HF_FI_2='Week 2 High Fat Diet Food Intake (g)'
HF_FI_3='Week 3 High Fat Diet Food Intake (g)'
HF_FI_4='Week 4 High Fat Diet Food Intake (g)'
HF_FI_5='Week 5 High Fat Diet Food Intake (g)'
HF_FI_6='Week 6 High Fat Diet Food Intake (g)'
```

```
Liver_Weight='Rat Liver Weight (g)'
Fat_Pad_Weight='Rat Epididymal Fat Pad Weight (g)'
Empty_Cecum_Weight='Rat Empty Cecum Weight (g)'
PDCAAS='PDCAAS values'
Liver_lipid_conc='Liver lipid conc (g lipids/g liver)'
Liver_chol_conc='Liver cholesterol conc (mmol cholesterol/g liver)'
LECT2_serum_conc='LECT2 serum concentration (ng/mL)'
pAMPK_AMPK_ratio='Ratio of pAMPK over AMPK, corrected for BActin
loading control'
ACC_pACC_ratio='Ratio of ACC over pACC, corrected for BActin loading
control'
serum_TAG='Serum Triacylglyceride levels (mg/dL)'
serum_cholesterol='Serum Cholesterol levels (mg/dL)'
lacto_abd='Lactobacillus relative abundance'
bifido_abd='Bifidobacterium relative abundance'
firm_bact_ratio='Firmicutes:Bacteroidetes Ratio'
prev_abd='Prevotella relative abundance'
akk_abd='Akkermansia relative abundance'
oscilli_abd='Oscillibacter relative abundance'
rose_abd='Roseburia relative abundance'
faeca_abd='Faecalibaculum relative abundance'
ali_abd='Alistipes relative abundance'
blautia_abd='Blautia relative abundance'
dorea_abd='Dorea relative abundance'
proteo_abd='Proteobacteria relative abundance'
actino_abd='Actinobacteriota relative abundance'
rumino_abd='Ruminococcus relative abundance'
;

Total_HF_FI = (HF_FI_1 + HF_FI_2 + HF_FI_3 + HF_FI_4 + HF_FI_5 +
HF_FI_6)/6;

Total_Liver_Lipids = (Liver_lipid_conc * Liver_Weight);

Total_Liver_Cholesterol = (Liver_chol_conc * Liver_Weight);

Change_in_HF_BW = (HF_BW_8 - HF_BW_0);

label
Total_HF_FI = 'Total High Fat Diet Food Intakes for entire High Fat
Feeding Trial (g)'
Total_Liver_Lipids = 'Total Liver Lipids for entire liver (g
Lipids/Liver)'
Total_Liver_Cholesterol = 'Total Liver Cholesterol for entire liver
(mmol cholesterol/liver)'
Change_in_HF_BW = 'The change in animal body weight over the High Fat
Feeding Trial (g)'
;

output all_data;
if diet = 4 or diet= 5 or diet=6 or diet=7 then output pea_protein;
if diet = 1 or diet = 3 or diet = 4 then output rice;

datalines;
```

```
1     1     1     73.86 91.4  90.1  90.1  151.9 202.3 260.1 304.3 340.1
      380.9 385.3 382.3 8.1   11.7  11.6  13.2  10.9  10.6  13.9  15.2
      16    16.2  17.3  14.5  10.208      2.546 0.545 0.70045
      0.08144     0.03121     .     0.063319386 5.731 .     .
      0.0026      0.0021      1.697972693 0.0075      0.3085
      0.0124      0.0026      0.0064      0.0135      0     0
      0.0221      0.0091      8.00E-04
2     1     1     83.41 97.2  98.6  98.6  169.2 225.4 292.7 331.7 370.3
      411.6 417.6 402.6 4.8   12.6  9.7   13.5  9.9   10.3  13.6  16.7
      21.2  20    19.4  15.9  10.379      2.196 0.452 0.69540
      0.08266     0.03954     2.936918304 0.059975816 3.333 30.79250993
      107.1459884 0.0096      0.0004      0.383809524 0.0021
      0.3729      0.0026      0.0033      8.00E-04    0.0288
      9.00E-04    0     0.0224      0.0011      0
3     1     1     78.89 92.4  93.6  93.6  162.6 229.1 295.7 348.5 391.8
      430.3 433.9 424.6 5.5   12.5  11.8  11.6  9.4   9.9   15.1  19.3
      23.6  21.3  18.4  11.9  11.566      2.839 0.387 0.67896
      0.15725     0.06279     2.611168563 0.056937799 3.754 35.40760356
      71.5872622  0.0125      0     0.810738255 6.00E-04    0.3641
      0.0049      0.003 8.00E-04    0.0205      0.0087      8.00E-04
      0.0425      7.00E-04    0
4     1     1     86.77 101.6 104.1 104.1 177   245.5 316.4 370.6 413.9
      455.6 481.9 492.4 8.3   12.4  13.4  12    13.1  12.7  16    20.1
      22.4  23    17.4  25.9  15.017      4.874 0.61  0.73247
      0.19446     0.06900     2.585315408 0.080909091 7.198 36.61811992
      80.54301075 0.0458      0.0005      1.299026426 0.0019
      0.2948      0.0101      0.0053      0.0016      0.0117      0
      0     0.0184      9.00E-04    9.00E-04
5     2     1     87.68 75.3  74    .     .     .     .     .     .
      .     .     .     1.4   7     6     4.3   3.4   4.9   .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
6     2     1     82.71 69.1  67.6  .     .     .     .     .     .
      .     .     .     2     6.9   5.7   4.4   4.5   4.8   .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
7     3     1     96.59 112.4 117.5 117.5 200.9 277.7 355.7 415.8 458.3
      492.9 512.2 504   8.7   11.1  13.2  14    14.4  14.2  16.3  25.5
      23    23.1  18.8  14.6  13.37 4.974 0.423 0.66863     0.14534
      0.04466     2.171664943 0.058601134 5.957 29.39285039 62.22043011
      0.0038      0     1.287396938 0.0016      0.3559      0.0039
      0.0027      4.00E-04    0.0149      4.00E-04    0     0.022
      7.00E-04    0
8     3     1     87.74 106.2 112.5 112.5 188.7 250.6 321.1 370.8 413
      455.8 480.2 465.9 8.3   13.6  13.8  13.4  13.1  13.5  19.3  19.6
      19.4  23.9  19.9  13.8  11.935      3.165 0.611 0.67610
      0.08678     0.03581     2.032057911 0.059685864 4.700 23.07546813
      96.5165426  0.0011      0     1.187614511 0.0022      0.3566
      0.0081      0.0026      0.0029      0.0193      0.0033
      2.00E-04    0.0362      0.0019      1.00E-04
9     3     1     78.01 99.9  104.1 104.1 170.6 234.3 300.1 356.9 385.5
      419.7 435.8 438.8 9.2   14.6  14.4  13.3  13.6  13.2  15.6  18.6
```

```
      21.3  25.4  15.6  16.4  13.236      2.39  0.567 0.66555
      0.08254     0.03565     1.641666667 0.041450777 5.670 22.20540949
      121.7394541 0.007 0.0002      0.978474201 0.004 0.3018
      0.0046      0.0016      0.0018      0.0298      0.0037
      2.00E-04    0.024 0.001 1.00E-04
10    3     1     88.05 104.5 110.1 110.1 194.9 266.3 337.7 402   445.5
      485.1 505.3 502   8.1   12.8  12.6  12.6  13.6  13.5  18.5  22.5
      19.9  20.2  24    24.7  14.043      4.333 0.512 0.68037
      0.14637     0.05691     2.843846949 0.08989071  4.453 46.60487989
      97.74979322 0.0007      0     1.085476025 0.0014      0.3252
      0.0073      0.0055      0.0016      0.0113      0.006 0.002
      0.0433      8.00E-04    0
11    4     1     84.45 114.6 121.3 121.3 202.1 267.6 335.8 376.1 404.2
      460.8 462.3 433.3 4.6   11.6  12.1  12.2  12.2  12.2  15.9  20.5
      17.1  26.4  23.6  4.8   14.246      3.269 0.522 1.11452
      0.16174     0.08249     3.076525336 0.053593947 5.290 36.65594855
      101.2733664 0.0002      0.0014      1.007689195 0     0.3952
      0.003 0.0089      4.00E-04    0.0365      0.0125      0
      0.0544      0.0031      0
12    4     1     79.79 110.4 116.3 116.3 196.7 255.2 312.6 353.4 397.7
      431.6 454.5 457.4 9.6   14    14.2  14.3  13.4  13.9  16.3  16.5
      21.4  17.7  17.9  22.5  15.453      2.29  0.535 1.11778
      0.21463     0.08998     2.641666667 0.041538462 4.226 18.15774541
      122.1799007 0.0019      0.0026      1.024590164 3.00E-04
      0.4235      0.0046      0.0004      5.00E-04    0.0153
      0.0041      2.00E-04    0.0389      0.0032      0.0013
13    4     1     94.79 122.2 127.9 127.9 216.6 292.2 360.1 408.5 447.9
      488.5 490.7 488.7 4.4   12.7  13.3  12.7  13    12.3  19.3  27.3
      20.6  24.4  15    17.8  15.572      2.58  0.525 1.12695
      0.18690     0.07540     2.569803516 0.052786885 3.601 63.32513713
      155.4483044 0.0024      0.0038      1.471458774 4.00E-04
      0.2046      0.0111      0.0108      0.0033      0.029 4.00E-04
      0     0.0326      0.0055      0.0024
14    4     1     80.04 110   117.3 117.3 194.3 260.9 330   381.6 424.9
      452.4 466.7 453.8 9.4   13.9  13.6  13.6  14.1  13.9  24.6  24.3
      26.3  29.8  16.5  21.8  15.279      3.365 0.738 1.12436
      0.22674     0.09133     2.414684592 0.097910448 5.956 16.19065633
      95.78246485 0.0002      0     0.70141844  0     0.4056
      0.0047      0.0082      0     0.0482      0.0158      0.0011
      0.0499      0.002 0
15    5     1     85.57 79.6  81.1  .     .     .     .     .     .
      .     .     .     4     4.7   3.1   5     4     6     .     .
      .     .     .     .     .     .     .     0.70196     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
16    5     1     87.93 80    81.4  .     .     .     .     .     .
      .     .     .     5.1   5.9   4     5.7   4.7   5.9   .     .
      .     .     .     .     .     .     .     0.67700     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
17    5     1     80.83 79.8  80.3  .     .     .     .     .     .
      .     .     .     5.5   6.7   5.7   6.7   5.6   7.1   .     .
      .     .     .     .     .     .     .     0.71094     .     .
```

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      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
18    5     1     93.12 88.8  89.3  .     .     .     .     .     .
      .     .     .     5.6   7.6   6     5.9   6.3   6.1   .     .
      .     .     .     .     .     .     .     0.73256     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
19    6     1     94.03 87.7  89.8  .     .     .     .     .     .
      .     .     .     5.2   6.9   5.9   6.3   5.2   7.4   .     .
      .     .     .     .     .     .     .     0.61636     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
20    6     1     93.62 94.4  96.1  .     .     .     .     .     .
      .     .     .     2.3   5.1   4.8   6     4.6   7.6   .     .
      .     .     .     .     .     .     .     0.65097     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
21    6     1     79.66 78.2  78.7  .     .     .     .     .     .
      .     .     .     5.6   7.8   7.3   7.2   5.6   6.5   .     .
      .     .     .     .     .     .     .     0.59969     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
22    6     1     89.05 86.7  87.9  .     .     .     .     .     .
      .     .     .     5     7.7   7.3   6.7   5.2   7.5   .     .
      .     .     .     .     .     .     .     0.65224     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
23    7     1     93.96 90.3  92.5  92.5  184.2 246.1 317.8 369.2 410.2
      454.8 486.4 514.1 6     6.4   7.3   7.4   5.4   7.6   19.3  24.6
      20.8  26.2  20.5  26.9  18.366      2.847 0.693 0.81358
      0.16604     0.05628     3.526370217 0.040213049 7.063 49.6311708
      96.16418528 0.0033      0.0028      1.047815617 0.0191
      0.3583      0.0024      0.0051      0.0021      0.047 0.0024
      8.00E-04    0.0191      0.0034      0.0017
24    7     1     85.93 84.8  87.5  87.5  168.1 229.4 303   364.4 416
      462   490.2 516.4 5.9   8.2   7.3   7.8   5.5   7.1   15.9  18.2
      18.7  24.8  22.5  19.3  18.809      2.568 0.795 0.76890
      0.11455     0.05666     2.673216132 0.040727903 5.504 11.61339134
      108.5554177 0.0052      0.0013      1.265859101 0.0222
      0.2229      0.0034      0.0132      0.0015      0.0462
      0.0064      3.00E-04    0.0401      0.0035      0.0018
25    7     1     90.42 87.7  88.3  88.3  175.8 248.5 319.8 382.7 430
      465.3 499.3 521.6 6.1   7.8   6.9   8     6.6   8.6   12.5  25.6
      24.6  20.8  20.4  21.2  18.239      3.24  0.661 0.77381
      0.22257     0.09286     2.84901758  0.049365079 7.445 32.22999811
      96.34036394 0.0005      0.0009      0.424085278 0.0135
      0.4003      0.0006      0.0015      4.00E-04    0.0347
      0.0083      7.00E-04    0.0349      9.00E-04    3.00E-04
26    7     1     84.26 87.9  89.5  89.5  171.7 237.2 311.5 376.7 430.7
      480.2 505.6 529.6 6.5   7.2   7.6   7.7   7.2   7.7   17.4  24.4
      22.8  24.3  18.5  16.8  16.33 2.914 0.482 0.74182     0.15438
      0.05959     3.940020683 0.038973214 3.936 88.44335162 86.82671629
      0.0007      0     0.413768116 0.023 0.2975      0     0.0022
```

```
      0     0.0319      0.0061      5.00E-04    0.045 0.0011
      7.00E-04
27    1     2     88.5  88.4  93.3  93.3  166.8 231.9 297.8 357.2 387.2
      411.3 417   442.7 4.1   12.6  5.4   4.8   4.7   5.2   17.5  20
      22.7  25.1  21.2  14.9  12.037      4.137 0.629 0.68492
      0.11461     0.04722     2.590486039 0.044778761 3.860 17.47682996
      112.5488007 0.0008      0.0001      0.51867292  0.034 0.0858
      0.0156      0.0016      0.0016      0.0195      0.0043
      5.00E-04    0.0025      2.00E-04    0
28    1     2     97.9  100.5 101.9 101.9 177.6 236.1 295   342.5 374.1
      386.4 397.3 415.2 6.5   10.9  6.9   8.8   7.8   6.8   14.2  19
      15.2  15    17.9  20    11.106      2.077 0.425 0.67823
      0.16161     0.04587     2.611168563 0.049166667 3.072 31.51125402
      104.2684036 0.0027      0.0009      0.888585472 0.0011
      0.2269      0.0114      0.0107      0.0025      0.0302
      0.0068      1.00E-04    0.036 0.0032      7.00E-04
29    1     2     108   119.8 121.4 121.4 230.4 306.1 385.6 472.4 490.7
      483.3 472.6 501   6.3   14.1  12.9  13.4  12.9  14.1  20    25.6
      20.9  13.8  17.4  14.4  16.213      4.297 0.557 0.69495
      0.17822     0.05419     2.171664943 0.02997543  2.477 63.66559486
      103.9747725 0.0011      0     0.379051701 0     0.2963
      0.0012      0.0033      0.0016      0.0312      0.0102
      3.00E-04    0.1133      8.00E-04    0
30    1     2     104.6 106.7 114.8 114.8 192.1 258.9 309.9 366.2 401.6
      382.7 411.9 423.3 4.1   13.2  7.2   11.7  11.4  12.3  18.6  20.5
      17.2  22.2  15.7  23.8  11.797      1.784 0.45  0.68437
      0.07758     0.03787     1.475 0.055030303 6.404 31.05731038
      118.3920596 0.0036      0.0002      1.703516091 0.0019
      0.0075      0.0178      0.0098      0.0024      0.017 0.0057
      3.00E-04    0.0404      0.0021      0
31    2     2     107.6 94.6  92.5  .     .     .     .     .     .
      .     .     .     1.1   9.5   6.6   6.9   5.8   5.3   .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
32    2     2     91.3  76.8  76.6  .     .     .     .     .     .
      .     .     .     1.7   7     5.9   6.9   6.6   5.5   .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
33    3     2     84.6  100.5 105.5 105.5 166.3 216.1 259.9 304.4 331.7
      356.3 371.6 394.4 8.7   13.5  13.8  12.8  14.3  14    16.3  17.1
      15.2  16.5  14.7  17.6  9.523 2.37  0.398 0.64535     0.07822
      0.02902     1.89762151  0.052529762 6.789 33.7809722  114.4574028
      0.004 0.0001      2.78125     0.0019      0.2983      0.0055
      0.0025      0.0043      0.0231      0.0085      0.0024
      0.0127      0.0032      0.0032
34    3     2     99.9  115.1 118.5 118.5 212.1 269.3 290.6 359.7 373.1
      401.7 420.6 440.6 8.6   14    13.2  13.4  13.1  13.2  18.4  16.5
      18.5  7.9   18.9  19.6  13.668      3.067 0.342 0.66229
      0.10428     0.03368     3.531540848 0.06352657  6.186 22.96198222
      83.56741108 0.0025      0     1.82696715  5.00E-04    0.1891
      0.0081      0.0033      0.0013      0.0192      0.0087
      8.00E-04    0.0377      6.00E-04    0
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35    3     2     89.3  105.7 109.2 109.2 181   239.9 294.4 351.4 394.1
      408.2 447.2 462.4 9.5   14.5  14.1  13.3  14.2  13.8  17.7  20.4
      18.6  19.6  15.6  19.5  13.117      3.534 0.429 0.65539
      0.10647     0.03897     3.350568769 0.036151603 4.172 32.45696993
      72.96732837 0.0058      0     2.790076336 9.00E-04    0.1603
      0.0032      0.0001      0.0037      0.0084      0.0093      0.001
      0.0258      0.0029      8.00E-04
36    3     2     102.7 117.8 121   121   206.3 274.6 324.6 374.8 416.3
      435.7 440.5 464.3 8.4   14.3  14.5  13.9  14.3  14.3  18.5  14.4
      20.4  22.1  21.5  15.3  13.22 3.077 0.392 0.63985     0.13466
      0.04582     2.507755946 0.026340426 2.794 30.33856629 76.5789909
      0.0023      0     1.265907569 0.0023      0.2564      0.0092
      0.0117      8.00E-04    0.0162      0.0088      0.0013      0.027
      0.0027      4.00E-04
37    4     2     86    116.2 122.5 122.5 202.9 272.9 335   392.7 430.8
      454.7 494.7 506.9 5.5   13.6  13    13.7  13.3  13.2  23.3  25.2
      19.6  17    17.6  22.7  16.089      4.133 0.413 1.10780
      0.17324     0.07172     4.296794209 0.062096774 3.702 37.03423492
      123.5599669 0     0     0.185621685 0     0.1443      0.0004
      0     2.00E-04    0.006 0.0032      0     0.0144      1.00E-04
      0
38    4     2     88.4  116.8 121.9 121.9 204.5 265.3 324.7 367.5 388.2
      406.2 424.5 436.6 9.1   13.3  14.3  13.3  14.2  13.9  18.5  19.7
      12    15.8  13.1  16.6  14.76 2.83  0.456 1.10881     0.15092
      0.07586     3.738366081 0.076557864 2.673 23.11329677 102.9470637
      0.0009      0.0004      1.535347244 0.001 0.0794      0.0126
      0.0042      0.001 0.0348      0.0022      0.0024      0.0398
      0.0036      0.0024
39    4     2     77.3  105   113.5 113.5 190.9 246.3 302.6 347.5 388.6
      412.3 442   458   10.5  14.4  14.3  13.5  13.7  13.6  15.4  19.2
      14.4  22.1  18.9  20.6  15.784      2.292 0.415 1.08545
      0.22787     0.06962     2.673216132 0.041925466 3.796 52.50485437
      104.2684036 0.0005      0.001 1.557628649 6.00E-04    0.0773
      0.0062      0.01  0.0032      0.0464      0.007 0.0033
      0.0242      0.0046      0
40    4     2     106.6 131.1 138.4 138.4 222.2 273.7 312.7 350   378
      396.3 421   437.8 8.4   14.4  14    13.4  14.3  14.4  18.4  16
      10.8  19.9  17    18.8  12.834      2.493 0.346 1.11633
      0.15123     0.06905     3.728024819 0.053258427 4.883 46.83495146
      103.4462366 0.0003      0.0005      1.528645833 4.00E-04
      0.2516      0.0034      0.0003      0.001 0.0309      9.00E-04
      0.001 0.0194      0.0021      2.00E-04
41    5     2     100.5 97.5  97.6  .     .     .     .     .     .
      .     .     .     4.5   9.2   5.8   7.4   6.3   6.4   .     .
      .     .     .     .     .     .     .     0.73204     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
42    5     2     101   97.4  98.4  .     .     .     .     .     .
      .     .     .     5.1   5.1   6.1   6     7.5   6.9   .     .
      .     .     .     .     .     .     .     0.72741     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
43    5     2     110.9 107.4 112.7 .     .     .     .     .     .
      .     .     .     5.6   11.3  7.5   6.9   8.9   9.3   .     .
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      .     .     .     .     .     .     .     0.69987     .     .
      .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
44    5     2     86.8  87.3  91.2  .     .     .     .     .     .
      .     .     .     2.6   8.8   6.8   8.2   8.2   7.6   .     .
      .     .     .     .     .     .     .     0.71632     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
45    6     2     81.6  80.7  82.3  .     .     .     .     .     .
      .     .     .     5     9.9   7.3   8.3   8.2   6.5   .     .
      .     .     .     .     .     .     .     0.63237     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
46    6     2     106   97    98    .     .     .     .     .     .
      .     .     .     4     8     6.8   6.9   7.3   7.2   .     .
      .     .     .     .     .     .     .     0.64084     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
47    6     2     106.6 98.3  105.9 .     .     .     .     .     .
      .     .     .     4.4   9.2   6.6   9.3   7.9   8.1   .     .
      .     .     .     .     .     .     .     0.62239     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .
48    6     2     101.4 92.3  95.3  .     .     .     .     .     .
      .     .     .     4.9   7.4   6.6   8.3   9.3   7.2   .     .
      .     .     .     .     .     .     .     0.63391     .     .
      .     .     .     .     .     .     .     .     .     .     .
      .     .     .     .     .     .     .     .
49    7     2     94.8  94.6  96.5  96.5  166   220   259.4 296.9 328.8
      360.2 375   393.3 6.2   7.6   7.4   10    10.9  8.6   13.5  17.2
      13.4  18.4  18.7  17.4  12.2  2.38  0.512 0.78670     0.12662
      0.04927     3.112719752 0.060828025 5.520 35.96116505 121.0934657
      0.0081      0.002 3.707917889 0.0021      0.16  0.0139
      0.0038      0.0013      0.0139      0.0031      0.0011
      0.0141      0.0068      0.0016
50    7     2     110.4 107.8 110.1 110.1 191.7 253.9 307   351   377.7
      415   428.1 448.4 5.2   10.3  6.9   7.8   7.8   8.1   19.2  20.8
      16.8  15.1  16.2  16.3  14.855      3.477 0.556 0.79265
      0.15686     0.06832     2.041666667 0.115086207 12.327
      39.72815534 85.91645988 0.0017      0.0001      0.433045702
      0.0141      0.417 0.0008      0.0052      1.00E-04    0.0376
      0.0115      8.00E-04    0.0433      7.00E-04    4.00E-04
51    7     2     96.9  95.8  97.3  97.3  171.4 230.5 285.8 329   373.5
      393.2 428.4 459.3 5.8   8.1   7.3   10.4  8.7   7.3   18.7  17.9
      15.9  23.6  25.4  21    14.1454     3.405 0.513 0.78427
      0.14336     0.06471     3.683333333 0.037943262 3.343 55.45631068
      87.64888337 0.0048      0     0.64640884  0.0319      0.1833
      0.0062      0.0021      0.0011      0.0391      0.0136      0
      0.0417      0.0018      0.0055
52    7     2     113.7 106.4 106   106   182.9 235.9 279.9 328.2 356.5
      386   381.7 409.8 5.6   11.6  6.6   7.2   6     6.5   15.3  13.9
      15.3  19.2  18.5  19.5  13.0859     2.817 0.447 0.78574
      0.12938     0.05972     2.575 0.030627178 5.290 53.24271845
      82.39288668 0.0102      0.0013      0.918418575 0.0031
```

```
      0.3216      0.0088      0.0032      0.0017      0.0512
      0.0126      0.0024      0.0402      0.003 7.00E-04
;
*Rats 3, 9, 12, 30, 50, 51, and 52 LECT2 serum concentrations were
redone due to improper replication. The new values have been listed
above EXCEPT FOR Rat 3, whose new value resulted in an outlier when
compared to liver fat, so the original value was kept.;

proc sort data=all_data; by Diet;

proc format;
value  dietfmt
            1='brown rice'
            2='protein-free'
            3='wild rice'
            4='casein control'
            5='PPI-salt'
            6='PPI-pH'
            7='Pea flour';
            run;
proc print data=all_data; by Diet; format diet dietfmt.; title 'All
groups'; run;

proc means data=all_data n mean stderr min max; by DIET; format diet
dietfmt.; title 'Means';

proc glm data=all_data;
title 'One-way ANOVA';
format diet dietfmt.;
class diet;
model  PDCAAS_BW_1 -- dorea_abd proteo_abd actino_abd rumino_abd
Total_HF_FI Total_Liver_Lipids Total_Liver_Cholesterol
Change_in_HF_BW=diet block;
Means diet / Duncan;
LSMEANS DIET / pdiff;
run;

proc glm data=pea_protein;
title 'One-way ANOVA - pea protein only';
format diet dietfmt.;
class diet;
model  PDCAAS_BW_1 -- dorea_abd proteo_abd actino_abd rumino_abd
Total_HF_FI Total_Liver_Lipids Total_Liver_Cholesterol
Change_in_HF_BW=diet block;
Means diet / Duncan alpha=.05 ;
LSMEANS DIET / pdiff;
run;

proc glm data=rice;
title 'One-way ANOVA - brown & wild rice only';
format diet dietfmt.;
class diet;
```

```
model  PDCAAS_BW_1 -- dorea_abd proteo_abd actino_abd rumino_abd
Total_HF_FI Total_Liver_Lipids Total_Liver_Cholesterol
Change_in_HF_BW=diet block;
Means diet / Duncan alpha=.05 ;
LSMEANS DIET/ pdiff;
run;
proc glm data=all_data;
title 'One-way COANOVA w/ FI';
format diet dietfmt.;
class diet block;
model  PDCAAS_BW_1 -- dorea_abd proteo_abd actino_abd rumino_abd
Total_HF_FI Total_Liver_Lipids Total_Liver_Cholesterol
Change_in_HF_BW=diet block Total_HF_FI;
Means diet / Duncan;
LSMEANS DIET / pdiff alpha=.05;
run;

proc glm data=all_data;
title 'One-way COANOVA w/ Final BW';
format diet dietfmt.;
class diet block;
model  PDCAAS_BW_1 -- dorea_abd proteo_abd actino_abd rumino_abd
Total_HF_FI Total_Liver_Lipids Total_Liver_Cholesterol
Change_in_HF_BW=diet block HF_BW_8;
Means diet / Duncan;
LSMEANS DIET / pdiff alpha=.05;
run;
proc corr data=all_data;
title 'Correlation of LECT2 with liver fat';
var LECT2_serum_conc;
with Total_Liver_Lipids Liver_lipid_conc;
run;
proc plot data=all_data;
title 'Plot of LECT2 with Liver Fat';
plot LECT2_serum_conc * (Total_Liver_Lipids Liver_lipid_conc);
run;
proc corr data=rice;
title 'Correlation of LECT2 with liver fat for Rice Groups Only';
var LECT2_serum_conc;
with Total_Liver_Lipids Liver_lipid_conc;
run;
proc plot data=rice;
title 'Plot of LECT2 with Liver Fat for Rice Groups Only';
plot LECT2_serum_conc * (Total_Liver_Lipids Liver_lipid_conc);
run;
QUIT;
```

Appendix 6: Microbiome Pre-Processing and R Code

This code was used in MSI by Linux

```
#use mangi.msi.umn.edu on puTTy login to get walltime, port 22
srun -N 1 --ntasks-per-node=4  --mem-per-cpu=16gb -t 16:00:00 -p
interactive --pty bash

egger228@ln1002 [~] % module load R/4.0.4
egger228@ln1002 [~] % R; version/4.0.4

#load dada2
>library(dada2)

#run this to install dada2, if not already installed
if (!requireNamespace("BiocManager", quietly = TRUE))
    install.packages("BiocManager")
BiocManager::install("dada2", version = "3.11")

#assign file path
> path <-
"/home/gallaher/data_release/umgc/miseq/210913_M04803_0436_000000
000-JWW96/Gallaher_Project_006"

> path <- "/panfs/roc/groups/6/gallaher/egger228/microbiome2"

#show files
> list.files(path)

#assign forward files
> fnFs <- sort(list.files(path, pattern="_R1_001.fastq",
full.names = TRUE))

#assign reverse files
> fnRs <- sort(list.files(path, pattern="_R2_001.fastq",
full.names = TRUE))

#assign sample name formula
> sample.names <- sapply(strsplit(basename(fnFs), "_"), `[`, 1)

#plot quality scores, forward & reverse
> plotQualityProfile(fnFs[1:2])
> plotQualityProfile(fnRs[1:2])

#assign filter paths
```

```
> filtFs <- file.path(path, "filtered1", paste0(sample.names,
"_F_filt.fastq.gz"))
> filtRs <- file.path(path, "filtered1", paste0(sample.names,
"_R_filt.fastq.gz"))

#apply sample names
> names(filtFs) <- sample.names
> names(filtRs) <- sample.names

#filter and trim based on quality scores
> out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs,
truncLen=c(240,160), maxN=0, maxEE=c(2,2), truncQ=2,
rm.phix=TRUE, compress=TRUE, multithread=FALSE)

#compute error rates, forward and reverse
> errF <- learnErrors(filtFs, multithread=TRUE)
129884880 total bases in 541187 reads from 3 samples will be used
for learning the error rates.
> errR <- learnErrors(filtRs, multithread=TRUE)
113727360 total bases in 710796 reads from 4 samples will be used
for learning the error rates.

#plot error rates
> plotErrors(errF, nominalQ=TRUE)

#dereplicate sequences
> derepFs <- derepFastq(filtFs, verbose=TRUE)

#name new files
> names(derepFs) <- sample.names
> names(derepRs) <- sample.names

#convert to dada2 to check reads
> dadaFs <- dada(derepFs, err=errF, multithread=TRUE)

> dadaRs <- dada(derepRs, err=errR, multithread=TRUE)

#check forwards
> dadaFs[[1]]

#merge processed reads
> mergers <- mergePairs(dadaFs, derepFs, dadaRs, derepRs,
verbose=TRUE)

#check mergers
> head(mergers[[1]])

#make sequence table of
mergers
```

```
> seqtab <- makeSequenceTable(mergers)
> dim(seqtab)
> table(nchar(getSequences(seqtab)))

#remove chimeras
> seqtab.nochim <- removeBimeraDenovo(seqtab, method="consensus",
multithread=TRUE, verbose=TRUE)
> dim(seqtab.nochim)
> sum(seqtab.nochim)/sum(seqtab)
> getN <- function(x) sum(getUniques(x))
> track <- cbind(out, sapply(dadaFs, getN), sapply(dadaRs, getN),
sapply(mergers, getN), rowSums(seqtab.nochim))
> colnames(track) <- c("input", "filtered", "denoisedF",
"denoisedR", "merged", "nonchim")
> rownames(track) <- sample.names
> head(track)


#assign taxonomy
> taxa <- assignTaxonomy(seqtab.nochim,
"/panfs/roc/groups/6/gallaher/egger228/microbiome2/silva_nr99_v13
8.1_wSpecies_train_set.fa.gz", multithread=TRUE)
> taxa.print <- taxa
> rownames(taxa.print) <- NULL
> head(taxa.print)

#export data to tables to use in native R session by desktop
> write.table(taxa,
"/panfs/roc/groups/6/gallaher/egger228/microbiome2/taxa_mbwrpf.tx
t")
> write.table(seqtab.nochim,
"/panfs/roc/groups/6/gallaher/egger228/microbiome2/seqtabnochim_m
bwrpf.txt")
> write.table(track,
"/panfs/roc/groups/6/gallaher/egger228/microbiome2/tracking_mbwrp
f.txt")
```

Now in R, the rest of the code using phyloseq and microeco plug-ins can be found on the public GitHub repository “ofriceandrats”:

ofriceandrats/JEE_RStudio_mbwrpf.R at main · egger228/ofriceandrats (github.com)