Case study of diet digestibility and gut microbiome changes in a dog undergoing CHOP chemotherapy for lymphoma

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ABSTRACT

Nutritional strategies in the management of canine cancer patients undergoing CHOP chemotherapy are still in its infancy. Studies on a novel diet containing cooked navy beans and rice bran have demonstrated to have cancer chemo-preventive properties and have a strong potential to support a healthy gut microbiome in both humans and animals. In this case study we evaluated how a diet rich in rice bran and navy bean affects a dog's gut microbiome and its function during CHOP chemotherapy. Stool samples were collected from an adult 12-year-old male castrated Labrador which is diagnosed with multicentric B-cell lymphoma with ocular involvement. The dog was undergoing a 15 week CHOP chemotherapy protocol combined with a 6 week dietary trial containing 25% cooked navy bean powder and heat stabilized rice bran. Nine breed matched control dogs undergoing the diet intervention without CHOP chemotherapy were used to evaluate diet digestibility. Four of the breed matched control dogs were used to evaluate microbial alterations.
The 16s rRNA gene was pyrosequenced, a digestibility analysis was performed and fatty acids were extracted and analyzed utilizing Gas Chromatography-Mass Spectrometry (GC-MS). We found that canine fecal samples collected after the diet intervention displayed notable increases in *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii* compared to fecal samples prior to the diet intervention. In addition a notable increase in Palmitic and Stearic acid and a decrease in Oleic acid and Linoleic acid was found. In this case study, the consumption of a diet containing rice bran and navy beans during chemotherapy was associated with an enhanced modulation of the canine gut microbial composition and function. Therefore a diet rich in rice bran and navy beans could potentially be combined with chemotherapy to support the gut microbiome and improve quality of life during chemotherapy.

1. Introduction

Canine lymphoma is one of the most commonly diagnosed canine neoplasms and accounts for up to 24 percent of all canine tumors.[1] Multidrug chemotherapy such as CHOP are at the moment the most effective treatments for canine lymphoma with remission rates up to 85% and survival times up to 12 months.[2] Chemotherapy has been shown to cause shifts in the gut microbiota in humans and therefore causes gastrointestinal side effects.[3–5] Most complications in dogs after CHOP chemotherapy include gastrointestinal side effects(vomiting, diarrhea, hyporexia) or hematological (e.g. neutropenia).[2,6] Approximately 25% of the dogs undergoing CHOP chemotherapy experience gastrointestinal system-related adverse effects, and these comprise around 40% of all adverse events encountered during therapy .[6] This high incidence of gastrointestinal signs during chemotherapy requires further research on the canine microbiome impact. Research indicates that in humans and animals these side effects are caused by gut dysbiosis.[4,7] Gut dysbiosis is defined as a shift in the gut microbiome composition with a loss of diversity and a lack of

balance in the commensal microbiota. which in humans can cause several gastrointestinal diseases such as inflammatory bowel disease.[4,7] Given the parallels between human and canine neoplastic disease, further evaluation of shifts in the gut microbiome during treatment with chemotherapy in dogs is warranted. Research displays that canine dysbiosis is caused by alterations in native phyla including *Firmicutes, Fusobacteria, Actinobacteria, Bacteroidetes, Proteobacteria, Bifidobacteria and Lactobacteria.*[8,9] These bacteria produce predominantly fatty acids that support digestion, sustain the mucosal and systemic immunity and have an anti-diarrheal effect which can aid in decreasing the morbidity related to neoplastic diseases.[8,9] Accordingly, a treatment to improve the gut microbiome during chemotherapy is essential to reduce treatment side effects. Nutritional strategies in the management of canine cancer patients undergoing CHOP chemotherapy and microbial production of fatty acids are still in its infancy. Studies on a novel diet containing cooked navy beans and rice bran have demonstrated to have cancer chemopreventive properties and have a strong potential to support a healthy gut microbiome in both humans and animals.[10,11] Research displays that this diet additionally enhances the abundances of a health promoting microbiome, with an increase in the Phylum *Firmicutes* and a decrease in the Phyla *Actinobacteria* and *Fusobacteria*.[12] This health promoting microbiome leads to a better digestion, intestinal epithelial barrier and immune response.[13,14] This probiotic improvement in gut health may be attributed to a displacement of intestinal pathogens and a production of antimicrobial substances produced during rice bran an navy bean digestion including fatty acids and amino acids.[14,15,16]. The aims of the current study were to evaluate how a rice bran and navy bean diet modulates the gut microbiome during CHOP chemotherapy. The overarching hypothesis is that in dogs undergoing chemotherapy, a diet rich in rice bran an navy beans will enhance or support the gut microbiome and its function.

2. Material and Methods

2.1. Study design

This case study was part of a larger 15-week clinical trial which was performed at the Colorado Teaching Hospital, to evaluate nutritional strategies in the management of canine cancer patients during chemotherapy. It involved an adult 12-year-old male castrated Labrador which was diagnosed with multicentric B-cell lymphoma with ocular involvement. The dog was undergoing a 15 week CHOP chemotherapy protocol (**Figure 1**) combined with a 6 week dietary trial containing 25% cooked navy bean powder and heat stabilized rice bran (Sahti).(Attachment 1).

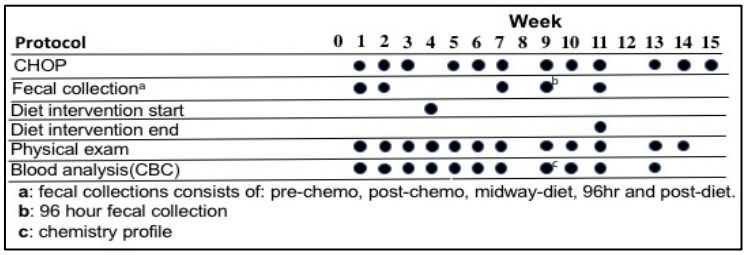

Figure 1: Study design scheme.
This figure displays a 15- week CHOP protocol divided in three cycles with a fourth treatment free week. Here fecal collections were performed on weeks 1, 2, 7, 9 and 11 which reflected pre-chemo/dietary intervention baseline, one week post-chemo, and two weeks into dietary intervention, intervention midpoint and intervention endpoints.

2.2. Inclusion & Exclusion criteria

Dogs were eligible for inclusion in this study if they had a recently confirmed diagnosis of lymphoma, an owner elected 15-week CHOP chemotherapy protocol, a minimum age of 12 months with a BCS between 4 and 7 on a 9-point scale and weighing at least 8 kg. Dogs were excluded if they had any reported gastrointestinal diseases, food allergies, other systemic diseases, other history of cancer and antibiotics use. (**See Attachment 3**)

2.3. Patients

This study included an adult 12-year-old male castrated Labrador which was diagnosed with multicentric B-cell lymphoma with ocular involvement undergoing CHOP chemotherapy combined with a diet trial containing 25% cooked navy bean powder and heat stabilized rice bran. This patient did not have any history of gastrointestinal diseases, food allergies and had no earlier cancer diagnosis or cancer treatment. The patient did not receive medications that were expected to alter the gut microbiome. To confirm the

health status, bloodwork and physical exams were performed as described in **Figure 1** (**and Attachment 2**). All blood and physical parameters were within normal limits. The patient's dietary history was noted to support the interpretation of microbiome alterations due to the nutritional intervention. The patient was previously consuming 2 cups per day of dry adult dog food 'Fromm salmon a la veg', 1 cup per day of Purina Veterinary Diets Gastroenteric "EN" wet food, and 10 Mother Biscuits per day. The dog was given the following supplements: a table spoon of salmon oil per day, three times per day Glucosamine-Glycoflex, and two times per day pill pockets. The transition to the Sahti Premium Adult Dog Food diet was performed on week 4 and fully transitioned in Week 5. This diet contains 25% navy bean powder and heat-stabilized rice bran, and has undergone canine feeding trials to establish its safety and digestibility in healthy adult dogs. An earlier non-published clinical trial which evaluated the diet digestibility in dogs was used to select breed matched control dogs. In total nine breed matched control dogs were used in this case study to evaluate diet digestibility. Four breed matched control dogs were used as controls to compare diet microbial changes.

2.4. Sample collection

Two fecal samples per dog per timepoint were collected by the owner after spontaneous defecation from the floor or from the glove. (see **Figure 1)** Owners were instructed to bring the fecal samples within 24 hours to the Veterinary Teaching Hospital while keeping them refrigerated. At the Veterinary Teaching Hospital, the samples were immediately frozen at -20 °C and transported to the laboratory on ice packs, and stored at -80 °C and frozen 8-12 weeks until further microbiome analysis was performed. The fecal samples of the lymphoma dog were collected during week 1, 2, 7 , 9, and 11. These fecal collections consists of pre-chemo, post chemo, midpoint nutritional intervention, a 96-hour collection and the endpoint of the nutritional intervention. No fecal collection was performed at the start of the nutritional intervention, to avoid the introduction of confounding alterations in microbiome caused by an adaptation to the new diet. A 96-hour pooled fecal sample was collected on week 9 to meet the guidelines of the Association of American Feed Control Officials(AAFCO) for digestibility analysis.[17]

2.5 Digestibility analysis

The 96hr total fecal collection of the Labrador was used for Apparent Tract Total Digestibility analysis, and compared to the fecal samples of nine healthy dogs undergoing the identical diet intervention without CHOP chemotherapy. Thereby proximate analysis of both the intervention diet as well as the 96-h pooled fecal sample were performed. The digestibility of protein and fat was calculated by using the following formula, where nutrients were measured in grams on a DM basis: Nutrient digestibility (%) = [(nutrient intake – nutrient in feces)/ nutrient intake] × 100.[18]

2.6 Fecal Fatty acid analysis

All samples were used for Targeted Fatty Acid analysis using a fatty acid methyl ester extraction (coupled with GC-MS) as previously reported.[18] This technique was used to assess fatty acid profiles and define the relative fatty acid proportions. Here the fecal samples were unfrozen and approximately 100mg from each sample was subjected for analysis. As a control for the extracted samples, 25 µL of 10 µg/mL C17:0 was added to all samples. The resulting raw spectral abundances were calculated to relative abundances by dividing the raw abundance of a sample by the total spectral counts in the data set**.**

2.7 Fecal Microbiome analysis

<u>DNA extraction & amplification</u>

The fecal DNA was extracted from all samples by using MoBio Powersoils DNA extraction kits **(see Attachment 4)** and approximately 0.25 grams of feces while following the kit manufacturer's instructions. Concisely, the defrosted fecal samples were dissolved in a 1X phosphobuffered saline and centrifuged for 10 minutes at 3000 revolutions per minute. After removal of the supernatant the fecal pellet was collected using a sterile swab. Sterile swabs containing a blank medium from the DNA extraction kit were used as negative controls. The extracted DNA was stored at -20^{o}C prior to the amplification steps, such as polymerase chain reaction sequencing .
For PCR a forward primer was used with a unique barcode sequence to associate sequences with parent samples. To remove known biases against *Archaeal Crenarchaeota*, *Thaumarchaeota* and *Alphaproteobacterial* clade SAR11 a degenerate

forward primer(515f) and a modificated reverse primer(806r) were used.
Subsequently 50 µl of extracted DNA and a 515/806 primer set were used to amplify the V4 region of the bacterial 16S rRNA by PCR. This amplification was done in triplicate for each fecal sample using the Fisher Hot Start Master Mix**.(See Attachment 5)** Twenty-five µl reactions containing 0.5 µl of forward and reverse primer, 1 µl of sample DNA, 12,5 µl Hot Master Mix, and 10,5 µl of H_2O were amplified at 95°C for 5 minutes. This was followed by 34 cycles of 94°C for 30 seconds, 62 °C for 90 seconds and 72 °C for 60 seconds and cooling at 72 °C for 10 minutes. Sterile Master mix and water were included as negative controls. The PCR reactions were purified using SPRI magnetic beads followed by fluorometrical DNA quantification by using a Kapa Biosystems Kit **(See Attachment 6)** while following the manufacturer's protocols.

Microbiome DNA Analysis

QIIME(Quantitative Insights Into Microbial Ecology) version 2.0.8 software was used to process the 16S rRNA raw DNA sequences.[19] Both forward and reverse sequences were merged for further paired-end sequence analysis and de-multiplexed using the demux plugin. Here forward sequences and reverse sequences were shortened to 200 and 130 basepairs respectively. The Demultiplexed samples were rarefied to a depth of 32,943 sequence counts; this depth was selected to retain all five, unique time point samples in this one dog pilot study. The negative control buffers contained negligible sequences counts(35 and 3031 counts respectively) and were not used for further analysis. Sequences were assigned by comparison to the Greengenes reference database.[4] We used an alpha_rarefaction.py script to calculate alpha diversity , which evaluates the differences in species richness and functional composition between fecal samples collected at each timepoint. Here the Shannon Diversity index was used, which in comparison to other alpha diversity indices such as the Simpson's index, is more sensitive to species richness.[20]

2.8 Statistical analysis

No statistical analysis was performed on the microbiome or fatty acid results due to a small sample size (n=1) and therefore low statistical power.

3. Results

The aims of the current study were to evaluate how a rice bran and navy bean diet modulates the gut microbiome and its function in one dog with multicentric B-cell lymphoma during CHOP chemotherapy. In **Figure 2** the digestibility parameters Crude Fat and Crude Protein are relatively similar between the lymphoma dog and the nine healthy control dogs which concludes that in this case the novel diet was digestible during CHOP therapy.

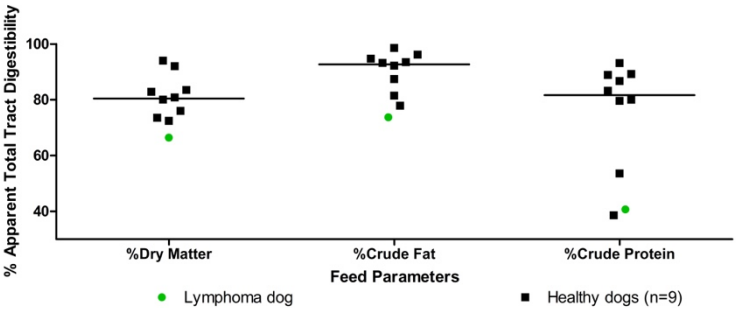

Figure 2: Apparent Total Tract Digestibility percentage of feed parameters in stool of the lymphoma dog and healthy dogs. The digestibility parameters of the lymphoma dog such as Dry Matter, Crude Fat and Crude Protein are relatively similar to the nine healthy dogs.

To compare the species richness also called alpha diversity the Shannon Diversity index was calculated. In **Figure 3** the Shannon Diversity Index displays an increase over time from 3.37 at Baseline Week 1 to Midway-Diet Week 7, a decrease at Week 9 and a further increase to 4.91 at Week 11 at the end of the dietary intervention.

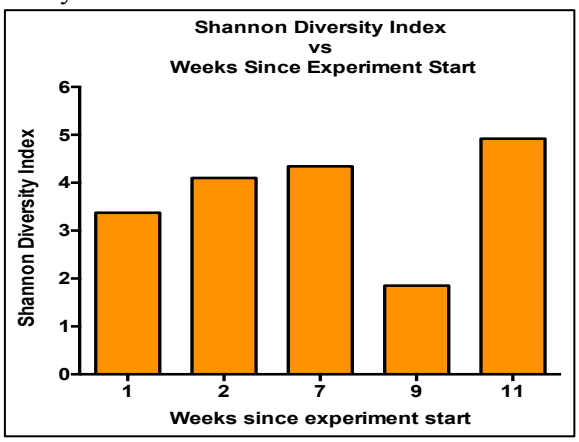

Figure 3: Shannon diversity index of feces during different timepoints. The Shannon Diversity Index increased over time with a peak at Week 11(End of diet intervention.

To further evaluate microbial diversity the taxonomic class microbiome abundances are displayed in **Figure 4.** The relative abundances at class level of four healthy retriever dogs undergoing a rice bran and navy bean diet are displayed in **Figure 4A.** Here *Firmicutes* (especially *Clostridiaceae, Erysipelotrichaceae*) were the most abundant bacterial phylum (73.8-91.7%) of all bacterial sequences; *Actinobacteria* were the next most abundant phyla (1.3-20.0%) followed by *Fusobacteria* (0.5-11.5%). **Figure 4B** displays notable changes in *Bacilli* and *Clostridial* relative abundances over time.

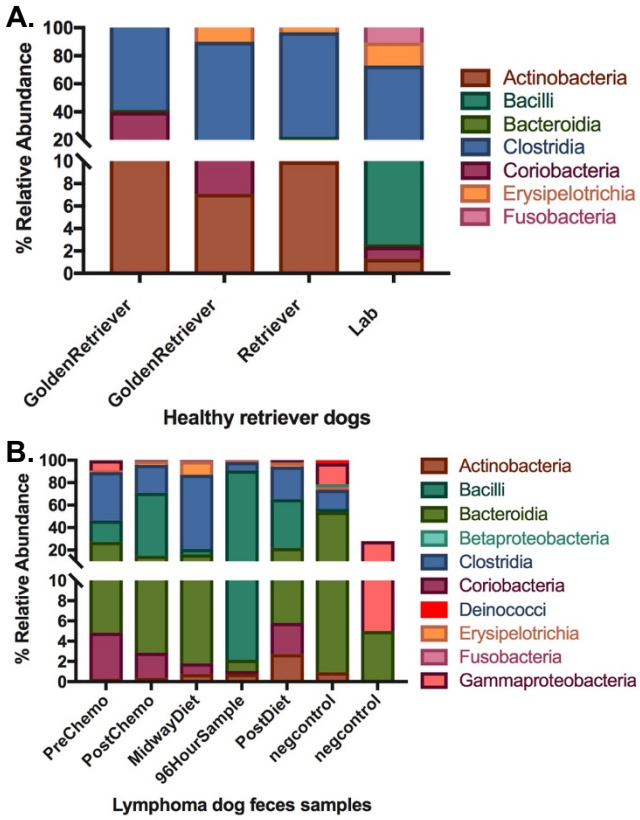

Figure 4A&B: Taxa relative abundance at class level of the canine stool microbiome
This figure displays microbiome class abundances in the stool microbiome of four healthy retriever dogs undergoing a rice bran diet(A) and the dog with multicentric B-cell lymphoma(B).

In the microbiome analysis two microbial species correlated to a healthy microbiome in dogs such as *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii* which are displayed in **Figure 5.** [14,21]
In **Figure 5a** the relative abundances are shown for *Bifidobacterium adolescentis* at different timepoints, with an increase in abundances of 1,31% between Week 7 (Midway-diet intervention) and Week 11 (End of diet intervention).
In **Figure 5b** the relative abundances are shown for *Faecalibacterium prausnitzii* at different timepoints, with an increase in abundance of 4,4% between Week 7 (Midway-diet intervention) and Week 11 (End of diet intervention).In both **Figure 4a** and **Figure 4b** a decrease in relative abundance of both microbiota species was measured at Week 9 but it re-increased at Week 11(End of diet intervention).

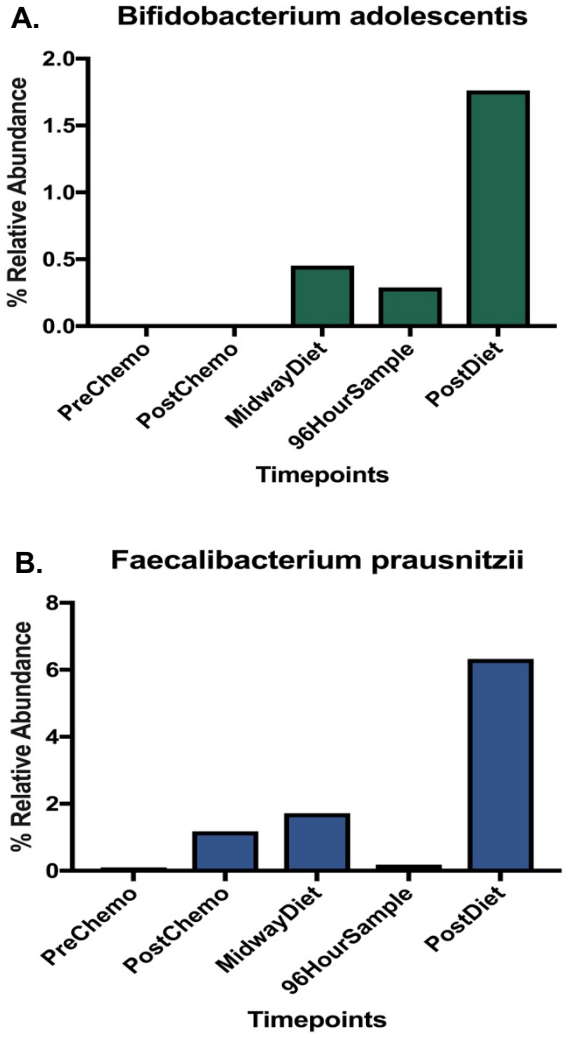

Figure 5A&B: Relative abundances of select probiotic microbial species during different timepoints. **The Relative abundances of (A) *Bifidobacterium adolescentis* and (B) *Faecalibacterium prausnitzii* are displayed for each timepoint. Here an overall increase of both microbial species is shown.**

In **Figure 6** the relative abundance of *E.coli* species are displayed which are considered as a marker of dysbiosis and/or intestinal inflammation[7,19] Here a decrease from 10,15% in Week 1 (Baseline) to 0,53% in Week 2 (Start CHOP chemotherapy) is seen and sustained from Week 7 (Midway-Diet) until Week 11 (End of diet intervention). A minor increase of E.coli relative abundance occurred in Week 7 till Week 9, from 0,20% to 0,29% and decreased again between Week 9 and Week 11 from 0,29% to 0,19%

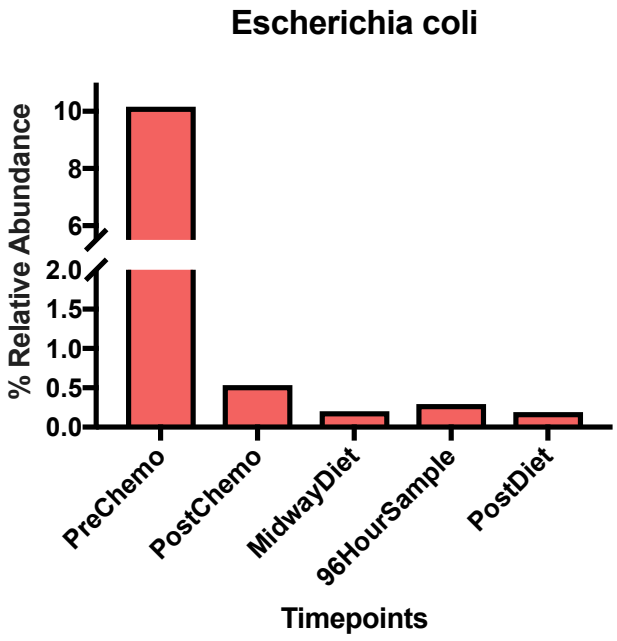

Figure 6: **Relative abundance of *E.Coli* Species during different timepoints.** The relative abundance of *E.coli* decreased after Week 1 and remained notably low during the next timepoints.

Figure 7a and **7b** display fecal fatty acid percent relative abundance at week 1 (baseline), week 7 (dietary intervention), and week 11 (end of dietary intervention).
In Figure **7b** overall, fatty acids did not vary appreciably over time. However, during the dietary intervention period, palmitate (C16:0) and stearate (C18:0), fatty acids with gastrointestinal health benefits[22,23],increased slightly and steadily in abundance from baseline levels, from 14.12% to 20.04% (palmitate) and 10.03% to 14.30% (stearate).

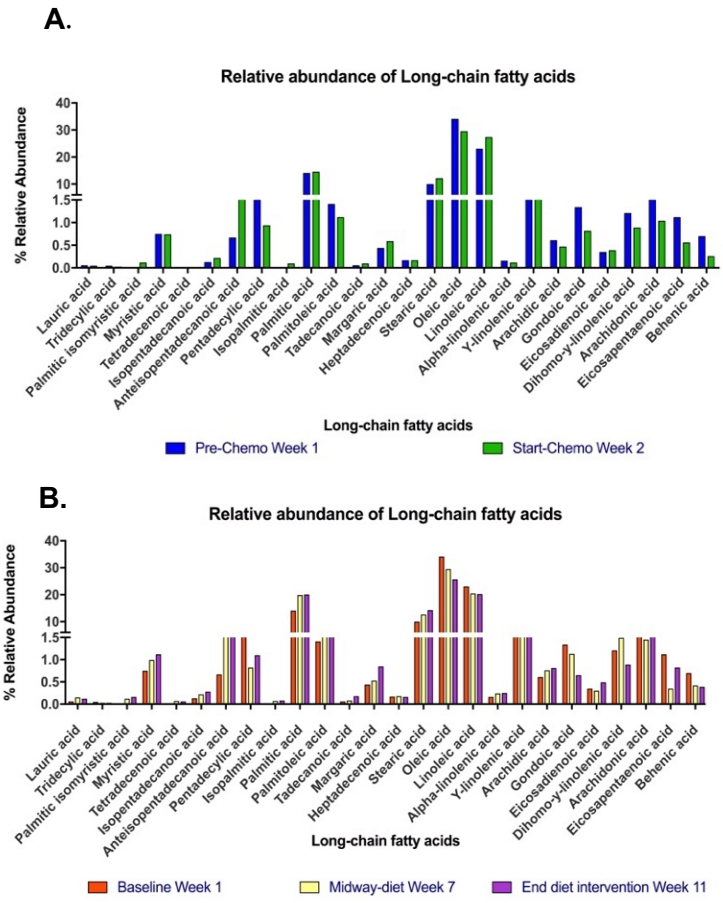

Figure 7A and 7B: Fatty acid relative abundances of feces during different timepoints. (A): a notable decrease of Oleic acid and an increase of Linoleic acid in relative abundance is displayed after one week of CHOP therapy. (B): A notable decrease of Oleic Acid and an increase of Palmitic and Stearic acid was observed between Baseline Week 1 and Post-diet Week 11

Discussion

The aims of this one-dog pilot case study were to evaluate how a diet rich in rice bran and navy bean affect a dog's gut microbiome and its function during CHOP chemotherapy. The results take part of a larger clinical trial involving dogs with neoplastic disease undergoing any form of chemotherapy combined with a cancer free group to examine gut microbiome modulations due to chemotherapy treatment. We hypothesized that in dogs undergoing CHOP chemotherapy, a rice bran and navy bean diet would enhance and or sustain gut microbiome composition

and function and therefore reduce chemotherapy side effects**. Figure 3** depicted the alpha diversity of the microbiome by using the Shannon diversity index which is a sensitive estimate of species richness and evenness.[20] This Shannon index increased over time with the greatest increase during the diet intervention period. However several research studies have confirmed that in humans and animals both chemotherapy and lymphoma significantly decrease alpha diversity.[4,24,25] This difference in alpha diversity could be related to the diet intervention containing rice bran and navy beans.

Besides increases in alpha diversity the results of the dietary intervention display an overall increase of both *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii* which are known to promote a healthy gut microbiome in dogs.[8,21]

Despite an overall increase of alpha diversity and abundance, a meaningful drop is observed at Week 9 in both Alpha diversity as relative abundance of microbial species. An explanation for this drop could be due to the chemotherapy treatment or due to supplemental feeding of non-diet intervention food such as yoghurt and a frozen lunch tray.(not shown). The yoghurt itself, which is both used as a probiotic and prebiotic could lead to an abundance in *Lactobacilli* species.[26] An increase in *Lactobacilli* relative abundance could result in dominance of this species at timepoint Week 9 as seen in the microbial taxa abundances, thereby decreasing the relative abundance of other bacterial species and thus a decreased alpha diversity. In future research on this subject strict measures should be taken to avoid the consumption of non-dietary foods and/or probiotics.

In addition to an increase in healthy bacteria, a decrease in the relative abundance of bacteria that promote inflammation and dysbiosis such as E.coli has been observed.[7,27] (**Figure 6**) The relative abundance of *E.coli* dropped after Week 1 and remained low in abundance. This could be explained due to an enhanced healthy gut microbiome and its probiotic commensals related. Although *E. coli* did not appreciably change in abundance after week 1 of the investigation, its sustained, markedly-low abundance in the microbiome may be related to increases in probiotic commensals and improved microbiome health in response to dietary intervention

A dysbiosis is not only caused by dysbiosis promoting bacteria but also through a decreased production of health promoting fatty acids.[28] Fatty acids produced by the microbiome can be divided into short to long chain fatty acids. These fatty acids are end products of food metabolized by the microbiome.[10] This metabolization reflects microbial function and is therefore used in this research as a marker. The relative abundance of long chain fecal fatty acids sustained at similar levels for most of the timepoints. Both Palmitic acid and Stearic acid display notable increases over during the diet intervention period. Research has shown that in animals these two fatty acids are anti-diarrheal metabolites and thus promote a healthy microbiome.[29] An explanation for the increase of stearic acid is that *Bifidobacteria* are known bacterial species that produce stearic acid.[22]

Besides an increase in fatty acids, a notable decrease is shown for Oleic acid (Omega 9) and Linoleic acid (Omega 6). A decrease in Linoleic acid and Oleic acid could be explained by the conversion to stearic acid by microbial species such as *Bacteroidetes* also shown in the human gut microbiome and other animals .[30-31] Here in addition to the diet intervention further research should be done to prescribe Omega fatty acids during chemotherapy.[32]

To conclude in this case, the consumption of a diet containing rice bran and navy beans during chemotherapy was associated with a general decrease in opportunistic pathogens (*E.coli*) and an increase in gut health promoting bacteria (*F.prausnitzzi and B.adolescentis*).[7,14] This could be considered as a positive modulation of the canine gut microbial composition and its function. Therefore a diet rich in rice bran and navy beans could potentially be combined with chemotherapy to improve the gut microbiome. It has to be taken into account that several bacteria did not resolve down to the species level. An explanation for this could be that the 16S rRNA sequencing may be biased during DNA extraction, primer selection, rRNA amplification or due to the sequencing quality.[7,33] In addition, due to the small sample size an interpretation of the results is not significant, but the notable differences in probiotic and dysbiotic species can be used for further research. These microbiome and fatty acid results will be used in a larger, controlled, three-armed clinical trial involving healthy dogs versus dogs undergoing chemotherapy. Further research will be necessary to assess significant dietary changes during chemotherapy that could influence the microbial function and thereby improve quality of life

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ATTACHMENT 1

ATTACHMENT 2

STUDY CHECKLIST

Day 0 is the first Day the patient eats 100% study diet.

Study (Day) Week	**Date** (DD/MM/YY)	**Procedures**
Pre-Enrollment		☐ Diet history ☐ Collect pre-chemo fecal sample
Week 1 Weight_____		☐ Owner consent ☐ Weekly owner survey ☐ Physical exam ☐ CBC ☐ Serum/plasma collection ☐ Vincristine ☐ Rx prednisone
Week 2 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ Collect post-chemo fecal sample ☐ CBC ☐ Rx cyclophosphamide and furosemide
Week 3 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ CBC ☐ Doxorubicin
Week 4 Weight_____		☐ CBC ☐ Weekly owner survey ☐ Serum/plasma collection ☐ Start transition to study diet
Week 5/Day 0 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ CBC ☐ Serum/plasma collection ☐ Vincristine Pet should be on 100% of study diet by this day
Week 6/ Day 7 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ CBC ☐ Rx cyclophosphamide and furosemide
Week 7/Day 14 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ CBC ☐ Serum/plasma collection ☐ Doxorubicin ☐ Collect midway diet fecal sample ☐ $500 VTH credit added to invoice
Week 8/Day 21		**OFF**
Week 9/Day 28 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ CBC ☐ Chemistry profile ☐ Serum/plasma collection ☐ Vincristine ☐ Owners start 4 day fecal sample collection
Week 10/Day 35 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ CBC ☐ Rx cyclophosphamide and furosemide
Week 11/Day 42 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ CBC ☐ Serum/plasma collection ☐ Doxorubicin ☐ Collect post-diet fecal sample ☐ Start transition back to normal diet
Week 12		**OFF**
Week 13 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ CBC ☐ Serum/plasma collection ☐ Vincristine
Week 14 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ CBC ☐ Rx cyclophosphamide and furosemide
Week 15 Weight_____		☐ Physical exam ☐ Weekly owner survey ☐ CBC ☐ Doxorubicin

ATTACH
MENT 3

Animal: _____ **Date:** _____
(Case ID) (DDMMMYY)

Pre-Enrollment Inclusion / Exclusion Criteria Form

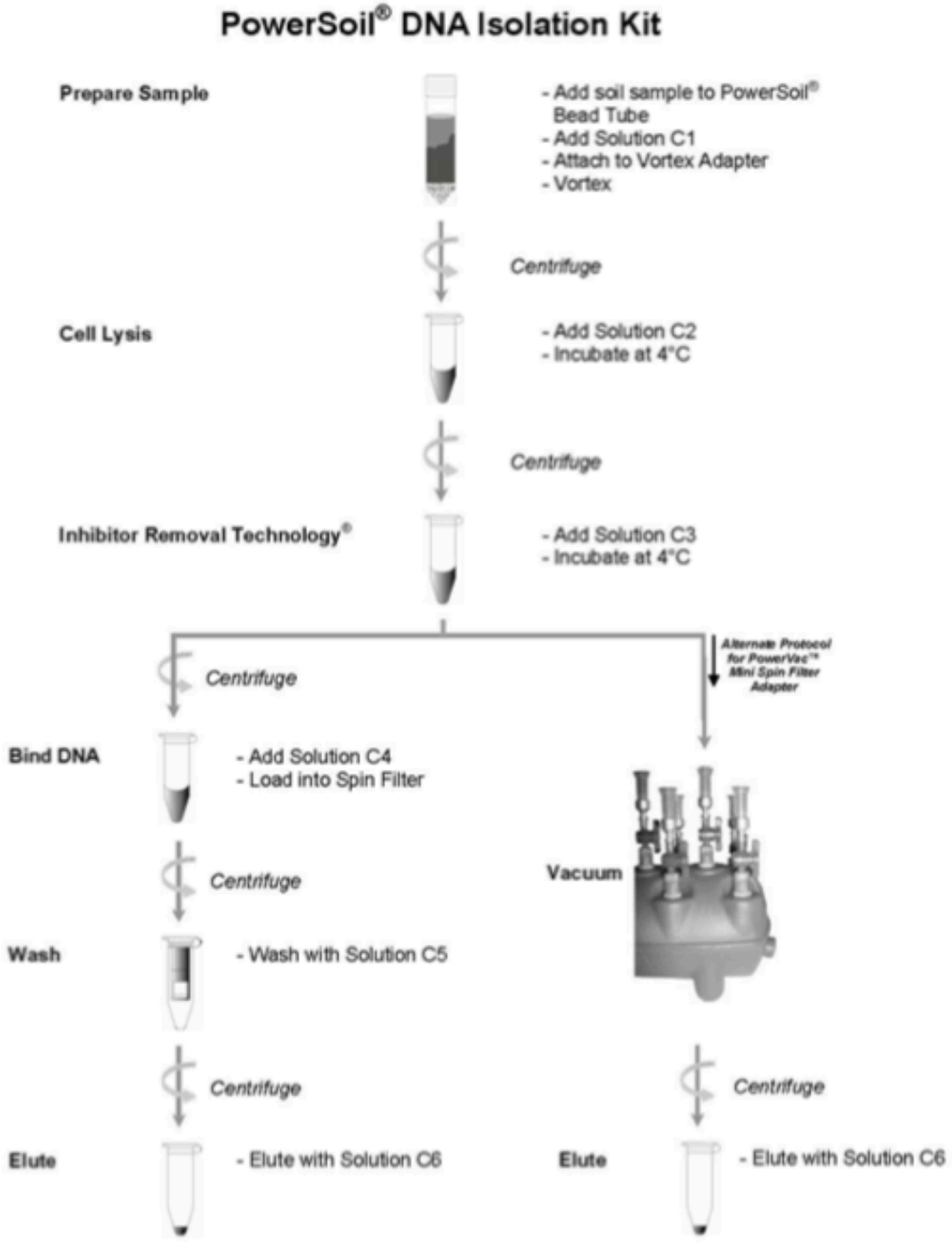
Inclusion Criteria

Age at least one year	**Yes**	**No**
Weight > 8kg	**Yes**	**No**
Body condition score of 4 to 7 out of 9	**Yes**	**No**
Confirmed diagnosis of lymphoma	**Yes**	**No**
ANC >2000 cells/μL; haematocrit >30%; platelets > 75.000/ μL; creatinine ≤ the upper limit of normal; bilirubin ≤ 1,5x the upper normal limit	**Yes**	**No**
Performance status of either 0 or 1 on Day 0, according to the modified ECOG Performance Scheme(Veterinary and Comparative Oncology, 2011)	**Yes**	**No**
Signed Owner Informed Consent	**Yes**	**No**

Exclusion Criteria

Concurrent medications and nutritional supplements will be considered case by case	**Yes**	**No**
Currently participating in other clinical trials	**Yes**	**No**
Concurrent malignancy or other serious systemic disorder incompatible with this study	**Yes**	**No**

ATTACHMENT 4



ATTACHMENT 5

Thermo SCIENTIFIC

PRODUCT INFORMATION

Thermo Scientific Maxima Hot Start PCR Master Mix (2X)

#K1051 100 rxns of 50 µl

Lot: **Expiry Date:**

Store at -20°C

Ordering Information

Component	#K1051 100 rxns of 50 µl	#K1052 500 rxns of 50 µl
Maxima Hot Start PCR Master Mix (2X)	2x1.25 ml	10x1.25 ml
Water, nuclease-free	2x1.25 ml	10x1.25 ml

www.thermoscientific.com/fermentas

Description

Thermo Scientific Maxima Hot Start PCR Master Mix (2X) is a ready-to-use solution containing Maxima® Hot Start *Taq* DNA Polymerase, optimized Hot Start PCR buffer, $Mg^{2+}$and dNTPs. The master mix retains all features of Maxima Hot Start *Taq* DNA polymerase. It is capable of high yield amplification of targets up to 3 kb from genomic DNA. This pre-mixed formulation saves time and reduces contamination due to a reduced number of pipetting steps required for PCR set up. The mix is optimized for efficient and reproducible hot start PCR.

Applications

- High throughput Hot Start PCR.
- RT-PCR.
- Highly specific amplification of complex genomic and cDNA templates.
- Amplification of low copy DNA targets.
- Generation of PCR products for TA cloning.

Maxima Hot Start PCR Master Mix (2X) composition

Maxima Hot Start *Taq* DNA polymerase is supplied in 2X Hot Start PCR buffer, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 4 mM Mg^{2+}.

PROTOCOL

1. Gently vortex and briefly centrifuge Maxima Hot Start PCR Master Mix (2X) after thawing.
2. Add the following components for each 50 µl reaction at room temperature:

Maxima Hot Start PCR Master Mix (2X)	25 µl
Forward primer	0.1-1.0 µM
Reverse primer	0.1-1.0 µM
Template DNA	10 pg - 1 µg
Water, nuclease-free (#R0581)	to 50 µl
Total volume	50 µl

3. Gently vortex the samples and spin down.
4. When using a thermal cycler that does not contain a heated lid, overlay the reaction mixture with 25 µl of mineral oil.
5. Perform PCR using the recommended thermal cycling conditions outlined below:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation / enzyme activation	95	4 min	1
Denaturation	95	30 s	25-40
Annealing	Tm-5	30 s	
Extension	72	1 min/kb	
Final Extension	72	5-15 min	1

GUIDELINES FOR PREVENTING CONTAMINATION OF PCR REACTION

During PCR more than 10 million copies of template DNA are generated. Therefore, care must be taken to avoid contamination with other templates and amplicons that may be present in the laboratory environment. General recommendations to lower the risk of contamination are as follows:

- Prepare your DNA sample, set up the PCR mixture, perform thermal cycling and analyze PCR products in separate areas.
- Set up PCR mixtures in a laminar flow cabinet equipped with an UV lamp.
- Wear fresh gloves for DNA purification and reaction set up.
- Use reagent containers dedicated for PCR. Use positive displacement pipettes, or pipette tips with aerosol filters to prepare DNA samples and perform PCR set up.
- Always perform "no template control" (NTC) reactions to check for contamination.

GUIDELINES FOR PRIMER DESIGN

Use the Thermo Scientific REviewer primer design software at www.fermentas.com/reviewer or follow the general recommendations for PCR primer design as outlined below:

- PCR primers are generally 15-30 nucleotides long.
- Optimal GC content of the primer is 40-60%. Ideally, C and G nucleotides should be distributed uniformly along the primer.
- Avoid placing more than three G or C nucleotides at the 3'-end to lower the risk of non-specific priming.
- If possible, the primer should terminate with a G or C at the 3'-end.
- Avoid self-complementary primer regions, complementarities between the primers and direct primer repeats to prevent hairpin formation and primer dimerization.
- Check for possible sites of undesired complementary between primers and template DNA.
- When designing degenerate primers, place at least 3 conserved nucleotides at the 3'-end.
- When introducing restriction enzyme sites into primers, refer to the table "Cleavage efficiency close to the termini of PCR fragments" located on www.fermentas.com to determine the number of extra bases required for efficient cleavage.

Estimation of primer melting temperature

For primers containing less than 25 nucleotides, the approx. melting temperature (Tm) can be calculated using the following equation:

$$Tm = 4 (G + C) + 2 (A + T),$$

where G, C, A, T represent the number of respective nucleotides in the primer.

If the primer contains more than 25 nucleotides we recommend using specialized computer programs e.g., REviewer™ (www.fermentas.com/reviewer) to account for interactions of adjacent bases, effect of salt concentration, etc.

COMPONENTS OF THE REACTION MIXTURE

Template DNA

Optimal amounts of template DNA for a 50 µl reaction volume are 0.01-1 ng for both plasmid and phage DNA, and 0.1-1 µg for genomic DNA. Higher amounts of template increase the risk of generation of non-specific PCR products. Lower amounts of template reduce the accuracy of the amplification.

All routine DNA purification methods can be used to prepare the template e.g., Genomic DNA Purification Kit (#K0512) or Thermo Scientific GeneJET Plasmid Miniprep Kit (#K0502/3). Trace amounts of certain agents used for DNA purification, such as phenol, EDTA and proteinase K, can inhibit DNA polymerases. Ethanol precipitation and repeated washes of the DNA pellet with 70% ethanol usually removes trace contaminants from DNA samples.

Primers

The recommended concentration range of the PCR primers is 0.1-1 µM. Excessive primer concentrations increase the probability of mispriming and generation of non-specific PCR products.

For degenerate primers we recommend higher primer concentrations in the range of 0.3-1 µM.

ATTACHMENT 6

should be kept at 4 µL, with 6 µL of KAPA SYBR® FAST qPCR Master Mix with Primer Premix. However, the amount of template (DNA Standard or diluted library) used per reaction may be scaled as required, provided that it can still be pipetted accurately. Always use the same volume of DNA Standard and diluted library. Ensure that plastic consumables, pipettes, and qPCR instruments are compatible with the reaction volume.

Internal Controls

The dilution of concentrated library DNA to fall within the dynamic range of this assay represents the biggest risk to accurate quantification, particularly if libraries are very concentrated and large initial dilutions are required.

If more than one dilution of each library is assayed (and falls within the dynamic range of the standard curve), the ΔCq value for consecutive dilutions is a good indication of the reliability of calculated library concentrations as in **Working Example** (step 5). However, ΔCq values for serial dilutions of a library do not provide any indication of the accuracy of the initial dilution.

For this reason, we recommend including at least one appropriate internal process or dilution control in every assay. These include:

- KAPA Library Quantification Dilution Control (KK4906). This is a quality-controlled 200 pM solution of the same linear, 452 bp dsDNA fragment comprising the DNA Standards and is also referred to as DNA Standard 0.
- An Illumina library that has previously been quantified with the KAPA Library Quantification Kit and that has been sequenced successfully.
- PhiX, a control library supplied by Illumina.

To be most effective, the internal control should be processed in the same way as the libraries to be assayed, i.e., the same initial dilution and serial dilutions should be prepared, and replicate reactions set up with each dilution of the internal control. Each of the internal controls listed above have advantages and disadvantages:

- The KAPA Library Quantification Dilution Control (DNA Standard 0) is subject to the same rigorous quality control as the set of DNA Standards supplied in the KAPA Library Quantification Kit. Absolute concentration and minimal lot-to-lot variation is guaranteed. At 200 pM, however, DNA Standard 0 is more dilute than most Illumina libraries. If DNA Standard 0 is diluted to the same extent as the samples to be assayed, the Cq scores for the dilutions are therefore likely to be a few cycles higher than for the libraries. This is acceptable, as long as at least one of the dilutions of Standard 0 falls within the dynamic range of the assay. Please note that the KAPA Library Quantification Dilution Control (DNA Standard 0) is not suitable as a sequencing control, as it is a homogenous solution of a single species of dsDNA and not a library.
- An existing, previously sequenced library is a valuable internal control, as both qPCR-based concentration and cluster density data will be available for such a control. The biggest risk of this control is degradation of DNA quality over time, particularly if the same library is used repeatedly as an internal control. The best approach is to select one or more internal controls from a pool of recently prepared and sequenced libraries, which have been stored in a buffered solution at -20°C, and have not been subjected to too many freeze-thaw cycles. Single-use aliquots of libraries can be prepared and stored at -20°C for use as controls.
- The use of PhiX as an internal library quantification control has similar advantages as that of a previously sequenced library. However, PhiX is not recommended if only one internal control is included in a library quantification assay, due to reported batch-to-batch variation in the given concentration and average fragment length of different lots.

Replicates, Data Reliability, Throughput, and Per-sample Cost

qPCR is an extremely sensitive measurement technique that is vulnerable to variation arising from a number of sources. Triplicate qPCRs are recommended for DNA Standards, library samples, and controls.

The number of replicates may be reduced to two in order to increase throughput and reduce per-sample cost. When selecting the best strategy for your workflow and throughput requirements, keep in mind that the reliability of data is inversely proportional to the number of replicates. Reducing the number of replicates increases the risk of having to re-assay libraries if reliable data was not obtained.

The risk of reducing the number of replicate qPCRs can be mitigated by designing workflows in such a way that at least two serial dilutions of each library are always assayed, provided that both of these dilutions fall within the dynamic range of the assay.

For high-throughput library construction pipelines, automated library quantification in 384-well format is highly recommended, as this offers the possibility of quantifying 96 libraries in triplicate in a single run, while reducing the per-sample cost by performing 10 µL qPCRs.

Assay Automation

Library quantification with the KAPA Library Quantification Kit is amenable to automation and the use of automated liquid handling platforms is highly recommended for high-throughput library quantification workflows.

Pre-validated KAPA Library Quantification methods are available from selected suppliers of automated liquid handling platforms. For more information, please contact Technical Support at **kapabiosystems.com/support**.

KAPA Library Quantification Kit
Illumina® Platforms Technical Data Sheet

Process Workflow