GUT MICROBIOME ANALYSIS IN DOGS WITH LYMPHOMA UNDERGOING CHOP PROTOCOL: A CORRELATIVE ANALYSIS

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Comparative Medicine and Integrative Biology—Master of Science

2022

ABSTRACT

Canine multicentric large cell lymphoma shares many similarities to Non-Hodgkin's Lymphoma in humans. Both human and canine large cell lymphoma require chemotherapy and chemotherapeutic toxicities can result in limiting factors for the compliance of the treatment and the better outcomes. The gut microbiome is the assembly of genomes of the microorganisms in the GI tract**.** The gut microbiome is compositionally changed by many factors, such as GI diseases, diet, and chemotherapy administration; the clinical significance of the changes remains unclear. Therefore, we sought to describe the change in gut microbiome in a clinically well-characterized population. Also, we tried to explore the correlations between the changes in the gut microbiome and chemotherapeutic toxicities. Twenty dogs were included. In this study, 32 GI toxicities and 42 neutropenia events were identified, but there was no correlation between the relative abundance of the gut microbiome and chemotherapy toxicities. We observed a dynamic compositional change in the gut microbiome over the first 10 weeks of the CHOP protocol. The relative abundance of *Lachnospiraceae* in the GI toxicity (P=0.0205) and Both (P=0.0089) groups significantly decreased and the relative abundance of *Fusobacterium.uncultured* significantly decreased (P=0.0197) in the Both group, compared to the No toxicity group. Further data analysis of the compositional change in the gut microbiome during chemotherapy is needed.

ACKNOWLEDGMENTS

First and foremost, I would like to express sincere gratitude to my primary advisor, Dr. Paulo Vilar Saavedra for his encouragement, constructive feedback, and continuous support and patience throughout my master's degree despite the pandemic of COVID-19. His solid scientific expertise, curiosity for translational medicine, and acceptance of new ideas have trained me for the right goal; it is a privilege that I could receive his guidance. My gratitude extends to the rest of my committee, Dr. Linda Mansfield, Dr. Nikolaos Dervisis, and Dr. Vilma Yuzbasiyan-Gurkan who dedicated their time to giving me their insights and guidance with their expertise. I am grateful to the former and current members of the Medical Oncology Service of Michigan State University Veterinary Teaching Hospital (i.e., former senior clinician – Dr. Honkisz –, interns, students who were rotating into our service, and veterinary technicians), the members of the Mansfield lab, other graduate students and interns/residents, and faculty and staff at the Department of Comparative Medicine and Integrated Biology for their support, collaborations, and friendships. In addition, I would like to thank my former supervisors and colleagues at Animal Medical Center, Gifu University. Especially, Dr. Takashi Mori, a professor of Gifu University, and the head of Veterinary Oncology Service, who contributed to my personal and professional development as a veterinary clinician specializing in veterinary oncology and triggered me to pursue an advanced degree in the field. Last not but least, I would also like to thank my family – my parents and grandparents, who supported me and believed in me throughout the program. I have been very fortunate to pursue my MS degree at Michigan State University, and I could not have done this work without all the people mentioned above in this COVID era.

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LIST OF ABBREVIATIONS

CBC	Complete blood cell count
CBD	Cannabidiol
CTX	Cytoxan: cyclophosphamide
dH2O	Distilled water
DLBCL	Diffuse large B cell lymphoma
DMAC	Dexamethasone, melphalan, actinomycin-D, cytosine arabinoside protocol
DNA	Deoxyribonucleic acid
DNTP	Deoxyribose nucleotide triphosphate
DSS	Dextran sodium sulfate
DXR	Doxorubicin (i.e., hydroxydaunorubicin, Adriamycin)
GI	Gastrointestinal
Hct	Hematocrit
IBD	Inflammatory bowel disease
IM	Intact male
IV	Intravenous
LOPP	Lomustine, vincristine, procarbazine, prednisone protocol
MDR1	Multi-Drug Resistance 1
$MgCl_2$	Magnesium chloride
MOPP	Mechlorethamine, vincristine, procarbazine, prednisone protocol
MSU	Michigan State University
NF-κB	Nuclear factor κB
NHL	Non-Hodgkin's Lymphoma

NM	Neutered male
OUT(s)	Operational Taxonomic Unit(s)
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PE	Physical examination
PO	Per os
PTCL	Peripheral T-cell lymphoma
rRNA	Ribosomal ribonucleic acid
SEER	Surveillance, Epidemiology, and End Results Program
SF	Spayed female
VCOG-CTCAE	Veterinary cooperative oncology group – common terminology criteria for adverse events
VCR	Vincristine
WHO	World Health Organization

INTRODUCTION

Canine and human lymphoma share a significant number of similarities. A review of human and canine lymphoma is provided below to set the stage for the goals and significance of this research endeavor for both species.

Non-Hodgkin Lymphoma in humans

Non-Hodgkin's Lymphoma (NHL) is a lymphoid neoplasm that is derived from B cell, T cell, or their precursors[1]. According to the Surveillance, Epidemiology, and End Results Program (SEER), the annual incidence rate is approximately 20 per 100,000 and the mortality rate is up to 5 per 100,000 in the United States[2]. There are various subtypes of NHL and each subtype has many distinct characteristics: clinical features, epidemiology, etiology, genetic immunophenotype, and response to therapy[1]. Based on the histopathological characteristics, Armitage and Weisenburger classified NHL into thirteen subtypes[3]. Among these, diffuse large B cell lymphoma (DLBCL) and peripheral T-cell lymphoma (PTCL) are reported to be the most frequently diagnosed with B cells comprising 31% and T cell 6% of the total[3].

The clinical signs of NHL include fever, weight loss, or night sweats (as known as B-symptoms), and more than two-thirds of the patients have painless peripheral lymphadenomegaly[1]. However, the clinical presentation of NHL can vary, depending on the anatomical site that is affected, the subtype, and/or the presence or absence of B-symptoms[4]. Complete blood cell count (CBC), chemistry profile, imaging diagnostics (e.g., CT scan), and tissue biopsy are commonly needed as part of the diagnostic workup of the patient[1]. The pathologic diagnosis and classification of lymphoma are made following the WHO classification system for lymphoid neoplasms[5]. Malignant lymphomatous cells grow diffusely with a high mitotic activity, destroy the normal architecture of lymph nodes, and sometimes have an interfollicular or intrasinusoidal growth

pattern[6]. The Ann Arbor staging classification system, which was originally used for Hodgkin's lymphoma staging, has been widely used to stage NHL **(Table 1.1)**[7].

The current standard of care for patients with DLBCL is a combination therapy of *r*ituximab, *c*yclophosphamide (CTX), and doxorubicin (DXR, generic name: *h*ydroxydaunorubicin), vincristine (VCR, also known as *O*ncovin), and *p*rednisone (R-CHOP) regimen[8]. Using this protocol, about 60–70% of patients with DLBCL are cured but approximately 25-30% of them relapse within 3 years after the R-CHOP regimen[8,9]. On the other hand, PTCL is less frequent (6% of NHL cases) and has a poor prognosis than DLBCL. Greer et al. reported that among patients with PTCL who underwent various combination chemotherapy (e.g., CHOP, BCOP or COMLA); only 24% of the patients achieved a complete remission and the median survival was 11 months[10].

Canine multicentric large cell lymphoma

Canine lymphoma is the most common hematopoietic malignancy diagnosed in dogs, which consists of 7 to 24% of all canine neoplasms reported in the veterinary pathology database[11]. Several comparative studies have used canine lymphoma as a spontaneous tumor-bearing animal model of non-Hodgkin's lymphoma in humans because canine lymphoma shares many significant similarities, including histopathologic classification, diagnostic work-up, and treatment[11,12]. Typical histopathological images of DLBCL and PTCL both in humans and in dogs are summarized in Figure 1.1. The most common clinical signs include non-painful generalized lymphadenomegaly and non-specific clinical signs, such as vomiting, diarrhea, hypo- or anorexia, and lethargy[11]. The diagnosis of canine multicentric lymphoma is made comprehensively, including physical examination, CBC, chemistry profile, cytology or tissue biopsy, and either PCR or flow cytometry is used for the immunophenotyping to determine B or T cell origin[11].

Multiagent chemotherapy is used as the current standard of care for canine lymphoma, and the treatment goal is to maximize anticancer effect and minimize adverse effects of chemotherapy. CHOP protocol (CTX, DOX, VCR, and prednisolone) with or without L-asparaginase, is reported as the most effective treatment regimen against multicentric, large cell lymphoma in dogs[11]. The complete response rates in canine multicentric B cell and T cell lymphoma, are approximately 80-90% and 39-88%, respectively[11,13-18]. The median disease-free interval is approximately 8-13 months for B cell lymphoma[11,13-15]. Simon et al. reported that the overall response rate of CHOP based protocol was 89%; median 1st remission duration, which corresponds to progressive free interval, in dogs with complete remission was 243 days (range: 19–1,191 days, 95% confidence interval: 199–287 days), and 85% of the dogs that had achieved complete remission relapsed[15]. About 60% of the dogs that relapsed after being treated with CHOP protocol, were treated with either the same CHOP based protocol or other rescue protocols[15]. The overall response rate in these relapsed dogs was 79% and the median duration of the second remission was 130 days (range: 17–606 days, 95% confidence interval:76–184 days) in dogs that achieve the complete response[15]. In the three dogs that achieve the partial response, the duration of the second remission lasted 39, 182, and 486 days, respectively[15]. When compared to B cell lymphoma, large T cell lymphoma has a poorer prognosis, with a disease-free interval of 52-200 days, and a median survival time of approximately 5-7 months[15,16,19-22]. When relapse occurs, various rescue protocols (e.g., MOPP, LOPP, DMAC, and a single agent rabacfosadine) have been reported (Table 1.2). However, there are no standardized guidelines and recommendations on protocols to follow, and which rescue protocol is used depends on the clinicians' preference.

CHOP based protocol consists of classic chemotherapeutic agents that are characterized by nonspecific cytotoxic activity[23]. Since the classic chemotherapeutic agents target rapidly dividing

cells, the toxicity (chemotherapy-induced toxicity) occurs frequently as gastrointestinal (GI) toxicity (e.g., vomiting, diarrhea, and hypoxia) and bone marrow suppression (e.g., neutropenia)[23]. Other than these, liver and kidney damage are also reported as chemotherapy-induced toxicity[23].

Chemotherapy-induced toxicities can be challenging for the management of multicentric large cell lymphoma. The occurrence of chemotherapy-induced toxicities at any grade is high in dogs with multicentric large cell lymphoma; approximately 50-70% of dogs have experienced at least one toxicity[14,15,19]. Specifically, GI toxicity (e.g., vomiting) and bone marrow suppression (e.g., neutropenia) are frequently found at approximately 40-50% and 60%, respectively, with differing severities[14,15]. Toxicity levels are graded according to the criteria outlined **(Table 1.3)**. Grade 3 or greater toxicities (either GI toxicity or neutropenia) occurs at approximately up to 30%. Moreover, dose modulation is required in approximately 40-67% of dogs with multicentric, large cell lymphoma due to chemotherapy-induced toxicities [13,19,24]. Approximately 10% of them require hospitalization because of either febrile neutropenia or severe GI toxicity[13-15,24].

The association of the gut microbiome with multiple morbidities in humans

Gut microbiota, which is composed of commensal microorganisms (e.g., bacteria, viruses, and fungi), has a stable and highly diverse ecosystem among individuals[25-27]. The gut microbiome is the assembly of genomes of the microorganisms that live in the GI tract[28]. The number of publications referring to the gut microbiome in humans has been explosively increasing since it emerged **(Figure 1.2, A).**

Changes in the gut microbiome have been associated with many diseases (e.g., Inflammatory bowel disease -IBD-, obesity, and cancer)[29]. Dysbiosis is defined as the compositional imbalance in the gut microbiome that relates to a pathologic state distinct from a healthy state[30]. The gut microbiome diversity has two major components: richness and evenness[31].

Richness is the number of phylotypes/taxa in the community, and evenness explains the difference in the relative abundance of species in the community[31,32]. For example, in IBD, Frank and colleagues reported that there were two distinct subsets (i.e., IBD subset and Control subset)[33]. The IBD subset predominantly consists of IBD, ulcerative colitis, and Crohn's disease, while the Control subset consists of non-IBD and that sequences representative of the *Bacteroidetes* and *Lachnospiraceae* were significantly depleted in IBD subset, and those of the *Actinobacteria* and *Proteobacteria* is more abundant in the IBD subset samples than the Control subset[33]. In obesity, Ley et al. used C57BL/6 mice and investigated the change of gut microbiome diversity by 16S rRNA analysis[34]. showed that the cecal microbiome in obese mice had a statistically significant decrease of *Bacteroidetes*, and a significantly higher concentration of *Firmicutes*, compared to lean mice[34].

In studies of carcinogenesis of colorectal cancer in C57BL/6 mice, Zackular et al. reported that gut inflammation leads to the development of colorectal cancer[35]. Using dextran sodium sulfate (DSS) administration to induce inflammation, they showed that a significant decrease in the diversity occurs in the gut microbiome after the first round of DSS administration inducing dysbiosis and resulting in developing more colorectal cancer than the control group with a healthy gut microbiome[35]. Moreover, gut microbiome is of key relevance in chemotherapy; various interactions between chemotherapy drugs and the gut microbiota relating to the efficacy, toxicity, and metabolism of the drugs have been reported. Those interactions resulted in variations of response to treatment, severity and or frequency of the chemotherapy-induced toxicities[26,36,37].

Chemotherapy causes an imbalance of the gut microbiome both in function and in composition, leading to or exacerbating gut inflammation[38]. For example, CTX, a cytotoxic chemotherapy drug frequently used to treat human and canine hematopoietic malignancies

including NHL, disrupts the intestinal barrier by shortening the villi, and increasing intestinal permeability by loosening tight junction between enterocytes, and inducing inflammation and accumulation of mononuclear cells in the lamina propria[39,40]. The damaged intestinal mucosa sometimes referred to as a "leaky gut", facilitates the bacterial translocation into the mesenteric lymph nodes and the spleen[40].This translocation is selective of Gram-positive bacterial species and secondary to the dysbiosis caused by CTX administration[40]**.**

Many clinical studies also have shown the importance of the compositional change in the gut microbiome during chemotherapy. Alexander et al. reported that the gut microbiome interacts with many chemotherapeutic agents in various mechanisms: translocation, immunomodulation, metabolism, enzymatic degradation, and reduced diversity[26]. Galloway-Peña et al. attempted to predict the risk of the infection during chemotherapy by the gut microbial community profiling[41]. In addition, Montassier et al. reported that chemotherapy has been shown to decrease bacterial diversity, richness, and metabolic functions and that severe dysbiosis in the gut microbiome by chemotherapy is associated with chemotherapy-induced GI toxicities[38].

Characteristics of the intestinal microbiome in dogs

In veterinary medicine, like in humans, the publications on the gut microbiome have been an increasing trend since 2009 **(Figure 1.2, B)**. In healthy dogs, the composition of the gut microbiome is relatively stable within the individual but more unstable among different individuals (interindividual) [27]. The intestinal microbiome is altered in composition by several factors, such as diet, antibiotics, probiotics, and co-morbidities. Raw food diets alter the abundance of *Lactobacillales*, *Enterobacteriaceae*, *Enterococcusona, Fusobacterium,* and *Clostridium* at different taxonomic levels[42,43]. Also, raw food diets significantly increase the abundance of *E.coli* and *Streptococcus* and decreased the abundance of *Faecalibacterium* in the fecal microbiome[43].

In some GI diseases (e.g., chronic inflammatory enteropathy), dysbiosis is often observed. Suchodolski et al. investigated the canine gut microbiome in dogs with diarrhea and reported that diseased dogs tended to have a decreased richness and diversity of gut microbiome in the fecal sample[44]. They also found dysbiosis in dogs with acute and chronic diarrhea, suggesting *E. coli, Isospora*, *Giardia/Cryptosporidium*, enterotoxigenic *C. perfringens*, and toxigenic *C. difficile* as potential pathogenic bacteria[44]. Gavazza et al. reported that dysbiosis was found in dogs with multicentric lymphoma with decreased abundance of *Faecalibacterium, Fusobacterium*, and *Turicibacter*[45]. In addition, dogs that received chemotherapy have been reported to have significantly increased pathogenic bacteria (i.e., *E.coli* and *Streptococcus*) in the gut microbiome at 8 weeks after the start of chemotherapy in a small study of 12 lymphoma dogs, compared to healthy dogs[46]. The dogs that have chemotherapy-induced toxicity, such as GI toxicity, might have a specific compositional change in the gut microbiome and different toxicities may cause different signatures of the gut microbiome profile. Therefore, profiling the gut microbiome is crucial for the better understanding, prediction, and management of chemotherapy-induced toxicity.

Methodology for the analysis of the intestinal microbiome

Many methodologies can be used to analyze the gut microbiome, including amplicon sequencing, shotgun sequencing (i.e., metagenomics), and metabolomics[47]. Amplicon analysis utilizes 16S rRNA sequencing and has been most commonly used in the past 15 years[48]. The differences between amplicon sequencing and shotgun sequencing are summarized by Allaband et al. and are presented in **Table1.4**[47]. The16S rRNA amplicon analysis can detect one or more of 9 hypervariable regions (V1–V9) that have sequence diversity in otherwise highly conserved 16S rRNA gene. Using primers that are targeted to the highly conserved regions flanking the variable regions, amplicons are obtained from a wide range of bacterial targets, and sequencing of the

amplicons can reveal nearly all bacterial taxa present. While the amplicon sequencing approach has been employed to detect one specific gene (16S rRNA gene for archaea), the shotgun sequencing can sequence all DNA fragments from a sample and then integrate and analyze these fragments, revealing in greater detail the bacterial community[47]. Thus, amplicon sequencing reveals which bacterial taxa are in a sample and the relative abundance of the bacteria in a sample, while shotgun sequencing reveals all genes that are coded by the bacteria in a sample.

Knowledge gap and the purpose of the study

The overarching hypothesis is chemotherapeutic agents alter the gut microbiota, linking to diarrhea and/or neutropenia, and the stability of the gut microbiome reflects the damage produced by chemotherapeutic agents. Although some studies have already shown an association between chemotherapy-induced toxicity, including GI toxicities and bone marrow suppression (e.g., neutropenia) and changes in gut microbiome resulting in dysbiosis in both humans and dogs, limited information is yet available on the canine microbiome, especially as it changes with chemotherapy. Therefore, the primary purpose of this study is to characterize the baseline gut microbiome in the dog with multicentric lymphoma and to describe the longitudinal changes in the gut microbiome induced by chemotherapy in dogs with large cell lymphoma undergoing CHOP protocol. The secondary purpose is to investigate if there is any correlation between the change in the gut microbiome and clinical and laboratory parameters (e.g., grade of chemotherapy-induced toxicity, neutrophil count, and percentile change of neutrophil count). The tertiary purpose is to identify which chemotherapeutic agent would cause the most severe dysbiosis in canine lymphoma.

MATERIALS AND METHODS

Case selections

Cases were enrolled between 2018 to 2021 at Michigan State University (MSU) Veterinary Teaching Hospital (MSU-VTH) with informed client consent under the IBR (VTH) approval for sample collection and completion of the study.

Client-owned dogs with lymphoma were included in the study based on the following inclusion criteria:1) dogs confirmed with large cell lymphoma based on cytology or histopathology, 2) fecal samples were to be collected and available for at least 7 out of the first 10 weeks of CHOP protocol (**Table S1.1**)[13], 3) medical records were available for review and data analysis, and 4) no previous cytotoxic chemotherapy were administered in the past three months. The use of other concomitant medication (e.g., antibiotics, corticosteroids, and probiotics) at the registration for this study were accepted and recorded for analysis.

CHOP based protocol

The first 10 weeks of CHOP are delivered according the previous report[13]. Briefly, VCR was administered intravenously at 0.5-0.7 mg/m^2 on Week 1, 3, 6 and 8, CTX was administered orally at 250-300 mg/m^2 on Week 2 and 7, DXR was administered intravenously either at 30 mg/m^2 if the dog is > 15kg or at 1mg/m^2 if the dog is < 15kg. Prednisone was prescribed at 2 mg/kg daily and tapering off over 4 weeks and the discontinuation was decided by the attending clinician.

Regarding concomitant medications, owners followed the instruction of clinicians when moderate to severe chemotherapy-induced toxicity occurs. Antibiotics, metronidazole, and/or antinausea medications, such as maropitant, were used to manage GI toxicity. For neutropenia, antibiotics were also used based on clinicians' judgment. Anti-anxiety medications were also used for the safe administration of chemotherapy, depending on dogs. Supplements were allowed to use

unless clinicians prohibited them.

Clinical data assessment

For clinical assessment, patient characteristics collected included: breed, gender, age, stage and substage of lymphoma at initial presentation, immunophenotype, body condition score, the presence or absence of cardiac murmur, the presence or absence of chemotherapy-induced toxicities (i.e., diarrhea, vomiting, anorexia, anemia, neutropenia, and/or thrombocytopenia), any other concomitant medication, serum chemistry profile and chemotherapeutic agent administered.

Staging diagnostics consisted of history, palpation, and cytology of blood smear, affected lymph node, and/or the spleen and liver. At initial presentation, the clinical stage was determined by the attending clinician following the WHO staging system **(Table 1.1)** [49]. The chemotherapeutic agent that was administered at last hospital visit was considered a "contributor" when developing chemotherapy-induced toxicity. The interval between chemotherapies, the percentile changes in white blood cell count, the percentile changes in neutrophil count, the percentile changes in platelet count, and the percentile change in the hematocrit were calculated. Chemotherapy-induced toxicities were evaluated and graded according to the VCOG – CTCAE v1.1[50]. All clinical data were reviewed in both electronic and paper-based form.

Fecal sample collection

Fecal samples were collected from the enrolled cases between 2018 and 2021 at every hospital visit on a weekly basis by attending clinicians and/or KK. The rectal examination, as part of the routine physical examination, was done in a minimally invasive manner, wearing examination gloves, using lubricant, and gently restraining the dog. A fecal sample was collected before chemotherapy administration during each hospital visit. To avoid bacterial contamination of the fecal samples from the hospital environment, fecal samples collected were transferred

promptly into a 2ml sterile, cryogenic vial. Then, the samples were immediately frozen at -20 °C, and then, transported to the laboratory after being placed on ice packs in a Styrofoam box, stored at -80 °C, and frozen until further microbiome analysis was done.

DNA extraction from fecal samples

Fecal DNA was extracted from all fecal samples by using the FastDNA^{TM} SPIN Kit for Soil (Mp Biomedicals LLC, California, USA) (**Figure S1.1**) following the kit manufacturer's instructions. In brief, 0.5 grams of each defrosted fecal sample, 978 µl of phosphate buffered saline (PBS), and 122 µl of MT buffer were added to a purple top tube provided by the kit, and the fecal samples were homogenized for 40 seconds at a speed setting of 6.0. Once the homogenization is done, the purple tube was centrifuged at 14,000g for 5 minutes. Then, the supernatant was transferred to a microcentrifuge tube, and 250 µl of Protein Precipitation Solution (PPS) was added and mixed well by inverting the microcentrifuge tube 10 times. After mixing the supernatant and PPS, the microcentrifuge tube was centrifuged again at 14,000g for 5 minutes. After the centrifuging, the supernatant was transferred to a clean 15ml tube. Then, 1ml of nucleic acid binding buffer was added to the 15 ml tube, and the supernatant and this binding buffer were mixed well by inverting the 15ml tube. After the reaction with the nucleic acid binding buffer, the supernatant of this mixture was discarded and the residue (i.e., DNA containing solution) was transferred to another tube with a spin column. The spin column was centrifuged at 14,000g for 1 minute. After the centrifuging, 500 µl of nucleic acid wash solution was added to wash away impurities, and then, the spin column was centrifuged at 14,000g minute. After that, the spin column was air dried for 5 minutes at room temperature. Then, DNA was eluted by adding 100 µl of elution buffer followed by centrifugation at 14,000 g for 1 min. Once DNA extraction was done, DNA concentration and purity were measured by NanoDrop (Thermo Fisher Scientific Inc.,

Waltham, Massachusetts, USA) and Qubit fluorometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The extracted DNA was stored at -80 °C prior to the amplification steps (e.g., PCR sequencing).

16S rRNA gene V4 region PCR analysis

Subsequently, 10 µl of extracted DNA and the primer set (Forward Primer = 5′ GTGCCAGCMGCCGCGGTAA; Reverse Primer = 5′TTAATCTWTGGGVHCATCAGG) were used to amplify the V4 region of the bacterial 16S rRNA by PCR[51]. This amplification was done for each fecal sample using the Master Mix. A total of 50 µl of reactants containing: 2.5 µl of forward and reverse primer, 5 µl of 10X PCR buffer, 1.5 µl of 50mM $MgCl_2$, 4 µl of 2.5 mM DNTPs, 0.5 µl of Taq polymerase, 26.5 µl of dH2O, and 10 µl of the extracted DNA. The amplification process was done at 94 ℃ for 3 minutes. This was followed by 35 cycles of 94℃ for 45 seconds, 50 ℃ for 60 seconds and 72 ℃ for 90 seconds and cooling at 72 ℃ for 10 minutes. Sterile-filtered, PCR grade water (Water – PCR reagent, Sigma-Aldrich, St. Louis, MO, USA) was used as negative template controls. DNA concentration was adjusted to ensure samples were approximately equal in concentration. Final concentrations were calculated using Qubit. Illumina MiSeq Amplicon sequencing was done by MSU Research and Technology Support Facility.

Illumina 16S rRNA gene sequencing and data analysis

16S rRNA gene amplicon analysis was performed using QIIME2 (v. 2019.1) and protocols available at reference or URL SOP https://docs.qiime2.org/2019.1/ accessed May 2019. Alignment was accomplished using the Silva 16S ribosomal gene database[52]. Chimeric sequences and any sequences classified as chloroplast, mitochondria, Archaea, or Eukaryota, were removed from the dataset using UCHIME. Sequences were clustered in Operational Taxonomic Units (OTUs) of 97% sequence identity yielding 79 OTUs. Analyses were performed

in PAST 3.07[53] and R v.4.06[54]. Following processing of sequences and chimera removal in QIIME2. In the sequence clean-up process, forward and reverse primers, reads with ambiguous bases or homopolymers greater than eight base pairs and chimeras were removed in QIIME2. As a result, high-quality reads remained. Sequence read data has been made available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) as documented in "Availability of data and materials".

Correlation analysis

The heatmap was generated on the relative abundance of 79 OTUs yielded to see if there is any difference in the gut microbiome between the dogs with and without chemotherapy-induced toxicity.

Among the 79 OTUs, *Lachnospiraceae, Bacteroides.uncultured*, *Prevotella.uncultured, Fusobacterium.uncultured., Streptococcus.uncultured, and Enterococcus* were selected because they had a dynamic change in the relative abundance analysis. Then, the 6 selected OTUs were used for the correlation analysis.

In the correlation analysis, regarding the chemotoxicity parameters, we categorized into four groups according to the chemotherapy toxicity status: No toxicity, GI toxicity, Neutropenia, and Both. No toxicity means the dogs didn't have either GI toxicity or neutropenia, GI toxicity means the dogs had GI toxicity but not neutropenia, Neutropenia means the dogs had chemotherapy induced neutropenia but not GI toxicity, and Both means the dogs had both GI toxicity and neutropenia. To determine whether there is a statistically significant difference in the relative abundance of the selected taxa, the relative abundance of each category was compared.

Statistical analyses

Kruskal-Wallis test was used for the Shannon Diversity Index. Principal Component

Analysis (PCA) was done using the Bray-Curtis test.

The Pearson correlation coefficient was calculated for the correlation between the relative abundance of selected bacteria species in the gut microbiome and the numerical clinical parameters. The previous statistical analyses were done with the R software package (ver. 4.2.0).

Mann-Kendall test was used for trends by using PAST software package (ver.4.03)

Brown-Forsythe and Welch's ANOVA test were done followed by Games-Howell's multiple comparisons test to compare the means of the relative abundance of the gut microbiome in the No toxicity group with that of each other group. This ANOVA test and following multiple comparisons were done by using GraphPad Prism 9.0 for Mac OS X (GraphPad Software, La Jolla, California USA). P-value < 0.05 was considered as significant.

RESULTS

Study population

A total of 20 dogs matched our inclusion criteria. The medical records of all study dogs were reviewed. Signalment and pretreatment conditions (i.e., breed, age, sex, the WHO stage and substage, immunophenotype) were summarized in **Table 1.5.** At presentation, the study dogs had a median age of 7 years (range: 3–14) with 13 castrated males, 1 intact male, and 6 spayed females. The male to female ratio was 2.3:1. Fifteen different breeds were identified with Mixed breed dog (3) as the most common breed, followed by Golden Retrievers (2), Goldendoodles (2), Rottweilers (2), and one each of Boxer, Bullmastiff, Chesapeake Bay Retriever, Coonhound, English Cocker Spaniel, Great Dane, German shepherd dog, Jack Russell Terrier, Labrador Retriever, Miniature Schnauzer, and Siberian Husky.

Regarding the staging of lymphoma, most dogs were categorized as stage III or higher. There were none of dogs with stage I, 1 with stage II, 7 with stage III, 5 with stage IV, and 7 with stage V. All dogs received palpation, abdominal ultrasound, and fine needle aspirate of affected lymph node for the diagnosis; not all dogs received the full diagnostic workup for the staging (e.g., fine needle aspirate in the spleen and the liver, or bone marrow aspirate)because of the discretion of the pet owner and the clinician[49]. There were 9 cases categorized as substage (a), and 11 as substage (b). In regards of cell immunophenotype, there were 13 of B cell lymphoma, 2 of T cell lymphoma, and 5 were not tested.

Regarding concurrent therapies, there were 6 dogs that had received corticosteroid prior to chemotherapy. Other concomitant medications at initial presentation were summarized in **Table S1.2** Fourteen out of 20 dogs received concurrent corticosteroids as part of the chemotherapy. Of them, 8 dogs discontinued corticosteroids within Week 5 and the remainder of 6 dogs discontinued

it within Week 10.

Multi Drug Resistance 1 (MDR1) gene, which encodes p-glycoprotein that efflux VCR and prednisolone that are used in CHOP based protocol[55], and therefore, it contributes one of the mechanisms of drug resistance[11]. The mutation of MDR1 gene is a prognostic indicator in canine large cell lymphoma. In this cohort, only one dog tested MDR1 gene mutation, and the result was negative (-/-) for the gene mutation.

Chemotherapy dose modulation

Thirteen of the 20 dogs (65%) experienced dose modulation (i.e., either dose delay or dose reduction) at any point within Week 10. Of them, 11 dogs had at least one dose delay, 7 dogs had at least one dose reduction, and 5 dogs had both dose delay and reduction. The cause of dose modulation is due to chemotherapy-induced toxicity (neutropenia or GI toxicity). None of the dogs experienced discontinuation of CHOP based protocol in the first 10 week.

GI toxicity and bone marrow suppression

There was 32 episodes of GI toxicities identified. Of them, three episodes resulted in dose delay. The grade and frequency of the vomiting, diarrhea, and anorexia were summarized in **Table 1.6.** The frequency of GI toxicity per Week is summarized in **Figure 1.3**. In addition to these 32 episodes of GI toxicity, one dog was diagnosed with a gray zone between GI toxicity and food intolerance/allergy. This dog had severe GI signs at 8 days after the previous vincristine administration and was given a different dog food the day before, and therefore, the true cause of the GI signs in this dog was undetermined. Regarding chemotherapy-induced neutropenia, 42 episodes were identified. Most of them were low grade and self-limiting **(Table 1.7).** Of them, 10 episodes caused dose delay and dose delay was done at all grades.

Intestinal microbiome profiling

Relative abundances were analyzed longitudinally in 8 dogs (**Figure 1.4**), and there are currently fecal samples from 13 dogs undergoing abundance analysis. Each dog showed a dynamic compositional change in the gut microbiome over the first 10 weeks of CHOP based protocol. The average of relative abundance of the gut microbiome is shown in **Figure 1.5**. When compared to the dog with vs without GI toxicity, dogs without GI toxicity tended to have a quicker stabilization of the gut microbiome composition than those with GI toxicity **(Figure 1.6 and 7).**

In the Shannon diversity index, there was not statistically significant in each arm grouped by chemotherapeutic agent **(Figure 1.8)**. The dogs with GI toxicity tended to have decreased diversity in the gut microbiome compared to those without GI toxicity **(Figure 1.9).** Also, the dogs receiving antibiotics have a significant decrease of diversity in the gut microbiome than those without antibiotics **(Figure 1.10).** When compared to the sample on Week 1, 6 of 10 top bacterial taxa contributing to PCA components 1 and 2 showed significant trends over the entire course of CHOP protocol (**Table 1.8**).

Correlation analysis

The heatmap was created to compare the microbiome composition between the dogs with and without GI toxicity, and those with and without neutropenia. There did not appear to be a distinct difference in the gut microbiome that is associated with either GI toxicity or chemotherapeutic neutropenia (**Figure 1.11**).

Next, the relative abundance of selected taxa was paired with the myelosuppression parameters (e.g., neutrophil count) to calculate a correlation coefficient. A total of 58 pairs of observations were available for the correlation analysis. The correlation coefficient was summarized in **Table 1.9.** There was no strong correlation found between the relative abundance

of selected taxa and the numerical clinical parameters.

We summarized the comparison of the relative abundance of selected taxa among the four groups that were categorized according to the chemotherapy-induced toxicity status in **Figure 1.12**. The relative abundance of *Lachnospiraceae* in GI toxicity (P=0.0205) and Both (P=0.0089) significantly decreased and the relative abundance of *Fusobacterium.uncultured* significantly decreased (P=0.0197), compared to No toxicity group. There was not any significant difference of the relative abundance of *Bacteroides.uncultured, Prevotella.uncultured Streptococcus.uncultured,* and *Enterococcus* when comparing No toxicity group to other categories. Although *Streptococcus.uncultured* and *Enterococcus* didn't show any statistical significance, there was the increasing trend in the chemo toxic groups (i.e., GI toxicity, Neutropenia, and Both).

DISCUSSION

In this study, we documented a dynamic compositional change of the gut microbiome over the first 10 weeks of CHOP based protocol. Each dog had a different diverse composition in the gut microbiome. Our study showed that interindividual diversity is greater than intraindividual diversity even in the state of lymphoma. This is in agreement with other reports where interindividual diversity in healthy dogs was shown to be greater than intraindividual diversity in the gut microbiome[27]

When compared to the dog with vs without GI toxicity, dogs without GI toxicity had a quicker stabilization of the gut microbiome composition than those with GI toxicity. Chemotherapy can cause taxonomic shift and increases the pathogenic bacteria such as *E. coli* and *Streptococcus*[38,46]. The relative abundance of *Bacteroides nordii, Ruminococcus sp*, and *Gardnerella vaginali*s are associated with severe toxicity of CTX[56]. Therefore, we expected that the relative abundance of pathogenic bacteria would increase compared to the baseline, and the compositional change in *Bacteroides, Ruminococcus,* and/or *Gardnerella* should be found in those dogs with severe toxicity of CTX in this cohort, too. Also, given that dogs with GI toxicity needed more time to stabilize the gut microbiome composition. This persistent destabilization in the gut microbiome may correlate with treatment response and/or prognosis. To test this, further studies are needed.

The dogs receiving antibiotics have a significant decrease in Shannon diversity index than those without antibiotics. Gut inflammation promotes *Enterobacteriaceae* and the combination of insult by enterobacteria and the gut inflammation reduces the richness of bacteria[57]. Also, in the study of human colorectal cancer, Fei et al. reported that decreased diversity in patients with GI toxicity was observed[58]. They considered that the decrease of microbial diversity may be related

to the imbalance of gut microbiome because the dominant pathogenic bacteria consume nutrients or produce bacterial toxic metabolites[58]. Clinically, the use of antibiotics at hospital visits suggests that the dogs receiving antibiotics had a recent moderate to severe GI toxicity and/or chemotherapy-induced neutropenia. Therefore, the decrease of Shannon diversity in antibiotic use may reflect the dogs' state that antibiotics were needed because of the severe GI toxicity or neutropenia, and like in humans, chemotherapy can reduce the diversity in the canine gut microbiome.

Although there was no statistical difference in the Shannon diversity index when comparing dogs with vs without GI toxicity, we noticed a decreasing trend in the diversity. In our study, most of the GI toxicity was low grade and self-limited. The degree of decrease in diversity may be related to the severity of GI toxicity. Another possible reason is that this may be a type 1 error due to the small sample size. Additional 13 dogs will shed light on the relationship between GI toxicity and microbiome diversity.

In our study, the relative abundance of *Lachnospiraceae* significantly decreased in the GI toxicity and Both groups. Based on the OTU assignment used in our study, *Lachnospiraceae* includes butyrate-producing bacteria. In human medicine, there was a decreased relative abundance of *Lachnospiraceae* in the IBD group, compared to the control[33]. Butyrate has an anti-inflammatory effect by the inhibition of nuclear factor κB (NF-κB) activation in human colonic epithelial cells and reinforces the colonic defense barrier[59]. Thus, these suggest that a decreased relative abundance of *Lachnospiraceae* is predominantly associated with GI toxicity in our study. The possible mechanism is that the decrease of butyrate producing *Lachnospiraceae* in the gut microbiome reduces the anti-inflammatory effect by failing to reinforce the gut barrier. This leads to GI inflammation, which results in GI toxicity (mainly diarrhea).

Also, there was a significantly decreased relative abundance of *Fusobacterium.uncultured* in the Both group. Jugan et al. reported that the abundance of *Fusobacterium* decreased in dogs with lymphoma that underwent CHOP based protocol[60]. Also, Gavazza et al. reported that the abundance of *Fusobacterium* decreased in dogs with multicentric lymphoma[45]. Our finding regarding the relative abundance of *Fusobacterium* is supportive of these previous reports. However, the relationship between the decreased relative abundance of *Fusobacterium* and chemotherapy-induced toxicity remains unclear. Moreover, the Both group had a very small sample size, so this may be a type 1 error. Therefore, further studies are needed to unveil the relationship between the relative abundance of *Fusobacterium* and chemotherapy-induced toxicity using a larger sample size.

Interestingly, there was an increasing trend in the relative abundance of *Streptococcus.uncultured* and *Enterococcus* in the chemo toxic groups in this study. Alessandra et al. showed that the pathogenic bacteria (e.g., *Streptococcus and E. coli)* increase after chemotherapy in dogs with lymphoma[46]. Stringer et al. reported that irinotecan administration to mice increases *Enterococcus,* and also, van Vliet et al. reported that a multiagent chemotherapy regimen used for pediatric patients with acute myeloid leukemia showed a drastically increased in *Enterococcus*[61,62]. These research on the increase of pathogenic bacteria by chemotherapy is consistent with our findings. Therefore, chemotherapy may increase the relative abundance of pathogenic bacterial taxa in canine gut microbiome. Specifically, CHOP protocol used for dogs with multicentric large cell lymphoma may also increase in *Streptococcus* and *Enterococcus.*

One limitation of the study is the clinical staging of lymphoma. In this study, not all dogs received the full work-up for the staging, including bone marrow aspirate. Although 9 dogs with stage II and III received an abdominal ultrasound, most of them did not receive fine needle aspirate

in the liver and spleen. Thus, these cases might be underscored in staging.

We failed to collect some fecal samples during this study because dogs had defecated just before the hospital visit or had severe GI toxicity. In these cases, it was impossible to collect relevant fecal samples of interest. Given the study design, the sample in Week 1 serves as a control for every dog included in this study. However, with Dogs 6 and 21, we failed to obtain samples in Week 1. Thus, for these two cases, the baseline gut microbiome status remains unclear. A bigger longitudinal study (sample size) which includes may contribute to the completeness of the data in this type of design that analyze the compositional change of the fecal microbiome over time.

CONCLUSIONS

In conclusion, there was a dynamic compositional change in the gut microbiome of dogs during the first 10 weeks of the CHOP based protocol. During CHOP protocol, dogs on antibiotics had a significant decrease in diversity in the gut microbiome. Also, there was a significant decrease in relative abundance in some bacterial taxa in relation to chemotherapy-induced toxicity. We haven't found any statistical significance in the correlation between the relative abundance in the gut microbiome and clinical laboratory parameters.

Future directions

The current level of analysis on 8 dogs studied longitudinally was not able to provide clear correlations with chemotherapy related toxicities. The microbiome analysis is being carried out in 13 additional dogs. By adding the samples from additional 13 dogs, the specific findings in the gut microbiome that correlate to or be associated with the chemotherapy-induced toxicity in dogs that underwent CHOP protocol would be found.

For further studies, the compositional change in the gut microbiome during chemotherapy should be examined for their relationship with prognosis and be explored for use as predictive markers for chemotherapy-induced toxicity.

ACKNOWLEDGEMENTS

This work was supported by Michigan State University College of Veterinary Medicine Endowed Research Fund 2018 (award $25,000). The author thanks veterinary technicians, veterinary students, and interns working in Medical Oncology Service for the support in fecal sample collections. The author -Keita Kitagawa- also thank Dr. Jason Couto, members and summer research students – Drs. Azam Sher, Eric Spilker, Hinako Terauchi, Nicole Mulready, Julia Bell, and Linda Mansfield – in Dr. Mansfield's lab for their fundamental work for this project and 16S rRNA sequencing. Their efforts will be clearly recognized as first co-authorship of the upcoming scientific manuscript.

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APPENDIX A:

TABLES AND FIGURES

Table 1.1 The comparison of the staging system in human and canine NHL[7,49]

Ann Arbor Staging system for human NHL		The WHO staging system for canine NHL	
Principal stages	Definition	**Stages**	Definition
I	Involvement of one lymph node or one extra nodal organ or site (IE)	**I**	Involvement limited to a single node or lymphoid tissue in a single organ
II	Involvement of two or more lymph node regions on the same side of the diaphragm, or localized involvement of an extra nodal site or organ (IIE) and one or more lymph node regions on the same side of the diaphragm	**II**	Involvement of many lymph nodes in a regional area (+/- tonsils)
III	Involvement of lymph node regions on both sides of the diaphragm, which might be accompanied by localized involvement of an extra nodal organ or site (IIIE) or spleen (IIIS) or both (IIISE); the spleen is regarded as nodal	**III**	Generalized lymph node involvement
IV	Diffuse or disseminated involvement of one or more distant extra nodal organs with or without associated lymph node involvement	**IV**	Liver and/or spleen involvement (+/- Stage III)
		V	Manifestation in the blood and involvement of bone marrow and/or other organ systems (+/- Stages I-IV)
Modifiers	Definition	**Substage**	Definition
A	Absence of B symptoms (listed below)	**a**	Without systemic signs
B	Temperature >38°C, night sweats, and weight loss of greater than 10% of bodyweight in the 6 months preceding admission are defined as systemic symptoms	**b**	With systemic signs

Table 1.2 The summary of the selected rescue protocol for canine lymphoma

	Chemotherapeutic agent(s)	Overall Response Rate	Median Duration of Complete Response	Reference
MOPP	mechlorethamine, vincristine, procarbazine, prednisone	65 %	63 days	Rassnick et al.[63]
LOPP	lomustine, vincristine, procarbazine, prednisone	61 %	not Reported	Fahey et al.[64]
DMAC	dexamethasone, melphalan, actinomycin-D, cytosine arabinoside	72 % 43 %	61 days not Reported	Alvarez et al.[65] Parsons-Doherty et al.[66]
Rabacfosadine	rabacfosadine	74%,	107 days	Saba et al.[67]

Table 1.3 Selected toxicity grading items defined by VCOG-CTCAE[50]

Grade	Anorexia	Vomiting	Diarrhea	Neutropenia
1	Coaxing or dietary change required to maintain appetite	<3 episode in 24 h	Increase of up to 2 stools per day over baseline	Neutrophil count: 1500 /ul to lower limit of normal
2	Oral intake altered (≤3 days) without significant weight loss; oral nutritional supplements/appetite stimulants may be indicated	3 – 10 episodes in 24 h ;<5 episodes/day for ≤48 h; parenteral fluids (IV or SC) indicated ≤48 h; medications indicated	Increase of 3–6 stools per day over baseline; medications indicated; parenteral (IV or SC) fluids indicated ≤48 h; not interfering with ADL	1000-1499 /ul
3	Of >3 days duration; associated with significant weight loss (≥10%) or malnutrition; IV fluids, tube feeding or force feeding indicated	Multiple episodes >48 h and IV fluids indicated>48h	Increase of >6 stools per day over baseline; incontinence >48 h; IV fluids >48 h; hospitalization; interfering with activities of daily living	500-999 /ul
4	Life-threatening; TPN indicated; >5 days duration	Life-threatening	Life-threatening	<499 /ul
5	Death	Death	Death	Death

Table 1.4 The summary of the differences between amplicon and shotgun sequencings

	Amplicon sequencing	Shotgun sequencing
Technical features	Most selected organisms present, depending on method used (no viruses)	Every organism present will have most of the genome sequenced: all bacteria, fungi, viruses etc.
Target	16S – bacteria and some archaea 18S – eukaryotes ITS – fungi	All organisms (including host tissues)
Method	High throughput	High throughput

Table 1.5 Demographic data of the enrolled dogs

Dog number	Breed	Age	Sex	WHO Stage	Substage	Immunophenotype
1	Goldendoodle	3	SF	4	b	N/A
2	Mixed Breed	11	NM	5	a	B
3	Miniature Schnauzer	13	SF	4	b	B
4	Mixed Breed	5	NM	4	b	N/A
5	Mixed Breed	8	SF	5	a	B
6	Bullmastiff	4	NM	4	a	B
7	Goldendoodle	8	NM	5	b	T
8	Golden Retriever	8	NM	3	b	T
9	Rottweiler	6	NM	3	b	B
10	Chesapeake Bay Retriever	10	SF	3	b	B
11	Coonhound	7	NM	3	a	B
12	Great Dane	7	NM	2	a	B
13	German Shepherd Dog	7	NM	3	a	B
14	Siberian Husky	6	NM	5	a	N/A
15	Jack Russel Terrier	14	NM	5	b	B
16	Golden Retriever	6	IM	5	a	N/A
17	Rottweiler	3	NM	3	a	B

Table 1.5 (cont'd)

18	English Cocker Spaniel	7	NM	3	b	B
19	Boxer	6	SF	4	b	N/A
20	Labrador Retriever	12	SF	5	b	B

Table 1.6 Summary of GI toxicity (events)

Grade	anorexia	vomiting	diarrhea
1	6	6	6
2	0	6	11
3	0	0	0
4	0	0	0
5	0	0	0

Table 1.7 Summary of chemotherapy-induced neutropenia

Grade	Definition (neutrophil count/ul)	Events	Dose delay
1	1500- 2,999	34	4
2	1000-1499	4	3
3	500-999	3	3
4	<499	1	1
	Total	42	10

Table 1.8 The summary of bacterial taxa that contributed to PCA components 1 and 2

			Taxa Showing Trends	
Dog number	**GI toxicity**	**Antibiotic at any point**	**Increase**	**Decrease**
1	No	No	*Lacnospiraceae* (P=0.0036*)	*Enterococcus* (P=0.0092*)
2	No	Yes	*Bacteroides.uncultured*	None
5	No	Yes	*Prevotella.uncultured* (P=0.0166*) *Fusobacterium.uncultured* (P=0.023*)	*Streptococcus.uncultured* (P=0.0187*) *Lacnospiraceae* (P=0.0166*)
9	Yes	No	None	None
3	Yes	Yes	None	*Enterococcus* (P=0.0046*)
6	Yes	Yes	None	None
7	Yes	Yes	None	None
8	Yes	Yes	*Lacnospiraceae* (P=0.023*)	*Prevotella.uncultured* (P=0.046*)

Note: The dogs are ordered by first the presence or absence of GI toxicity, and then, the antibiotic use. It shows then if there was any significant increasing/decreasing trend in the gut microbiome taxa compared to the sample on Week 1.

Table 1.9 Correlation coefficient between relative abundance of the selected OTUs and numerical clinical variables

	Lachnospiraceae	***Bacteroides.uncultured***	***Prevotella_uncultured***	***Fusobacterium.uncultured***	***Streptococcus_uncultured***	***Enterococcus.***
Neutrophil count	-0.0611517	0.21075359	-0.00969	0.04540302	-0.10473	-0.06718
Platelet count	0.25756698	0.06335429	-0.25824	-0.0864545	-0.26436	0.168598
Hct value	-0.1942566	0.10650308	0.333786	0.12566997	0.117599	-0.03016
Percentile change of neutrophil count	-0.0203185	0.12595068	-0.0611128	0.38534031	-0.0684224	-0.06779
Percentile change of platelet count	0.06561663	0.10747033	-0.07893	-0.0771521	0.093838	0.048584
Percentile change of Hct value	0.12001666	0.16635226	0.061099	0.03902454	-0.03104	-0.30184

Note: 1st row means the selected OTUs, and 1st column means numerical clinical variables, respectively. The intersection of each row and column is the corresponding correlation coefficient [r].

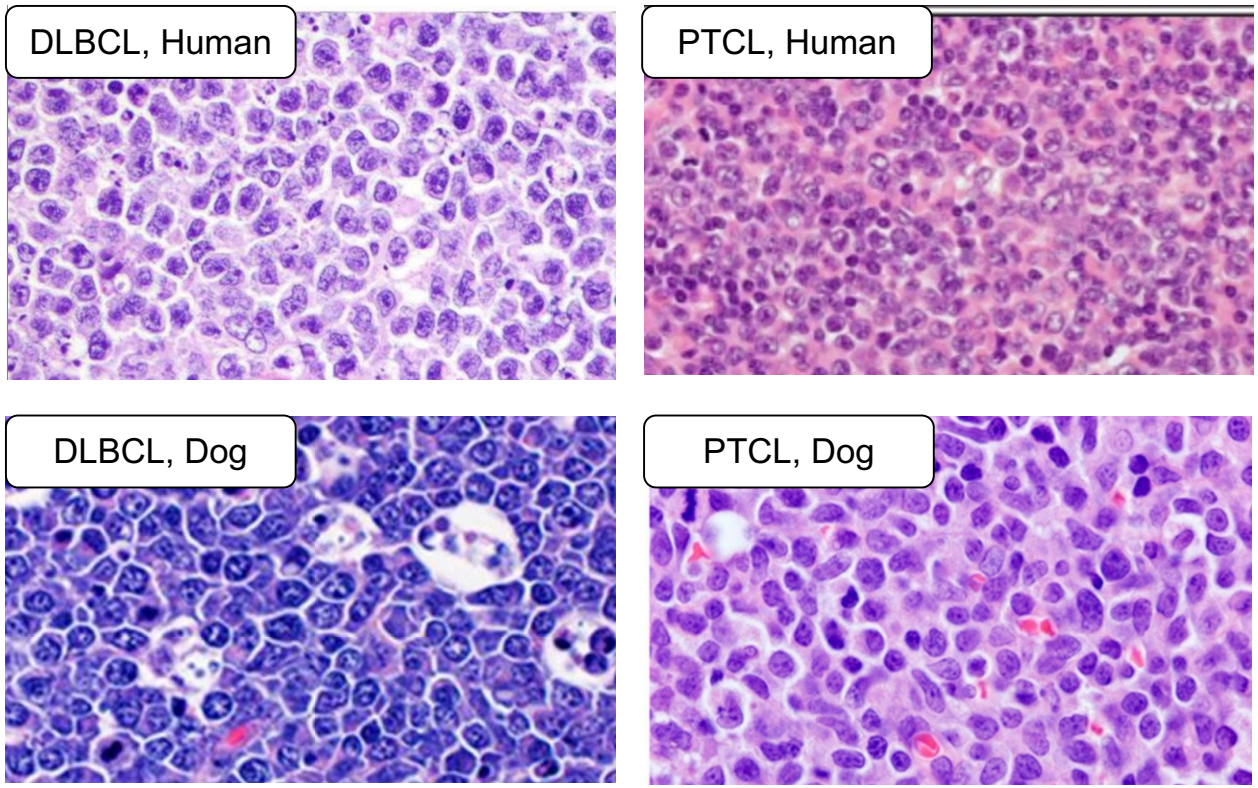

Figure 1.1 The comparative aspects of DLBCL and PTCL in histopathology[68-70]
The pictures show typical histopathologic finding of DLBCL and PTCL both in humans and in dogs

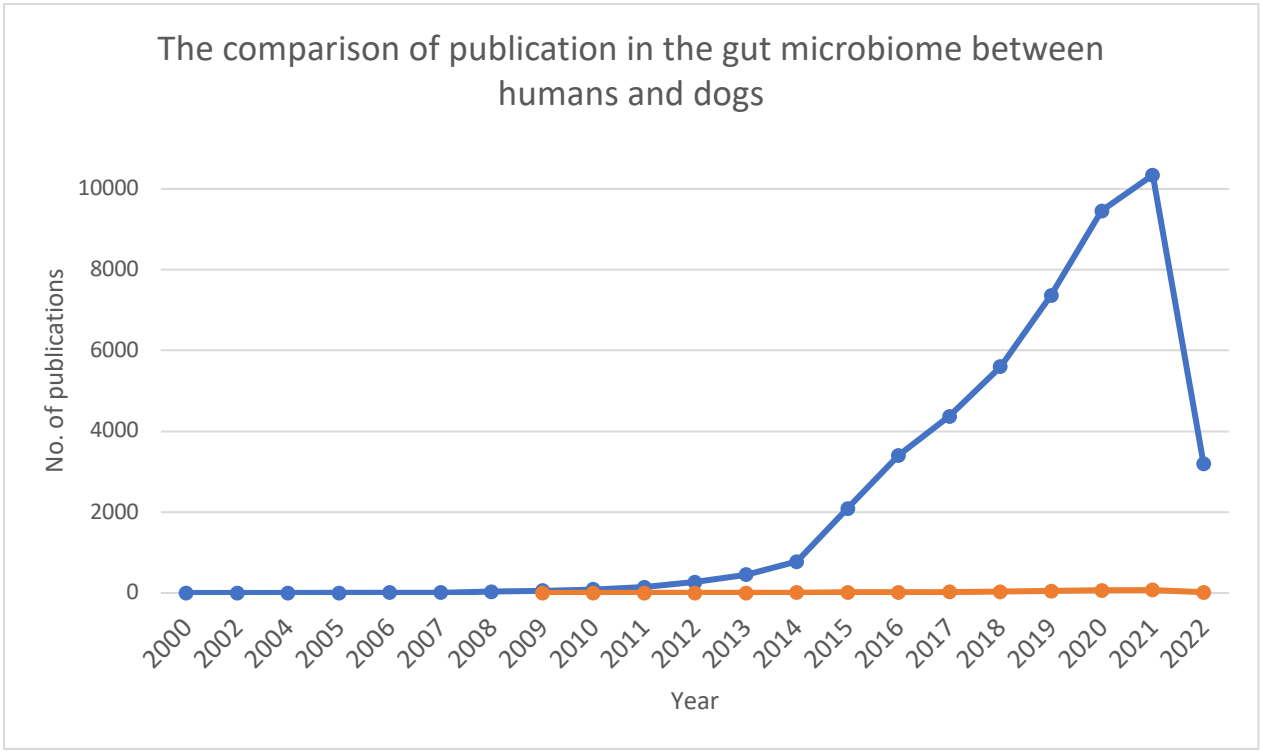

Figure 1.2 The comparison of publication in the gut microbiome between humans and dogs
Blue: the number of publications of the gut microbiome in humans
Orange: the number of publications the gut microbiome in dogs

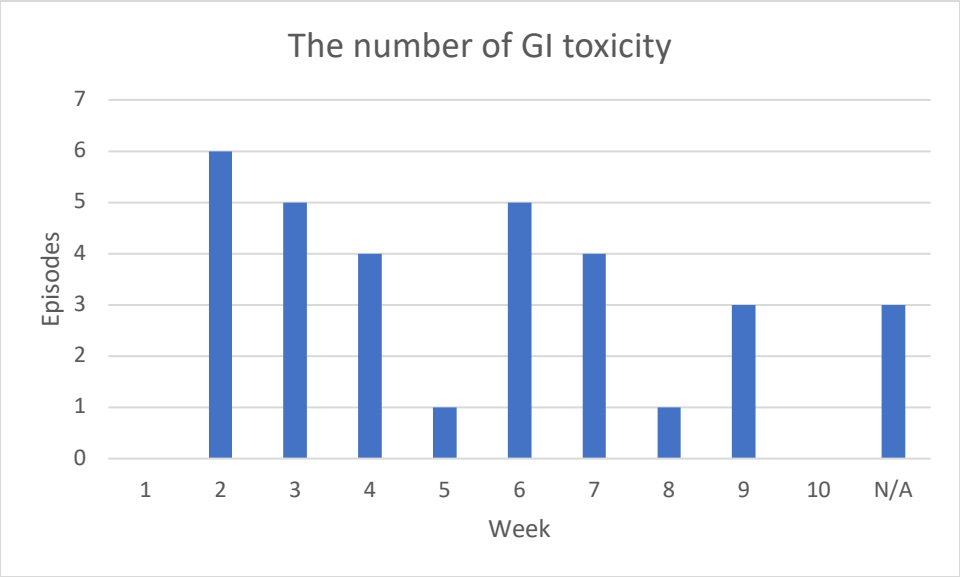

Figure 1.3 The number of GI toxicity identified in the study X-axis is Week (based on CHOP based protocol) and Y axis is the number of episodes. N/A means hospital visit out of the first week of CHOP protocol.

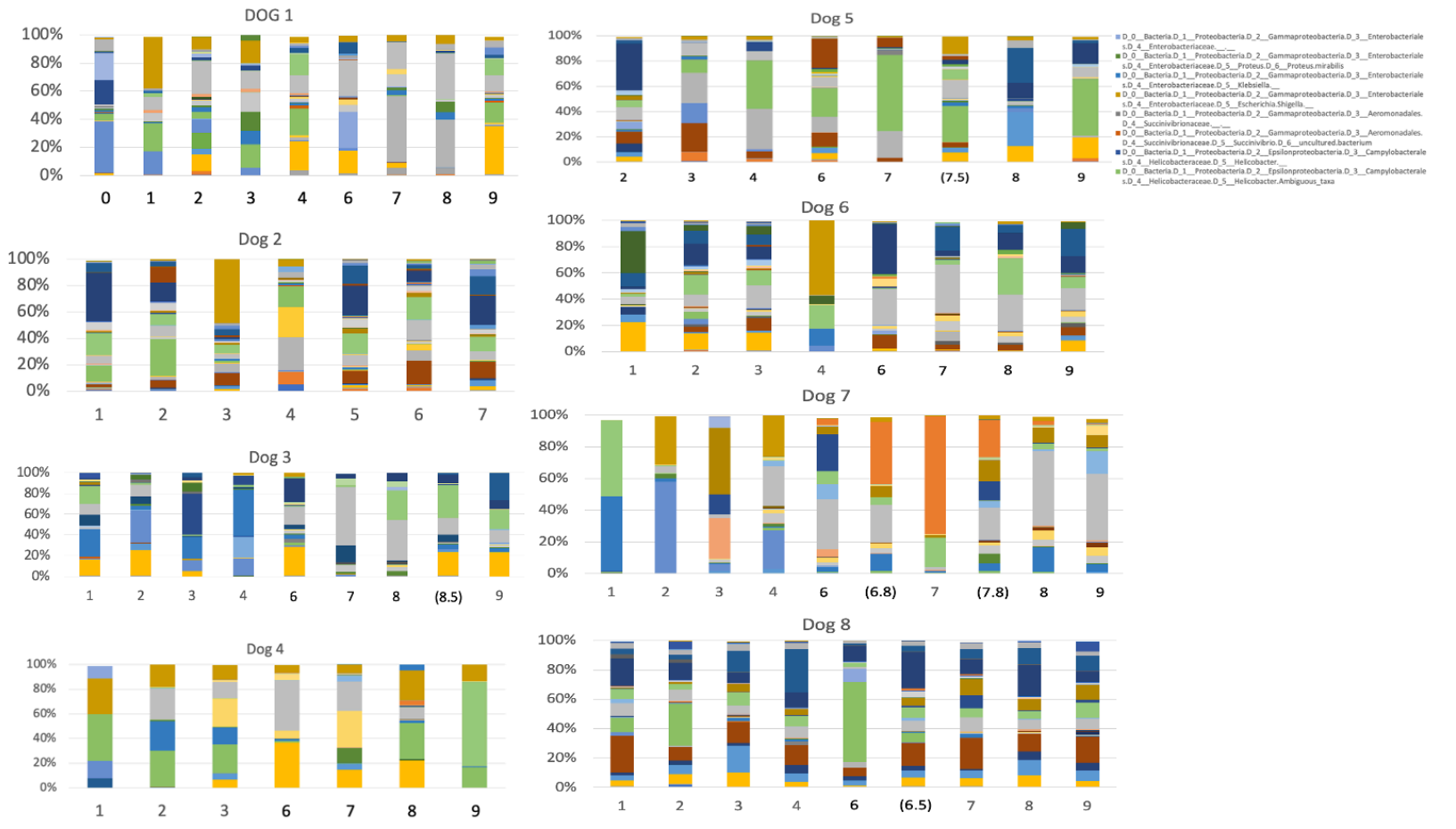

Figure 1.4 Longitudinal relative abundance of the gut microbiome in 8 dogs (Dog 1-8)
Each column shows the relative abundance of the gut microbiome in each dog. X axis means Week (based on CHOP based protocol). N/A means the week which was not applicable for the protocol.

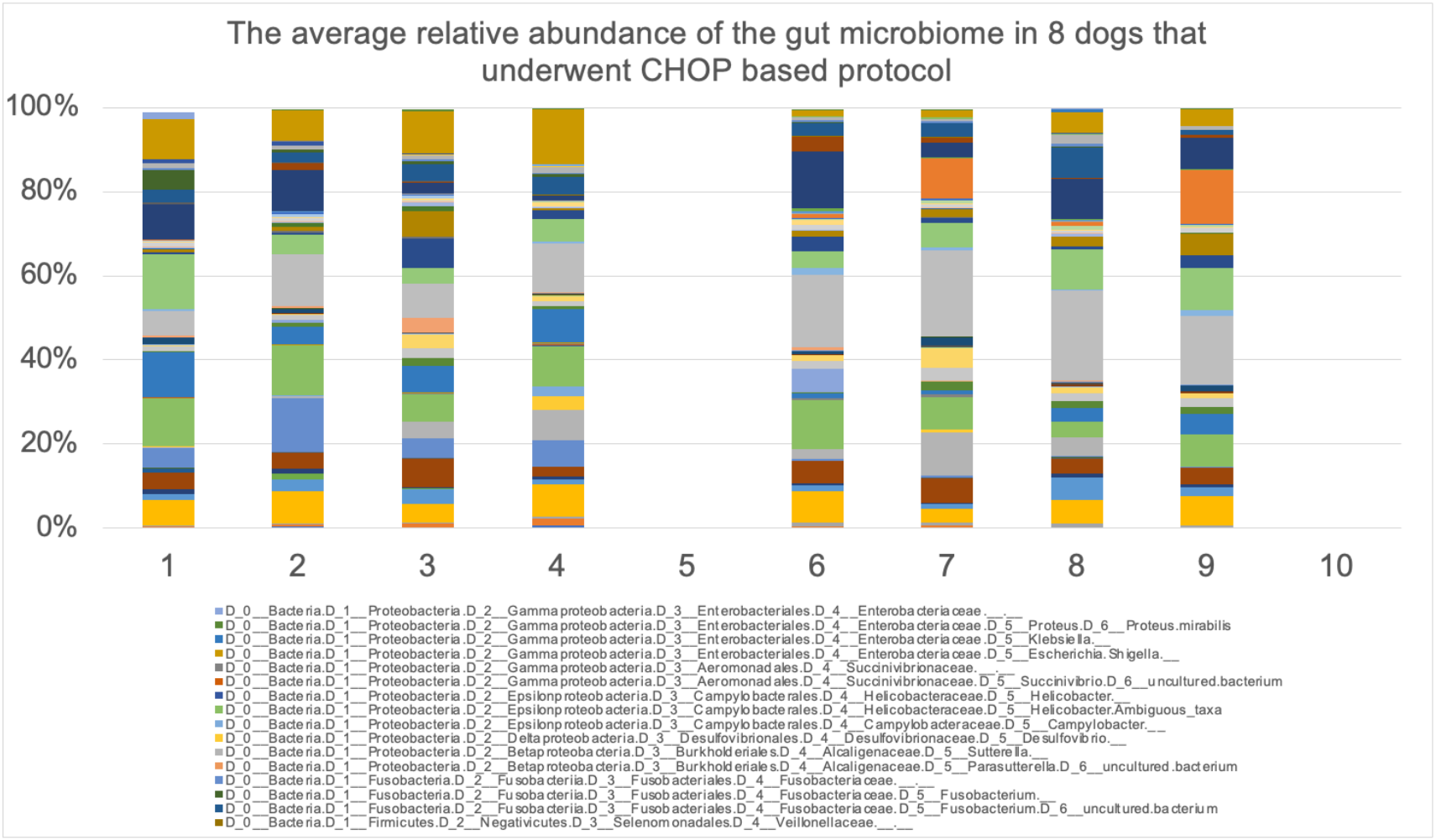

Figure 1.5 The average relative abundance of the gut microbiome in 8 dogs that underwent CHOP based protocol
X-axis means the Week of CHOP protocol, and Y-axis means the percentage of the gut microbiome.

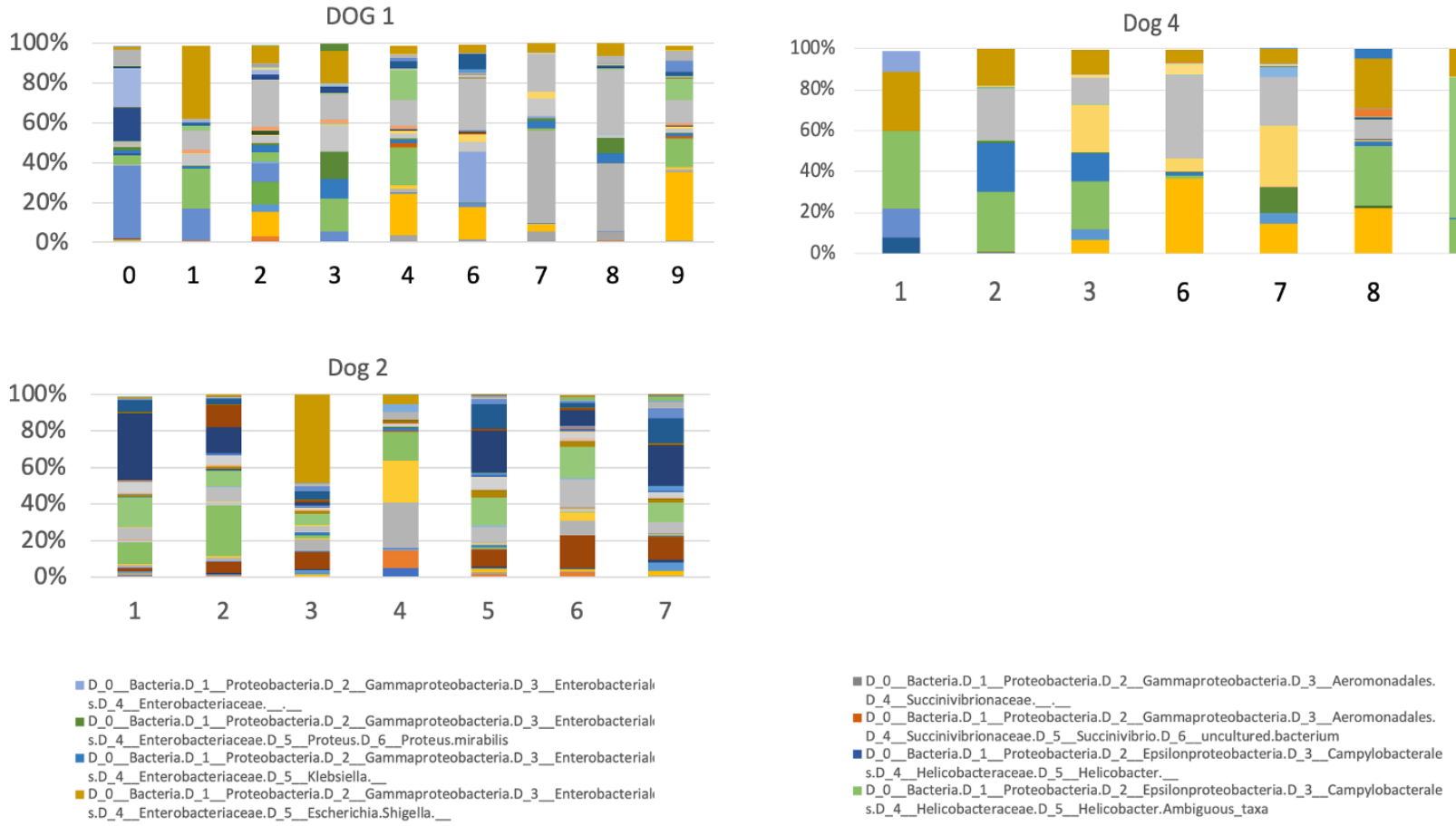

Figure 1.6 The relative abundance of the dog without GI toxicity

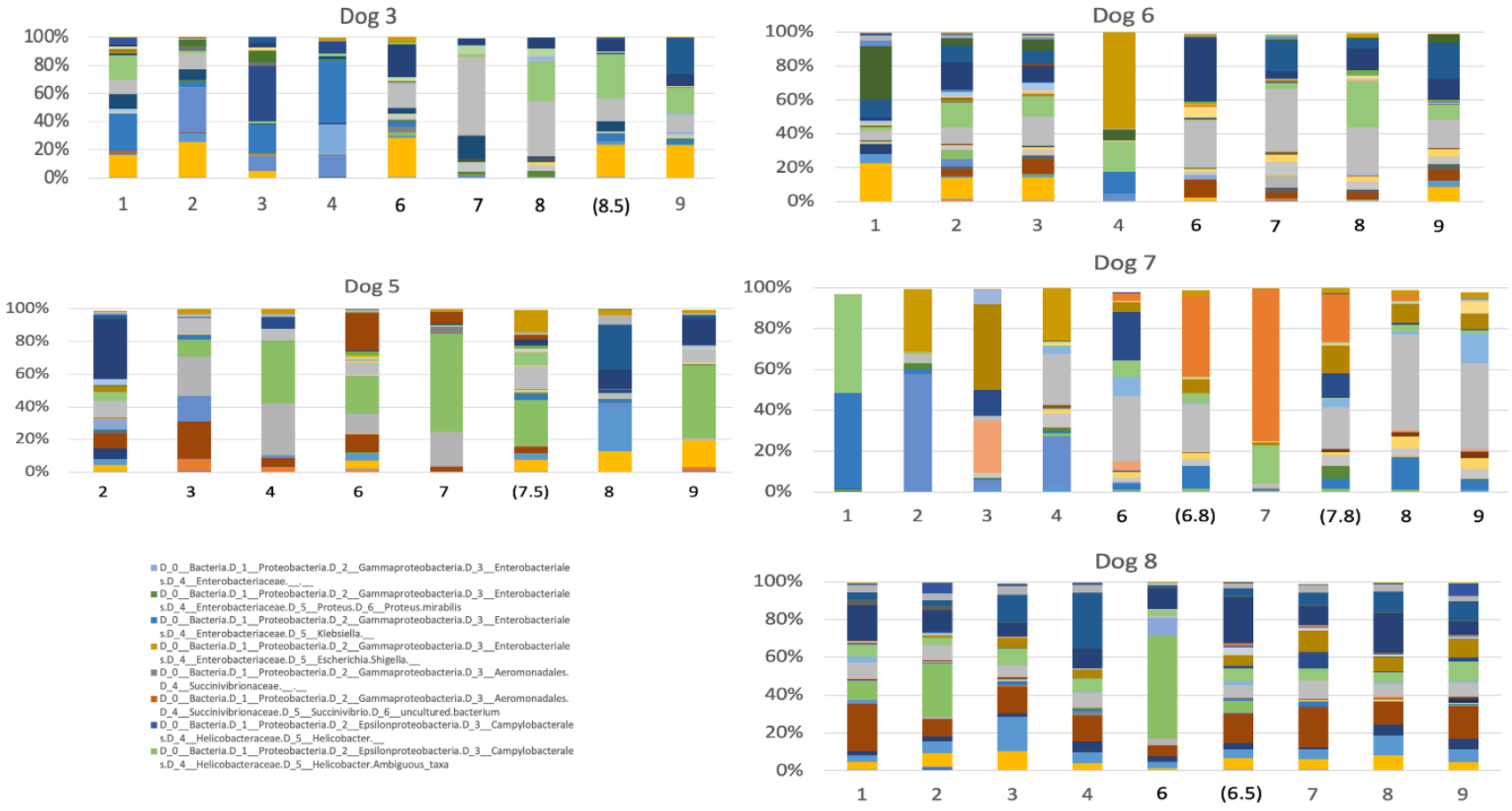

Figure 1.7 The relative abundance of the dog with GI toxicity

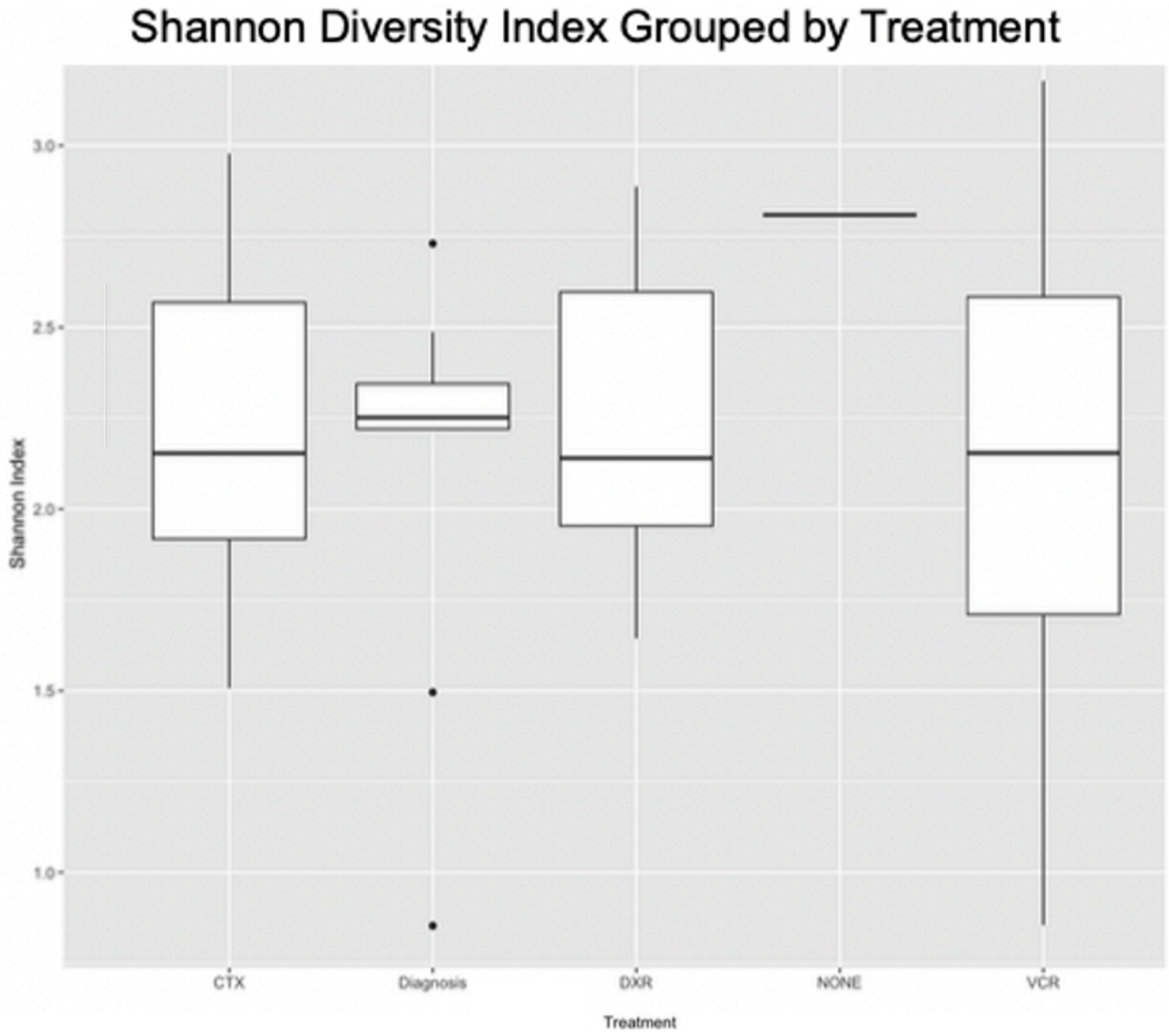

Figure 1.8 The Shannon Diversity grouped by treatment received

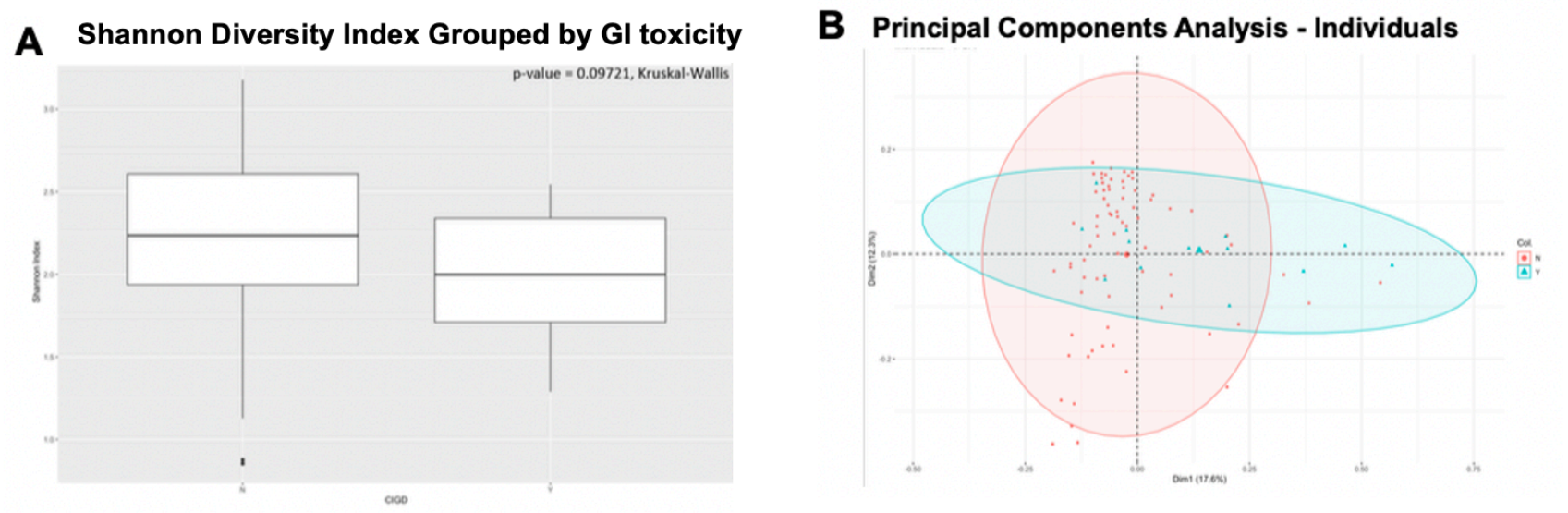

Figure 1.9 Shannon Diversity Index and PCA analysis in the dogs with vs without GI toxicity
(A) Shannon Diversity Index grouped by the presence or absence of GI toxicity showed a trend towards a difference in microbiome diversity. (B) PCA plot of the presence or absence of GI toxicity.

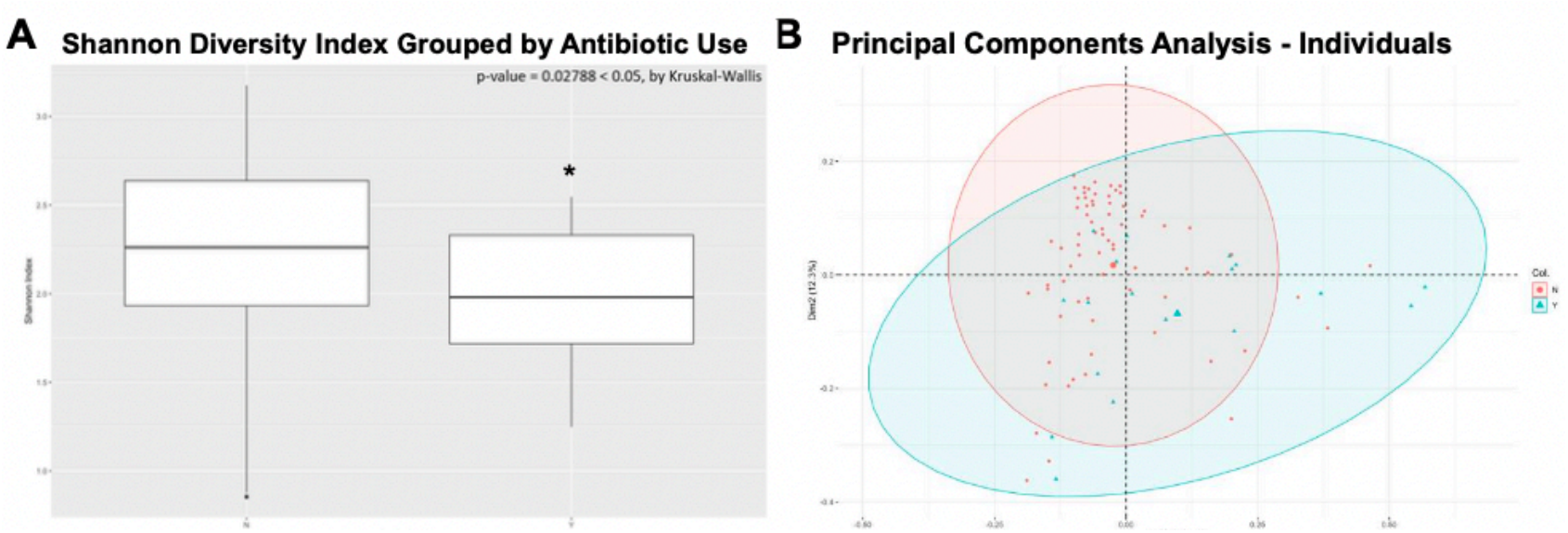

Figure 1.10 Shannon Diversity Index and PCA analysis in the dogs with vs without antibiotic use
(A) Shannon Diversity Index grouped by the administration of antibiotics during treatment and shows a significant difference, p-value<0.05 by Kruskal-Wallis test. (B) PCA plot of the administration of antibiotics.

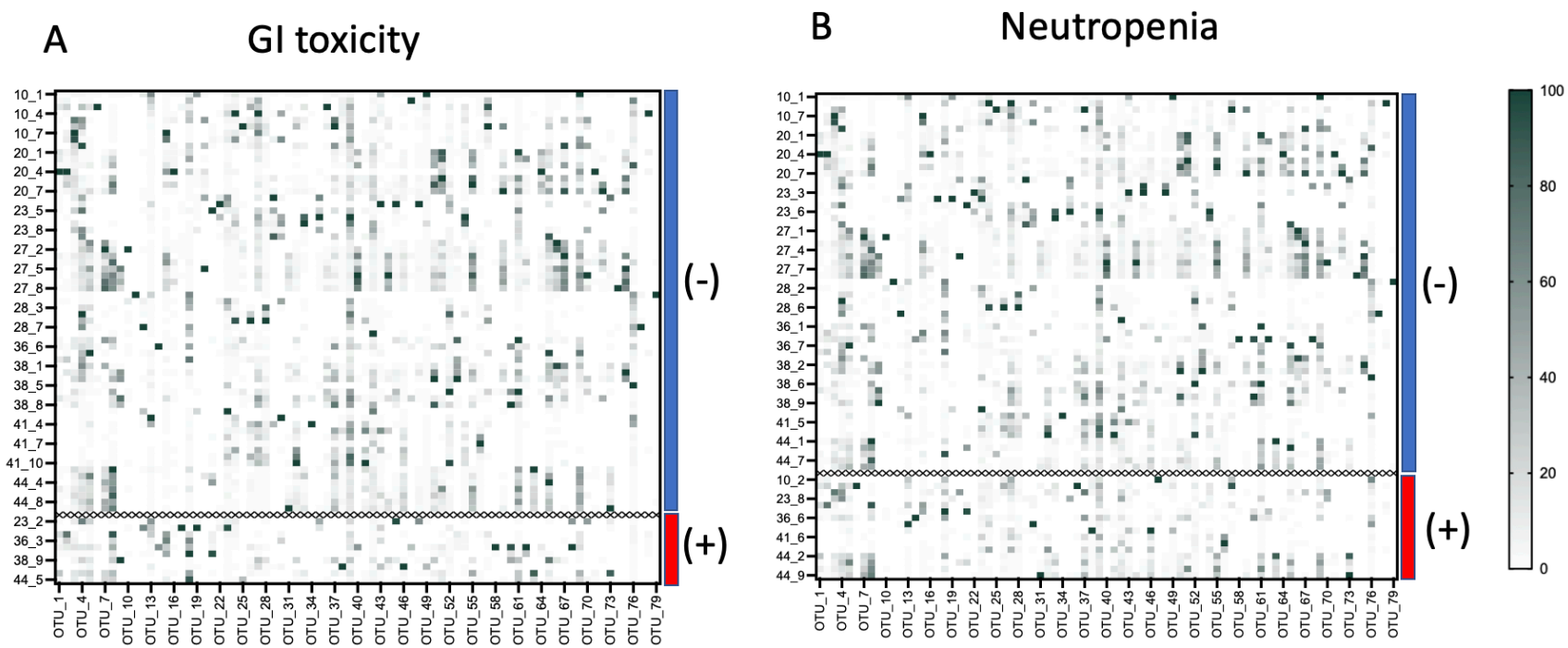

Figure 1.11 Heat map of relative OTU abundance across samples (A) heatmap of GI toxicity (B) heatmap of neutropenia Abundances were measured as proportions of samples and all the 79 abundant OTUs are shown. Samples and OTUs were clustered hierarchically based on relative abundance profiles.
On the left y-axis labels represent individual sample code. The color-coded on the right y axis groups the presence or absence of toxicity. OTUs are represented on the x-axis with corresponding relative abundances shown in the heatmap grid with increasing abundance from white to dark green.

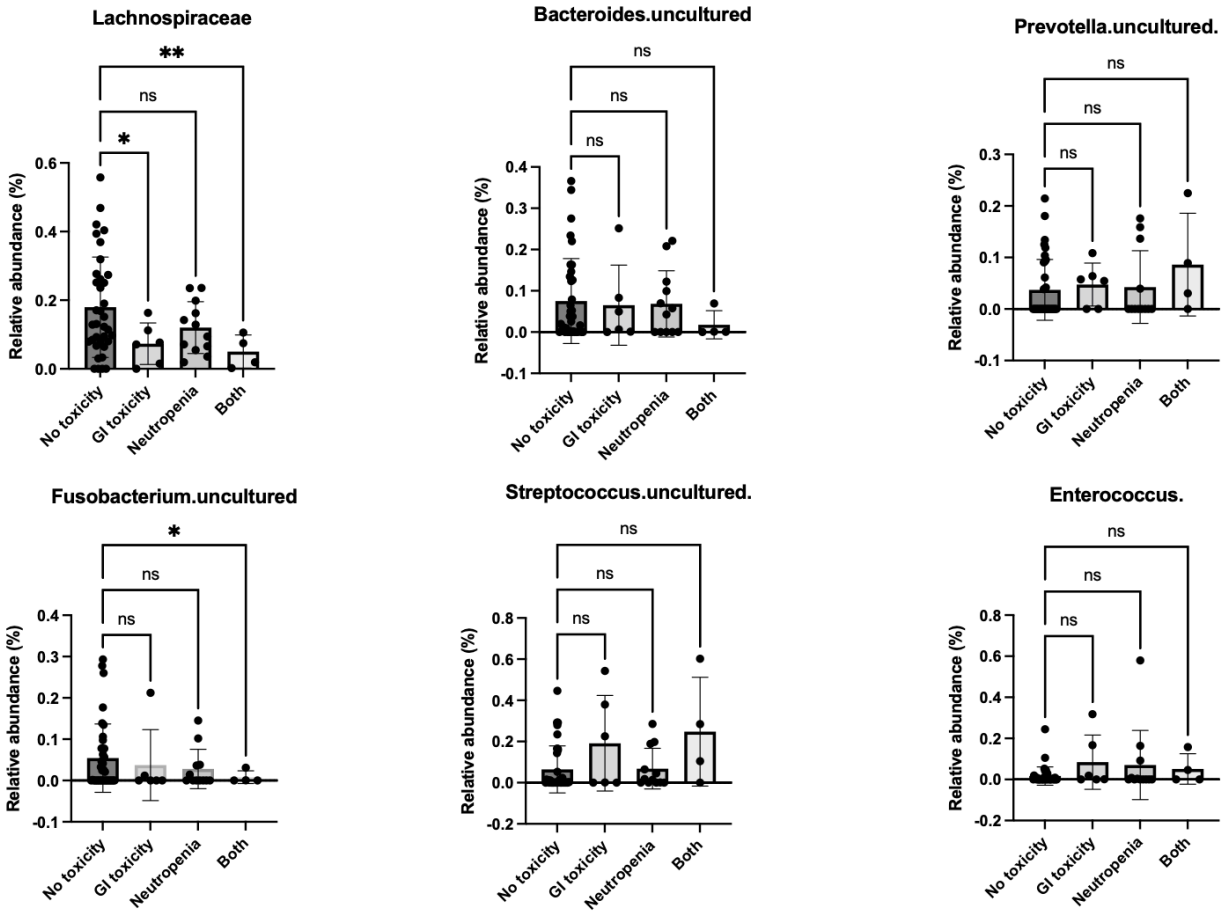

Figure 1.12 The comparison of the means of the relative abundance between dogs with chemotherapy induced toxicity and without it X axis means chemotherapy induced toxicity categories (No toxicity, GI toxicity, Neutropenia, or Both). Y axis means the relative abundance of the gut microbiome.

APPENDIX B:

SUPPLEMENTAL TABLES AND FIGURES

Table S1.1 The summary of the first 10 week of CHOP based protocol

Drug	Route	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
VCR	IV	x		x			x		x		
CTX	PO		x					x			
DXR	IV[a]				x					x	
Prednisone[b]	PO	x	x	x	x						
Fecal sample collection		x	x	x	x	(x)[c]	x	x	x	x	(x)[c]
CBC		x	x	x	x	x	x	x	x	x	x
PE		x	x	x	x	x	x	x	x	x	x

Note:
a. Doxorubicin is intravenously administered by free dripping for 15 minutes or longer. Dose is adjusted at 30 mg/m^2 when weight is >15kg, at 1mg/kg when weight is <15kg.
b. Prednisolone is prescribed at 2mg/kg daily, gradually decrease to 0.5mg/kg daily, and discontinue at Week 4.
c. Fecal sample collection at this point is optional.

Table S1.2 The summary of concomitant medications in 20 dogs included in the study

	Week										
Medications	0	1	2	3	4	5	6	7	8	9	10
Antibiotics	0	1	4	2	3	0	2	0	0	0	0
Steroid	0	3	12	14	11	1	5	0	0	0	0
Metronidazole	0	0	1	3	1	0	1	0	0	0	0
Probiotics	1	1	1	1	1	0	3	0	0	0	0
Maropitant	0	2	2	1	0	0	2	0	3	1	0
Omeprazole	0	1	1	1	3	0	1	1	1	1	0
Pantoprazole	0	1	0	0	1	0	0	0	0	0	0
Ondansetron	0	0	0	0	1	0	2	1	1	1	0
Famotidine	0	0	4	4	3	0	1	1	2	1	0
Diphenhydramine	0	1	0	0	0	0	0	0	0	0	0
Trazodone	0	0	2	2	1	0	3	4	4	7	0
Fluoxetine	0	1	0	0	0	0	0	0	0	0	0
Phenylpropanolamine	0	1	1	1	1	0	1	0	0	0	0
levothyroxine	0	1	1	1	1	0	1	1	1	1	0
Vitamin B12	0	0	0	1	1	0	1	1	1	1	0
CBD	0	1	0	2	1	0	0	1	1	0	0
Clopidogrel	0	0	0	0	0	0	0	0	1	1	0
Total	1	14	29	33	29	1	23	10	15	14	0

Note: the row means the type of the concomitant drugs. The column means the Week of CHOP based protocol.

Figure S1.1 The picture of FastDNA SPIN Kits for Soil