Investigating diet as a confounder to asparaginase treatment of acute lymphoblast leukemia

by

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Submitted in partial fulfillment of the requirements

for the degree of Master of Science

at

Dalhousie University

Halifax, Nova Scotia

July 2023

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Abstract

Pegaspargase (P-ASP) is an integral part of the treatment of Acute Lymphoblastic Leukemia. P-ASP works by depleting serum asparagine (Asn), killing leukemic but not healthy cells. Whether confounding sources of Asn, for example, from diet and bacteria in the gut, impact the levels in blood has not been explored. We provided mice with an Asn rich (AR, 4%) or Asn depleted (AD, 0%) diet for 72 days prior to P-ASP injection. Neither diet change blood Asn levels. P-ASP depleted blood Asn in mice consuming either diet. In a second experiment, mice were provided with the diet one day prior to P-ASP injection. AD mice all had to be euthanized 3 days after injection due to an acute loss of weight. The remaining AR mice, despite also losing weight, completed the study; however, Asn levels were not depleted by P-ASP. The outcomes provide evidence that there may be an acute harmful effect of combing a diet lacking in Asn with Asn depletion by P-ASP. On the other hand, it is feasible that a diet high in Asn can overcome a single injection of P-ASP. Future studies should confirm these results, as well as include approaches to determine the source of Asn in the blood while ingesting diets with no or high amounts of Asn.

List of Abbreviations Used

AD	Asparagine Depleted Diet
ALL	Acute lymphoblastic leukemia
AR	Asparagine rich diet
Asn	L-Asparagine
ASNS	Asparagine Synthetase
Asp	L-Aspartic Acid
CACF	Carleton Animal Care Facility
CNS	Central Nervous System
COREs	Centralized Operation of Research Equipment & Supports
CSF	Cerebral Spinal Fluid
Gln	L-Glutamine
Glu	L-Glutamic Acid
GS	Glutamine Synthetase
HP1	High Asn diet High Pegaspargase dose batch 1
HP2	High Asn diet High Pegaspargase dose batch 2
ILIS	Isotope labelled internal standards
IM	Intramuscular
IP	Intraperitoneal
IV	Intravenous
L-ASP	L-Asparaginase
LC-MS-MS	liquid chromatography with tandem mass spectrometry
LP	High Asn diet Low Pegaspargase dose batch 1

MRD	Minimal Residual Disease
P-ASP	Pegaspargase
PCA	Principal Coordinate Analysis
Ph+ ALL	Philadelphia Chromosome Positive Acute Lymphoblastic Leukemia
WBC	White Blood Count

Acknowledgements

I am extremely grateful to my supervisors Dr. Andrew Stadnyk and Dr. Ketan Kulkarni, for their mentorship and support throughout my MSc program. The experience I gained from them is invaluable.

I would also like to thank my committee members Dr. Shashi Gujar and Dr. Mahboubeh Rahmani for their help and advice given during committee meetings. As well as Dr. Tamara MacDonald for her advice and expertise surrounding pegaspargase therapy.

I am also grateful to the granting agencies, IWH Health, Beatrice Hunter Cancer Research Institute, Dalhousie Medical Research Foundation, and the Canadian Institute of Health Research, for funding my project and making my research possible.

Finally, I would like to thank my family for always being there to support me while completing my MSc.

Chapter 1 Introduction:

1.1 Acute Lymphoblastic Leukemia

1.1.1 Diagnosing ALL

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer, accounting for ~28% of all cancer diagnoses (*Incident Cases - Cancer in Young People in Canada Data Tool | Public Health Infobase - Public Health Agency of Canada*, n.d.). ALL can also occur in adults, but it is much less common, accounting for 0.3% of all new cancer cases (*Acute Lymphocytic Leukemia - Cancer Stat Facts*, n.d.). ALL cells may be B or T cell lineage but, in both cases, lymphoid cells pause differentiation at the lymphoblast stage, yet the cells continue to rapidly proliferate. Lymphoblasts overwhelm the patient's bone marrow, displacing healthy cells, and can migrate into the peripheral circulation. On initial presentation, patients' symptoms can consist of fever, increased bruising or bleeding, weakness, swollen lymph nodes, and decreased appetite, among others (*Signs and Symptoms of Acute Lymphocytic Leukemia (ALL)*, n.d.). Complete blood counts and peripheral blood smears are routinely the first tests to indicate a possible ALL diagnosis, as patients will typically present with pancytopenia. Bone marrow aspiration and biopsy are used to diagnose the patient with ALL by the presence of blasts (*Tests for Acute Lymphocytic Leukemia (ALL)*, n.d.). Pancytopenia can lead to patients experiencing anemia, infections, and increased bleeding from wounds (*Childhood Acute Lymphoblastic Leukemia Treatment (PDQ®)–Patient Version - NCI*, 2022). Without treatment ALL is lethal.

1.1.2 ALL Staging

Pediatric patients undergo staging for their disease, involving multiple tests to identify the patients as standard, high, or very high risk (*Childhood Acute Lymphoblastic Leukemia Treatment (PDQ®)–Patient Version - NCI*, 2022). Patients' bone marrow samples are sent to a Hematopathologist to determine if the cells are immature or mature, and if they are B or T cells. T cell ALL is considered a higher risk disease in comparison to B cell ALL (Teachey & Pui, 2019). The cells will undergo further genetic characterization including fluorescent in situ hybridization, polymerase chain reactions, or other genetic tests which can identify specific sub-types of ALL that may be more or less responsive to treatment. One example is Philadelphia chromosome positive ALL (Ph+ ALL) in which patients are considered very high risk due to a tyrosine kinase signaling protein being constitutively activated and supporting the leukemia (Foà & Chiaretti, 2022). Lumbar punctures are conducted on ALL patients to determine if the blasts have entered the cerebral spinal fluid (CSF). Patients are identified as either CNS1, meaning there are no blasts in the CSF or CNS 2/3 indicating there are blasts in the CSF (Lee, n.d.-a). Staging is further completed by the white blood cell count (WBC) at diagnosis, with a lower WBC indicating a better prognosis. Age is another important prognostic marker as children over the age of 10 are considered high risk and over 13 considered very high risk (Lee, n.d.-b). Staging has consequences for a patient's treatment, with higher risk patients requiring more intensive therapy. After the first phase of therapy, induction, pediatric ALL patients will have their minimal residual disease (MRD) measured, the percent of leukemic cells in the bone marrow. An MRD <0.01% indicates the patient is in remission and can continue therapy on the same protocol. If the MRD is >0.01%, the treatment was not fully effective

and the patients risk staging will be increased and protocol changed to include more intensive treatment (Lee, n.d.-b). Adults do not undergo staging and instead are referred to as “untreated ALL”, “adult ALL in remission”, or “recurrent adult ALL” (*Adult Acute Lymphoblastic Leukemia Treatment (PDQ®)–Health Professional Version - NCI*, 2022). Staging for pediatric and adult ALL does not include any drug specific tests for resistance, which leaves the space for further testing in risk assessment.

1.2 Treatment

1.2.1 Treatment Overview

The first-time chemotherapy was offered to children with ALL was in 1948 and consisted of a single drug, aminopterin, intended to induce remission. Aminopterin is no longer used in the treatment for ALL. In 1961, a trial was established examining a combination of 6-mercaptopurine and methotrexate which induced remission in 44% of children and 30% of adult patients (Frei et al., 1961). Despite treatments becoming available at the time for pediatric ALL patients, most patients still succumbed to the disease. Over the subsequent decades, chemotherapeutic agents continued to be systematically added to the treatment protocols of ALL patients. Currently, the standard risk protocols for pediatric patients consist of multiple therapeutics including antineoplastic agents and corticosteroids. Pediatric treatment protocols can last between 2-4 years depending on the risk stage. Additionally, certain subtypes of ALL like Ph+ ALL, patients that do not respond well to therapy, or relapsed patients, may receive additional treatment. Additional treatment may include radiation, stem cell transplants, or targeted therapies like tyrosine kinase inhibitors for Ph+ ALL (Lee, n.d.-e, n.d.-d). Adult ALL protocols have been modified from pediatric protocols. The standard treatment may include

chemotherapy, radiation, chemotherapy with stem cell transplants, or targeted therapy. The length of treatment for adult patients can range from 1.5 to 3 years (*Adult Acute Lymphoblastic Leukemia Treatment (PDQ®)–Health Professional Version - NCI*, 2022). Considering the burden of illness, the complexities behind treatments, and the length of treatment, there is room for improvement.

1.2.2 Survival Outcomes

Currently, the five-year observed survival rate for combined pediatric ALL patients of both lymphocyte types is 94% (Lee, n.d.-c). Of the 6% of patients that do not survive, most succumb to their cancer (Lee, n.d.-c). Despite this high survival rate, 15-20% of pediatric patients will experience a relapse (*Relapsed Childhood Acute Lymphoblastic Leukemia (ALL) Overview - Dana-Farber Cancer Institute | Boston, MA*, n.d.). Adult ALL patients have a much lower 5-year survival rate, 40%, and a higher relapse rate, ~50% (*Leukemia - Acute Lymphocytic - ALL - Statistics*, 2012; *Relapsed ALL Booklet*, n.d.). Patients that experience a relapse will be required to undergo additional years of therapy and more intensive treatment to try to achieve remission. This greatly affects the quality of life for patients, requiring more hospital visits, injections, time sick, and increased stress relating to relapse. The high relapse rate for both pediatric and adult ALL patients is an indication that treatment is still sub-optimal.

1.2.3 History of Asparaginase

One therapeutic agent, among many, used to treat pediatric ALL is L-asparaginase (L-ASP). The decision to use L-ASP has an interesting origin. In 1953, mice were injected with malignant lymphoma cells in the groin to create subcutaneous lymphomas. Mice were then injected with guinea pig, rabbit, horse serum, or saline. Surprisingly, the mice injected

with guinea pig serum achieved remission while all other mice were killed by the disease (Kidd, 1953). Almost a decade later, in 1961, the protective factor in the guinea pig serum was identified to be L-ASP (Broome, 1963). L-ASP converts L-asparagine (Asn) to L-aspartic acid (Asp), and less effectively converts L-glutamine (Gln) into L-glutamate (Glu). Most leukemic cells are unable to synthesize Asn, in contrast to healthy cells, and are entirely reliant on exogenous (blood) Asn. When L-ASP depletes Asn in the patient's blood, the lack of Asn triggers apoptosis of leukemia but not healthy cells. Noteworthy is the fact that humans lack any L-ASP and therefore the first preparation used to treat ALL was a cloned, recombinant enzyme from bacteria (Mashburn & Wriston, 1964; Schwartz et al., 1966; Wriston, 1967). The first study testing the therapeutic efficacy of L-ASP in children was conducted in 1977. The Children's Oncology Group added L-ASP derived from *Escherichia coli* to the established induction protocol consisting of vincristine and prednisone. The authors found that the addition of L-ASP increased the remission rate to 93% compared to 86% in the control group (Ortega et al., 1977), and L-ASP was permanently added to the treatment of pediatric ALL. The use of L-ASP required multiple doses due to the short half-life of the protein in blood, and hypersensitivity reactions were common (Heo et al., 2019). Pegylation was added to the protein to increase the half-life in blood and reduce hypersensitivity reactions. The current pegylated drug is marketed pegaspargase (P-ASP, Oncaspar™) (Avramis et al., 2002). P-ASP was approved in the USA in 1994 for use in patients with a hypersensitivity reactions to L-ASP but by 2006 it replaced L-ASP as the primary therapeutic option (European Medicines Agency Oncaspar Assessment, 2016). Pediatric patients receive 2 doses of P-ASP if they are diagnosed with standard risk B Cell ALL, with the number of doses required increasing with risk. The

highest risk patients being T-cell ALL patients who receive 9 doses. All pediatric patients, except for infantile ALL patients, receive 2500 IU/m^2 of P-ASP per dose. Recent studies have looked at the survival outcome of patients who had missed doses of P-ASP. Pediatric and young adults were both shown to have a decrease in event free survival associated with missed doses (Aldoss et al., 2023; Gupta et al., 2020). The introduction of P-ASP into adult ALL protocols is only underway (Gupta et al., 2020). Currently, young adults up to the age of 40 can be treated using pediatric protocols in which they receive P-ASP. This was shown to lead to an increased overall 3-year survival rate of 73% in comparison to non-pediatric protocols of 58% (Daley et al., 2021). More recent studies have examined the use of P-ASP in adult ALL patients over 40 years. They found that although side effects are common, P-ASP can be safely tolerated if monitored correctly (Stock et al., 2019). These outcomes highlight the importance of P-ASP in the treatment of ALL in both pediatric and adult patients, although the lack of personalized medicine considerations when dosing P-ASP leaves opens the door to further optimizing P-ASP therapy.

1.2.4 Adverse Reactions to Asparaginase

As with all chemotherapeutics, there are multiple side effects associated with the use of P-ASP. The most common side effects, according to the United States Food and Drug Administration, are hypersensitivity reactions to the drug, hyperglycemia, pancreatitis, central nervous system thrombosis, coagulopathy, hyperbilirubinemia, and elevated transaminases (Highlights of prescribing information 2020). Stock et al. (2011) convened a panel of experts to conduct a literature search on adverse events related to using P-ASP. They found that 20% of pediatric patients had elevated liver enzymes, 10% had hypersensitivity reactions, 7% had neuropathy, and 7% had hyperglycemia. When

reviewing adverse events in adult patients, an increased risk of elevated liver enzymes (36%) and hyperglycemia (25%) predominated with only 1% reporting an allergy/hypersensitivity reaction (Stock et al., 2011). The adverse events associated with P-ASP are quite high, dosing modifications and controlling other sources of Asn may help to reduce side effects.

1.2.5 Monitoring of P-ASP

Following intravenous administration, P-ASP in the patient is measured using an assay of P-ASP activity (Salzer et al., 2018). Measuring blood Asn levels would presumably provide a more direct measure of efficacy but is confounded by any P-ASP present in the patient's blood catabolizing Asn in the sample, possibly leading to inaccurate, false negative results (Asselin et al., 1991; Lanvers-Kaminsky et al., 2014). (A protocol has been published that quenches P-ASP activity in rodent blood, allowing the measurement of Asn but there is no equivalent in use for human blood samples (Horvath et al., 2019).) Therefore in the clinic, a trough level of activity ≥0.1 IU/mL ASP is recommended (van der Sluis et al., 2016). Clinically P-ASP activity levels are taken one week after P-ASP injection. Patients that do not meet the trough level of activity will be more closely monitored. In some cases, patients may experience silent inactivation where there is no clinical sign of hypersensitivity but the P-ASP activity levels are decreased (Burke & Zalewska-Szewczyk, 2022). The level of maximal efficiency is still unknown due to individual differences and potentially confounding sources of Asn (Pike et al., 2019; van der Sluis et al., 2016).

1.3 Possible Confounding Sources of L-Asparagine

1.3.1 Healthy Cells

Possibly the most significant confounding source of Asn to control comes from the patients' healthy cells. Healthy cells possess Asparagine synthetase (ASNS) which uses Gln and Asp to catalyze the synthesis of Asn and Glu. Leukemic cells in the presence of P-ASP therapy can increase glutamine synthetase activity, providing one of the necessary substrates for ASNS to mesenchymal stromal cells (MSC) in the patient's bone marrow. A study in 2020 provided evidence that MSC cells from ALL patients secreted more Asn than MSC from healthy controls, resulting in greater protection of ALL cells (Chiu et al., 2021). Other research has shown that another antineoplastic agent, vincristine, used in conjunction with P-ASP in current ALL protocols, can suppress the secretion of Asn by MSC cells (Fung et al., 2010). . Whether the dynamics are this simple in a patient's bone marrow and blood remains to be proven but controlling healthy cell synthesis of Asn during treatment will certainly be difficult to achieve.

1.3.2 Diet and ALL

Diet is arguably the second-most important source of patient blood Asn, and there is some history of how diet may impact the disease. Diet has been shown to impact patients with ALL in multiple, albeit indirect ways. Studies have indicated that maternal diets high in sugars and syrups are linked to increased risk of a child developing ALL, while diets high in vegetables and proteins were linked with reduced risk (Kwan et al., 2009; Petridou et al., 2005). Pediatric ALL patients experience high obesity rates (15-43%) by the end of therapy and hypertriglyceridemia post-induction (Salvador et al., 2012; Van Dongen-Melman et al., 1995). Four years after treatment cessation, obesity (17-44%) and low bone

mineral density (21%) continue to affect pediatric ALL patients (Kaste et al., 2001; Van Dongen-Melman et al., 1995). Obesity has been linked to a poorer prognosis, affecting both adults and children. Obesity leads to increased mortality and relapse risk (Amankwah et al., 2016; Castillo et al., 2012). Dietary and exercise studies are underway, aiming to reduce obesity in ALL patients; early results have shown that decreasing obesity reduced the minimal residual disease for patients (Orgel et al., 2021). Therapeutic agents can change patients' hunger and cause changes in taste. One study found 30% of pediatric ALL patients reported moderate changes in taste and 7% found the change to be severely bothersome (Loves et al., 2019). Apart from these indirect effects of diet, dietary Asn could directly affect P-ASP therapy. As with all amino acids from digestion, Asn has been shown to enter the bloodstream through intestinal absorption (Knott et al., 2018). In 1966, Halikowski et al. trialed a low-protein diet in 13 pediatric ALL patients. Low-protein diets showed an increase in treatment response (Halikowski et al., 1966). Asn is rich in animal proteins and this study may have indirectly been lowering the amount of Asn available to the patients through their diet. Specialized diets aimed at potentially controlling Asn could lead to improved outcomes in patients, if not fewer side effects of P-ASP if lower doses are permitted.

1.3.2 Gut Bacteria

Diet as a confounder of blood Asn levels cannot be discussed without considering the microbes in the gut as well. The digestive tract is inhabited by a multitude of microorganisms including bacteria, fungi, phage, viruses, and possibly protozoa and metazoans, constituting the microbiome. The bacteria within the gut microbiome are diverse and highly individualized and can be modified by antibiotics as well as diet. During

ALL therapy patients become immunocompromised and susceptible to infection due to treatment. Patients are given antibiotics during their treatment to prevent severe infections. Despite this precaution, overuse of antibiotics and antifungal agents can decrease microbial diversity and increase the abundance of opportunistic pathogens (Dunn et al., 2022). Additionally, there is a dynamic relationship between the blood and the microbiome (Visconti et al., 2019). A study by Visconti et al. in 2019 found that 46% of blood metabolites are associated with the microbiome. There are bacteria that can synthesize and catabolize Asn (recalling that L-ASP is a bacterial protein). Research has shown that communities of bacterial species can be associated with serum P-ASP activity (Dunn, Connors, et al., 2021). A study by Dunn et al. analyzing stool metabolites determined that patients had significantly lower stool Asn levels 1 week after P-ASP therapy compared to weeks 2 and 3. P-ASP should not enter the blood stream and so these changes in the gut may be due microbial changes. In support of this, the authors found microbial changes in genes related to Asn, Asp, Glu, and Gln metabolism were also present (Dunn, Forbrigger, et al., 2021). Therefore, the (bacterial) microbiome may be an additional confounding factor to the treatment of P-ASP therapy.

1.4 Mice and P-ASP

Mice are often used to model disease, and ALL is no exception. Many studies have been conducted looking at the impact of P-ASP in mice. However, the dose and route are not standardized between studies, nor is the choice of asparaginase, with many researchers choosing to use less expensive L-ASP. Still, P-ASP has been administered intravenous (IV), intramuscular (IM), and intraperitoneal (IP). Dosing for IV has been as low as 100 IU/kg to 1000 IU/kg and 200 to 1200 IU/kg for IM (European Medicines Agency Oncaspar

Assessment, 2016; Hinze et al., 2019; Poppenborg et al., 2016; Schewe et al., 2017). IP injections are the most common route but have the most variability with some studies using as few as 20 IU/kg once compared to others that dose at 1500 IU/kg twice a week (Bendich et al., 1982; European Medicines Agency Oncaspar Assessment, 2016.; Kumar et al., 2021; C. Liu et al., 2016; Y. Liu et al., 2019; Reiff et al., 2001; Sea et al., 2020). A toxicity study looking at the maximal dose in mice showed that at ≥5000 IU/kg mice experienced a decrease in body and spleen weight, ≥10000 IU/kg mice had significant liver changes, and the approximate lethal dose was shown to be ≥25000 IU/kg (European Medicines Agency Oncaspar Assessment, 2016). Despite the studies conducted using P-ASP in mice, there is no consensus on the amount that should be injected, nor is there any information on the dose required for sustained Asn depletion. Thus, we have identified a gap in using mice, both in terms of testing efficacy of P-ASP but also using mice to test the possibility of using diet to control blood Asn levels to improve P-ASP efficacy.

A study by Knott et al which was published in 2018, looked at the effects of dietary Asn on mice with breast cancer. They determined that limiting dietary Asn resulted in a decrease in metastasis. The results demonstrate the importance of diet on cancer response and give a precedent for trialing Asn diets in mice.

1.5 Hypothesis and Aims

In this study, I aimed to address the potentially confounding factors on blood Asn arising from the gut, through a dietary mouse model. The hypothesis is that diet can be used to influence blood Asn levels and that mice consuming a Asn Rich (AR) diet will overcome P-ASP therapy sooner than mice on an Asn depleted (AD) diet. My aims were to:

- Determine whether diets with different amounts of Asn influenced blood levels of Asn.
- Determine the effect of dietary asparagine on the depletion of Asn by P-ASP.
- Determine if a diet high in Asn will overcome P-ASP depletion sooner than mice on a diet low in Asn.
- Determine changes in the microbiome in mice on the two diets.

The standard institutional chow for mice was replaced with custom diets prepared with "high" or "low" Asn and the effects on the metabolome and microbiome post diet and post-P-ASP injection were observed.

Chapter 2 Materials and Methods:

2.1 Experiment 1: Modifying Diet Prior to P-ASP Injection

2.1.1 Mice

All experiments involving mice were approved by the Dalhousie University Committee on Laboratory Animals, who apply the Canadian Council on Animal Care's guidelines for the ethical treatment of animals. Eleven 5-week-old C57BL/6 female mice (Charles River, Saint Constant, QC) were sorted into 3 cages by staff at IWK Health who were unrelated to the study. The mice acclimated to the facility for 2 weeks. In order to account for possible microbial drift, mice were re-sorted into 2 cages of 5 mice each. Female mice were chosen for all experiments due to the aggression shown between males combined into cages with new males.

2.1.2 Sample Timepoints

Baseline stool and saphenous blood vein samples were collected from all mice. After sample collection, 1 cage of mice was started on an AD diet (0% Asn) and the other cage was given an AR diet (4% Asn), purchased from Dyets (Bethlehem, PA product #519592 and #519593 respectively, Appendices 1 and 2). After 35 days, stool and blood samples were collected for metabolome analysis. On day 36 stool samples were collected for microbiome analysis. On day 72, blood and stool samples were collected, and the mice injected with 200 IU/kg of P-ASP (Servier Canada, Laval, QC). Two (day 74) and 5 days (day 77) post-P-ASP injection, blood and stool samples were collected for metabolome analysis and 4 days post-P-ASP (day 76) stool microbiome samples were collected. After sample collection on day 77, mice were euthanized (Figure 1). Mice were weighed weekly.

2.2 Experiment 2 Batch 1: Modifying Diet Concurrent with P-ASP Treatment

2.2.1 Mice

Twenty-four 5-week-old C57BL/6 female mice (Charles River) were sorted into 5 cages by staff at the Carleton Animal Care Facility (CACF), who were unrelated to the study. The mice acclimated to the facility for almost 3 weeks then started on the study, at 8 weeks old. The mice were then combined into 8 cages of 3 mice each.

2.2.2 Sample Timepoints

Baseline stool and saphenous blood vein samples were collected from all mice. Four cages of mice were then started on the AD diet and the other 4 on the AR diet. The diet was not the same as in the first experiment but was a diet reported by Knott et al., (2018) (Teklad diets, Madison WI. Product numbers TD.160365 and TD.160366, Appendices 3 and 4) (Knott et al., 2018). After 1 day on diet, 2 cages were started on each treatment protocol and all mice were injected with P-ASP (Servier Canada) as follows:

- AD diet 450 IU/kg P-ASP (n=6)
- AD diet 900 IU/kg P-ASP (n=6)
- AR diet 450 IU/kg P-ASP (LP, n=6)
- AR diet 900 IU/kg P-ASP (HP1, n=6)

Seven-, 14-, 21-, and 28-day post-injection blood and stool samples were collected for metabolome analysis. After 28 days mice were euthanized (Figure 2). Mice were weighed at baseline, then daily starting 3 days post P-ASP injection.

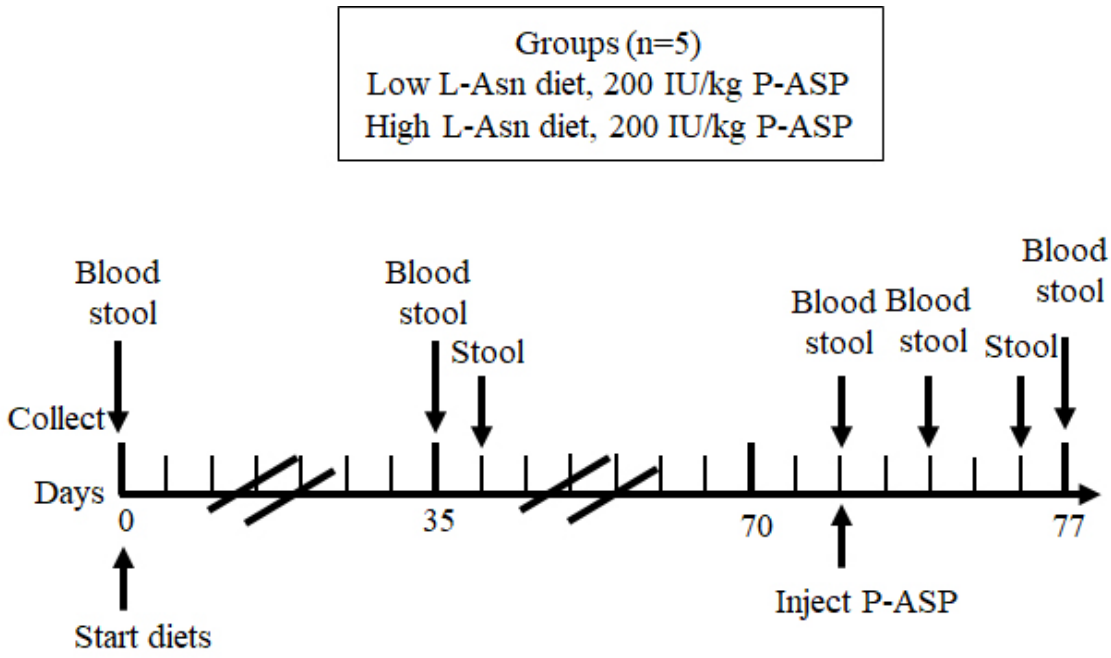

Figure 1 Methodology for mouse experiment 1. Mice were treated with a diet of low L-asparagine (L-Asn, 0%) or high L-Asn (4%). Blood and stool samples were collected from mice throughout the study for metabolome and microbiome analysis. On day 72 mice were injected with P-ASP and on day 77 all mice were euthanized after sample collection.

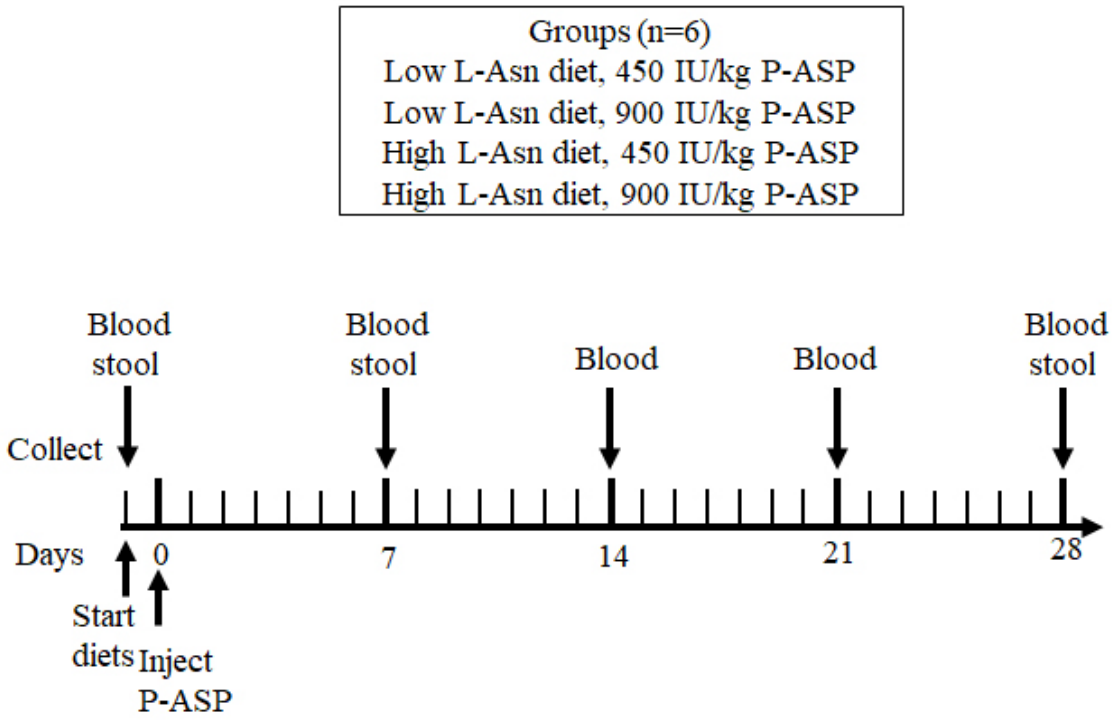

Figure 2 Methodology for mouse experiment 2 batch 1. Mice were started on either a low L-asparagine diet (L-Asn, 0%) or a high L-Asn diet (4%). On day 0, mice were injected IP with either 450 IU/kg or 900 IU/kg of pegaspargase (P-ASP). Blood and stool samples were collected throughout the study for metabolome analysis. On day 28, the study was complete, and mice were euthanized.

2.3 Experiment 2 Batch 2: Modifying Diet Concurrent With P-ASP Treatment, Repeat

2.3.1 Mice

Twelve 6-8-week-old C57BL/6 female mice (Charles River) were sorted into 3 cages by staff at the CACF. The mice acclimated for 1 week and were then separated into 4 cages of 3 mice each and started on the study.

2.3.2 Sample Timepoints

Baseline stool and saphenous blood vein samples were collected from all mice. Two cages of mice were then started on the AD diet and 2 cages on the AR diet (HP2). After 1 day on the diet, all mice were injected with 900 IU/kg of P-ASP. Five, 7-, 14-, and 21-days post injection blood and stool samples were collected from mice. After 21 days, mice were euthanized (Figure 3). Mice were weighed daily throughout the study.

2.4 Sample Collection and Processing

2.4.1 Stool Sample Collection

Stool samples were collected by placing individual mice into an empty cage for up to 1 hour. This ensured the samples collected could be related back to a specific mouse. Microbiome analysis required 50 mg of stool and metabolome analysis required 100 mg. For experiment 1 ~150mg of stool was collected from each mouse at every timepoint for both metabolome and microbiome analyses. For experiments 2 and 3 ~100mg was collected for metabolome analyses only. All stool samples were stored at -80 C.

2.4.2 Blood Sample Collection

Blood samples were collected from mice through their saphenous veins. Mice were lightly anesthetized using isoflurane then securely placed headfirst into a 50mL conical

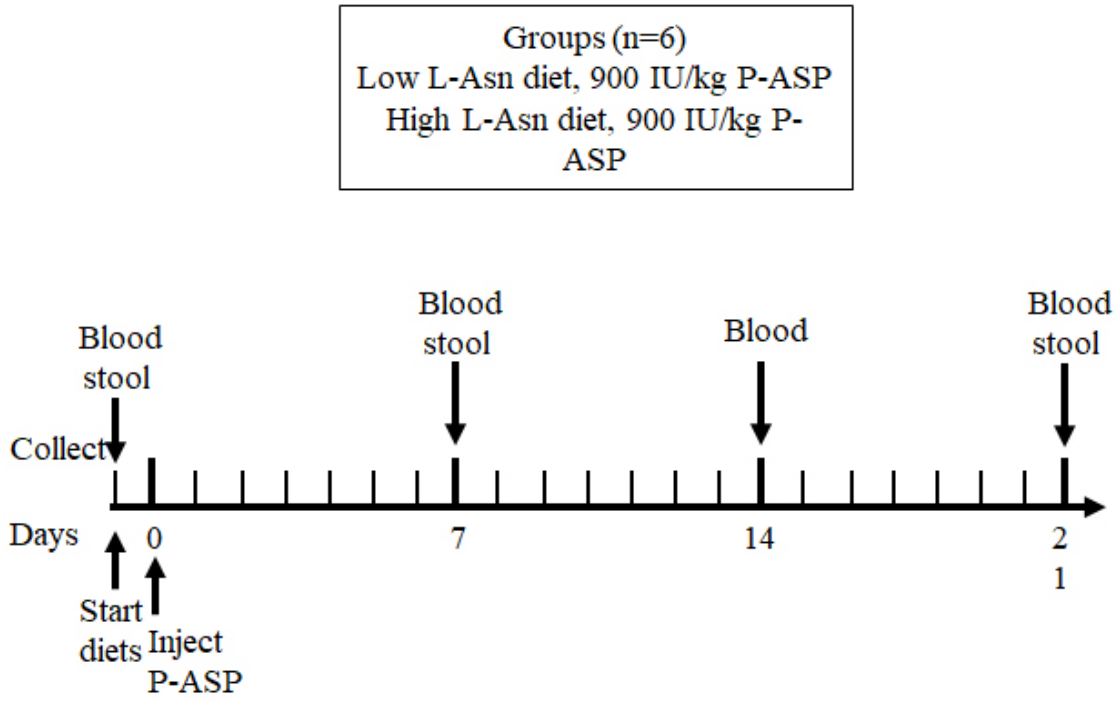

Figure 3 Methodology for mouse experiment 2 batch 2. Mice were started on either a low L-asparagine diet (L-Asn, 0%) or a high L-Asn diet (4%). On day 0, mice were injected IP with 900 IU/kg of pegaspargase (P-ASP). Blood and stool samples were collected throughout the study for metabolome analysis. On day 21, the study was complete, and mice were euthanized.

tube, leaving their hind leg accessible. The thigh was shaved, and a 23-gauge needle was used to pierce the saphenous vein and the blood was collected into a microhematocrit heparinized capillary tube (Fisherbrand) microhematocrit. In experiment 1, 50 μL of blood was taken from each mouse at every timepoint and in experiments 2 and 3, 25 μL was collected. Any P-ASP in the blood samples was immediately quenched post collection with 20% formic acid in ultrapure water following the methodology published by Horvath et al. (2019). Blood samples collected from mice not injected with P-ASP were similarly treated. The samples with formic acid added were held on ice until further processing.

2.4.3 Blood Sample Preparation

Blood samples were prepared for metabolome analysis immediately after all the samples from the timepoint were available, using a protocol reported by Horvath et al. (2019). In short, after the quenching at collection, a solution of methanol containing 1% (v/v) formic acid was added to precipitate the protein fraction. The samples were then vortex mixed, centrifuged (16000g), and evaporated to dryness using a Savant ISS110 SpeedVac Concentrator. Isotope labelled internal standards (ILIS) was added at 10 μL per sample during the methanol stage for experiment 1. For experiments 2 and 3 ILIS was added to the evaporated sample, which was then re-evaporated. Samples were stored at -80°C.

In preparation for analyses, the samples were reconstituted using a solution of ultrapure water with 1% formic acid. The samples were then vortexed mixed, and a solution of acetonitrile with 1% formic acid was added, then vortex mixed and centrifuged (16000g). “Pooled” samples were created by adding 10 μL of each sample into a single tube. The samples were then sent to the Centralized Operation of Research Equipment &

Supports (COREs, Faculty of Medicine, Dalhousie University) for analysis by liquid chromatography with tandem mass spectrometry (LC-MS-MS). Experiment 1 was batched and analyzed at the same time and experiments 2 and 3 were batched and analyzed later.

2.4.4 Stool Sample Preparation LC-MS-MS

Stool samples were prepared using a procedure provided by the COREs facility. One hundred milligrams of stool were added to a 1:3 ratio (w/v) of methanol. The samples were homogenized until the stool was in suspension. Stool samples were then stored at -20°C for 1 hour. After an hour, the samples were centrifuged, and the methanol fraction was collected. Ten microliters of each sample were recovered and placed into a separate microcentrifuge tube for the pooled sample. ILIS was then added (5 µL) to each sample. Samples were prepared a couple of days prior to analysis. Experiment 1 samples were batched and analyzed at the same time and experiments 2 and 3 were batched and analyzed together.

2.4.5 Stool Sample Preparation 16S and Metagenome

Stool sample DNA was isolated using the Norgen Biotek Corp. (Thorold, Ontario) Stool DNA Isolation Kit. Stool samples with the lysis solution and lysis additive were added to a bead tube. The samples were vortexed, centrifuged (13000g) and the lysate collected. A binding solution was then added, and samples were incubated on ice for 10 minutes. The sample was then centrifuged (13000g) and the supernatant collected. An equal volume of 70% ethanol was added, and the sample was vortex mixed. A spin column was assembled, and the supernatant was centrifuged in the spin column with a collection tube. The sample was then washed twice with each washing solution (I and II) and centrifuged. The flowthrough was discarded. The column was then placed into an elution

tube and an elution buffer was added and the sample centrifuged. The DNA sample was then stored at -20°C.

2.4.6 Sample Analysis

Stool samples were sent to the Biological Mass Spectrometry COREs facility at Dalhousie University for metabolome analysis using LC-MS-MS. The results were sent to the National Research Council of Canada (Oxford Ave, Halifax, NS) for analysis. Microbiome samples were sent to Integrated Microbiome Research (IMR, https://imr.bio/) for V3-V4 16S Ribosomal DNA sequencing and metagenome analysis. Samples were run in duplicate. Preliminary data analysis was completed at the IMR where the samples were normalized.

2.4.7 Statistical Analysis

Independent t-tests were run to test weight gain/loss significance. Metabolome analysis was run using an ANCOVA with a p value of <0.05 indicating significance. For day 35 and 72 samples, day 0 samples were used for the covariate. For day 77 samples, day 72 samples were used as the covariate. Metaboanalyst 5.0 was used to identify affected pathways.

Microbiome 16S and metagenome data were analyzed using STAMP (https://beikolab.cs.dal.ca/software/STAMP). PCA, box plots, and heatmaps for the 16S analysis were run at the genus or species level. Metagenome data was analyzed by looking at the pathways and functions of bacteria.

Chapter 3 Results

3.1 Experiment 1

3.1.1 Sample Collection

Our approach to addressing whether dietary Asn can be used to influence blood levels was to restrict the diet of mice to 1 of 2 diets, each diet with substantially different Asn content. Mice on the 2 diets did not have a significant difference in their average weight gains over the study period, indicating there were no unanticipated or profound effects of changing the animals' diets (Figure 4). At the time P-ASP was injected, the mice weighed an average of 24.8g for the AR diet and 26.9g for the AD diet. This was within or above the standard deviation of Jackson laboratories weight guide for mice of the same strain and age (23.5 ± 2.3g) (*Body Weight Information for C57BL/6J (000664)*, n.d.). On day 41 of the study mouse A1 escaped and had to be euthanized and thus is missing from day 72 to 77 samples. Mouse A2 received roughly half the amount of P-ASP as the other mice and its days 74, 76, and 77 samples were excluded from analysis. It was noted that once starting mice on their diet, both mice from the high and low Asn diet stools turned white.

3.1.2 Metabolomics: PCA Analysis

LC-MS/MS was used to determine changes in metabolites present in both blood and stool samples for mice. Metabolome results were run in duplicate and normalized. Isotope labelled internal standards (ILIS) were used to ensure accuracy. Principal Coordinate Analysis (PCA) was used to provide an overview of similarities among the sample metabolomes between groups and across timepoints. The blood PCA figure showed

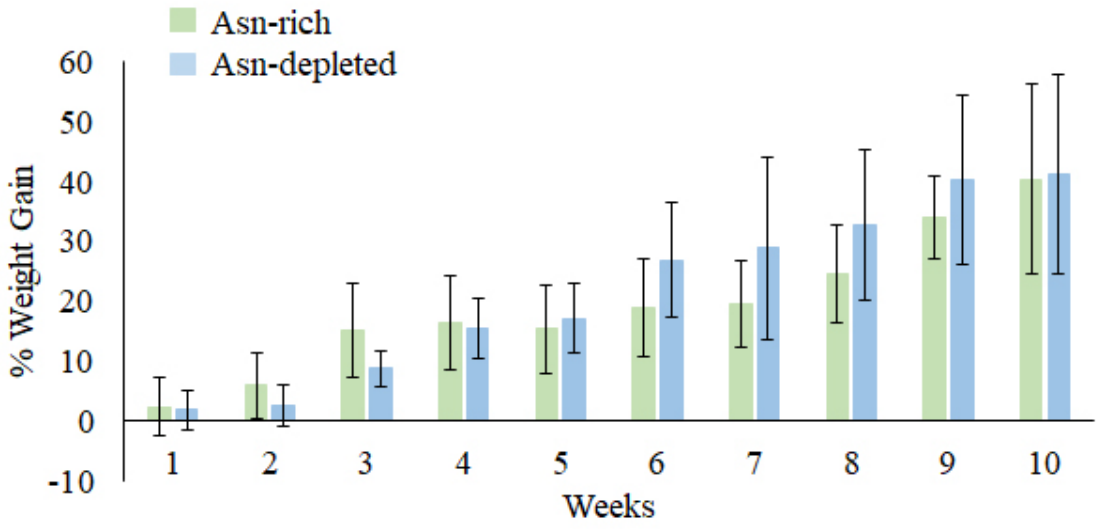

Figure 4 Average weight for mice across the study period for experiment 1 for the asparagine (Asn) rich and depleted treatment groups. Mice were weighed weekly, starting on the day they commenced their diets. There was no significant difference between the Asn rich and depleted group at the end of the study.

3 distinct clusters of samples (Figure 5). The day 0 samples clustered together as did the day 35 samples. Days 72, 74 and 77 also clustered together, showing that there was not a major effect of P-ASP on the whole metabolome profile. From the PCA plot, it appears that diet had the most profound effect on the metabolites although the samples did not separate based on diet.

Metabolome PCA plots of stool samples showed two distinct clusters (Figure 6). Day 0 samples clustered, and all other stool samples were in a second cluster. Maintaining the mice on diet for 35 and 72 days did not have a further effect on the metabolome, nor did the administration of P-ASP. This pattern indicated that both groups of mice experienced similar changes from a common pattern prior to starting the 2 diets. This outcome is a strong indication of diet-based though not diet-specific changes. Also noteworthy, the changes due to diet occurred at the first time point then remained consistent.

3.1.3 Metabolomics: Metabolite Levels

Blood Asn levels were depleted to the level of detection 2- and 5-days post P-ASP injection in both the AR and AD groups. On day 77 stool Asn levels were depleted for all but one mouse in the AD group, which registered a sharp increase in Asn (Figure 7). The dietary differences in Asn did not affect the extent to which P-ASP was able to deplete Asn from the blood, up to 5 days post-injection. Despite not expecting the P-ASP to enter the gut, the AD stool samples had decreased Asn post-P-ASP indicating that there could be feedback between the blood and gut for Asn levels. The one mouse that had increased Asn post-P-ASP demonstrates that there are still individual differences that can affect P-ASP therapy.

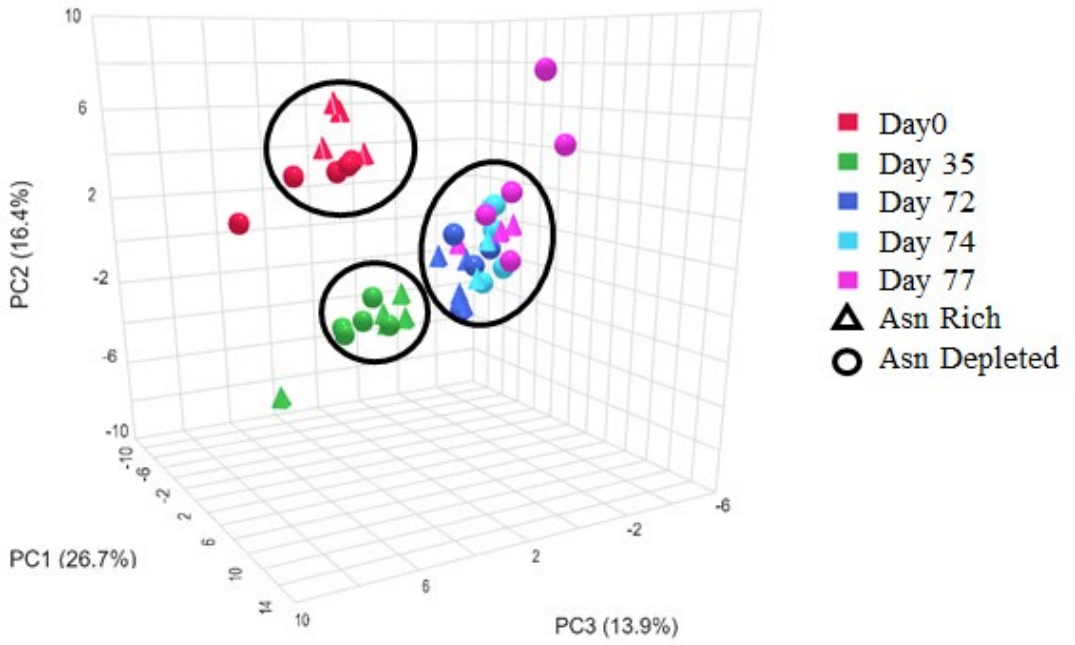

Figure 5 Experiment 1 blood metabolome PCA plot for samples collected throughout the study. Day 72, 74, and 77 samples clustered together. Day 0 samples and day 35 samples clustered separately. The asparagine (Asn) rich and depleted diet did not cluster separately.

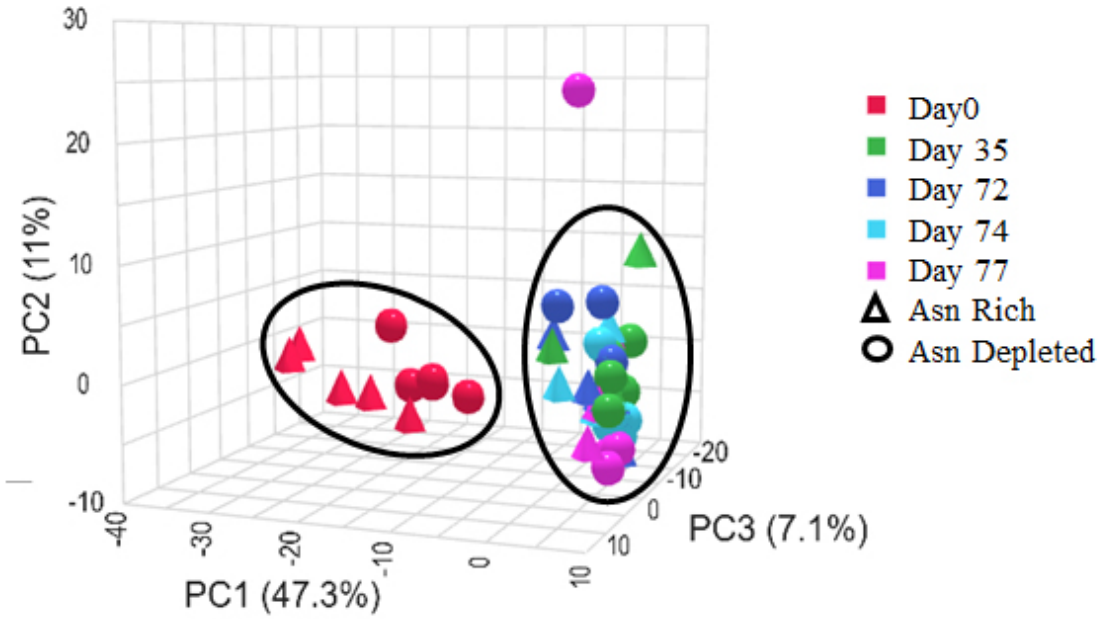

Figure 6 Experiment 1 stool metabolome PCA plots. Day 0 samples clustered separately. Day 35, 72, 74, and 77 samples clustered together. The samples did not cluster separately based on treatment group.

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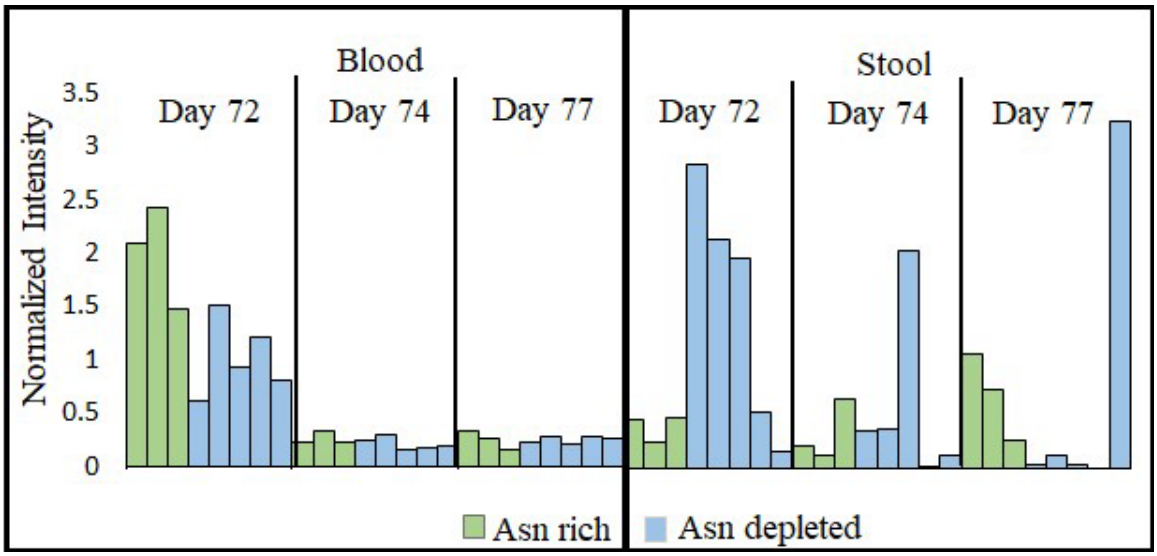

Figure 7 Experiment 1 blood and stool asparagine (Asn) levels for each individual mouse prior to pegaspargase (P-ASP) injection (day 72) and post P-ASP injection (days 74 and 77). Bars are color coded based off whether they were on the Asn rich or depleted treatment group. Blood Asn levels were depleted for all mice, up to 5 days post P-ASP. Stool Asn levels in the Asn depleted diet were lowered for all but one mouse that had a sharp increase in stool Asn.

Asn is metabolized to Asp, and Gln to Glu by P-ASP so it is reasonable to predict that Asp and Glu levels may be elevated and Gln levels decreased. Pair-wise t-tests were used to examine within group differences (Table 1). Interestingly, the AR group had similar Asp, Glu, and Gln levels post P-ASP when compared to the day 72 samples. The AD diet did show a difference in Gln when comparing days 72 and 77 but unexpectedly, there was an increase in blood Gln levels post P-ASP. ANCOVA was then used to identify between diet differences. Blood Asp, Gln and Glu levels were not significantly different between diet groups post-P-ASP (Figure 8).

When looking at the pre-P-ASP samples (days 0-72), the AD diet could have increased Glu due to the diet being higher in Glu to account for the lack of Asn. There was no significant difference between groups for blood Glu or Gln while on the diet. There was, however, what appeared to be an acute effect of the AD diet where blood Glu and Gln levels increased on day 35. Blood Asp levels were significantly higher in the AR group on day 72 in comparison to the AD group. When looking at within a diet, it became clear that the difference was due to a significant increase of Asp in the AR group ($p=0.043$).

Stool Asp, Glu and Gln levels were not significantly different between groups throughout the study period. When examining withing a group differences, both AR and AD mice had significantly less Glu on day 35 in comparison to day 0 ($p=0.03$ and $p=0.013$, respectively. The AR group also had an increase in Gln after P-ASP (day 72 to 77, $p=0.045$) (Table 2). Stool Asn levels were significantly lower in the AR group on days 72 and 77 in comparison to the AD group (Figure 9). Additional metabolites were measured, and significance tested (Appendix Tables 1 and 2). The fact that there was no significant difference in Asn levels in the stool 35 days comparing diets indicates that the changes

Table 1 Experiment 1 blood metabolome results for the asparagine (Asn) rich and depleted diets within group analysis. P values are displayed for days that were significantly different within a group. P value <0.05 was taken for significance. Blood Asn levels were depleted for mice on both treatment groups 5 days post pegaspargase (day 77). Aspartic acid levels were significantly increased after 72 days on diet for the Asn rich group.

Asn rich					
Days	0 vs 35	35 vs 72	0 vs 72	72 vs 74	72 vs 77
L-Asparagine				0.02	0.019
L-Aspartic Acid			0.043		
L-Glutamine					
L-Glutamate					
Asn depleted					
Days	0 vs 35	35 vs 72	0 vs 72	72 vs 74	72 vs 77
L-Asparagine		0.042		0.005	0.007
L-Aspartic Acid					
L-Glutamine		0.014			0.036
L-Glutamate	0.03	0.039			

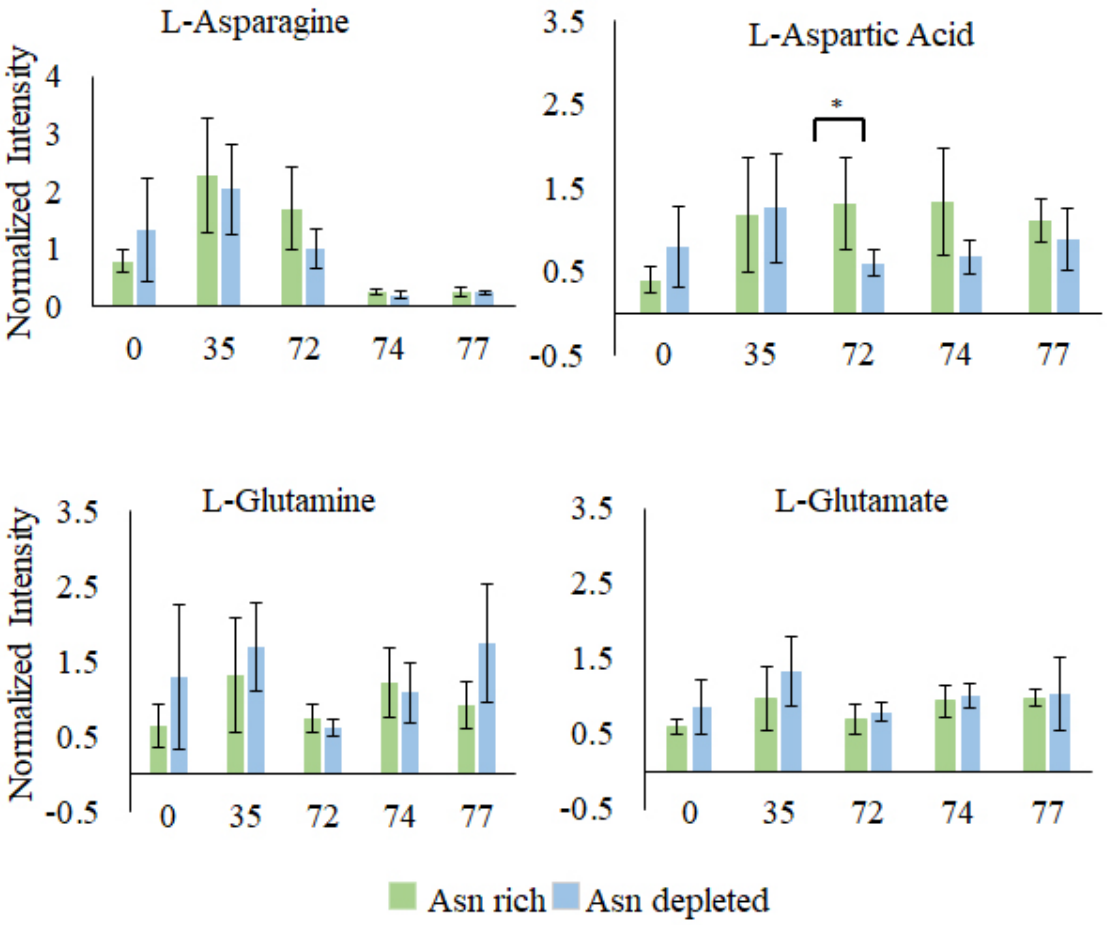

Figure 8 Experiment 1 average blood levels for mice treated on the asparagine (Asn) rich or depleted diets across the study period. Significance is shown for between groups differences, p value <0.05 was taken as significant. Aspartic acid levels were significantly different between groups on day 72.

Table 2 Experiment 1 stool metabolome results for the asparagine (Asn) rich and depleted diets within group analysis. P values are displayed for days that were significantly different within a group. P value <0.05 was taken for significance. Stool Asn levels were not significantly affected throughout the study period.

Asn rich					
Days	0 vs 35	35 vs 72	0 vs 72	72 vs 74	72 vs 77
L-Asparagine					
L-Aspartic Acid					
L-Glutamine					0.045
L-Glutamate	0.03				
Asn depleted					
Days	0 vs 35	35 vs 72	72 vs 74	74 vs 77	0 vs 72
L-Asparagine					
L-Aspartic Acid					
L-Glutamine					
L-Glutamate	0.013				

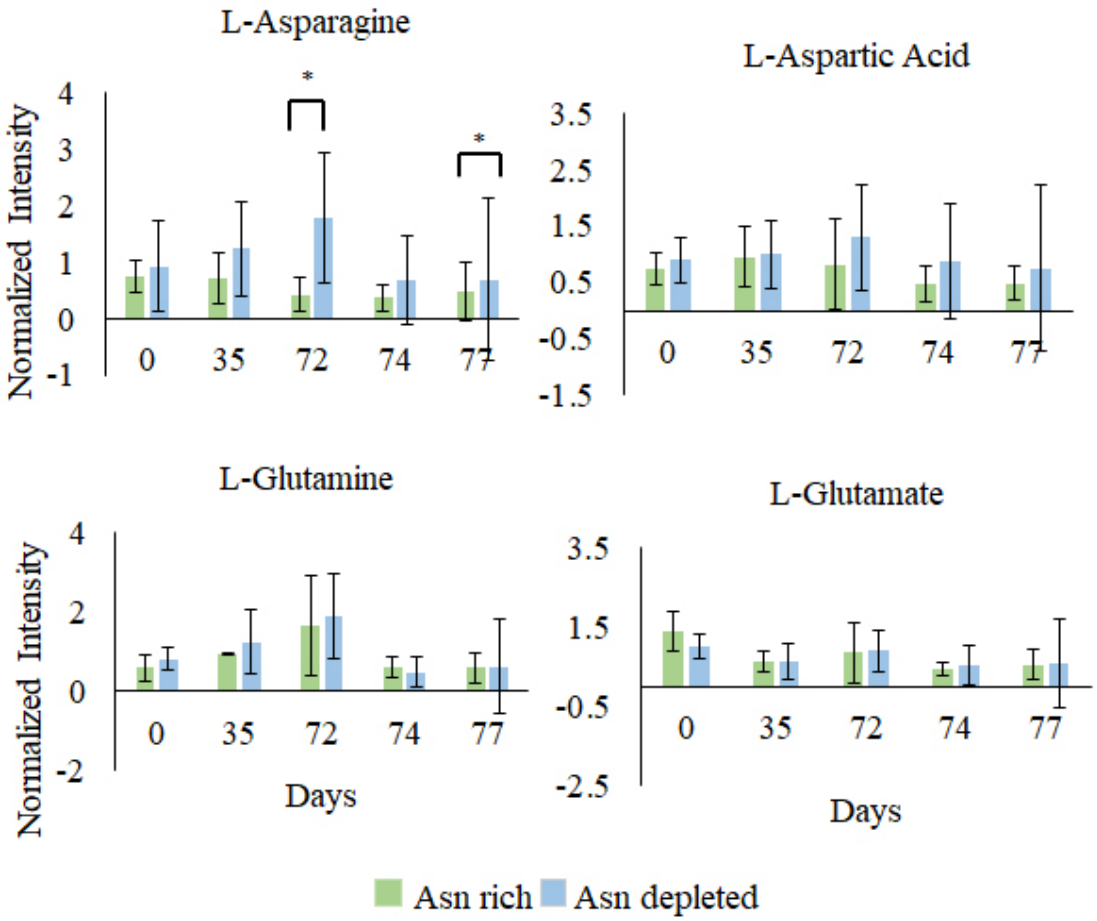

Figure 9 Experiment 1 average stool levels for mice treated on the asparagine (Asn) rich or depleted diets across the study period. Significance is shown for between groups differences, p value <0.05 was taken as significant. Stool Asn levels were significantly different between groups after 72 days on diet and 5 days post pegaspargase.

resulting in a difference at day 72 had a slow onset. Surprisingly, mice on the high Asn diet had less Asn in their stool after 72 days. This indicates that the mice may have undergone biological changes aimed to remove the excess Asn in the gut. The corresponding increase in blood Asp on day 72 in the AR diet provides one possible explanation.

3.1.4 Metabolomics: Asn vs Asp and Glu vs Gln Correlations

Knowing that P-ASP catabolizes Asn to Asp, and Glu to Gln, I next investigated any correlations between Asn vs Asp and Glu vs Gln within each mouse at each timepoint for both blood and stool samples, predicting there will be negative correlations. The AR diet showed no relationship between Asn and Asp in the blood on day 0 (Figure 10). Once the mice were on diet for 35 and 72 days, as well as post-P-ASP, the relationship became linear. The opposite was true for the AD diet which showed a linear relationship on day 0 but showed no relationship between Asn and Asp on days 35, 72 and 77. The stool samples showed a linear relationship in both diets on day 0 and 77, but not during days 35 and 72. When examining the relationship between Gln and Glu in the blood, the AR diet, like with Asn vs Asp, showed no linear relationship on day 0 while the AD diet did. On day 77 the AD diet showed a linear relationship in the blood, unlike the AR diet. In the stool, the relationship between Gln and Glu was not linear on day 35. On days 72 and post-P-ASP both treatment groups showed a linear relationship (Figure 11). The AR and AD treatment groups showed different trends at different timepoints, showing that the diet can have a profound effect on the relationship between Asn and Asp and Gln and Glu. We also observed changes in linearity due to P-ASP, where in the stool both groups were highly linear but, in the blood, only the AR diet showed linearity. Since there were differences

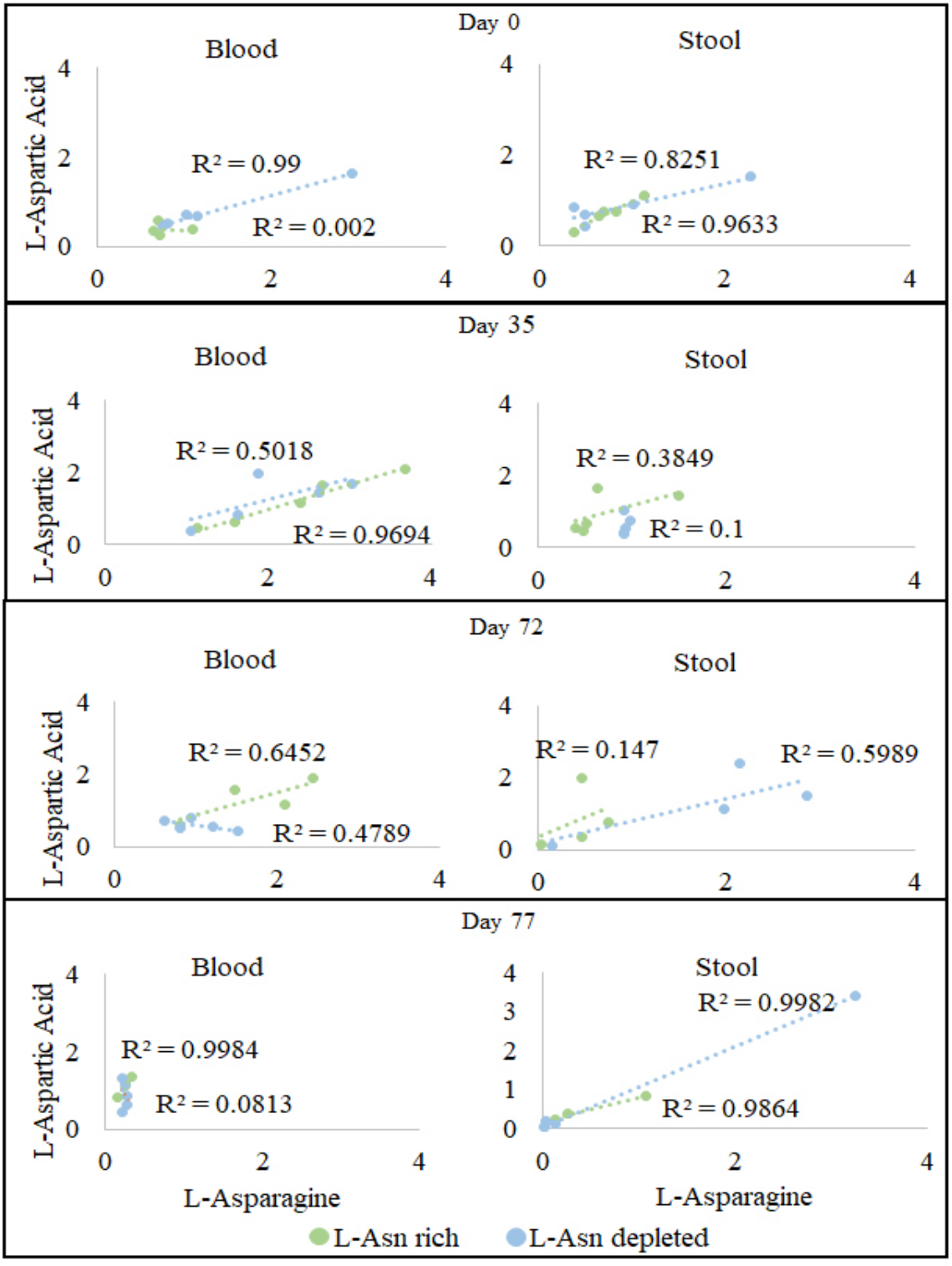

Figure 10 Experiment 1 plots for L-Asn vs L-Asp across sampling periods for each mouse. Linear regression performed on each group of mice, and r^2 values reported.

post P-ASP between dietary groups, this is a possible indication that P-ASP could be affected by diet.

3.1.5 Metabolomics: Metabolic Pathways

Metaboanalyst was used to identify metabolic pathways that were impacted during the experiment. There were 5 pathways highlighted in the blood during pre-diet, 10 on day 35, 12 on day 72, and 4 on day 77 that were significantly different between diets (Table 3). One noteworthy pathway was on day 72 of diet, the Alanine, Asp and Glu metabolism pathway, with the Asp metabolism affected. In this pathway, Asp was significantly higher in the AR group and L-acetylaspartylglutamate was higher in the AD group (Figure 12).

In the stool there were 20 pathways pre-diet, 5 on day 35, 4 on day 72, and 1 on day 77 that were significantly different between diets. Pathways affected in the stool were different from pathways affected in the blood at the same sample time point (Table 4). Prior to starting the diet, mice had many metabolic pathways that differed. As the mice remained on diet and post P-ASP, they became more similar in terms of their stool metabolites.

3.1.6 Bacterial Microbiome 16S

The metabolomic outcomes in the stool were suggestive of changes in bacterial metabolism of amino acids in the gut. 16S Ribosomal DNA sequencing was used to identify the relative abundance of bacteria at multiple taxonomic levels, on multiple days post P-ASP between dietary groups, this is a possible indication that P-ASP could be affected by diet.

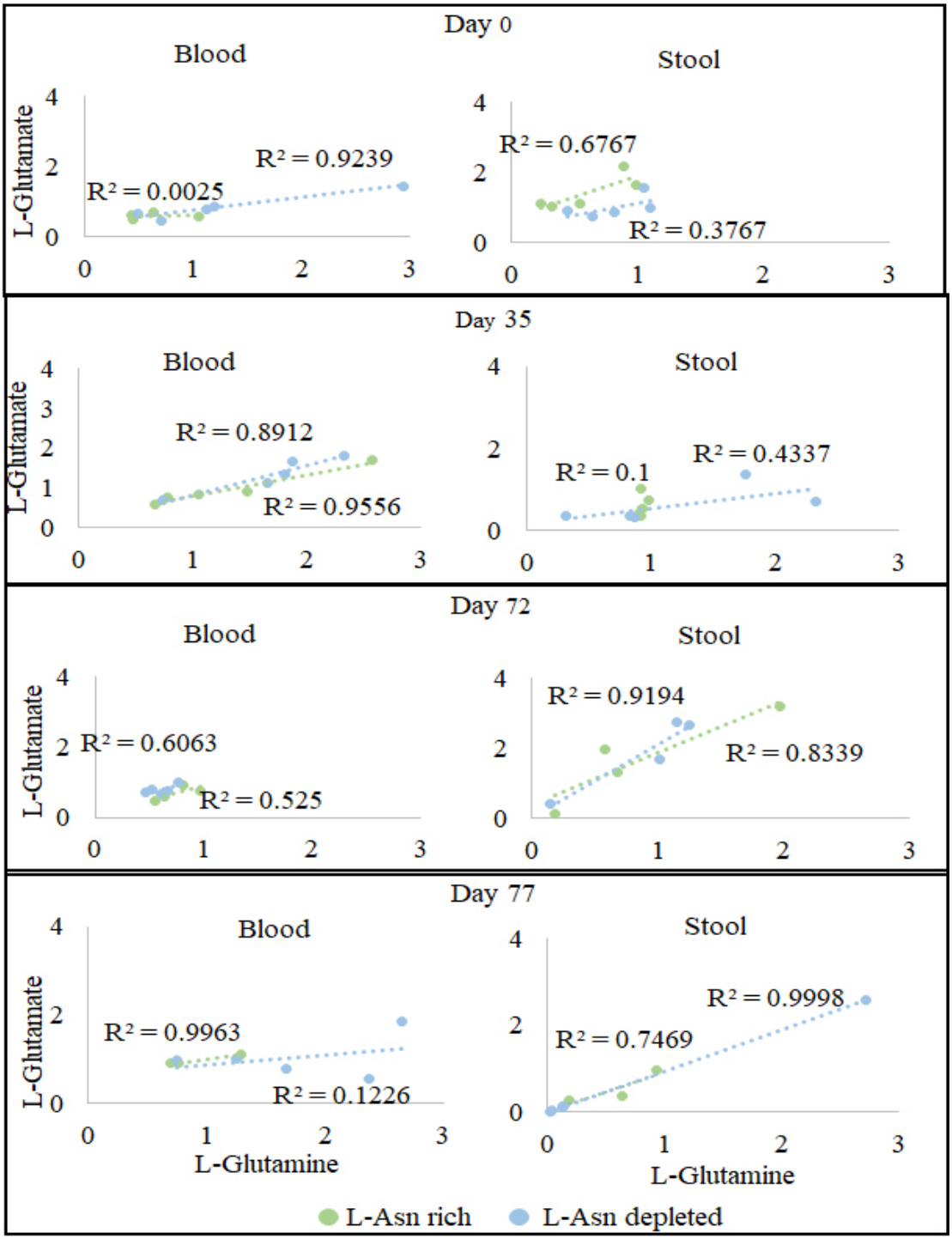

Figure 11 Experiment 1 plots for L-Gln vs L-Glu across sampling periods for each mouse. Linear regression performed on each group of mice, and r^2 values reported.

3.1.6 Bacterial Microbiome 16S

The metabolomic outcomes in the stool were suggestive of changes in bacterial metabolism of amino acids in the gut. 16S Ribosomal DNA sequencing was used to identify the relative abundance of bacteria at multiple taxonomic levels, on multiple days of the experiment. Stool DNA samples were run in duplicate as a measure of confidence in the signal. PCA plots on the PC1 vs PC2 axis demonstrated four distinct clusters (Figure 13). Samples clustered based on treatment group as well as the day 0 samples clustering separately from the day 36, 72, and 76 samples. The PCA plots demonstrate that the microbiome was different between the two groups of mice prior to starting the study. Days 36, 72, and 76 clustered into 2 groups based on diet, showing the microbiome remained different across treatment groups. Since days 36, 72, and 76 clustered together for each diet the PCA plots indicate that there was no overall difference post-diet and post-P-ASP.

To improve the resolution on differences, heatmap data at the genus level was prepared and showed the AR diet mice had a high abundance of *Muribaculaceae* prior to starting the study (Figure 14). After 36 days on diet the *Muribaculaceae* abundance significantly decreased ($p<0.001$), and the reduced abundance persisted after 72 and 77 days. The AD diet mice began with a low abundance of *Muribaculaceae* which continued throughout the study. The AD group had a high abundance of *Bacteroides* pre-diet, but this significantly declined ($p<0.001$) after being on diet for 36 days and the low level persisted for the rest of the study period. The AR group started with a low abundance of *Bacteroides* which increased in abundance after 36 days on both diets ($p<0.001$). On day 36 the relative increase in *Bacterioides* by the AR group and decrease by the AD group resulted in a

Table 3 Experiment 1 pathway blood analysis results from Metaboanalyst. P values listed for significant pathways by timepoint. On day 72, the alanine, aspartate, and glutamate metabolism pathway were significantly different between groups.

Pathway Affected	**Day 0**	**Day 35**	**Day 72**	**Day 77**
Alanine, aspartate and glutamate metabolism			0.019	
Arginine biosynthesis			0.046	
Biotin metabolism		0.024		
Citrate cycle (TCA cycle)		0.029		
Glycerolipid metabolism			0.005	
Glycerophospholipid metabolism			0.006	
Glycine, serine, and threonine metabolism		0.021		
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	0.046		0.006	
Glyoxylate and dicarboxylate metabolism		0.039		
Lysine degradation		0.016		
Mannose type O-glycan biosynthesis	0.027			
Nicotinate and nicotinamide metabolism			0.044	0.046
Pantothenate and CoA biosynthesis			0.032	
Phenylalanine metabolism			0.049	0.013

Pathway Affected	Day 0	Day 35	Day 72	Day 77
Phenylalanine, tyrosine and tryptophan biosynthesis				0.016
Propanoate metabolism		0.027		
pyrimidine metabolism				
Retinol metabolism			0.043	
Sphingolipid metabolism				
Starch and sucrose metabolism			0.030	
Thiamine metabolism		0.016		0.017
Tryptophan metabolism		0.018	0.016	
Ubiquinone and other terpenoid-quinone biosynthesis	0.044			
Valine, leucine and isoleucine degradation	0.014	0.026		
Valine, Leucine, and isoleucine biosynthesis	0.011			
Vitamin B6 metabolism		0.031	0.017	

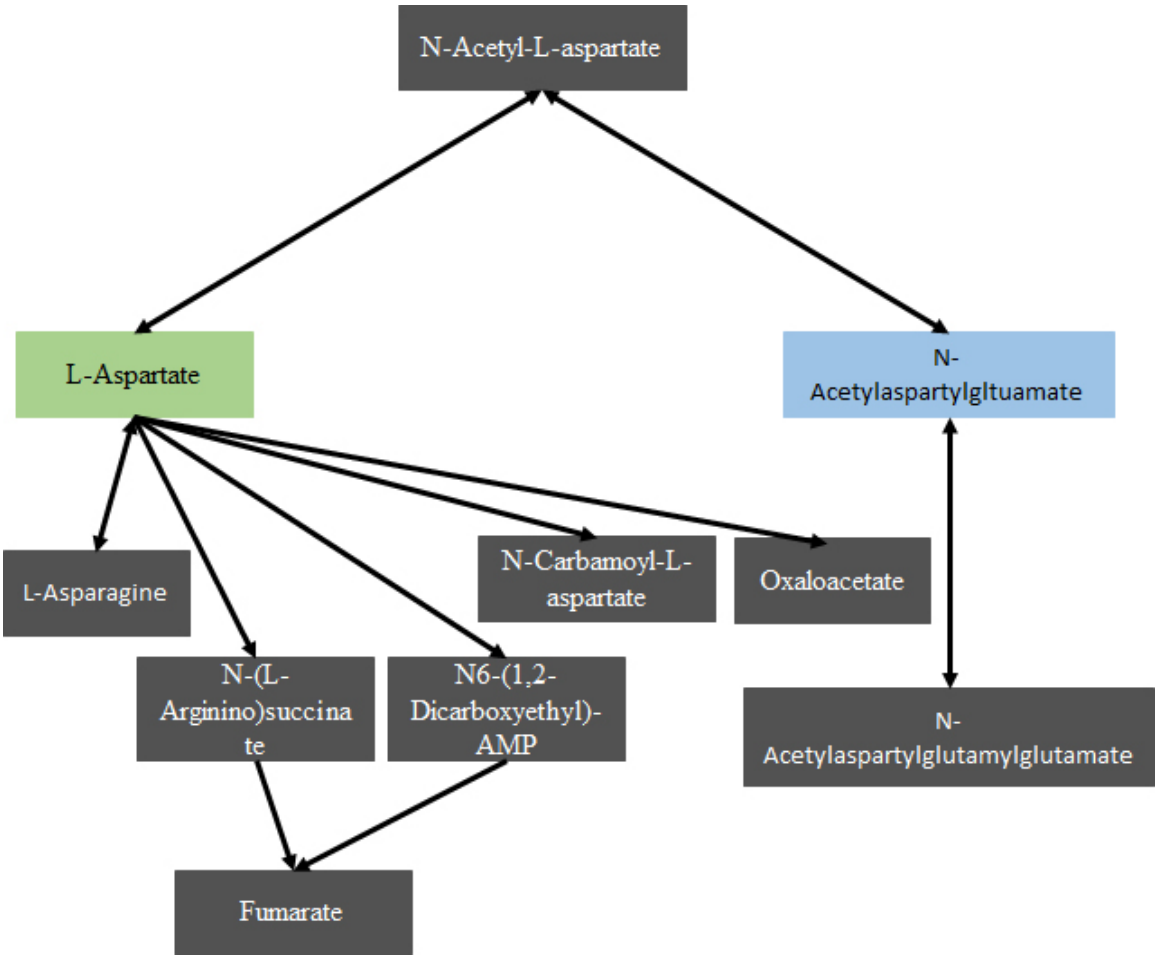

Figure 12 Experiment 1 aspartate metabolism pathway in the blood after 72 days on diet. The green box was significantly higher for the Asn rich diet, and the blue box was significantly higher for the Asn depleted diet.

Table 4 Experiment 1 pathway stool analysis results from Metaboanalyst. P values listed for significant pathways by timepoint

Pathway Affected	**Day 0**	**Day 35**	**Day 72**	**Day 77**
Arachidonic acid metabolism	0.041			
Ascorbate and aldarate metabolism		0.030		
Biosynthesis of unsaturated fatty acids		0.002		
Cysteine and methionine metabolism	0.003			
Folate biosynthesis		0.001		
Glutathione metabolism	0.008			
Glycerophospholipid metabolism	0.020			
Glycine, serine and threonine metabolism	0.021			
Inositol phosphate metabolism	0.017			
Linoleic acid metabolism		0.003		
Mannose type O-glycan biosynthesis	0.007			
Neomycin, kanamycin and gentamicin biosynthesis	0.017			
Nicotinate and nicotinamide metabolism	0.002			

Pathway Affected	Day 0	Day 35	Day 72	Day 77
Phenylalanine metabolism	0.037			
Phenylalanine, tyrosine and tryptophan biosynthesis	0.037			
Phosphatidylinositol signaling system	0.004			
Propanoate metabolism			0.014	0.021
Purine metabolism	0.008			
Pyrimidine metabolism			0.041	
Retinol metabolism	0.026			
Starch and sucrose metabolism	0.018			
Taurine and hypotaurine metabolism	0.002			
Terpenoid backbone biosynthesis			0.041	
Thiamine metabolism	0.007			
Tryptophan metabolism	0.033			
Tyrosine metabolism	0.005	0.004		
Ubiquinone and other terpenoid-quinone biosynthesis	0.038			

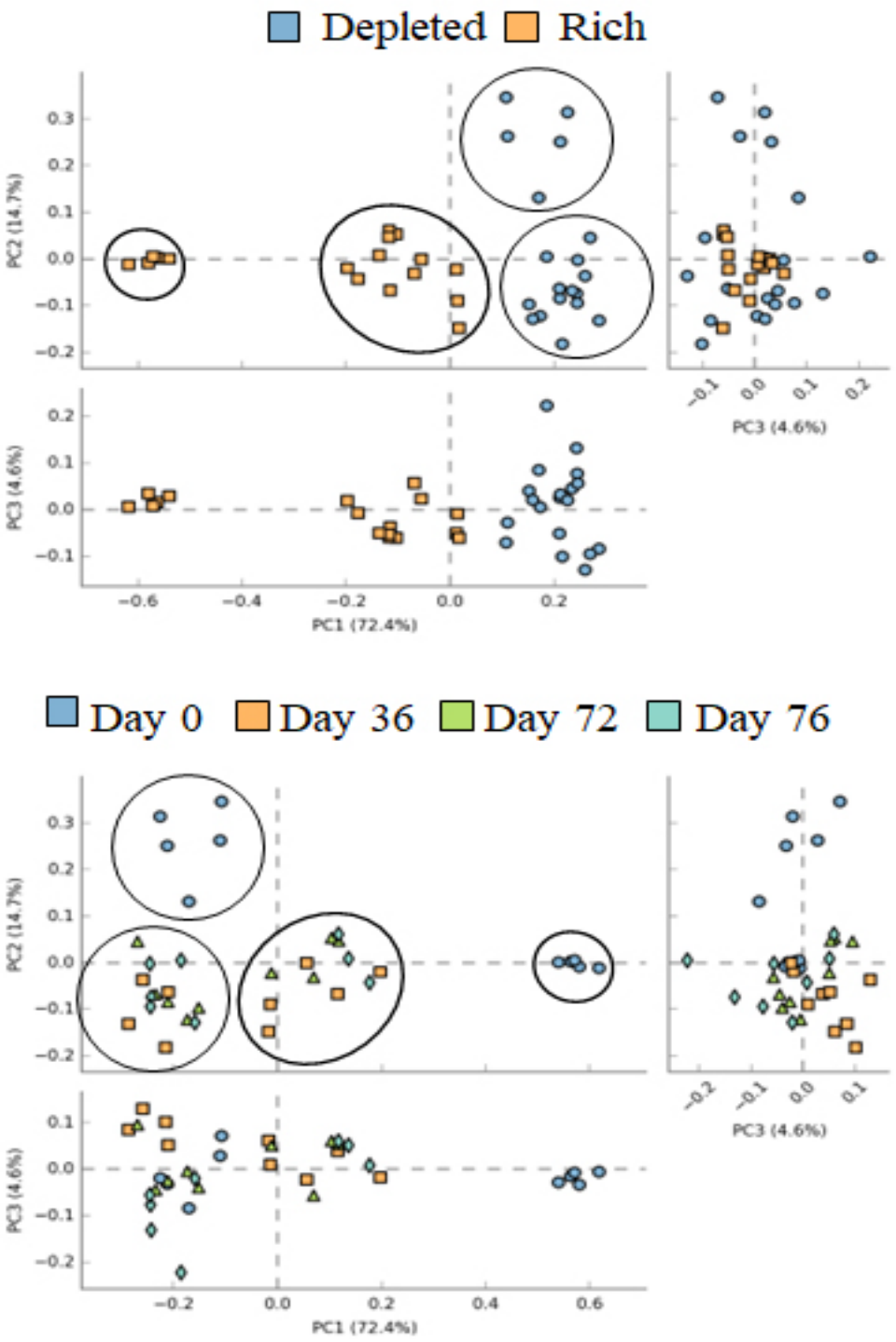

Figure 13 Experiment 1 stool 16S microbiome PCA plots for experiment 1. The top plot is color coded by diet and the bottom by day. Samples clustered into four distinct groups. Day 0 samples clustered separately based off treatment group. Day 36, 72, and 76 samples clustered together within a treatment group.

similar abundance between groups (p=0.242). Mice on both diets had undetectable *Akkermansia* pre-diet but the bacteria increased in abundance after 36 days on diet (p=0.005 and p<0.001 respectively). As the mice remained on diet (day 72) the abundance of *Akkermansia* continued to significantly increase (p=0.009) for AD mice but plateaued for the AR mice (p=0.651). *Akkermansia* abundance did not change significantly post-P-ASP for either the AR (p=0.573) or AD (p=0.557) diets. The AD diet had a higher abundance of *Parabacteroides* after 72 days (p=0.048) and post-P-ASP (p=0.026) in comparison to the AR diet (Figure 14). Multiple bacterial species were significantly different between diets as well as within a diet at all timepoints (Supplementary materials 5-7). Despite there being an unanticipated but significant difference between mice on day0, we were able to identify gut bacterial genus and species that were affected by both the diet and P-ASP therapy.

3.1.4 Bacterial Microbiome Metagenome

We hypothesized that the difference in blood Asp and stool Asn levels in the high Asn diet mice would be due to asparaginase producing bacteria, and that the low Asn diet would favour the emergence of bacteria with asparagine synthetase. Day 72 samples were sent for metagenome analysis. There were 57 bacterial functional differences between the AR and AD diets (Table 5). When comparing asparaginase (p=0.705) and asparagine synthetase (p=0.783) there was no significant difference between groups (Figure 15). Nevertheless, there were 16 bacterial pathways that were significantly different between groups; however, when comparing the PCA plots, the samples did not cluster together suggesting the specific organisms constituting the differences were variable (Table 6 and Figure 16). The metagenome results showed that the hypothesis was not correct and there

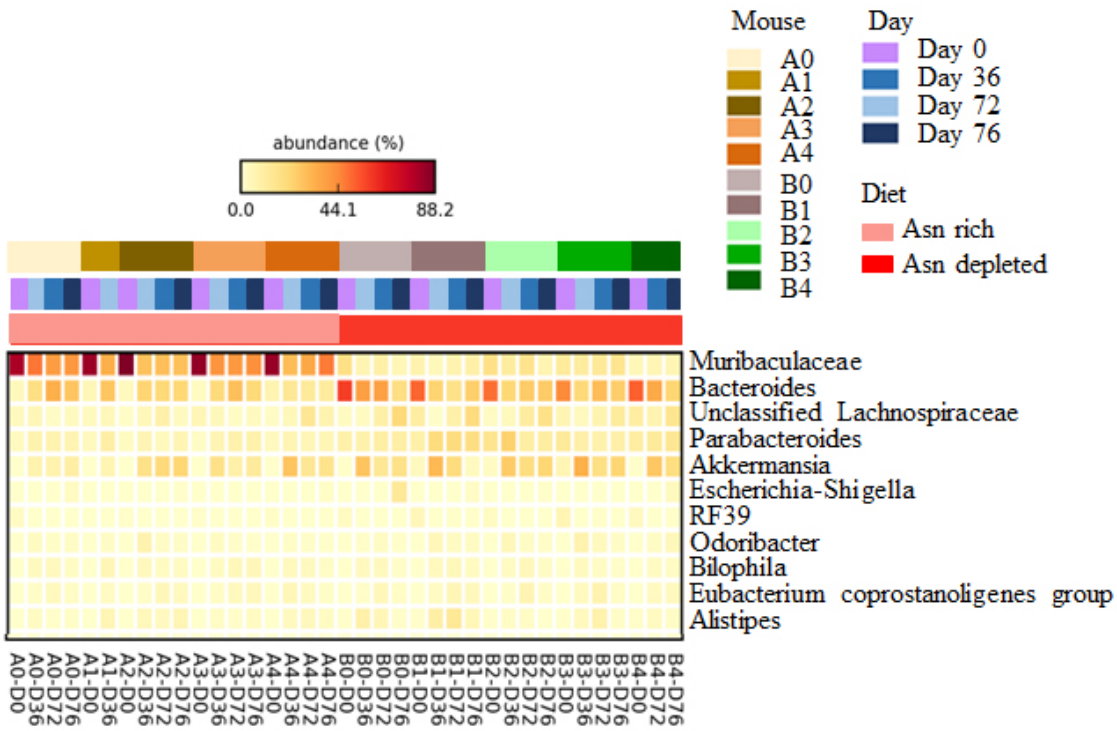

Figure 14 Experiment 1 microbiome heatmap at the genus level for bacteria that changed during the study period. The heatmap is colored coded by diet, day, and mouse. Muribaculaceae were in high abundance in the asparagine (Asn) rich treatment group on day 0 but were present in a low abundance in the Asn depleted group. Bacteroides were present in high abundance in the Asn depleted group on day 0 but not in the Asn rich group.

must be another biological response that can explain why the high Asn diet had significantly less stool Asn compared to the low Asn diet after 72 days.

3.2 Experiment 2

3.2.1 Lessons Learned from Experiment 1

We learned from the first experiment that varying dietary Asn for weeks prior to P-ASP injection does not modify the mouse's blood Asn levels. It is unclear from the first study if an acute change in diet prior to starting P-ASP could have a more profound effect on Asn metabolism. We also learned that 200 IU/kg of P-ASP is sufficient to fully deplete blood Asn for 5 days. The Asn levels did not recover during the sampling period post P-ASP despite a diet high in Asn. It remained to be determined whether blood Asn levels would return at different rates, as P-ASP levels declined, in mice on diets with differing levels of Asp.

An experiment was planned to extend the period of observation while the mice consumed diets with differing levels of Asn and had P-ASP in their system (Figure 2). Studies reporting the half-life of P-ASP in mice are sparse. One study by Abuchowski in 1984 in mice reported the half-life to be 3.73 days. Another study reported the terminal half-life as 1.43 days (Poppenburg 2016). Both studies reported half-life based off of the drugs presence and activity in the blood, but Asn levels in the blood were not reported. There have not been many studies conducted looking at the half-life of P-ASP in mice. Thus, we included two doses of P-ASP (450 IU/kg and 900 IU/kg) to determine how different doses would affect the length of Asn depletion. The experiment could extend to 28 days if no early endpoints emerged during the study. The experiment would also take

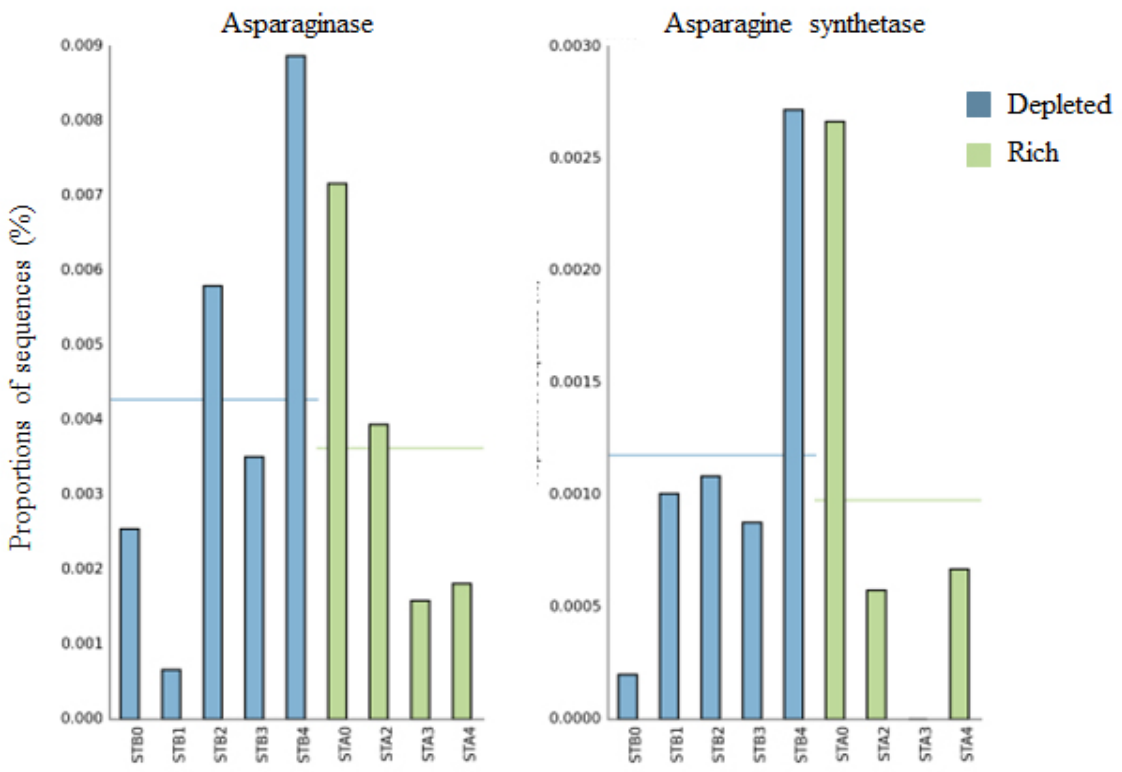

Figure 15 Experiment 1 metagenome analysis for experiment 1. Asparaginase and asparagine synthetase microbial functional differences between the L-Asn rich and L-Asn depleted groups. There was not a significant difference in asparaginase or asparagine synthetase genes between groups.

Table 5 Experiment 1 metagenome results for bacterial functions that were significantly different between groups. P value <0.05 was taken for significance and the diet with significantly more bacteria with the specified function is listed in the table.

Name	P value	Higher
EC:3.2.1.89|Arabinogalactan endo-beta-1,4-galactanase	<0.001	rich
EC:3.5.1.5|Urease	<0.001	rich
EC:4.2.1.130|D-lactate dehydratase	<0.001	rich
EC:4.1.2.9|Phosphoketolase	<0.001	rich
EC:3.4.21.26|Prolyl oligopeptidase	<0.001	depleted
EC:4.1.3.3|N-acetylneuraminate lyase	<0.001	rich
EC:4.2.1.59|3-hydroxyacyl-[acyl-carrier-protein] dehydratase	<0.001	rich
EC:2.7.1.82|Ethanolamine kinase	0.004	depleted
EC:5.1.1.1|Alanine racemase	0.004	rich
EC:2.7.11.32|not_found	0.005	rich
EC:2.7.9.1|Pyruvate, phosphate dikinase	0.006	rich
EC:6.3.4.5|Argininosuccinate synthase	0.007	rich
EC:1.1.1.264|L-idonate 5-dehydrogenase (NAD(P)(+))	0.007	rich
EC:5.3.1.6|Ribose-5-phosphate isomerase	0.007	rich
EC:2.3.1.157|Glucosamine-1-phosphate N-acetyltransferase	0.008	rich
EC:2.4.1.313|not_found	0.009	depleted
EC:3.6.1.7|Acylphosphatase	0.013	rich
EC:2.7.7.72|CCA tRNA nucleotidyltransferase	0.013	rich
EC:2.7.7.56|tRNA nucleotidyltransferase	0.015	rich
EC:2.4.1.7|Sucrose phosphorylase	0.016	rich
EC:4.1.1.101|not_found	0.016	rich
EC:2.7.1.199|not_found	0.017	rich
EC:1.12.1.3|Hydrogen dehydrogenase (NADP(+))	0.018	depleted
EC:1.8.5.3|Dimethylsulfoxide reductase	0.018	rich
EC:5.6.2.2|not_found	0.018	rich
EC:3.4.14.11|Xaa-Pro dipeptidyl-peptidase	0.019	rich
EC:1.5.1.3|Dihydrofolate reductase	0.02	depleted
EC:3.5.1.24|Choloylglycine hydrolase	0.02	rich
EC:4.2.1.77|Trans-L-3-hydroxyproline dehydratase	0.02	rich
EC:2.1.1.198|16S rRNA (cytidine(1402)-2'-O)-methyltransferase	0.021	depleted
EC:2.7.7.n1|not_found	0.021	depleted
EC:4.4.1.15|D-cysteine desulfhydrase	0.022	depleted
EC:2.4.2.6|Nucleoside deoxyribosyltransferase	0.023	rich
EC:1.1.1.25|Shikimate dehydrogenase	0.024	rich
EC:2.7.1.176|UDP-N-acetylglucosamine kinase	0.025	depleted

Name	P value	Higher
EC:3.4.22.16|Cathepsin H	0.026	depleted
EC:6.3.5.3|Phosphoribosylformylglycinamidine synthase	0.026	rich
EC:3.1.26.5|Ribonuclease P	0.028	rich
EC:2.1.1.148|Thymidylate synthase (FAD)	0.029	rich
EC:2.3.1.89|Tetrahydrodipicolinate N-acetyltransferase	0.03	rich
EC:3.2.1.122|Maltose-6'-phosphate glucosidase	0.034	rich
EC:3.2.1.151|Xyloglucan-specific endo-beta-1,4-glucanase	0.035	depleted
EC:1.1.1.22|UDP-glucose 6-dehydrogenase	0.035	rich
EC:2.1.3.15|not_found	0.036	rich
EC:2.7.1.16|Ribulokinase	0.036	depleted
EC:1.8.4.11|Peptide-methionine (S)-S-oxide reductase	0.039	rich
EC:3.2.2.23|DNA-formamidopyrimidine glycosylase	0.039	rich
EC:5.1.3.9|N-acylglucosamine-6-phosphate 2-epimerase	0.039	rich
EC:1.1.1.27|L-lactate dehydrogenase	0.04	rich
EC:3.5.4.13|dCTP deaminase	0.04	depleted
EC:6.2.1.54|not_found	0.04	rich
EC:5.1.3.2|UDP-glucose 4-epimerase	0.041	rich
EC:1.1.5.3|Glycerol-3-phosphate dehydrogenase	0.042	depleted
EC:1.3.98.1|Dihydroorotate oxidase (fumarate)	0.043	rich
EC:4.2.1.75|Uroporphyrinogen-III synthase	0.043	depleted
EC:2.6.1.2|Alanine transaminase	0.046	rich
EC:1.7.99.1|Hydroxylamine reductase	0.047	rich

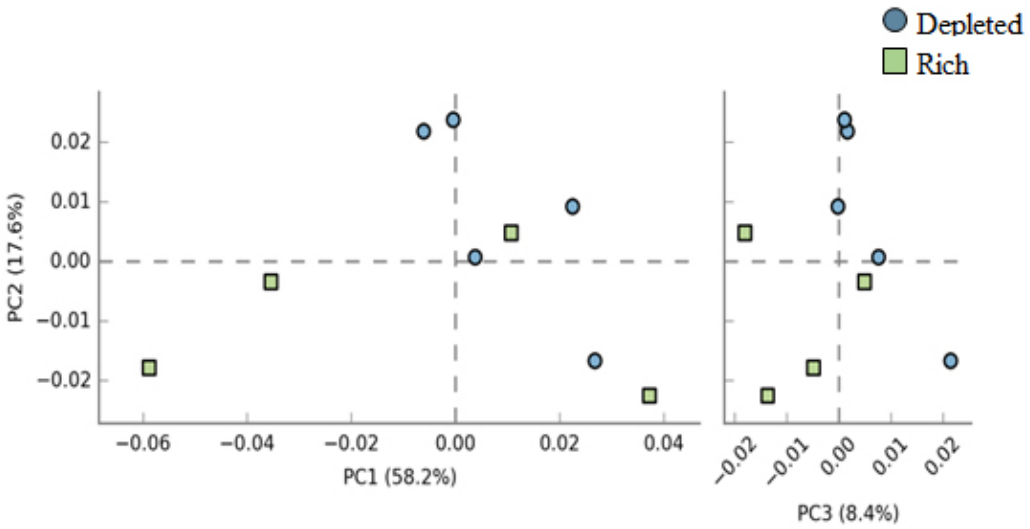

Figure 16 Experiment 1 PCA plot for metagenome bacterial pathways for both the L-Asn depleted and L-Asn rich group. The samples did not cluster.

Table 6 Experiment 1 Metagenome results for bacterial functions that were significantly different between groups. P value <0.05 was taken for significance and the diet with significantly more bacteria is listed in the table under "higher".

Name- IMR	Name- MetaCyc	P Value	Higher
PWY-5384	sucrose degradation IV (sucrose phosphorylase)	0.006	rich
PWY-6590	superpathway of Clostridium acetobutylicum acidogenic fermentation Inferred from experiment Traceable author statement to experimental support	0.009	rich
CENTFERM-PWY	pyruvate fermentation to butanoate	0.009	rich
PWY-5189	tetrapyrrole biosynthesis II (from glycine)	0.021	depleted
NONOXIPENT-PWY	pentose phosphate pathway (non-oxidative branch) I	0.030	rich
PWY-6163	chorismate biosynthesis from 3-dehydroquinate	0.037	rich
PWY-5177	glutaryl-CoA degradation	0.044	rich

into consideration the possibility of a “cage effect” by including 2 cages of identically treated mice. As a secondary objective, the Oncaspar would be used in another cohort of mice several days after opening the vial, to test whether the activity declines after opening (the current practice in the clinic is to discard opened vials). Finally, it was decided to change to a more defined diet in which there was no casein present, (the diet used in experiment 1 contained casein which could potentially confound the asparagine levels). The diet used by Knott et al. 2018 was selected.

3.2.2 Sample Collection

Mice from the first batch of experiment 2 were injected with P-ASP the day the vial was opened. Mice from this batch were divided into 2 groups. One day post P-ASP injection, 1 mouse (E1) on the HP1 treatment arm was hunched and was isolating, indicating stress and/or pain. This mouse was monitored until day 3 when it was determined to be losing weight. It had lost >20% of its weight from the start of the experiment and was euthanized. While the other mice were active, all on the low Asn diet had to be euthanized due to them reaching a weight loss early endpoint (Figure 17). Mice E1 and E2 from the HP1 group were also euthanized on day 3. When comparing the mice weights by cage, a cage effect was noticeable as mice in cage E lost more weight than mice in cage F, despite both being on the HP1 treatment arm (Figure 18). During dissection of mouse E1, it appeared that constipation was the issue as food was observed in the mouse intestines although there was a lack of stool in the cage. All surviving mice were changed to a mash diluted in water which was refreshed daily starting on day 3. On day 4, one more mouse, A3, needed to be euthanized. By day 7, the remaining mice began to gain weight which continued through the remainder of the study (Figure 17).

Due to the acute circumstances arising from batch 1 mice, which seemed to be solved using food in a mash preparation, the mice from batch 2 started on the study roughly 2 months later. On day -1, all mice were started on a mash of their respective diets with the hope to reduce weight loss in the first week. Mice were injected using the same vial of P-ASP (which happened to be the day that the vial expired). Unfortunately, by day 3 all mice on the low Asn diet again lost enough weight that they had to be euthanized (Figure 19). One mouse, K1, that was treated on the high Asn diet had to be euthanized on day 4. Mice from batch 2 took longer to re-gain weight, as by day 7, the average weight gain was still negative. By day 14, mice had begun gaining weight again which continued until the end of study.

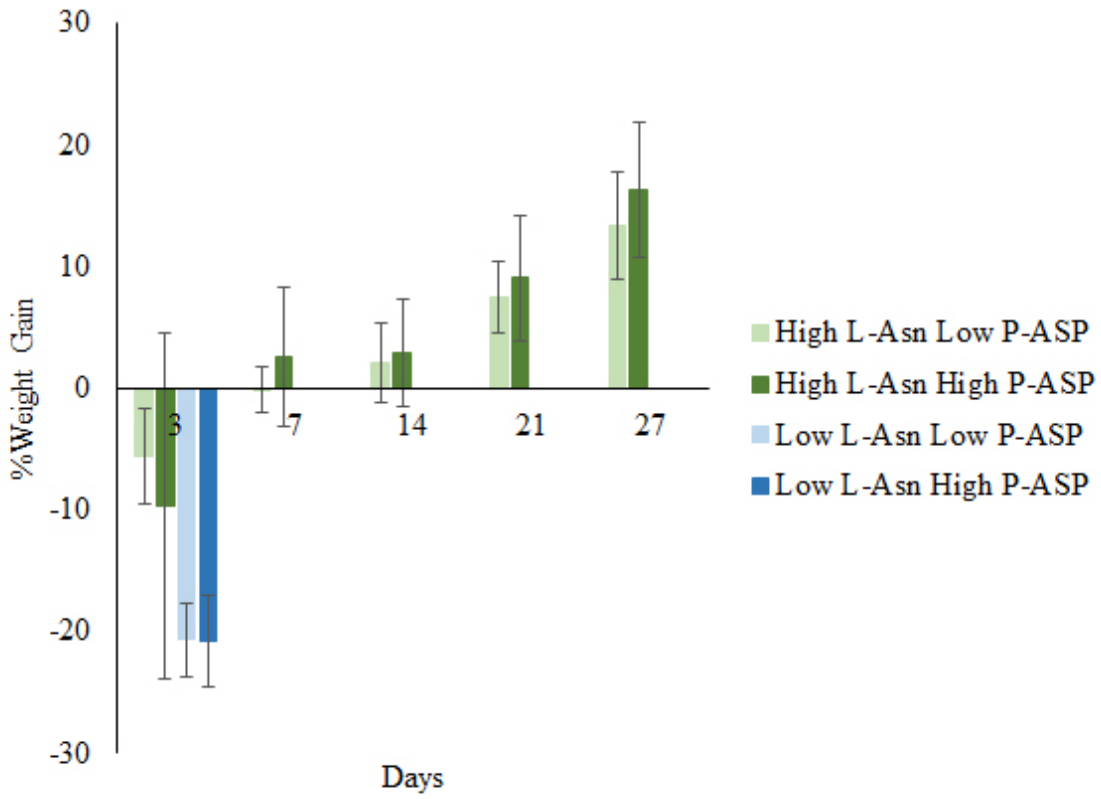

Figure 17 Experiment 2 batch 1 %weight gain by group. On day 3, all mice from the low asparagine (Asn) diet treated with both the high pegaspargase (P-ASP) and low P-ASP dose needed to be euthanized due to weight loss. The high Asn diet mice started gaining weight on day 7.

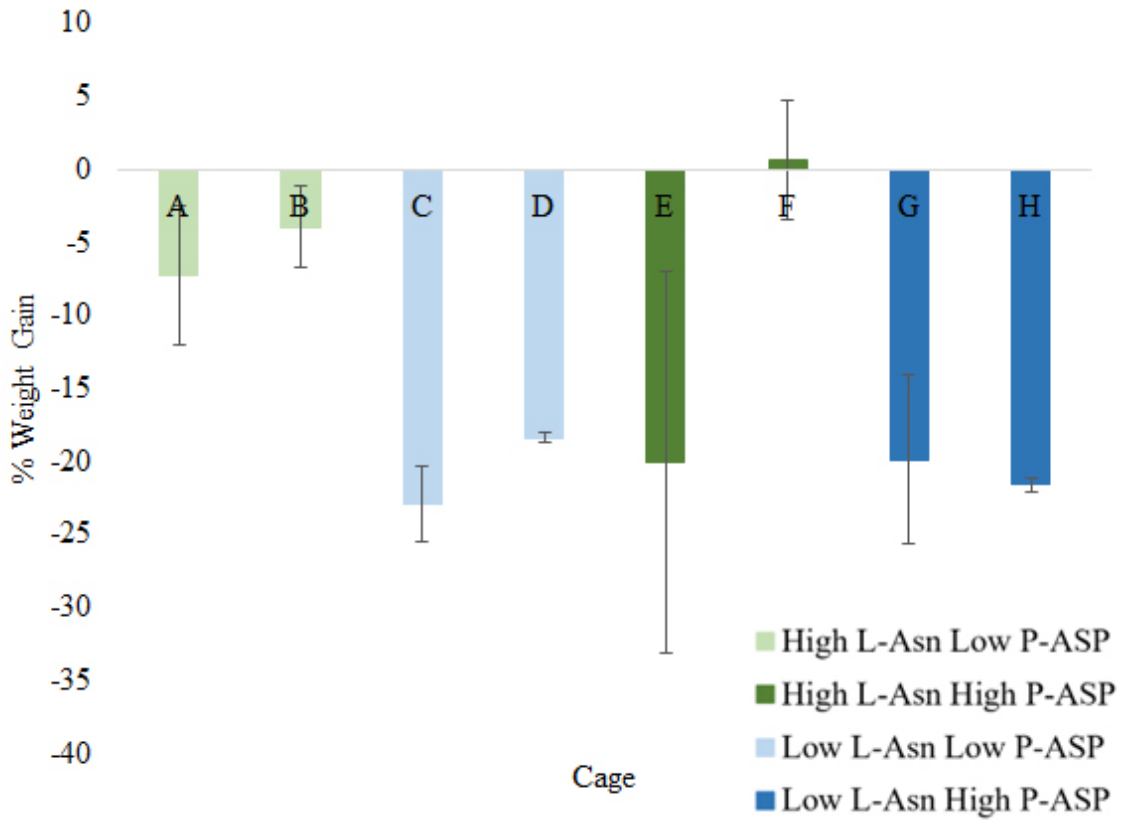

Figure 18 Experiment 2 batch 1 day 3 % weight gain by cage. Mice in the high asparagine (Asn) and high pegaspargase (P-ASP) group had differences in weight loss based off of the cage they were in.

3.2.3 Metabolome: PCA Analysis

Blood PCA plots combining the data from both batches of mice. All samples clustered along the PC1 axis by the day they were collected. Along the PC3 axis, days 7 and 21 clustered into two groups, which represented batch 1 and 2 mice (Figure 20). As the mice remained on diet and the time from P-ASP injection increased, the mice metabolome continued to become more different from day -1. When comparing the high P-ASP samples from batch 1 and 2, it becomes clear that the separate clusters on day 7 are due to the different batches of mice although it represents only 8.8% of all variances (Figure 21). When looking at the stool samples, mice from both batches clustered together on day -1. Mice in batch 1 clustered together 7 days post P-ASP injection but mice in batch 2 did not cluster together and showed wide variation in their stool metabolites (Figure 22). Based on the results from the PCA plots, there appears to be a difference in the blood and stool metabolism between batches of mice. As the treatments were similar between batches on day -1, it could be due to how long the vial of P-ASP was opened.

3.2.4 Metabolome: Metabolite Levels

I was informed by the service provider that data for Asp was unreliable and therefore not available for analyses. Between-group analysis was conducted using an ANCOVA. Blood Asn levels were significantly different on day 14 between the LP group and HP2 groups, with the HP2 group having significantly less Asn ($p=0.02$, Figure 23). Mice from batch 1 had significantly more Gln than the HP2 group on day 7 with HP1 having greater Gln levels ($p=0.044$) and with LP having greater levels on day 21 ($p=0.01$). Similarly, the Gln levels were higher for batch 1 when comparing HP1 on day 7 ($p=0.006$) and LP on day 14 (0.033). On day 21 all groups were significantly different from each

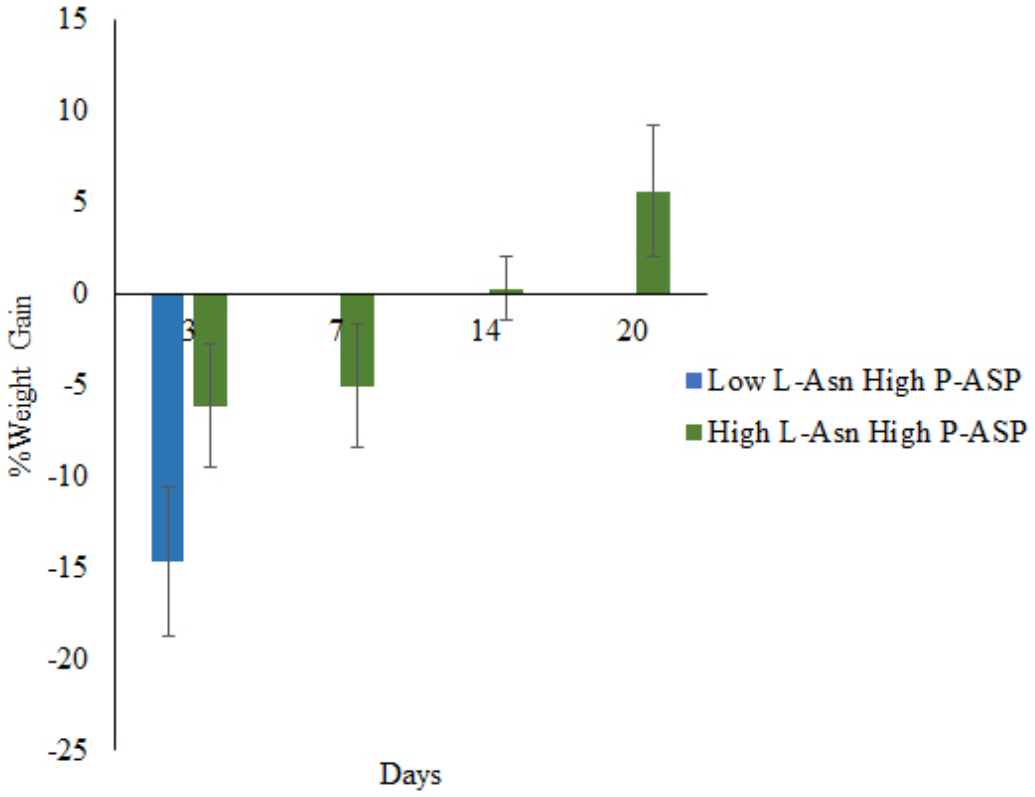

Figure 19 Experiment 2 batch 2 percent weight gain. All mice on the Low asparagine (Asn) diet had to be euthanized due to weight loss, independent on whether they received the high or low pegaspargase (P-ASP) dose. Mice on the high Asn diet began gaining weight on day 14.

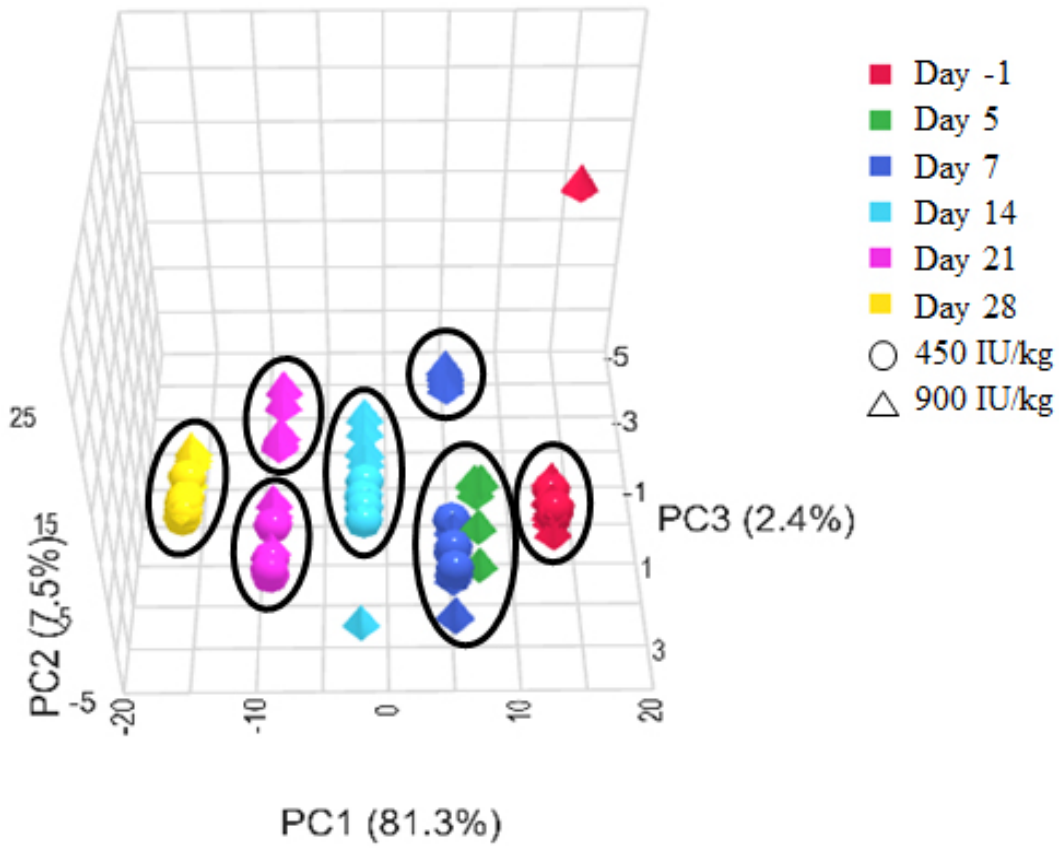

Figure 20 Blood PCA plot for experiment 2 metabolites for both batches 1 and 2. Samples clustered separately based off treatment day.

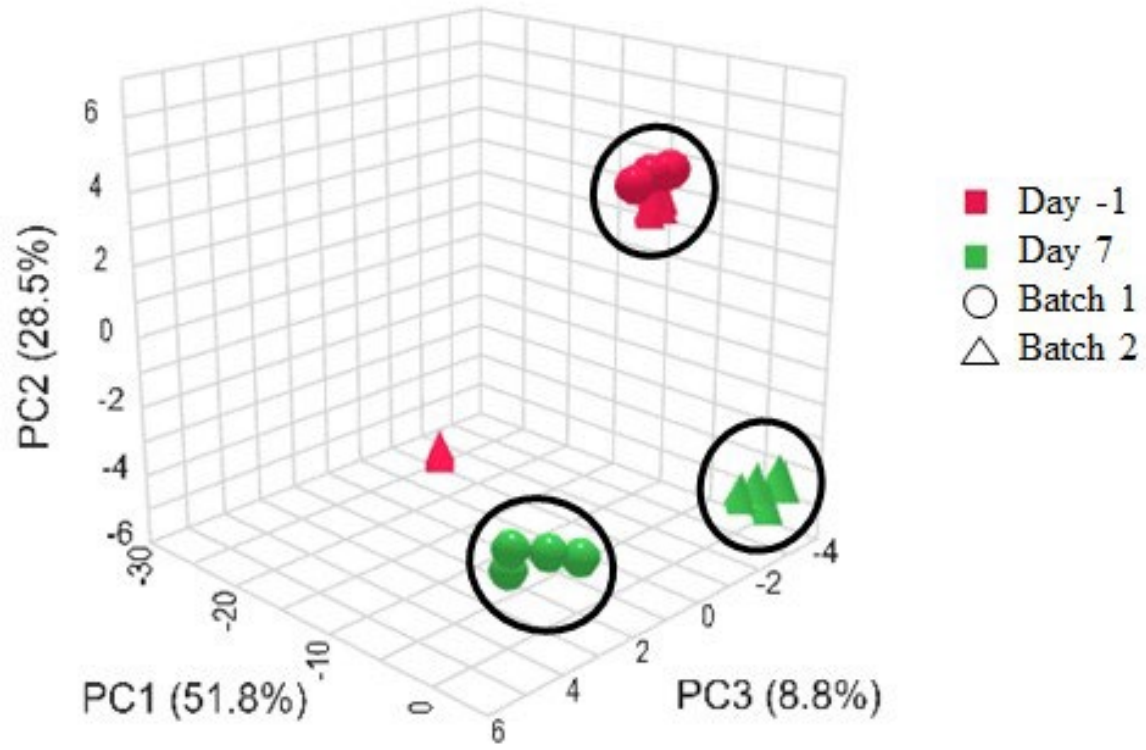

Figure 21 Experiment 2 blood metabolite PCA plot for the high Asn high P-ASP group in both batches 1 and 2. Day -1 samples clustered together but by day 7 they clustered by batch.

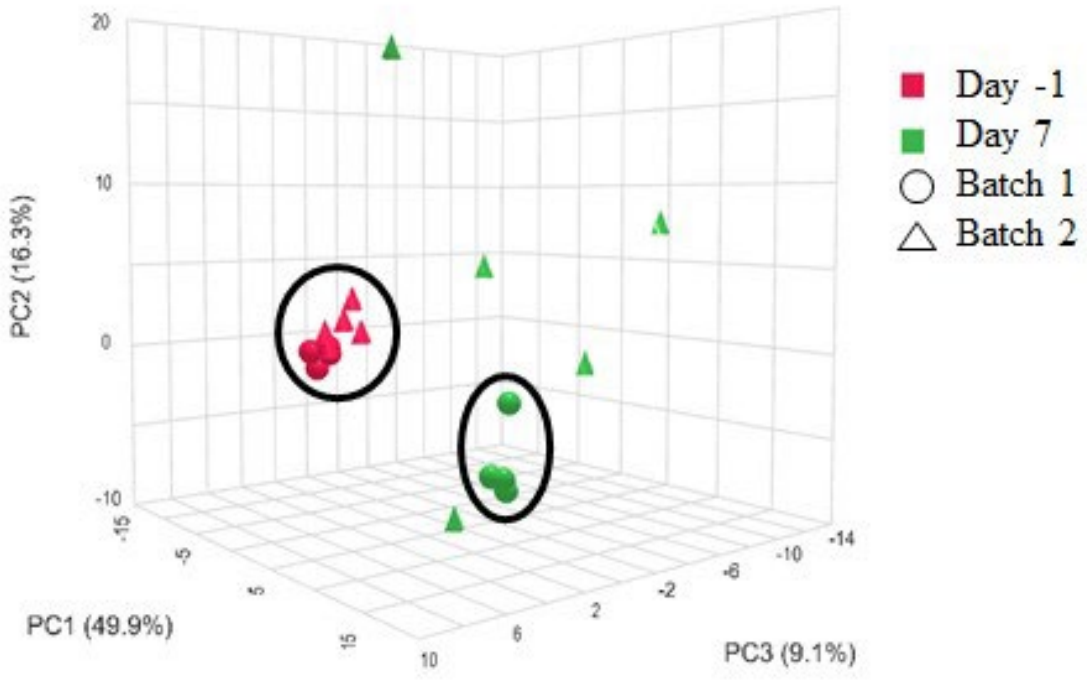

Figure 22 Experiment 2 stool metabolite PCA plot for the high asparagine (Asn) high pegaspargase (P-ASP) group in both batches 1 and 2. Day -1 samples clustered together. Batch 1 day 7 samples clustered together but batch 2 day 7 samples did not cluster.

other with LP having the highest glutamate levels followed by HP1 and then HP2 (Figure 23). Day 7 samples did not show a significant difference between groups for their Asn levels, which could be an indication that despite the dose and when the vial was opened there is no effect on acute Asn depletion. However, Asn was not depleted in any groups making it difficult to determine if this is accurate.

Within-group blood differences are shown in Table 7. Surprisingly, none of the groups had a significant difference in Asn levels between their pre-P-ASP samples (day -1) and the samples collected 7 days post-injection (day 7), indicating that the P-ASP failed to deplete Asn. All 3 groups had a significant increase in the amount of serum Gln 7 days post P-ASP, which is unexpected when considering P-ASP converts Gln to Glu (the results from the first experiment also did not show a significant increase in Gln in the AR diet but, interestingly, it was shown in the AD diet.) Despite the serum Gln rising after 7 days, the Glu levels were similar to baseline levels across groups. The results from the blood metabolome were not as predicted as P-ASP was predicted to deplete serum Asn, yet no difference was seen.

The difference in stool Asn, Gln, and Glu levels between the HP1 and HP2 group were examined. There was no significant difference between the groups for Asn or Gln. The Glu levels were significantly different between groups on days -1 and 7, with Glu being higher in HP1 for both days. Within group comparisons showed that the HP1 group had significantly more stool Asn and lower Gln and Glu 7 days post P-ASP injection. The HP2 group had significantly less Glu after 7 days but no significant change in Asn and Glu (Figure 24). As predicted, stool Asn in group HP1 mice increased after initiating the high Asn diet, surprisingly this trend was not repeated with the HP2 group.

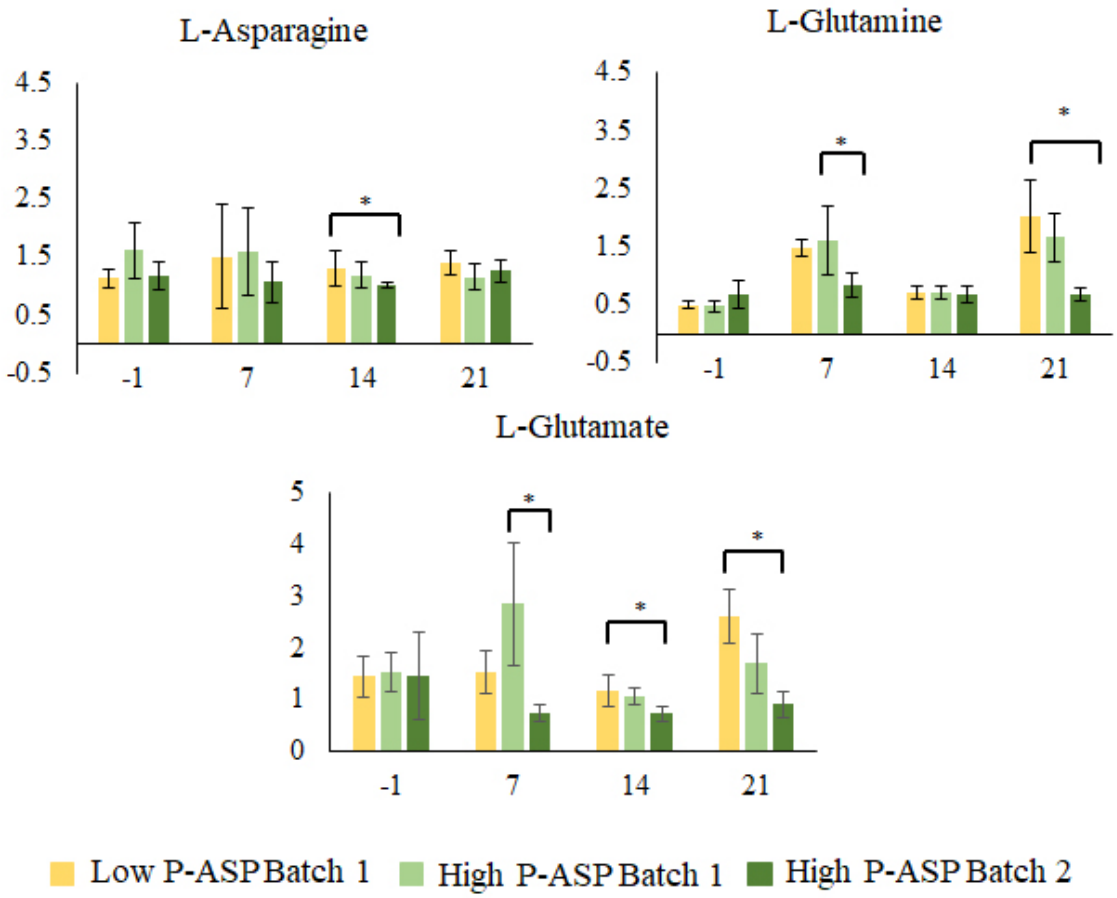

Figure 23 Experiment 2 blood levels for the three treatment groups. Significance is reported for between group analysis, with a p value <0.05 indicating significance. Glutamine and glutamate levels were significantly different between the high pegaspargase (P-ASP) batch 1 and 2 mice on day 7.

Table 7 Experiment 2 blood metabolome results for the high L-Asn low P-ASP (LP), high L-Asn high P-ASP batch 1 (HP1), and the high L-Asn high P-ASP batch 2 (HP2) within group analysis. P values are displayed for days that were significantly different within a group. P value <0.05 was taken for significance. Glutamine levels significantly increased in all groups on day 7.

LP					
Days	1 vs 7	1 vs 14	1 vs 21	7 vs 14	14 vs 21
L-Asparagine			0.032		
L-Glutamine	0.001			0.002	0.036
L-Glutamate			0.018		0.006
HP1					
Days	1 vs 7	1 vs 14	1 vs 21	7 vs 14	14 vs 21
L-Asparagine					
L-Glutamine	0.039		0.006		0.027
L-Glutamate				0.044	
HP2					
Days	1 vs 7	1 vs 14	1 vs 21	7 vs 14	14 vs 21
L-Asparagine					0.024
L-Glutamine	0.011				
L-Glutamate					

When comparing the Gln levels on day 7 the Gln levels significantly increased in the blood and decreased in the stool for both HP1 and HP2. For group HP1, the Glu levels also significantly decreased in the stool while increasing in the blood. This may be evidence of a link between Gln and Glu levels in the gut and blood in the presence of P-ASP.

The metabolic levels in experiment 2 were not as predicted. The first major difference was our anticipation that the higher doses of P-ASP would be able to fully deplete Asn levels in the blood after 7 days. Instead, the two higher doses of P-ASP (450 IU/g and 900 IU/kg) were unable to deplete blood Asn while the lower P-ASP dose (200 IU/kg) was. The next surprising finding in the blood was that Gln levels increased 7 days post P-ASP, when we expected them to either stay the same, as they did in experiment 1, or decrease due to the glutaminase activity of P-ASP. Despite the increasing Gln levels we did not see a corresponding decrease in Glu in the blood, indicating a different pathway or different biological source could be responsible for the increased Gln. One possible explanation could come from the stool where we saw a significant decrease in stool Gln on day 7. The data also showed significant differences between the HP1 and HP2 group. Both groups received the same treatment, but the batches were two months apart. The groups had similar blood Gln and Glu levels on day -1 but by day 7, HP2 had significantly less Glu and Gln than HP1. This indicates that there is a different effect on blood Glu and Gln levels the longer the P-ASP vial has been open, despite there being no effect on blood Asn. The findings from experiment 2 were not as expected, but we were able to identify more possible links between stool and serum metabolite levels as well as potential differences in P-ASP as the drug approaches its expiry date.

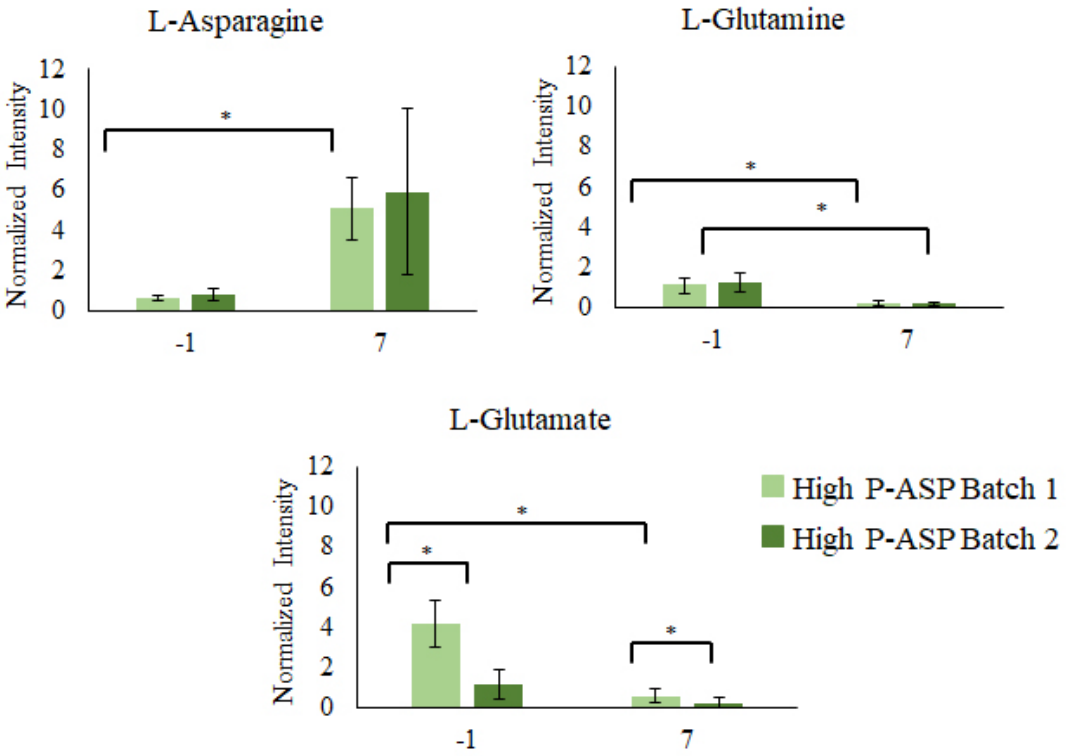

Figure 24 Experiment 2 stool levels for the high pegaspargase (P-ASP) treatment groups. Significance is reported for between group analysis, with a p value <0.05 indicating significance. Stool glutamine levels significantly decreased in both batches after 7 days. Stool asparagine levels significantly increased in the batch 1 mice after 7 days.

Chapter 4. Discussion

The experiments in this study had no direct precedent and consequently were planned around several predictions drawn from the known activity of asparaginase and the few precedents from limited elements of the full study. The Discussion will be structured around the predictions and observed outcomes and explain any difference between the two.

4.1 Experiment 1: Blood Asn Levels Will be Linked to the Diet.

The key prediction for these experiments relies on blood Asn levels being linked to gut Asn levels. A paper by Knott et al. in 2018 found that Asn levels in the blood were significantly higher for mice consuming a diet rich in Asn (4%) compared to mice on either a control (0.6% Asn) or depleted diet (0% Asn), after 7 days on the diets. This precedent contributed to our hypothesis that blood Asn levels would be modified by diet. Asn is closely related to Asp, Gln, and Glu levels since all these amino acids are products of Asn metabolism.

4.1.1 Prolonged Diet Did Not Modify Serum Asn Levels.

In experiment 1 we maintained mice on the diet for 72 days prior to P-ASP injection, to determine if prolonged exposure (contrasting Knott et al. 2018 “acute” 7-day period) to the diet would modify blood Asn. On day 35 of the diet, the AD group had a significant increase in blood Asn but by day 72, the blood levels were back to baseline. This may indicate that the animals’ metabolism reacted to low Asn with robust synthesis. Contrary to the prediction, the AR diet did not result in significantly higher blood Asn levels. We therefore conclude that a long period of diet Asn modification (measured in weeks) does not impact blood levels, and the animals’ metabolism rebalanced levels, presumably through synthesis. The gut microbiome may also have adapted to the AR diet

by increasing metabolism of Asn. Another adaptation may be reduced uptake by intestinal epithelial cells. Noteworthy, our protocol neglected to test earlier time points including after one week. Taking this into account, we planned our second experiment to have sampling periods closer to the start of the diet.

4.2 Experiment 1: Dietary Differences in Asn Levels Will Result in Differences in Blood Asp.

Asn can be hydrolyzed to Asp in the gut by bacteria, but not in the blood as neither mouse nor human cells possess asparaginases. The reaction is reversible, as Asp is also one of the key substrates, along with glutamine, in the synthesis of Asn through ASNS. The close relationship between these two amino acids makes it likely that changes in Asn would also affect Asp. This was observed on day 72, when there was a significant difference in Asp levels in the blood between the AR and AD diet. The difference was driven by Asp levels significantly increasing in the Asn rich group while on diet. This result cannot be explained by asparaginase activity in the blood so we sought to determine whether it could be attributed to differences in microbial function between the two groups on day 72.

4.2.1 Dietary Asn levels Did Not Lead to an Enrichment of Gut Bacteria With ASNS or Asparaginase.

Day 72 mouse stool samples were processed for bacterial metagenome analysis. The result was no significant difference between groups in either enzyme, meaning it is unlikely that bacteria caused changes in the Asp levels. One caveat is that we only ran metagenome analysis on the day 72 samples, so it is unclear whether the day 72 bacterial asparaginase and ASNS could have changed over time within a group. A second caveat is that the metagenome data does not provide absolute gene copy numbers, and a third caveat

is that many bacteria remain associated with the mucosa and are not necessarily represented in the stool.

4.2.2 Stool Asn Levels Will be Altered by Dietary Asn.

Comparing the stool levels of Asn between the 2 diets, there were no significant changes at day 35 but at day 72 there was a significant difference between the AR and AD group. Surprisingly, the AD diet had significantly more stool Asn than the AR group. This is the opposite of the prediction as the diet with high Asn would presumably contribute to higher stool Asn levels. As there were no changes in bacterial ASNS or asparaginase, it is difficult to interpret how the AR diet had a decrease in Asn and the AD had an increase. One possible explanation could be the transport of Asn from the gut into bloodstream, leading to a decrease in stool Asn; however, we did not find a corresponding increase in serum Asn on day 72, which showed that mice's body may have had time to rebalance their blood Asn levels. This leads to the question; could dietary Asn be replacing the need for cells to synthesize Asn, resulting in an accumulation of Asp? Alternatively, despite there being no difference in bacterial asparaginase, could there have been enough asparaginase activity to hydrolyze the Asn in the gut into Asp? Future studies measuring ASNS activity prior to and during the diet as well as completing pulse/chase studies where Asn is tracked from the gut into the blood stream may answer this question.

4.3 Mouse Microbiome: Changes Due to Dietary Asn

Bacterial diversity and abundance in the gut is unique to the individual yet there is bacterium that are common in the gut of healthy mice. Wang et al (2019) reviewed studies conducted on mice that reported gut bacteria. They identified 37 genera that were present in most samples. These contained bacteria genus like *Bacteroides, Lachnospiraceae,* and

Parabacteroides which were also identified in high abundance for mice in our first experiment. *Akkermansia* was prevalent in our mice but was not among the 37 genera in Wang et al. (2019) despite being present in 44.6% of mice, as it fell below the threshold of >50%. In our experiment, mice in the AR diet had a high abundance of *Muribaculaceae* on day 1. *Muribaculaceae* was not mentioned in the paper indicating it may not be a common gut bacterium in mice or speaks to the fact that there are strong regional/facilities differences.

Wang et al. (2019) also identified bacterial genus that were associated with metabolic syndrome. One bacterium that was found to be decreased in abundance in these mice was *Turicibacter*. We found that mice on both the AR and AD diet had a decrease in *Turicibacter*, which was maintained post P-ASP. Alone, this finding does not confirm that mice on both the AR and AD diets were sick as the other bacteria associated with diseased mice in the Wang et al. (2019) review were unchanged in the mice.

4.4 P-ASP Asn Depletion Will be Affected by Dietary Asn

4.4.1 Experiment 1: Prolonged Effects of Dietary Asn on P-ASP Depletion

We predicted that prolonged exposure to the diet would be necessary to change the blood metabolites and gut microbes. Instead, we found that blood Asn levels were unchanged in the mice after 35 or 72 days. Asn levels were significantly decreased in both the AR and AD group after P-ASP therapy in experiment 1 and maintained up to 5 days following P-ASP injection, indicating that diet was unable to overcome the P-ASP. Blood Asn levels were able to re-balance prior to P-ASP injection, which could explain why the diet did not affect P-ASP depletion. The Asn diets in the first experiment also had casein in the ingredients. Casein is a protein additive that has Asn (Fox et al., 2015). The casein

in the first experiment could have confounded the results by providing a confounding dietary source of Asn to the AD mice; however, that seems unlikely since even mice on the AR diet had their Asn blood levels reduced by P-ASP. Another potential confounder in the first experimental diet is that Glu was used to balance the Asn depleted diet. Glu can be converted to Gln which is a substrate for ASNS, which could have also indirectly increased the dietary Asn in the AD mice. Thus, when planning our second experiment we chose to look at an acute dietary effect and change the diet to eliminate casein as well as use multiple amino acids to balance the AD diet rather than just Glu.

4.4.2 Experiment 2: Explain the Catastrophic Weight Loss

Experiment 2 was undertaken with the knowledge that P-ASP reduced blood Asn levels in mice that consumed the AR diet for weeks, and the diet potentially having even more Asn, should casein be digested. It was also learned that depleting blood Asn was not associated with the predicted changes in levels of Asn metabolites. Therefore, in experiment 2, mice were started an AR or AD diet 1 only day prior to P-ASP injection, to determine the acute effects of diet on P-ASP Asn depletion. Unexpectedly, mice on the AD diet all reached their HEP weight within 3 days of the P-ASP injection and had to be euthanized. Weight loss is a well-known side effect of P-ASP in mice at high doses (Bendich et al., 1982; Kumar et al., 2021; C. Liu et al., 2016). Studies using an IP dose of P-ASP (1500 IU/kg), greater than used in the present study, showed an average of 10% weight loss in C57BL/6 male mice 3-5 days post injection (Kumar et al., 2021), making it unlikely that the P-ASP alone was responsible for the weight loss. Most mice on the AR diet in Experiment 2 survived on either dose of P-ASP, further supporting that the weight loss was unlikely entirely due to P-ASP.

Another cause of weight loss in the mice must be the diet, as all mice on AD but not AR diet needed to be euthanized. The diets in experiment 2 were the same products used by Knott et al (2018). With this precedent and no report of adverse events, particularly no report of weight loss, by Knott et al, there was no plan to include a "diet only" arm to the study. The drastic weight loss in mice treated on the AD diet was unexpected but is possible evidence that acute P-ASP is highly toxic in the absence of dietary Asn despite Asn being a nonessential amino acid.

4.4.3 Experiment 2: Asn Rich Diet P-ASP Depletion

Another unpredicted outcome was the finding in experiment 2 that P-ASP was unable to deplete serum Asn levels in any dietary treatment groups. It is difficult to conclude that the dietary Asn alone prevented the depletion by PASP considering there are no blood metabolite results for the AD groups. However, evidence that dietary Asn could have prevented the blood Asn depletion by P-ASP comes indirectly, from examining the stool. In the second experiment we observed a sharp increase in stool Asn levels in all the batch 1 mice (batch 2 mice as a group did not have a significant increase in Asn but some individual mice experienced a steep increase, up to 12X). It appears that the mice were unable to balance Asn levels during an acute exposure to the diet and the gut Asn was diffusing into the blood, explaining why the blood Asn levels in the second experiment were not depleted. The acute effect of dietary Asn could have flooded the bloodstream to a concentration where the P-ASP, despite the increased dose, was unable to deplete blood Asn. Future research using a labelled amino acid could be used to determine if the blood Asn, despite P-ASP, is dietary or from cellular sources.

Another potential explanation, although less likely, is that the increased dose resulted in an increase in cellular ASNS activity. In the first experiment we used a low dose of P-ASP at 200 IU/kg. The second experiment used doses of 450 IU/kg or 900 IU/kg. Hypothetically, the increase in P-ASP dose should have increased the serum Asn depletion, as more P-ASP would be available to hydrolyze Asn. We know that ASNS is upregulated in cells when there is extracellular Asn depletion (Chen et al., 2004, p. 200; Kilberg et al., 2005). It is possible that there could be a dose-dependent activation of ASNS where higher doses of P-ASP activate the production of ASNS whereas the low dose, in the first experiment, did not. To preserve blood Asn, it was necessary to quench P-ASP activity at the time of blood collection. One downside to our quenching methodology for the blood samples is that the quenching process lyses and cells, making it difficult to determine if intracellular or extracellular metabolite levels are being measured. A paper by Purwaha in 2014 demonstrated that when L-ASP is added to cell cultures, there is a rapid efflux of Asn from cells. This is evidence that the Asn measured in the blood of the mice may be cell derived. It would be beneficial to include ASNS testing in combination with labeling amino acids to determine if the Asn is coming from the diet or if it a product of ASNS.

A final explanation is that the activity of the P-ASP was considerably lower than in experiment 1. We believe this to be unlikely because of the precipitous weight loss all the mice experienced, although again, we did not have a diet-only arm in the study.

4.5 Gln Levels Are Predicted to Increase After P-ASP Injection

4.5.1 Glutamine Synthetase

In congruence with our hypothesis, we found an increase in blood Gln levels post P-ASP in most treatment groups. The AD group in experiment 1 and all mice on the AR

diet in experiment 2 had increased blood Gln levels post P-ASP. Horvath et al. (2019) reported blood metabolites during L-ASP (we used P-ASP, presumably with a longer half-life) but without dietary modifications. They found that Gln levels in the blood increased post L-ASP without a corresponding decrease in Glu. In their study, mice were treated with either 1000 IU/kg or 5000 IU/kg every 24 hours for three days, so substantially more L-ASP than our dose of P-ASP. Cells can increase the production of glutamine synthetase (GS) in response to amino acid deprivation, which could explain the increased Gln levels in the blood post P-ASP (Rotoli et al., 2005). In experiment 1 the diet AD had increased Gln while the AR group did not. In experiment 2 the dose of P-ASP was increased and Gln levels increased in all mice. Blood samples were collected, at the earliest, two days post P-ASP. It is unclear whether there was an acute effect of Asn and Gln depletion that was missed. Thus, there is the potential for GS to be activated based on how strong the Asn and Gln depletion is either through the combination of diet and P-ASP, as seen in the diet low in Asn in experiment 1, or P-ASP alone as seen in experiment 2.

A study by Chan in 2019 showed that the glutaminase activity of P-ASP is necessary for an anti-leukemia effect even in leukemias without ASNS. It is troublesome for the treatment of leukemia that in vivo Gln levels seem to be increased post P-ASP, as Gln can be converted in Asn, and GS is important even without ASNS.

4.5.2 Stool Gln Levels

An additional explanation for the increase in blood Gln levels can be found in the stool. Experiment 2 had a significant decrease in stool Gln levels for the HP1 and HP2 group. Gln can be transported from the gut into the blood stream. As with Asn, there is no literature around how selective the gut epithelial cells are. The Asn rich diet from

experiment 1 mirrored this finding with a significant decrease in stool Gln post P-ASP but did not have a corresponding increase in blood Gln. Conversely, the AD diet from experiment 1 did have an increase in blood Gln but stool Gln levels remained the same. It is unclear why the stool and blood results from experiment 1 did not correlate. Similarly, to Asn and ASNS, future studies should include measuring GS and tracking the movement of Gln from the blood to the gut.

4.6 Storage Life of P-ASP

P-ASP is an expensive drug, costing over $7,000 for a 5 mL vial. The shelf life of an opened vial of P-ASP has not been well documented. P-ASP is provided reconstituted and is suggested to be used immediately following opening of the vial (European Medicines Agency Oncaspar Assessment, 2016) due to potential microbial contamination. This is despite a "best before" date. Our second experiment sought to address whether P-ASP lost activity after opening the vial. The HP1 cohort was administered P-ASP the day the vial was opened and HP2 was injected using the opened vial on the expiry day, roughly two months later (the delay due to reconciling how to prevent the acute weight drop observed in the HP1 cohort). The HP1 and HP2 group had similar Asn levels throughout the study period. The P-ASP did not deplete serum Asn levels in either group, likely due to the high dietary Asn content. This makes it difficult to directly confirm that P-ASP was still working optimally. It would be beneficial to conduct a study in the future looking at the shelf-life of an opened vial of P-ASP in vivo without high levels of dietary Asn. Moreover, asparaginase activity can be directly measured, as done in human blood samples; however, whether that can be achieved in mouse serum is unknown. On the other

hand, the precipitous weight drops in both cohorts of mice in experiment 2 suggest that the P-ASP was active.

From the PCA plots of blood metabolites for HP1 and HP2, the groups clustered separately along the PC3 axis. Gln and Glu serum levels were significantly higher in the HP1 group in comparison to the HP2 group post P-ASP, despite both groups having increased Gln. The lower level of Glu in the HP2 group may indicate that the glutaminase effects of P-ASP may decline over time. The higher levels of Gln in the HP1 group would seem to contradict this if we had not already demonstrated an increase in Gln across groups post P-ASP. This could be due to GS not being activated due to the decrease in glutaminase activity in P-ASP over time.

4.7 Microbiome and P-ASP

Through the initial experiment we were able to identify bacterial species that changed due to diet and P-ASP. Multiple studies have been conducted in both mice and humans showing that diet can influence the microbiome (Beam 2021); however, few studies have shown the effects of P-ASP on the gut microbiota in humans or mice. A paper published by our Oncologists in 2021 looked at the gut bacterial changes of pediatric ALL patients post P-ASP therapy (Dunn 2021). The only bacterial family that changed in both the mice and human patients with P-ASP was *Ruminococcacea,* which increased in both species. One potential reason would be differences in the bacterial genera that make up the gut microbiome in humans and mice (Nguyen et al., 2015). The other main difference is the study by Dunn et al. inferred which bacteria were present using metagenomic data.

4.8 Limitations

Several limitations have been discussed under each prediction. One limitation in these experiments was the fundamental differences between experiment 1 and 2. Another potential limitation of this study was we did not measure serum ASNS or GS levels, which could have helped to determine what was happening physiologically in the mice. Mice were not fasted prior to blood and stool sample collection, so the time of day and whether or not the mouse had eaten recently could have affected the metabolites in their blood and stool. Another limitation was the use of stool samples as a surrogate measure of the gut microbiome and metabolome. Unfortunately, DNA we prepared from mucosal scrapings failed to sequence. The microbial community in the stool has been shown to be like the gut, but it does not fully represent it and can only be significantly correlated at the phylum level (Yan et al., 2019). It is also obvious that the stool specimens for metabolites would likely not represent metabolites at the mucosa and may be affected by multiple factors like the period between the animal's last meal, time of day, and others. Experiment 1 was intended as a pilot to gain insights into the question of the diet and efficacy of P-ASP in an acute setting. Results from experiment 1 informed changes for Experiment 2 but the acute weight loss in Experiment 2 was unpredicted, and consequently the sample size and treatment groups were negatively affected.

There were no tumors in the mice, a step closer to ALL. The experiments were exploratory and "proof of principle" in nature. Including cancer in the model introduces a major variable that will have to be systematically analyzed in terms of the impact on the animals' health, P-ASP dosing and effect, even which strain of mice can be used, etc.

Tumors have atypical metabolic requirements for amino acids and mice and humans likely have additional requirements when sick with cancer.

4.9 Conclusions and Relevance to Human ALL

P-ASP is an integral component for the treatment of ALL; this study sought to determine if dietary Asn could be a confounder to P-ASP therapy and whether dietary intervention could improve the efficacy of the drug. The idea to modify dietary Asn was published in an editorial prior to the use of asparaginase to treat ALL (Halikowski et al., 1966). Our findings demonstrated that maintaining mice on an Asn diet for weeks prior to P-ASP would not affect serum Asn levels or the depletion of Asn by P-ASP. The potential for diet to change the efficacy of P-ASP became clear when we started mice on the diet the day prior to P-ASP therapy. Mice without any dietary Asn experienced acute weight loss requiring euthanasia, possibly showing the nonessential amino acids may become essential when combined with P-ASP. We also showed that high dietary Asn could overcome P-ASP depletion. Clinically, patients start treatment within a couple of days of being diagnosed and receive their first dose of P-ASP on day 4. The acute effect of diet on blood Asn levels is beneficial as patients would not need to be maintained on the diet long in advance of their P-ASP therapy.

It is necessary to use whole animals for dietary studies, as conducted in this thesis. It is also important to use live animals to best represent the multiorgan involvement in cancer growth and treatment. For example, using mice to drive the mutation evolution of cancers may yield outcomes different from an effort in vitro.

There is no published information available on the activity of an opened vial of P-ASP. The high cost of P-ASP often leads researchers to utilize commercial L-ASP, making

comparisons between studies more difficult. Perhaps our most convincing evidence that activity does not decay significantly is the fact that mice of both cohorts in Experiment 2 suffered (similar, meaning the 0 Asn group all had to be euthanized) acute weight loss. However, similar blood Asn levels in mice injected two months apart and the fact that Asn was not depleted do not help answer the question of efficacy with time. We did note some differences in the glutaminase activity of P-ASP. The balance of evidence is indicative that the enzyme did not noticeably decay, and we would continue to use an opened vial in future experiments.

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Appendix 1 Asn depleted diets purchased from Dyets, used in the first experiment.

DYET# 519592

Modified L-Amino Acid Defined AIN-93G Diet
(Control for 4% Asparagine Diet #519593)

L-Alanine			4.5		
L-Arginine, fb			6.3		
L-Aspartic Acid			11.3		
L-Cystine*			3.7		
L-Glutamic Acid			78.4		
Glycine			3.1		
L-Histidine, f b			4.5		
L-Isoleucine			8.4		
L-Leucine			15.3		
L-Lysine-HCl			16.1		
L-Methionine			4.5		
L-Phenylalanine			8.7		
L-Proline			20.4		
L-Serine			9.4		
L-Threonine			6.6		
L-Tryptophan			2.1		
L-Tyrosine			9.2		
L-Valine	kcal/g		9.9		Kcal/kg
	4	total L-AA*.....	222.4		889.6
Ingredient				gm/Kg	
Sucrose	4			57.8	231.2
Cornstarch	3.6			399.886	1439.59
Dyetrose	3.8			145	551
Soybean Oil	9			70	630
tBHQ	0			0.014	0
Cellulose	0			50	0
Salt Mix #210030	0.88			35	30.8
Sodium Bicarbonate	0			7.4	0
Vitamin Mix #310025	3.87			10	38.7
Choline Bitartrate	0			2.5	0
		total.....		1000.00	3810.89

Appendix 2 Asn rich diet purchased from Dyets and used in the first experiment

DYET# 519593

Modified L-Amino Acid Defined AIN-93G Diet with 4% Asparagine

	L-Alanine			4.5	
	L-Arginine, fb			6.3	
	L-Asparagine H2O			40	
	L-Aspartic Acid			11.3	
	L-Cystine*			3.7	
	L-Glutamic Acid			0.0	
	Glycine			3.1	
	L-Histidine, f b			4.5	
	L-Isoleucine			8.4	
	L-Leucine			15.3	
	L-Lysine-HCl			16.1	
	L-Methionine			4.5	
	L-Phenylalanine			8.7	
	L-Proline			20.4	
	L-Serine			9.4	
	L-Threonine			6.6	
	L-Tryptophan			2.1	
	L-Tyrosine			9.2	
	L-Valine	kcal/g		9.9	Kcal/kg
		4	total L-AA*……..	184	736
Ingredient				gm/Kg	
Sucrose		4		96.2	384.8
Cornstarch		3.6		399.886	1439.59
Dyetrose		3.8		145	551
Soybean Oil		9		70	630
tBHQ		0		0.014	0
Cellulose		0		50	0
Salt Mix #210030		0.88		35	30.8
Sodium Bicarbonate		0		7.4	0
Vitamin Mix #310025		3.87		10	38.7
Choline Bitartrate		0		2.5	0
			total……..	1000.00	3810.89

Appendix 3 Asn depleted diet from Envigo and used in experiments 2 and 3.

Teklad Custom Diet

TD.160365

Asparagine Deficient Diet (Red)

Formula	g/Kg
L-Alanine	4.85
L-Arginine HCl	12.1
L-Aspartic Acid	5.515
L-Cystine	3.5
L-Glutamic Acid	42.23
Glycine	24.44
L-Histidine HCl, monohydrate	4.5
L-Isoleucine	8.2
L-Leucine	11.1
L-Lysine HCl	18.0
L-Methionine	8.2
L-Phenylalanine	7.5
L-Proline	5.242
L-Serine	5.09
L-Threonine	8.2
L-Tryptophan	1.8
L-Tyrosine	5.0
L-Valine	8.2
Sucrose	340.813
Corn Starch	150.0
Maltodextrin	150.0
Soybean Oil	80.0
Cellulose	30.0
Mineral Mix, AIN-93M-MX (94049)	35.0
Calcium Phosphate, monobasic, monohydrate	8.2
Vitamin Mix, AIN-93-VX (94047)	19.5
Choline Bitartrate	2.7
TBHQ, antioxidant	0.02
Red Food Color	0.1

Appendix 4 Asn rich diet from Envigo and used in experiments 2 and 3.

Teklad Custom Diet

TD.160366

Asparagine Diet (4%, Blue)

Formula	g/Kg
L-Alanine	1.4733
L-Arginine HCl	12.1
L-Asparagine	40.0
L-Aspartic Acid	0.4775
L-Cystine	3.5
L-Glutamic Acid	8.824
Glycine	7.3945
L-Histidine HCl, monohydrate	4.5
L-Isoleucine	8.2
L-Leucine	11.1
L-Lysine HCl	18.0
L-Methionine	8.2
L-Phenylalanine	7.5
L-Proline	0.8873
L-Serine	1.1147
L-Threonine	8.2
L-Tryptophan	1.8
L-Tyrosine	5.0
L-Valine	8.2
Sucrose	368.0087
Corn Starch	150.0
Maltodextrin	150.0
Soybean Oil	80.0
Cellulose	30.0
Mineral Mix, AIN-93M-MX (94049)	35.0
Calcium Phosphate, monobasic, monohydrate	8.2
Vitamin Mix, AIN-93-VX (94047)	19.5
Choline Bitartrate	2.7
TBHQ, antioxidant	0.02
Blue Food Color	0.1

Appendix 5 Experiment 1 bacteria that were significantly different between treatment groups pre-diet (day 0), after 72 days on diet, and 5 days post-P-ASP (day 77). The diet with the higher abundance of the bacteria is reported in the table. Greyed boxes were not significantly different between diets. A p value ≤0.05 was considered significant.

Species	**Day 0**	**Day 36**	**Day 72**	**Day 77**
Alistipes finegoldii	Depleted			
Alistipes obesi	Depleted			
Alistipes shahii	Depleted			
Bacteroides caccae	Depleted			
Bacteroides dorei	Depleted			Rich
Bacteroides fragilis	Depleted			
Bacteroides vulgatus	Depleted		Depleted	Depleted
Clostridiales bacterium				Depleted
Clostridium fusiformis				Depleted
Clostridium leptum			Depleted	Depleted
Lachnospiraceae bacterium				Depleted
Lactobacillus murinus			Rich	
Mouse gut	Depleted			
Parabacteroides distasonis	Depleted			Depleted
Parabacteroides goldsteinii	Depleted			
Parabacteroides merdae	Depleted			
Unclassified Acetatifactor				Depleted
Unclassified Acetivibrio ethanolgignens				Depleted
Unclassified Adlercreutzia	Depleted	Rich	Rich	
Unclassified Akkermansia		Depleted		
Unclassified Alistipes				Rich
Unclassified Anaerotruncus				Depleted
Unclassified Bacteroides	Depleted	Depleted	Depleted	Depleted
Unclassified Christensenellaceae R-7				Depleted
Unclassified Clostridia UCG-014	Depleted			
Unclassified Clostridia vadinBB60	Depleted			
Unclassified Clostridium sensu stricto	Depleted	Depleted	Depleted	Depleted
Unclassified Erysipelatoclostridiaceae				Depleted
Unclassified Erysipelatoclostridium	Depleted			Depleted
Unclassified GCA-900066575	Depleted			Depleted
Unclassified Lachnospiraceae		Rich		Depleted
Unclassified Lactobacillus				Rich
Unclassified Muribaculaceae	Rich	Rich	Rich	Rich

Species	Day 0	Day 36	Day 72	Day 77
Unclassified Negativibacillus		Rich		
Unclassified Odoribacter			Depleted	
Unclassified Oscillospirales	Depleted	Rich		
Unclassified Parabacteroides	Depleted		Depleted	Depleted
Unclassified Paraprevotella	Depleted			Depleted
Unclassified Parasutterella	Depleted			
Unclassified Peptococcaceae	Depleted			Depleted
Unclassified RF39	Depleted			
Unclassified Rhodospirillales		Depleted		
Unclassified Romboutsia	Depleted		Depleted	Depleted
Unclassified Ruminococcaceae				Depleted
Unclassified Ruminococcus	Depleted			
Unclassified Turicibacter	Depleted		Depleted	
Unclassified Tuzzerella				Depleted
Unclassified UCG-005	Depleted			

Appendix 6 Experiment 1 bacteria differences within a group for the Asn-rich diet when comparing pre-diet (day 0) to 72 days on diet and 72 days on diet to 4 days post P-ASP (day 76). Only significant results ($p \leq 0.05$) are shown and the day with the higher abundance of bacteria is indicated.

Name	Day 0-36	Day 0-72	Day 36-72	Day 72-76
Alistipes finegoldii	Day 0			
Alistipes onderdonkii	Day 36			
Alistipes shahii	Day 0	Day 0		
Bacteroides caccae	Day 36	Day 72		
Bacteroides dorei		Day 72	Day 72	
Bacteroides fragilis	Day 36			
Bacteroides vulgatus	Day 36	Day 72	Day 36	
Blautia coccoides	Day 0	Day 0		
Lachnospiraceae	Day 0	Day 0		
Lachnospiraceae bacterium	Day 36			
Lactobacillus murinus		Day 72		
Parabacteroides goldsteinii	Day 0			
Unclassified Adlercreutzia		Day 72	Day 72	Day 72
Unclassified Akkermansia	Day 36	Day 72		
Unclassified Alcaligenes	Day 0			
Unclassified Bilophila	Day 36	Day 72		
Unclassified Clostridia vadinBB60	Day 0			
Unclassified Clostridium sensu stricto	Day 0			
Unclassified Colidextribacter	Day 36			
Unclassified Erysipelatoclostridium				Day 72
Unclassified Eubacterium coprostanoligenes	Day 36	Day 72		
Unclassified Intestinimonas	Day 0			
Unclassified Lachnospiraceae NK4A136	Day 0			
Unclassified Lachnospiraceae UCG-001	Day 0	Day 0		
Unclassified Lactobacillus			Day 72	
Unclassified Muribaculaceae	Day 0	Day 0		
Unclassified Odoribacter	Day 36	Day 72		
Unclassified Oscillibacter	Day 36		Day 36	
Unclassified Parabacteroides	Day 36	Day 72		
Unclassified RF39	Day 0			
Unclassified Rhodospirillales	Day 36			
Unclassified Romboutsia	Day 36		Day 36	
Unclassified Roseburia	Day 36			
Unclassified Ruminococcaceae	Day 36			
Unclassified Turicibacter	Day 0	Day 0		
Unclassified Tyzzerella	Day 0	Day 0		

Appendix 7 Experiment 1 bacteria differences within a group for the Asn-depleted diet when comparing pre-diet (day 0) to 72 days on diet and 72 days on diet to 4 days post P-ASP (day 76). Only significant results ($p \leq 0.05$) are shown and the day with the higher abundance of bacteria is indicated.

Name	Day 0-36	Day 0-72	Day 36-72	Day 72-76
Alistipes finegoldii	Day 0	Day 0		Day 76
Alistipes obesi	Day 0	Day 0		
Alistipes onderdonkii	Day 36	Day 72		
Alistipes shahii	Day 0	Day 0		
Bacteroides dorei	Day 0	Day 0		
Bacteroides fragilis	Day 36			
Blautia coccoides	Day 0	Day 0		
Clostridiales bacterium				Day 76
Clostridium				Day 76
Clostridium leptum		Day 72		
Clostridiumfusiformis				Day 76
Lachnospiraceae bacterium	Day 36	Day 72		
Mouse gut	Day 0	Day 0		
Parabacteroides goldsteinii	Day 0			
Parabacteroides merdae	Day 0	Day 0	Day 72	
Unclassified Acetatifactor				Day 76
Unclassified Acetivibrio ethanolgignens		Day 72		Day 76
Unclassified Adlercreutzia	Day 0			
Unclassified Akkermansia	Day 36	Day 72	Day 36	
Unclassified Alcaligenes	Day 0	Day 0		
Unclassified Bacteroides	Day 0	Day 0		
Unclassified Bilophila	Day 36	Day 72		Day 72
Unclassified Clostridia UCG-014	Day 0	Day 0		
Unclassified Clostridia vadinBB60	Day 0	Day 0		
Unclassified Clostridium sensu stricto		Day 0		
Unclassified Colidextribacter				Day 76
Unclassified Erysipelatoclostridium		Day 72	Day 72	
Unclassified Eubacterium coprostanoligenes	Day 36	Day 72	Day 72	Day 72
Unclassified Eubacterium siraeum	Day 0	Day 0		
Unclassified GCA-900066575		Day 72		Day 76
Unclassified Lachnospiraceae	Day 0		Day 72	Day 76
Unclassified Lachnospiraceae NK4A136		Day 0		
Unclassified Lactobacillus	Day 0	Day 0	Day 72	
Unclassified Negativibacillus	Day 0			
Unclassified Odoribacter	Day 36	Day 72		

Unclassified Oscillibacter		Day 72		
Unclassified Oscillospiraceae				Day 76
Unclassified Oscillospirales	Day 0	Day 0		
Unclassified Parabacteroides	Day 36	Day 72		
Unclassified Paraprevotella		Day 0		Day 76
Unclassified Parasutterella	Day 0	Day 0	Day 36	
Unclassified Peptococcaceae	Day 0	Day 0		Day 76
Unclassified RF39	Day 0	Day 0		
Unclassified Rhodospirillales	Day 36		Day 36	
Unclassified Romboutsia		Day 72		
Unclassified Ruminococcaceae		Day 72		Day 76
Unclassified Ruminococcus	Day 0	Day 0		
Unclassified Turicibacter	Day 0	Day 0		
Unclassified Tuzzerella				Day 76