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The effects of torula yeast as a protein source on apparent total tract digestibility, inflammatory markers, and fecal microbiota dysbiosis index in Labrador Retrievers with chronically poor stool quality

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Abstract

This study examined the effects of varying protein sources on apparent total tract digestibility, inflammatory markers, and fecal microbiota in Labrador Retrievers with historically poor stool quality. Thirty dogs (15 male, 15 female; aged 0.93 to 11.7 yr) with stool quality scores ≤2.5 on a 5-point scale (1 representing liquid stool and 5 representing firm stool) were randomly assigned to 1 of 3 nutritionally complete diets with differing protein sources and similar macronutrient profiles: 1) chicken meal ($n = 10$); 2) 10% brewer's yeast ($n = 10$); or 3) 10% torula yeast ($n = 10$). Another 10 dogs (five male, five female) with normal stool quality (scores ranging from 3 to 4) received diet 1 and served as negative control (NC). All dogs were fed diet 1 for 7 days, then provided their assigned treatment diets from days 7 to 37. Daily stool scores and weekly body weights were recorded. On days 7, 21, and 36, blood serum was analyzed for c-reactive protein (CRP), and feces for calgranulin C (S100A12), α_1-proteinase inhibitor (α_1-PI), calprotectin, and microbiota dysbiosis index. Apparent total tract digestibility was assessed using the indicator method with 2 g titanium dioxide administered via oral capsules. Stool scores were greater in NC ($P < 0.01$) as designed but not affected by treatment × time interaction ($P = 0.64$). Body weight was greater ($P = 0.01$) and CRP lower ($P < 0.01$) in NC dogs. Dry matter and nitrogen-free extract digestibility did not differ among groups ($P \geq 0.14$). Negative controls had greater fat digestibility compared to BY (94.64 ± 1.33% vs. 91.65 ± 1.25%; $P = 0.02$). The overall effect of treatment was significant for protein digestibility ($P = 0.03$), but there were no differences in individual post hoc comparisons ($P \geq 0.07$). Treatment did not affect S100A12 or α_1-PI ($P \geq 0.44$). Calprotectin decreased at a greater rate over time in TY ($P < 0.01$). The dysbiosis index score for BY and TY fluctuated less over time ($P = 0.01$). *Blautia* ($P = 0.03$) and *Clostridium hiranonis* ($P = 0.05$) abundances were reduced in BY and TY. Dogs with chronically poor stool quality experienced reduced body weights and increased serum CRP, but TY numerically increased protein digestibility, altered the microbiome, and reduced fecal calprotectin. Torula yeast is a suitable alternative protein source in extruded canine diets, but further research is needed to understand the long-term potential for improving the plane of nutrition and modulating gut health.

Lay Summary

Pet and human populations continue to grow and compete for nutritious, sustainable protein sources. The incorporation of alternative proteins like torula yeast can provide a solution to this problem. Torula yeast also may have additional health benefits like reducing gut inflammation. To test its effects in dogs, we fed Labrador Retrievers with chronically poor stool quality either a control diet with chicken meal, a diet with 10% brewer's yeast, or a diet with 10% torula yeast. We compared their responses to dogs with normal stool quality fed the control diet. Dogs with chronically poor stool quality had lower body weights and increased systemic inflammation compared to those with good stool quality. Calprotectin, a marker of gut inflammation, was reduced more in dogs fed torula yeast than in dogs fed chicken meal. Torula and brewer's yeast also changed the abundance of certain gut bacteria. Torula yeast may be added to dog diets with no negative effects and can alter the gut environment in Labrador Retrievers with chronically poor stool quality.

Key words: digestibility, dog, gut health, Labrador Retriever, torula yeast

Abbreviations: AAFCO, the Association of American Feed Control Officials; ADC, apparent digestibility coefficient; AOAC, Association of Official Analytical Chemists; BY, poor stool dogs on a diet containing 10% brewer's yeast; CON, control diet with chicken meal as a main protein source; CRP, c-reactive protein; ELISA, enzyme-linked immunosorbent assay; IBD, inflammatory bowel disease; ME, metabolizable energy; NC, normal stool dogs on a control diet with chicken meal as a main protein source; NFE, nitrogen-free extract; NRC, National Research Council; PC, poor stool dogs on a control diet with chicken meal as a main protein source; S100A12, calgranulin C; TY, poor stool dogs on a diet containing 10% torula yeast; α_1-PI, alpha$_1$-proteinase inhibitor

Introduction

Numerous new pet food formats have been introduced to the rapidly growing pet food industry, as environmentally conscious consumers influence market trends. Protein quality and the sustainability of protein sources are of mounting concern for pet owners and consumers, who are increasingly choosing

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novel and responsibly sourced ingredients that reflect their own food purchases (Joseph et al., 2020; Kerwin, 2023). Protein is an essential dietary component that can be animal-based or plant-based, but not all protein sources are created equal in terms of amino acid or nutrient digestibility when accounting for overall crude protein content (Bednar et al., 2000; Sousa et al., 2020). In addition, consumers are also becoming progressively interested in functional ingredients that support their pet's well-being, such as improving stool quality or maintaining a healthy gut microbiome. As more consumers shift away from animal-based proteins in both their own diet as well as their pets' diet, technology is evolving to sustainably meet the growing demand for alternative protein sources. Dietary yeast has emerged as a promising functional protein source in companion animal diets. Previous studies have shown beneficial modulatory effects of *Saccharomyces cerevisiae* yeast products on colonic microbiota in canines (Lin et al., 2019). Torula yeast (*Cyberlindnera jadinii*, anamorph name *Candida utilis*), another alternative dietary yeast, is unique in its robust fermentative capacity and strong ability for amino acid synthesis and protein secretion (Sousa-Silva et al., 2021). Torula yeast is driving an emerging technology that upcycles forestry and agriculture co-products to generate a protein-rich food ingredient (Reed and Nagodawithana, 1991; Arbiom, 2020). Ease and flexibility of cultivation combined with the potential to convert low-value non-food waste from industry into a useful protein, while avoiding additional pressure on existing agricultural systems, makes torula yeast a promising alternative to traditional plant-based protein sources such as corn and soy, or emerging plant proteins such as pea.

Torula yeast has historically been viewed as a functional animal feed additive and protein source for poultry, pigs, and aquaculture diets (Øverland and Skrede, 2017; Cruz et al., 2020; Lagos and Stein, 2020). Pig diets containing torula yeast provided greater amino acid digestibility than similar diets containing fishmeal as the protein source (Lagos and Stein, 2020). Its protein content and amino acid profile make it a suitable candidate for inclusion in dog food (Øverland and Skrede, 2017). In addition, dietary torula yeast has beneficial health-modulating effects on the intestinal tract, as observed in vitro and in vivo. Using a Caco2/THP-1 co-culture in vitro model for intestinal cell health, researchers demonstrated that aqueous extracts of torula yeast improved cellular health, as seen through increased transepithelial electrical resistance, and increased anti-inflammatory cytokines (Marzorati et al., 2023). Weanling pigs fed diets with torula yeast in place of animal-based protein sources had improved fecal scores and reduced instances of diarrhea (Espinosa et al., 2023). Furthermore, weanling pigs consuming torula yeast had reduced circulating inflammatory cytokines and increased gain-to-feed ratios (Espinosa et al., 2020, 2023). The improved digestibility and beneficial modulatory effects on gut health make torula yeast an appealing alternative protein source for today's pet food consumer. However, there have been limited studies investigating the digestibility, bioactive functions, and acceptability of torula yeast when used as a significant protein source in an extruded diet for canine companion animals.

We, therefore, aimed to assess the effects of a commercially available torula yeast protein product when used in extruded canine kibble diets. Given the previous data on swine, we hypothesized that torula yeast would improve stool quality and modulate gut health and inflammation when compared to animal-based proteins or other yeast sources. This study investigated the effects of torula yeast on apparent total tract digestibility, stool quality, inflammation markers, and the fecal microbiota dysbiosis index in Labrador Retrievers with chronically poor stool quality.

Materials and Methods

All experimental procedures were approved by the Institute of Animal Care and Use Committee at Four Rivers Kennel under Protocol FRK-40.

Animals and Housing

Forty working Labrador Retrievers (20 male and 20 female) were enrolled in this study. Ages ranged from 11 months to 11 years and 8 months, with an average of 4.42 ± 3.66 years (mean ± SD). Thirty dogs in the colony with a history of poor-quality stool, but otherwise healthy, were identified before the start of the study based on kennel stool quality records. Dogs were confirmed as actively having poor stools by assessing stool quality for seven consecutive days before treatment assignments. Dogs with an average score of 2.5 or less on a scale of 1 to 5 (see Stool quality methods section) were blocked by sex and stool score and randomly assigned to one of the three treatment diets. Another 10 dogs with an average stool score between 3 and 4 after 1 wk were considered normal stool quality dogs and selected as a negative control. All dogs were individually housed in indoor, temperature-controlled kennels made from galvanized chain-linked fencing (1.22 m × 1.83 m × 1.83 m × 1.83 m) and were provided daily access to outdoor socialization yards for 6 to 8 h per day, weather permitting. Animals had free access to automatic waterers in both the kennels and the yards. All animals were up to date on vaccinations and received monthly prophylactic heartworm and parasite prevention. Body weights were measured weekly on each animal. All dogs in this study, regardless of stool scorings, were classified as healthy at the time of their annual vaccinations and general veterinary wellness exam, and considered healthy at the start of the study if there were no recorded health issues within that 4-mo span.

Diet and treatments

Diet compositions are listed in Table 1 and nutrient analysis of diets are listed in Table 2. Three extruded kibble diets were formulated to meet the Association of American Feed Control Officials (**AAFCO**) nutrient requirements for adult dog maintenance (AAFCO, 2022) with varying sources of protein: 1) control diet with chicken meal as a primary protein source (**CON**, $n = 20$); 2) diet containing 10% brewer's yeast (Brewer's EXL, ICC Brazil, São Paulo, State of São Paulo, Brazil; **BY**, $n = 10$); 3) diet containing 10% torula yeast (SylPro, Arbiom, Durham, NC; **TY**, $n = 10$). The CON diet was fed to both the negative control group (dogs with normal stool, **NC**, $n = 10$) and the positive control group (dogs with poor stool quality, **PC**, $n = 10$). Diets were produced with similar levels of metabolizable energy, crude protein, acid-hydrolyzed fat, and crude fiber. Poor stool quality dogs were randomly assigned to one of the three diets. All dogs were given a 1-wk acclimation period on the control diet beginning on day 0, then switched to their assigned treatment diets which were fed from day 7 until the end of the study at day 37. The diets were weighed out in grams each day and dogs were provided 30 min to consume diets. Orts remaining after 30 min

Table 1. Composition of diets on an as-fed basis

Ingredient, %	Treatment diets[1]		
	CON[2]	BY	TY
Corn, ground	38.96	38.96	38.96
Chicken meal	26.36	16.36	16.36
Wheat, ground	11.64	11.64	11.64
Torula yeast[3]	–	–	10.00
Brewer's yeast[4]	–	10.00	–
Rice, brewer's	5.00	5.00	5.00
Beet pulp	5.00	5.00	5.00
Egg, dried	1.01	1.01	1.01
Flaxseed	1.01	1.01	1.01
Salt, plain	0.54	0.54	0.54
Potassium chloride	0.50	0.50	0.50
Mixed tocopherols	0.20	0.20	0.20
L-Lysine	0.20	0.20	0.20
DL-Methionine	0.17	0.17	0.17
Dog and cat vitamin premix[5]	0.12	0.12	0.12
Dog and cat mineral premix[5]	0.10	0.10	0.10
Choline chloride 60%	0.10	0.10	0.10
Poultry fat (top coating)	9.09	9.09	9.09

[1]CON: control diet; BY: 10% brewer's yeast diet; TY: 10% torula yeast diet.
[2]CON diet fed to both negative control (NC) and positive control (PC) dogs.
[3]SylPro (Arbiom, Durham, NC, USA).
[4]Brewer's EXL (ICC Brazil, São Paulo, State of São Paulo, Brazil).
[5]Nutra Blend LLC, Neosho, MO, USA.

Table 2. Analyzed values for diet compositions. Items, except for moisture and metabolizable energy (ME), are presented on a dry-matter basis

Item	Treatment diets[1]		
	CON[2]	BY	TY
Moisture, %	7.22	7.12	6.99
Crude protein, %	26.62	26.06	26.13
Acid-hydrolyzed fat, %	13.26	13.14	12.69
Crude fiber, %	2.16	2.11	2.11
Ash, %	6.60	6.60	6.63
Calcium, %	0.88	0.83	0.87
Phosphorus, %	0.96	0.96	0.95
ME, kcal/kg[3]	3796.66	3796.38	3781.50

[1]CON: control diet; BY: 10% brewer's yeast diet (Brewer's EXL, ICC Brazil, São Paulo, State of São Paulo, Brazil); TY: 10% torula yeast diet (SylPro, Arbiom, Durham, NC).
[2]CON diet fed to negative control (NC) and positive control (PC) dogs.
[3]Estimated using NRC equations for prepared dog foods (NRC, 2006).

were collected, weighed, and recorded. Males were offered on average 585 ± 16.7 g of feed per day and females were offered 480 ± 13.8 g of feed per day. Feed amounts were offered to satisfy the maintenance energy requirements on all dogs based on historical feeding records. Males weighed more than females (31.18 ± 0.65 kg vs. 26.62 ± 0.65 kg) and therefore required higher caloric intake to maintain body weight.

Digestibility

In the last week of the trial, apparent total tract digestibility was estimated using the indicator method (Alvarenga et al., 2019; AAFCO, 2022). A slight modification of the AAFCO published method was used because the indicator was not initially included in the extruded diets: dogs received 2 g of titanium dioxide orally via gelatin capsules daily for 5 days. Fecal samples were collected every day beginning 24 h after the first titanium dioxide dose for 4 days and thoroughly homogenized. After stool collections, equal aliquots from each daily sample were mixed and homogenized for proximate analysis testing at an ISO 17025:2017 accredited outside laboratory (Nestle Purina Analytics Labs, St. Louis, MO) using methods approved by the Association of Official Analytical Chemists (AOAC, 2023). Samples of each diet were also sent to the same laboratory for concurrent testing. Moisture was analyzed by loss on drying at 133 °C (AOAC 930.15), protein by Kjeldahl (AOAC 988.05), fat by acid hydrolysis (AOAC 954.02), crude fiber (AOAC 962.09), ash at 600 °C (AOAC 942.05), and titanium dioxide by inductively coupled plasma atomic emission (AOAC 990.08). Nitrogen-free extract (**NFE**) was calculated as 100 – (% moisture + % crude protein + % fat + % crude fiber + % ash). Metabolizable energy was estimated using National Research Council (**NRC**) published equations for prepared dog food (NRC, 2006). Apparent digestibility coefficients (**ADC**) were calculated for dry matter, crude protein, acid-hydrolyzed fat, and NFE using the following published equations (Bosch et al., 2009):

$$Nutrient_{flow} = Nutrient_f \; x \; \frac{TiO_{2i}}{TiO_{2f}}$$

$$ADC\ (\%) = \frac{Nutrient_i - Nutrient_{flow}}{Nutrient_i} \; x \; 100\%$$

where $Nutrient_{flow}$ is measured g/d, $Nutrient_f$ represents the nutrient content of the feces in g/kg, TiO_{2i} represents the consumed TiO_2 in g, TiO_{2f} represents the TiO_2 content of the feces in g/kg, and $Nutrient_i$ is the nutrient intake in g/d. All values were analyzed on a dry matter basis.

Stool quality

Stool quality was recorded each day for the duration of the trial. Study technicians were trained and evaluated for proper stool scoring before beginning the trial to ensure consistency. Stool quality was scored based on what each dog had produced overnight in individual kennels. If a dog had not produced a stool sample overnight, the dogs were accompanied outside individually to obtain a stool score. Scoring was performed as follows: 1—liquid diarrhea, no form, could be poured; 2—loose diarrhea, no form, will take the shape of a container; 3—very moist, soft, and partially formed; 4—firm, well-formed, easy to pick up and leaves no marks on floor; 5—little to no moisture, hard and crumbled easily.

Sample collection and analysis

Blood samples were collected around 9 a.m. prior to feeding on days 7, 21, and 36 via jugular venipuncture by a trained technician into vacutainer tubes containing clot activator and separator gel additive (BD Vacutainer SST Tubes, BD, Franklin Lakes, NJ). Technicians collected 5 to 10 ml of blood for

each dog at each time point. Tubes were left to clot for at least 30 min at room temperature, then centrifuged at 1,500 × *g* for 15 min. Serum was transferred to 2.0 ml polypropylene tubes and frozen at −80 °C until analysis. C-reactive protein (**CRP**) was analyzed with a canine-specific enzyme-linked immunosorbent assay (**ELISA**) kit (#ab157698, Abcam, Cambridge, UK). Serum for CRP analysis was diluted 1:1000 and the protocol was followed per the manufacturer's instructions.

Fecal samples were collected on days 7, 21, and 36 for microbiota dysbiosis index, α_1-proteinase inhibitor (**α_1-PI**), calprotectin, and calgranulin C (**S100A12**) analysis. Stool was collected beginning at 7:30 a.m., placed in a Ziplock bag, and refrigerated for no more than 2 h until processed. The majority of stool samples collected for processing were produced at an unknown time while the dogs were housed in their individual kennels overnight. Roughly 1 g of sample was collected and stored in a 2.0 ml polypropylene tube for analysis of microbiota dysbiosis index through Texas A&M Veterinary Medical Diagnostic Lab (College Station, TX) through methods as previously described (AlShawaqfeh et al., 2017). Briefly, a quantitative polymerase chain reaction panel is used to identify the abundance of total bacteria, *Faecalibacterium, Turicibacter, Escherichia coli, Streptococcus, Blautia, Fusobacterium,* and *Clostridium hiranonis*. Data are expressed as log amount of DNA (fg) for each bacterial group per 10 ng of total isolated DNA. Calculation of a single numerical value, the dysbiosis index, is computed through a mathematical algorithm (AlShawaqfeh et al., 2017) where values less than 0 indicate eubiosis, values between 0 and 2 are equivocal, and values above 2 indicate significant dysbiosis (Pilla et al., 2020). Another sample, approximately 1 g, was collected in pre-weighed stool collection tubes, frozen at −80 °C, and sent to Texas A&M University Gastrointestinal Laboratory (College Station, TX) for analysis of α_1-PI via radioimmunoassay as previously described (Heilmann et al., 2011, 2013). Calprotectin and S100A12 were extracted from 0.1 g of wet feces by diluting with 1 ml of phosphate-buffered saline (pH 7.4, calcium chloride and magnesium chloride free; Sigma–Aldrich, St. Louis, MO), vortexing for 30 s, then centrifuging at 2,500 × *g* for 20 min. Supernatant was collected and stored at −80 °C until in-house analysis using canine-specific ELISA kits following manufacturer's protocols (Calprotectin: #MBS2706865; S100A12: #MBS1607820; MyBioSource, San Diego, CA). No further sample dilution was used in these two assay kits.

Statistical analysis

Statistical analysis of apparent digestibility and feed refusal data was performed in JMP Pro 16.0.0 (SAS Institute, Cary, NC). Feed consumption data were analyzed first as a proportion of feed consumed to feed offered, given an arcsine square root transformation. Feed consumption was then also analyzed by the amount of feed consumed in grams and feed consumption in grams per kg of body weight, both averaged for individual dogs over the entire study. Feed consumption and ADC were analyzed with one-way ANOVA followed by Tukey's HSD post hoc test. Stool quality, weights, biomarkers, and microbiota dysbiosis endpoints were analyzed in SAS Studio 3.8 (SAS Institute, Cary, NC) using a repeated measures mixed model (PROC MIXED) with Tukey–Kramer adjustment. Covariance structure selection was based on variance and covariances of the unstructured model and the lowest corrected Akaike information criterion and lowest Bayesian information criterion. All models included the effects of sex, treatment, time, and treatment × time interaction. Any model with a significant effect of sex was further analyzed to include the full factorial sex × treatment × time interactions. Serum CRP data and fecal α_1-PI data were given a log transformation to satisfy the normality of errors assumption. Significance was set at $P \leq 0.05$. Data are presented as least square means with their standard errors. Transformed data are presented as the back-transformed means with standard error estimations.

Results

Feed consumption and digestibility

Although numerically minor, there were significant differences in the average percentage of daily feed consumption from feed offered across groups during the study ($P < 0.01$). The experimental diet containing BY consumed more of their daily ration throughout the study, while the PC animals had the greatest refusal (Table 3). Both yeast-supplemented diets increased feed consumption among dogs with poor stool quality. The total amount of feed consumed in grams was also greater in BY while the NC group consumed the least ($P < 0.01$, Table 3). On a g/kg body weight basis, the NC dogs had lower consumption compared to PC, BY, and TY dogs ($P < 0.01$, Table 3).

Apparent digestibility coefficients are presented in Table 3. There were no significant changes in apparent dry matter digestibility ($P = 0.14$) nor apparent NFE digestibility ($P = 0.36$) among treatments. Treatment diet significantly affected protein ($P = 0.03$) and fat ($P = 0.02$) digestibility. While the overall effect for protein ADC was significant, there were no significant differences among treatments with Tukey's post hoc ($P \geq 0.07$). Comparison between the NC and PC dogs was closest to approaching significance ($P = 0.07$). Fat digestibility was reduced in BY compared to NC with TY and PC groups intermediate ($P = 0.02$).

Stool quality

As was anticipated from our study design, stool quality scores significantly differed among treatment groups ($P < 0.01$, Figure 1), with the NC group having better stool quality scores (3.31 ± 0.12) than PC (2.39 ± 0.12), BY (2.27 ± 0.12), and TY (1.97 ± 0.12) groups. There were no significant differences in stool quality scores among PC, BY, and TY, but there was tendency by post hoc analysis for PC to be slightly better than TY ($P \geq 0.07$). There was an effect of time ($P < 0.01$, Figure 1), although this reflected more day-to-day variation than any general overarching trends, as there were no differences by Tukey's post hoc analysis between days 1 and 36 ($P = 0.84$). There was no treatment × time interaction ($P = 0.64$).

Body weights

Body weights differed significantly among treatments, with normal stool quality dogs in the NC group weighing more than the poor stool quality dogs in all other treatments ($P < 0.01$, Table 4). Weights recorded 2 wk prior to the start of the study followed a similar pattern, with NC weighing more than groups PC, BY, and TY ($P = 0.01$). There was an effect of sex, as expected, with males weighing more than females (31.18 ± 0.65 kg vs. 26.62 ± 0.65 kg); however, sex did not affect any of the outcomes as observed by insignificant

sex × time, sex × treatment, or sex × time × treatment interactions ($P \geq 0.34$). Lastly, body weights differed over time ($P < 0.01$). This was mainly attributed to week 3 having significantly lower weights than any other week. This may have been attributed to equipment or technician error during weight measurements in that week, as there were no major changes to dogs' diets or exercise habits throughout the study. Weights did not differ between the first and last week of the study ($P = 0.86$).

Serum CRP

There was an effect of sex and treatment on serum CRP levels (Figure 2). Males had higher levels of CRP overall compared to females (5.33 ± 0.49 mg/l vs. 3.06 ± 0.86 mg/l; $P = 0.02$). Negative control dogs had significantly lower levels of serum CRP compared with PC, BY, and TY groups (1.63 ± 0.38 mg/l vs. 5.22 ± 1.19 mg/l, 4.51 ± 1.03 mg/l, 6.78 ± 1.56 mg/l; $P < 0.01$). There were no effects of time or any interactions among sex, time, or treatment ($P \geq 0.09$). General veterinary reference intervals classify healthy dogs as having CRP concentrations less than 10 to 20 mg/l (Klenner et al., 2010; Hillström et al., 2016; Hindenberg et al., 2018). Treatment averages remained below this threshold, and individual dogs fell below the 20 mg/l threshold.

Fecal biomarkers

All of the observed fecal biomarkers were significantly affected by time. Fecal α_1-PI decreased at day 36 in comparison to days 7 and 21 ($P = 0.05$, Figure 3A). Concentrations of fecal S100A12 increased from days 7 to 36 ($P = 0.01$, Figure 3C). Calprotectin levels significantly declined across all time points ($P < 0.01$, Figure 3B). Neither treatment nor sex affected fecal biomarker concentrations ($P \geq 0.09$). There was also no significant treatment × time interactions for S100A12 or α_1-PI ($P \geq 0.21$), but there was for calprotectin concentrations ($P < 0.01$). The TY diet was able to reduce fecal calprotectin levels in poor stool quality dogs by day 36 at a greater rate than occurred in the NC and PC dogs, with BY intermediate.

Microbiota dysbiosis index

All dogs at all time points had microbiota dysbiosis indexes less than 2, indicating at no point did any animal experience significant dysbiosis (Figure 4). There were no effects of sex or treatment overall ($P \geq 0.21$). There was a significant effect of time ($P < 0.01$) where all samples had a reduction in dysbiosis index by day 21 but then increased back to baseline by day 36 of the study. There was a significant treatment × time interaction ($P = 0.04$). The dysbiosis index decreased at day 21 significantly for the NC and BY groups ($P < 0.01$). All values fell below 0 on day 21, indicating there were no shifts in the microbiota diversity associated with chronic enteropathies at that time. The dysbiosis index significantly increased from days 21 to 36 for the NC and PC groups ($P < 0.01$). All dysbiosis indexes were around 0 at day 36, indicating minimal shifts in the microbiota diversity.

The individual taxa *Faecalibacterium, Turicibacter, Streptococcus, Escherichia. coli, Blautia, Fusobacterium*, and *Clostridium hiranonis* were identified as part of the dysbiosis index analysis (Table 5). As the dysbiosis index shifted with time point, so did the abundance of certain taxa. *Faecalibacterium* ($P < 0.01$) and *Fusobacterium* ($P < 0.01$) abundances increased after day 7. *Streptococcus* decreased from days 7 to 21, but then increased back to starting levels by day 36 ($P < 0.01$). *E. coli* abundance was greater at day 36 in all groups compared to day 7 ($P = 0.05$), with day 21 intermediate. *Blautia* abundance decreased after day 7 ($P < 0.01$). *Clostridium hiranonis* decreased at day 21 and then decreased further by 36 ($P < 0.01$). *Turicibacter* was the only genus not significantly affected by time, treatment, or treatment × time interaction ($P \geq 0.06$). There was no effect of sex on any bacterial genus ($P \geq 0.21$).

Three taxa were affected by treatment. *Blautia* was decreased in BY and TY groups compared to NC and PC ($P < 0.01$). *Fusobacterium* was reduced in TY compared to PC, with NC and BY groups intermediate ($P = 0.03$). There was also a significant treatment × time interaction ($P = 0.01$, Figure 5), where TY diets increased *Fusobacterium* abundance at a slower rate than NC or PC from days 7 to 21 and continued to increase from days 21 to 36 while NC and

Table 3. Apparent total tract digestibility coefficients and average feed consumption by treatment group.

Item	Treatment[1]				*P*-value
	NC	PC	BY	TY	
Digestibility, %[2]					
Dry matter	85.67 ± 2.53	82.65 ± 2.40	83.65 ± 2.40	90.76 ± 2.68	0.14
Crude protein[3]	75.52 ± 5.01	58.91 ± 4.68	58.26 ± 4.42	72.18 ± 5.01	0.03
Acid-hydrolyzed fat	94.64 ± 1.33[a]	91.65 ± 1.25[ab]	88.63 ± 1.17[b]	92.69 ± 1.33[ab]	0.02
Nitrogen-free extract[4]	87.98 ± 2.21	85.18 ± 2.21	82.62 ± 2.21	86.89 ± 2.05	0.36
Consumption, %[5]	98.5 ± 0.01[b]	95.7 ± 0.01[c]	99.8 ± 0.01[a]	99.3 ± 0.01[b]	<0.01
Consumption, g/d	433.56 ± 20.46[b]	489.56 ± 20.46[ab]	544.75 ± 20.46[a]	513.54 ± 20.46[a]	<0.01
Consumption, g/d per kg BW[6]	13.62 ± 0.68[b]	17.70 ± 0.68[a]	19.42 ± 0.68[a]	18.82 ± 0.68[a]	<0.01

[1]NC: negative control; PC: positive control; BY: 10% brewer's yeast (Brewer's EXL, ICC Brazil, São Paulo, State of São Paulo, Brazil); TY: 10% torula yeast (SylPro, Arbiom, Durham, NC).
[2]Crude protein, acid-hydrolyzed fat, and nitrogen-free extract ADC are presented on a dry-matter basis.
[3]Tukey's post hoc analysis did not yield significant ($P < 0.05$) differences among groups.
[4]Nitrogen-free extract % = 100 – (% moisture + % crude protein + % acid-hydrolyzed fat + % crude fiber + % ash).
[5]Percentage of feed offered.
[6]BW: body weight.
[abc]Differing letter superscripts within rows denote significant differences among treatments ($P < 0.05$).

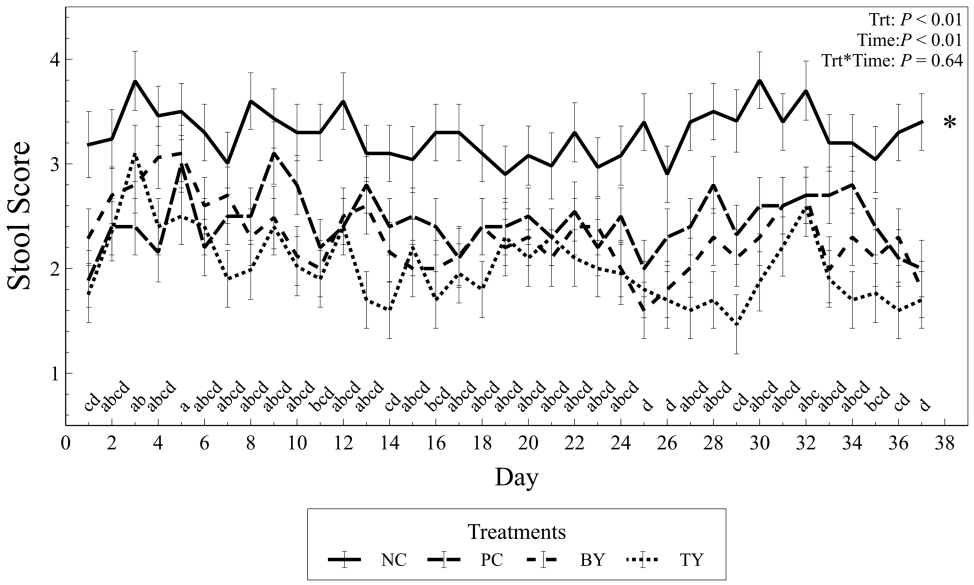

Figure 1. Average stool scores by day and treatment over the course of the study. Treatments were as follows: (NC) dogs with normal stool quality fed control diet; (PC) dogs with poor stool quality fed control diet; (BY) dogs with poor stool quality fed diet with 10% brewer's yeast; (TY) dogs with poor stool quality fed diet with 10% torula yeast. Asterisks represent statistical significance by treatment ($P < 0.01$). Differing superscripts (abcd) represent statistical significance by time ($P < 0.01$).

Table 4. Body weights by sex, treatment group, and week, as well as overall average for body weight by treatment

Time point	Treatment[1]								SEM
	NC		PC		BY		TY		
	Male	Female	Male	Female	Male	Female	Male	Female	
Week 0	34.27	29.34	30.62	25.82	31.06	25.07	28.54	26.90	1.35
Week 1	35.06	29.20	29.29	25.46	30.74	25.40	28.08	26.63	1.35
Week 2	34.61	29.54	30.23	25.66	31.75	25.56	29.18	26.44	1.35
Week 3	34.28	28.33	29.26	25.56	30.25	24.24	28.79	25.34	1.35
Week 4	34.94	29.83	30.25	26.00	31.45	25.79	29.17	25.87	1.35
Week 5	34.98	29.67	31.32	25.85	31.60	25.90	28.65	25.53	1.35
Overall[2]	32.01[a]		27.94[b]		28.23[b]		27.43[b]		0.92

[1]NC: negative control; PC: positive control; BY: 10% brewer's yeast (Brewer's EXL, ICC Brazil, São Paulo, State of São Paulo, Brazil); TY: 10% torula yeast (SylPro, Arbiom, Durham, NC).
[abc]Differing superscripts within rows denote significant differences between treatments ($P < 0.01$).

PC had leveled out. *Clostridium hiranonis* was reduced in TY compared to NC, with PC and BY groups intermediate ($P = 0.05$). Decreases in these particular genera have been associated with increased dysbiosis; however, it should be noted both these genera fell within normal ranges considered for the dysbiosis index in canines (AlShawaqfeh et al., 2017), and the average dysbiosis score per treatment never exceeded 0.5 which remains in the equivocal range.

Discussion

This study aimed to evaluate the effects of torula yeast on stool quality, fecal, and serum inflammatory markers, and fecal microbiota dysbiosis index in Labrador Retrievers with a history of poor stool quality. Expanding human and pet populations puts increasing pressure on food systems to sustainably support both humans and animals. Protein is the most demanding nutrient in terms of economic and ecological impact, which makes it an effectual target to modulate the sustainability of pet foods (Swanson et al., 2013). The inclusion of alternate protein sources such as torula yeast can help alleviate this demand. Torula yeast was previously utilized in feline diets with good palatability and digestibility (Holt and Aldrich, 2022); however, to the authors' knowledge, no work has been published on the inclusion of torula yeast in canine diets and its potential as a functional protein source.

The addition of torula yeast to an extruded kibble diet did not improve stool quality in dogs with chronically poor stool consistency. We hypothesized that torula yeast would improve stool quality given that previous research in weanling pigs demonstrated reduced incidences of diarrhea and improved fecal consistency after being fed diets containing torula yeast (Espinosa et al., 2023). Stool scores varied by day, but there were no overall trends in stool quality, and scores did not differ from the first to last days of the study. Discrepancies from day-to-day could be partially attributed to scoring technician error or natural fluctuations in daily stool quality. While all technicians were trained in stool assessment, the scoring

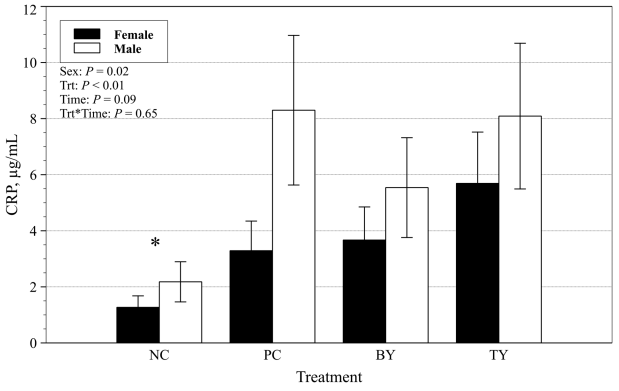

Figure 2. Average serum c-reactive protein (CRP) concentrations (µg/ml) by treatment and sex over the course of the study. Treatments were as follows: (NC) dogs with normal stool quality fed control diet; (PC) dogs with poor stool quality fed control diet; (BY) dogs with poor stool quality fed diet with 10% brewer's yeast; (TY) dogs with poor stool quality fed diet with 10% torula yeast. Asterisks represent statistical significance by treatment ($P < 0.01$).

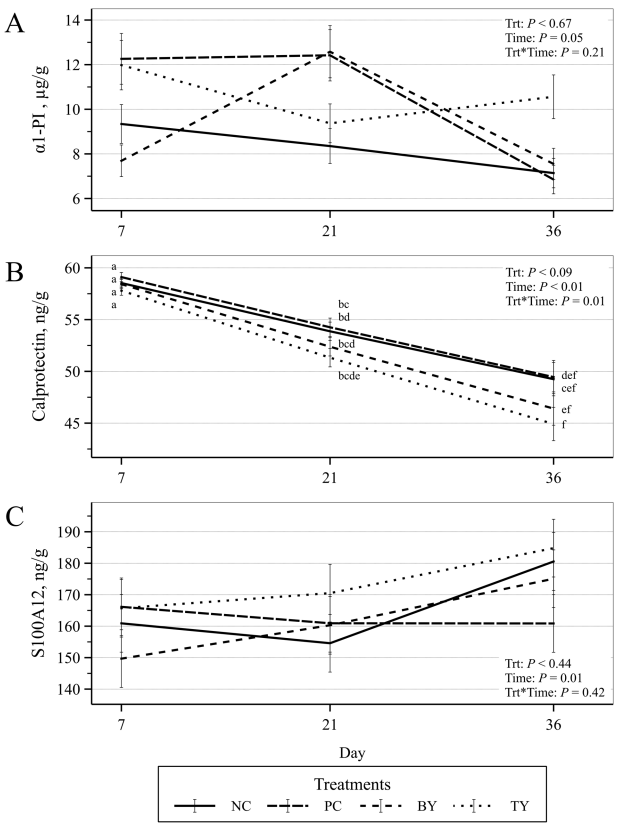

Figure 3. Concentrations of fecal biomarkers, (A) alpha 1 proteinase inhibitor (α_1-PI), (B) calprotectin, and (C) calgranulin C (S100A12), per gram of wet feces by treatment and day. Treatments were as follows: (NC) dogs with normal stool quality fed control diet; (PC) dogs with poor stool quality fed control diet; (BY) dogs with poor stool quality fed diet with 10% brewer's yeast; (TY) dogs with poor stool quality fed diet with 10% torula yeast. Superscripts (abcdef) represent statistical significance within treatment × time interaction ($P = 0.01$).

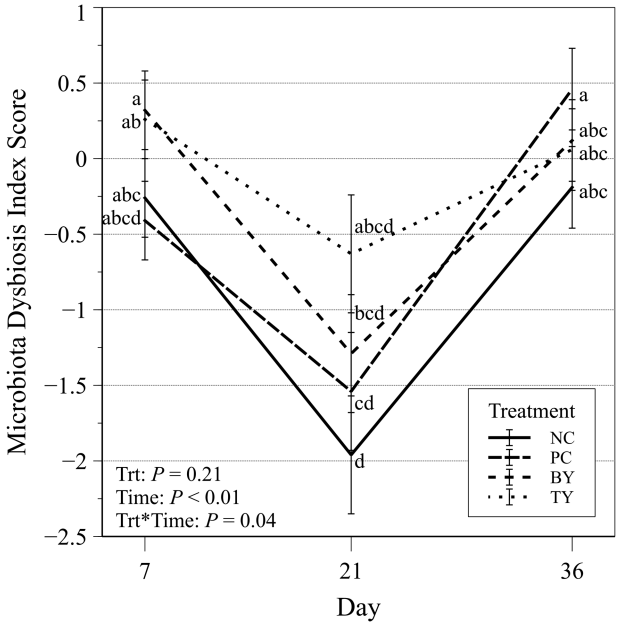

Figure 4. Average microbiota dysbiosis index score by treatment for days 7, 21, and 36 of the study. Dysbiosis index scores < 0 represent normal dysbiosis index with no shifts in the overall diversity. Scores of 0 to 2 are equivocal or potential mildly increased dysbiosis. Scores > 2 represent significant dysbiosis. Treatments were as follows: (NC) dogs with normal stool quality fed control diet; (PC) dogs with poor stool quality fed control diet; (BY) dogs with poor stool quality fed diet with 10% brewer's yeast; (TY) dogs with poor stool quality fed diet with 10% torula yeast. Superscripts (abcd) represent statistical significance within treatment × time interaction ($P = 0.04$).

method used is subjective. We did not formally characterize the underlying causes for the poor stool quality in the PC, BY, and TY dogs, but our model may be inherently different from the weanling pig model resulting in differing results. Diarrhea in weanling pigs has a primarily infectious basis, brought on by the acute stresses of weaning and subsequent functional changes in the gut (Montagne et al., 2007; Rhouma et al., 2017). Our dogs, on the other hand, did not have poor stool quality resulting from infectious disease or acute stressors and could be more reflective of a chronic enteropathy or inflammatory bowel disease (**IBD**) model. However, no clinical diagnosis was made. While it is still unclear how torula yeast modulates the gut environment, different underlying causes of loose stools or gut inflammation may respond differently to dietary torula yeast.

Although there were no clinical cases of IBD in our study dogs, there were distinct physical differences between normal and poor stool dogs. Dogs with chronically poor stool quality in this study had increased serum CRP which corresponds to increased inflammation, reduced body weights, and numerically reduced macronutrient digestibility. However, fecal biomarkers did not differ between our NC and the PC, BY, or TY groups. Typically, levels of α_1-PI, S100A12, and calprotectin are elevated in dogs with significant gut inflammation and chronic enteropathies (Murphy et al., 2003; Heilmann et al., 2016, 2018). Previous studies have demonstrated there can be substantial overlap in these biomarkers between healthy and clinically ill animals, particularly in those without histologic lesions (Melgarejo et al., 1998; Grellet et al., 2013; Heilmann et al., 2016; AlShawaqfeh et al., 2017). Increased inflammation and gut permeability in a pre- or subclinical phase are linked to occurrences of Crohn's disease in humans (Turpin et al., 2020; Lundgren et al., 2021). Poor stool quality dogs in this study, while considered healthy from general physical examination, could be in a pre-clinical or subclinical

state of chronic enteropathies. Further monitoring of animals is needed to confirm any formal diagnosis of IBD, but the consistent loose stools and elevated CRP may provide a model for studying IBD issues such as systemic inflammation or nutrient malabsorption in canines.

While there was an overall effect of treatment on protein digestibility, Tukey's post hoc analysis did not reveal any significant pair-wise comparisons. The large standard errors for protein ADC compared to other digestibility parameters likely reduced power, causing insignificant post hoc comparisons. The ADC in this study for crude protein in NC dogs was similar to previously reported values for a commercial premium diet (75.52 ± 5.01 vs. 77.2 ± 1.44) with similar crude protein values (26.62% vs. 24.4%) (Chiofalo et al., 2019). Numerically, NC had the greatest ADC (75.52 ± 5.01) and PC a low ADC (58.91 ± 4.68) despite being on the same diet, which could be indicative of impaired nutrient absorption. Protein ADC was numerically greater in torula yeast (72.18 ± 5.01) compared to brewer's yeast (58.26 ± 4.42) and PC (58.91 ± 4.68), and not statistically different from the NC (75.52 ± 5.01; $P = 0.24$). Studies have previously reported an increase in protein and amino acid digestibility of torula yeast compared to fish meal in growing pigs (Lagos and Stein, 2020) and compared to brewer's yeast in Atlantic salmon (Øverland et al., 2013). Woody torula yeast also alters gene expression controlling protein and vitamin digestion and absorption in the intestinal mucosa of weanling pigs (Espinosa et al., 2023). In Atlantic salmon, dietary torula yeast significantly increased the expression of the stem-cell marker *Lgr5*, suggesting improved plasticity, resistance to damage, and maintenance of homeostasis in the distal intestine (Hofossæter et al., 2023). Torula yeast extract improved cell barrier function in vitro in Caco-2/THP-1 cell co-cultures (Marzorati et al., 2023). Torula yeast has demonstrated beneficial effects on the gut environment in other species, so it is plausible that it can impart beneficial effects in canines. Replication of this work is needed to reaffirm the observed numeric differences in protein digestibility for dogs with normal and poor stool quality, as well as further investigate the functional properties of torula yeast in canines.

Many of the significant findings in the fecal biomarkers and microbiota were in relation to changes over time and might have resulted from the diet change at the beginning of the study. All treatment diets, including the control diet, differed from the dogs' previous maintenance diet, which may also explain the changes in biomarkers and microbiome of the control animals. The fluctuations in microbiota dysbiosis index scores were likely driven by *Faecalibacterium* increasing and *Streptococcus* decreasing at day 21, which correspond to reduced dysbiosis scores, and then returning to elevated or similar levels by day 36. Previous studies have demonstrated that the canine fecal microbiome shifts rapidly after dietary modifications, but unlike our current study, have suggested the microbiome stabilizes after 2 to 4 wk (Allaway et al., 2020; Lin et al., 2022). This study demonstrated alterations in the microbiome as far as 5 wk out from the diet change. Lin et al. (2022) reported that the stabilization time of the microbiome was dependent on individual taxa and species. Both studies took a metagenomic approach, while this study utilized a more targeted approach of select genera/species associated with chronic enteropathies. The inclusion of dietary yeast in this study, both brewer's and torula, decreased the abundance of *Blautia*. Decreases of *Blautia* in response to feeding yeast have been observed in raccoon dogs and weanling pigs (Iakhno et al., 2022; Zhao et al., 2022); however, other studies in pigs and dogs have reported increases in *Blautia* from yeast supplementation (Lin et al., 2019; Lagos et al., 2020). Another study showed reductions in the *Clostridiaceae* family, containing the *Clostridium* genus, but the authors did not specify if there were any alterations in *Clostridium hiranonis* (Iakhno et al., 2020). One study examined the effects of a probiotic containing *Saccharomyces cerevisiae* (brewer's yeast) in dogs and also utilized the same dysbiosis index score analysis as was used in this study (Bastos et al., 2023). Bastos et al.'s (2023) yeast postbiotic reduced *E. coli* and tended to reduce *Streptococcus* while torula yeast inclusion did not affect either taxon; however, several differences between this study and ours, such as diet composition and the use of classifications based on stool quality, may account for differences in the microbiota outcomes.

Limitations to this study concerning microbiota analysis include the breadth of sequencing analysis and sample collection procedures. Analysis of the microbiota dysbiosis index focuses on only seven bacterial taxa, while more extensive sequencing methods quantify the entirety of the microbiome. As full sequencing was not performed in the current study, changes occurring at the alpha and beta diversity levels, which can be more descriptive of the microbiome's overall functionality, were not determined. Therefore, future studies examining torula yeast in canine diets would benefit from full microbiome 16s or shotgun sequencing. Fecal samples for this study were collected around 7:30 a.m. when kennel staff arrived, but stool may have been produced any time between 5 p.m. the previous day and the morning of collection. Short-term storage (<3 days) of canine and feline fecal samples at room temperature may not significantly impact overall richness, and diversity, but may present changes in individual taxa (Weese and Jalali, 2014; Tal et al., 2017). Collection of samples immediately after defecation or the use of fecal swabs could improve the consistency of microbiota results across studies.

Fecal calprotectin and fecal S100A12 concentrations have been strongly correlated in dogs with chronic inflammatory enteropathies and decreases in both markers are typically associated with improved gut health (Heilmann et al., 2018). The current study demonstrated decreases in calprotectin but increases in S100A12 over time. Further work is needed to examine how environmental aspects can modulate fecal biomarkers in healthy dogs. Heilmann et al. recently reported significantly different levels of S100A12 dependent on the reproductive status and size of dogs (Heilmann et al., 2021). One noted limitation of their study was that because pet dogs were used as opposed to kennel dogs, details on dietary composition were lacking to determine any influences of diet. In addition, pets were on different food brands which could confound any dietary influences. In humans, factors like diet and obesity status affect fecal calprotectin levels (Poullis et al., 2004; Mendall et al., 2016). Considering that there were significant changes in both the NC and PC groups over time as well, the significant time effects in fecal biomarkers and microbiome are likely attributed to acclimation to dietary changes at the start of the study. The treatment × time interaction in calprotectin levels, which decreased at a greater rate in the yeast diets than in the control diet, provides further evidence that dietary adjustment can influence fecal calprotectin.

Table 5. Log amount of DNA (fg) per 10 ng of total isolated DNA of identified bacterial taxa by timepoint in the microbiota dysbiosis index analysis

Item	Treatment[1]				SEM	*P*-value		
	NC	PC	BY	TY		Trt	Time	Trt × Time
Faecalibacterium	5.98	6.03	5.94	5.87	0.15	0.89	<0.01	0.62
Day 7	5.21^{b}	5.19^{b}	5.15^{b}	4.76^{b}	0.20			
Day 21	6.66^{a}	6.50^{a}	6.68^{a}	6.45^{a}	0.14			
Day 36	6.07^{a}	6.39^{a}	6.00^{a}	6.41^{a}	0.32			
Turicibacter	8.22	7.59	7.98	7.68	0.19	0.10	0.06	0.64
Day 7	8.21	7.55	8.07	7.84	0.22			
Day 21	8.32	7.77	8.09	7.59	0.22			
Day 36	8.12	7.43	7.78	7.61	0.22			
Streptococcus	7.23	7.38	7.43	7.46	0.16	0.77	<0.01	0.33
Day 7	7.39^{a}	7.29^{a}	7.79^{a}	7.68^{a}	0.22			
Day 21	6.57^{b}	6.79^{b}	6.83^{b}	6.93^{b}	0.22			
Day 36	7.75^{a}	8.07^{a}	7.66^{a}	7.76^{a}	0.22			
E. coli	5.47	5.41	5.58	5.40	0.25	0.95	0.05	0.28
Day 7	5.26^{b}	4.92^{b}	5.71^{b}	5.24^{b}	0.32			
Day 21	5.29^{ab}	5.41^{ab}	5.46^{ab}	5.50^{ab}	0.32			
Day 36	5.86^{a}	5.91^{a}	5.56^{a}	5.45^{a}	0.32			
Blautia	10.42^{x}	10.43^{x}	10.25^{y}	10.21^{y}	0.05	<0.01	<0.01	0.10
Day 7	10.44^{a}	10.53^{a}	10.35^{a}	10.31^{a}	0.06			
Day 21	10.42^{b}	10.46^{b}	10.18^{b}	10.09^{b}	0.06			
Day 36	10.40^{b}	10.30^{b}	10.22^{b}	10.24^{b}	0.06			
Fusobacterium	8.09^{xy}	8.19^{xy}	7.94^{x}	7.78^{y}	0.10	0.03	<0.01	0.01
Day 7	7.00^{b}	7.06^{b}	6.97^{b}	7.04^{b}	0.14			
Day 21	8.66^{a}	8.78^{a}	8.34^{a}	7.95^{a}	0.14			
Day 36	8.60^{a}	8.73^{a}	8.53^{a}	8.37^{a}	0.14			
Clostridium hiranonis	6.45^{x}	6.44^{x}	6.36^{xy}	6.23^{y}	0.06	0.05	<0.01	0.07
Day 7	6.58^{a}	6.64^{a}	6.63^{a}	6.61^{a}	0.09			
Day 21	6.44^{b}	6.50^{b}	6.32^{b}	6.04^{b}	0.09			
Day 36	6.32^{c}	6.17^{c}	6.14^{c}	6.04^{c}	0.09			

[1]NC: negative control; PC: positive control; BY: 10% brewer's yeast (Brewer's EXL, ICC Brazil, São Paulo, State of São Paulo, Brazil); TY: 10% torula yeast (SylPro, Arbiom, Durham, NC).
^{abc}Differing superscripts within columns denote significant differences over time ($P < 0.05$).
^{xyz}Differing superscripts within rows denote significant differences among treatments ($P < 0.05$).

Calprotectin is primarily secreted by macrophages, while S100A12 is produced by classical monocytes (Singh and Ali, 2022). The differential changes in these biomarkers in this study may reflect changing immune cell populations within the gut, with increasing S100A12 representing an increased classical monocyte presence and decreasing calprotectin representing decreased macrophage populations. This change could be driven by reduced monocyte-to-macrophage differentiation. To date, there is little information on the roles of these cytokines in relation to gut homeostasis as opposed to stimulating the inflammation cascade. Future studies may help clarify the underlying immune mechanisms responsible for the effects of dietary modifications on calprotectin and S1000A12.

Additionally, some limitations concerning animals and diets existed in this study. Of the 40 dogs used in this study, 4 were 11 months of age at the start of the study and had not reached the 1-yr mark typically classifying dogs as adults (Harvey, 2021). All four dogs were from the same litter, distributed as equally among treatments as possible, and reached 12 months of age by the end of the study. The four dogs were chosen to proceed with the study because they had reached their mature body weight and there was limited availability of poor stool dogs in the kennel. Previous studies have reported changes to the canine microbiome in relation to aging (Omatsu et al., 2018; Mizukami et al., 2019), including minor changes between adolescents (0.5 to 1 yr) and adults (2 to 5 yr) (You and Kim, 2021). The extent of microbiota changes occurring between 11 and 12 mo are still unclear. A change in diet would likely impact the microbiota more than naturally occurring changes while aging. Another limitation was that body condition scores were not collected during this study. Body condition scores provide better insight into an animal's nutritional state compared to body weight alone. At the time of this study, kennel staff were not sufficiently trained in this evaluation and thus it was not performed when collecting body weights. This data would be crucial in confirming that the reduced body weights in poor stool dogs were indeed related to metabolic status rather than general frame size. Finally, the diets did not reach the 1:1 Ca:P ratio as required by AAFCO (2022); instead, the analyzed values showed a 1:1.1 Ca:P ratio. They were originally formulated to satisfy

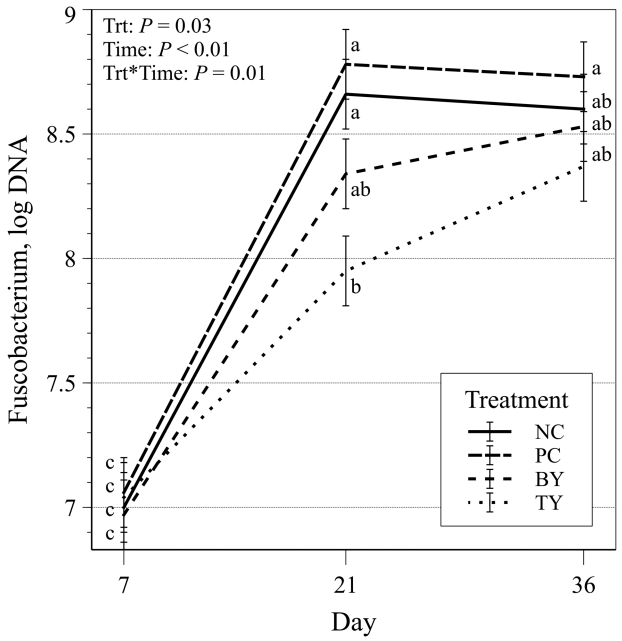

Figure 5. Abundance of *Fusobacterium* expressed as log amount of DNA (fg) per 10 ng of total isolated DNA. Treatments were as follows: (NC) dogs with normal stool quality fed control diet; (PC) dogs with poor stool quality fed control diet; (BY) dogs with poor stool quality fed diet with 10% brewer's yeast; (TY) dogs with poor stool quality fed diet with 10% torula yeast. Superscripts (abc) represent statistical significance within treatment × time interaction ($P = 0.01$).

this requirement. Discrepancies in formulation could have occurred during diet preparation and extrusion or analysis of the kibbles. Only one sample of each diet was sent for testing at the end of the study during digestibility assessment.

Conclusion

The inclusion of 10% torula yeast did not improve stool quality or inflammatory biomarkers, but it did modify gut microbiota and numerically increased protein ADC. The dietary inclusion of a commercial torula yeast protein product in extruded canine diets did not produce any detrimental effects on dog health or maintenance. While there were no significant differences among treatments as assessed by Tukey's post hoc analysis, there was an overall significant difference in crude protein ADC across treatment means. The TY diet was numerically greater compared to PC and was more similar to NC. It also reduced fecal calprotectin at a greater rate than the negative and positive control animals, which has been associated with improved gut health and chronic diarrhea status. However, there were no significant changes in other biomarkers, which could explain why we did not see changes in stool quality scores. The dogs in this study were considered healthy and had not been diagnosed with IBD which was confirmed by the lack of differences in fecal inflammatory markers among treatments. However, the increased systemic inflammation and trending nutrient digestibility may place the poor stool animals in a pre- or subclinical state, falling within the overlapping areas of healthy and chronic disease that is not well discussed in the literature. The addition of brewer's and torula yeast resulted in modifications to select taxa within the gut microbiome. The TY group had less pronounced changes to *Fusobacterium* and dysbiosis index scores than the NC and PC groups, although no dogs were experiencing dysbiosis. Torula yeast is a suitable alternative protein source for canine companion animal diets with potential as a functional protein source as seen through its ability to significantly alter select microbiota taxa and fecal calprotectin. Further research is needed to confirm the trending observations in macronutrient digestibility, as well as the potential for improving canine gut health in the long term.

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Conflict of Interest

Ricardo Ekmay is an employee of Arbiom. The remaining authors declare no other real or perceived conflicts of interest.

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