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Assessing non-protein nitrogen sources in commercial dry dog foods

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ABSTRACT

Protein is a macronutrient required by dogs for growth and maintenance metabolism. However, a portion of the crude protein listed on pet foods may actually arise from non-digestible organic nitrogen or potentially toxic inorganic non-protein nitrogen sources. Neither non-protein source is retained or used by the animal. However, these compounds may result in adverse effects such as methemoglobin formation and increased oxidative stress or potentially beneficial effects such as improved vascular distensibility and decreased inflammation. To analyze nitrogen retention and screen for non-protein nitrogen, four commercial, dry kibble dog foods and one laboratory-made diet were evaluated and then fed to beagles during two separate feeding trials. During the first trial, dogs were randomly assigned each diet (n = 4 dogs/diet) and fed chromium oxide-coated diets for 48 h, followed by total urine and marked fecal collection, as well as plasma collection for total nitrogen, nitrate, ammonia, and urea determination. The amount of nitrogen retained (93%–96%) did not differ among commercial diets. Protein total tract apparent digestibility (TTAD) ranged from 69% to 84%, with the high protein diets significantly higher than the laboratory-made and mid-ranged diets (1-way ANOVA: $P < 0.05$). The high protein diet also contained the highest concentration of nitrate with subsequent elevations in plasma nitrotyrosine levels (indicator of oxidative stress). During the second trial, eight dogs (n = 8) were fed the same diets for 6 d, after which echocardiography was completed with blood, urine, and feces collected. For health end-points, methemoblobin, plasma nitrotyrosine, and C-reactive protein (CRP; indicator of inflammation) levels were measured. Methemoglobin levels were significantly lower in the high protein diet ($P > 0.05$), possible due to the stimulation of methemoglobin reductase while nitrotyrosine was unchanged and CRP was undetectable. Furthermore, there was a positive relationship between crude protein, crude fat (simple linear regression: $P = 0.02$, $r^2 > 0.6$), price ($P = 0.08$, $r^2 > 0.6$), and caloric density ($P = 0.11$, $r^2 > 0.6$). There were no significant cardiovascular differences among any of the diets ($P > 0.05$). Ultimately, this study shows that in commercial diets, price does reflect protein content but that feeding dogs high protein diets for a long period of time may provide an excess in calories without a change in cardiovascular function or detectable increases in inflammation.

Key words: cardiovascular function, dogs, nitrogenous compounds, pet food, protein

Abbreviations: AAFCO, Association of American Feed Controls Organization; CRP, C-reactive protein;ELISA, enzyme-linked immunosorbent assay;FDA, Food and Drug Administration;Hz, hertz;kDa, kilodalton;MER, maintenance energy requirement;ND, not detectable;ppm, parts per million

INTRODUCTION

Protein, as a nitrogen-containing compound, is essential for growth and maintenance metabolism in dogs (Dzanis, 1994). However, a portion of the crude protein listed on pet food labels may be from non-protein nitrogen sources. Non-protein nitrogen can be found both as organic non-digestible nitrogen from plant sources (Li et al., 2015) and also as toxic inorganic sources like nitrate, nitrite, ammonia, and urea. Incorporated as a meat preservative or bound within plant products, these compounds may have toxic effects on the animal, such as methemoglobin formation and subsequent reduced oxygen carrying capacity in blood (Carriker et al., 2018). Even at subclinical levels, these compounds affect physiological processes such as nitrogen retention and digestibility. The Association of American Feed Controls Organization (AAFCO) and Food and Drug Administration (FDA) have set nutritional limits in order to avoid this type of toxicity and ensure proper nutritional maintenance in pet foods (AAFCO, 2013; FDA, 2018).

In contrast, meta-analyses of studies examining dietary nitrate and nitrite have reported notable positive influences on the cardiovascular system through conversion into nitric oxide in humans (Stanaway et al., 2017). Once converted, nitric oxide subsequently acts as a vasodilator to increase blood flow throughout the body (Daiber et al., 2019). Thus, dietary nitrate and nitrite could be indirectly linked to improvements in vascular endothelial function and reduced blood pressure (Carlstrom and Montenegro, 2019). Aside from direct effects to increase nitric oxide levels, oral or intravenous nitrate has been reported to both decrease oxidative stress and inflammatory responses in a multitude of rodent models of disease (Cui et al., 2020; Hu et al., 2020; Peleli et al., 2020), whereas other studies have found no effect on inflammation (Fischer et al., 2020) or have even reported increased oxidative stress (Bruning-Fann and Kaneene, 1993; Mohiuddin et al., 2006). Beneficial effects of nitrite and urea, if any, are less clear in humans, and effects of non-protein nitrogenous compounds are virtually unknown in dogs.

The purpose of this study was to assess the protein quality of commercial pet foods and screen for effects of toxic nitrogenous compounds. A secondary objective of this study was to assess the therapeutic potential of dietary nitrate and nitrite on the cardiovascular system. C-reactive protein (CRP) was

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used as an indicator of inflammation, whereas nitrotyrosine was assessed as an indicator of peroxynitrite formed after interaction of nitric oxide with oxidative stress. It was hypothesized that due to regulations in the pet food industry, protein quality and nitrogenous compound concentrations will be similar among all diets. Furthermore, after being fed diets containing ingredients high in nitrate and nitrite, dogs would have improved vascular distensibility with neither methemoglobin increases nor any subclinical signs of toxicity.

MATERIALS AND METHODS

All procedures and handling involving dogs were completed according to a protocol approved by the University of Saskatchewan's Animal Research Ethics Board according to guidelines established by the Canadian Council on Animal Care.

Animals

Eight adult beagle dogs (9.64 ± 0.24 kg; four spayed females and four neutered males) of 5 ± 0.5 yr of age at the time of this study were originally obtained from a certified scientific breeder (Marshall Farms, NY). Dogs had their own individual kennels for feeding and overnight, but were kept together in open kennels during the day to socialize with each other, with access to outdoor runs and taken on daily walks. When not on trial, dogs were fed a standard commercial adult maintenance pet food diet (Hills Pet Nutrition Inc, Topeka, KS). The weight of food fed per animal per day varied for each individual, but portions were adjusted for each animal as needed to maintain ideal body condition score (4–6 on 9-point Purina body condition scale). Dogs were clinically healthy prior to and throughout the study.

Diet Selection

Four commercial pet food brands were selected based on a range of price points and crude protein content. All diets were selected for adult animal health maintenance and included similar macronutrients, with chicken as the major animal protein source. These commercial diets were compared to an experimental diet, formulated in laboratory for both dogs and cats during previous experiments (Briens et al., 2021). Feed weights were calculated based on body condition score and body weight, with reference to labeled digestible energy per weight to produce isocaloric portions during testing. At the start of the experiment, meal portions were allocated to each animal based on each individual's history of energy needs to maintain optimal condition and caloric density of the diet, in order to maintain optimal body condition score throughout the trial.

Nitrogen Retention and Protein Utilization

Prior to feeding trials, diets were coated in a non-digestible marker, chromium oxide (VWR, Mississauga, Canada) at 0.01% (w/w Cr_2O_3 to feed), to aid determination of transit time of the diet and to aid in the determination of protein total tract apparent digestibility (TTAD; Peachey et al., 2000). During the first feeding trial, dogs were fed one of the five different diets in a randomized fashion such that each diet was tested in four different animals (n = 4 dogs/diet). Animals were acclimated to the uncoated diets for 2 d prior to sample collection. After acclimation period, all dogs were housed in individual metabolic cages to allow for total urine collection and fed chromium coated diet for 48 h (Bingham et al., 2004). Total fecal output resulting from the diet during this 48-h period was collected based on presence of the non-digestible marker in the feces (turns feces green), with fecal collection extending beyond the 48-h period as needed until all marked feces had passed. After 48 h, animals were maintained on uncoated test diet and kept alone in their home kennel until all marked feces passed (an additional 2 d was sufficient; Carciofi et al., 2007). At 96 h after starting this trial, blood (1.0 mL) was collected into ethylene diamine tetraacetate tubes from a sub-sample of animals (n = 4/species), spun at 5,000 × g for 10 min and plasma aliquoted, and then stored at −80 °C until use in nitrite/nitrate determination assays. Animals were then returned to regular husbandry. Feed and fecal samples were dried in an oven at 65 °C for 7 d or until dry, ground and stored at room temperature until analyzed for macronutrients and total nitrogen levels by a commercial laboratory (Central Testing Laboratories, Winnipeg, MB, Canada). Urine was stored at −20 °C until total nitrogen analysis (Central Testing Laboratories, Winnipeg, MB, Canada). Nitrogen retention was calculated according to the equation used by Tome et al. (2000), based on intake of nitrogen via the feed vs. nitrogen loss in urine and feces.

Nitrogen retention was calculated as

$$\text{Nitrogen Retention} = \frac{\begin{array}{c}\text{total dietary nitrogen intake}\\ \text{- (fecal nitrogen } + \text{ urine nitrogen)}\end{array}}{\text{otal dietary nitrogen intake}}$$

Presence of the chromium oxide marker in the feed and feces was determined using atomic absorption spectroscopy (Central Testing Laboratories, Winnipeg, MB, Canada). Protein TTAD was calculated in all of the diets based on the presence of the chromium oxide marker (Hernot et al., 2006).

Protein TTAD was calculated as

$$\text{TTAD}\ \ (\%) = 1 - \left[\frac{\%\ \text{crude protein in feces} \times \%\ \text{marker in feed}}{\%\ \text{crude protein in feed} \times \%\ \text{marker in feces}}\right]$$

Cardiovascular Ultrasound

In a second round of feeding trials, dogs were fed for 6 d on each diet in their home kennels, followed by ultrasound testing on dogs fasted overnight, in the morning of day 7. During this second feeding trial, each dog was fed five different diets (n = 8) in a randomized crossover design. Feed portions were calculated based on body condition score and body weight, with reference to formulated digestible energy per weight to produce isocaloric portions during testing and avoid errors in cardiovascular measurements due to metabolic differences between the animals. Therefore, individual dogs received a slightly different dose of nitrate and nitrite per diet (Figure 1). All dogs were previously acclimated to all blood collection and ultrasound procedures by providing positive attention during testing and treats after all procedures were done. Thus, the dogs were highly cooperative and we were able to examine the dogs without stress or any sedation for these procedures. Prior to ultrasound, dogs were weighed and blood pressure was taken using a high definition canine/feline oscillometer on the tail (S + B medVET GmbH, Babenhausen, Germany). Endpoints of flow mediated dilation included brachial artery diameter during baseline, during inflation of a

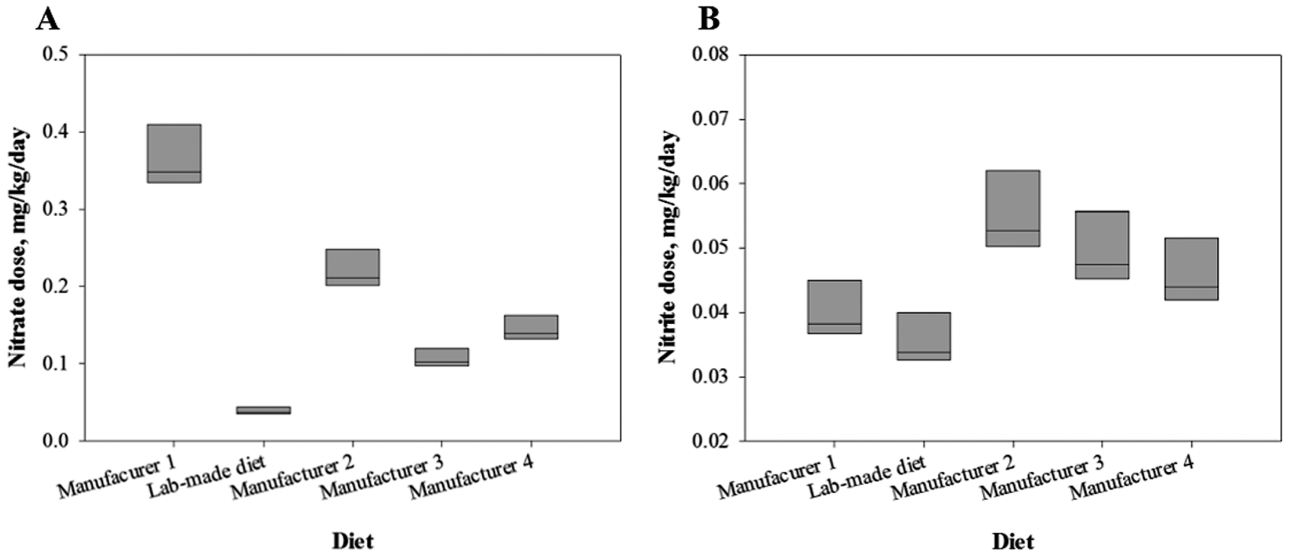

Figure 1. Dose of nitrate (A) and nitrite (B) fed to dogs in commercial diets for 6 d. Data shown as minimum, maximum, and median concentration per diet. Diets are shown from left to right in order of decreasing crude protein content.

blood pressure cuff placed distal to the brachial artery and at the time of peak dilation (30 s) after cuff release previously determined using B-mode ultrasound in longitudinal view of the brachial artery (Raitkatari et al., 2000; Adolphe et al., 2012). Echocardiography endpoints to assess cardiac function included heart rate, stroke volume, and cardiac output (Otto et al., 2019; Adolphe et al., 2012). Flow-mediated dilation and echocardiography were measured using SonoSite Edge II ultrasound (Fujifilm SonoSite Inc., Bothell, USA), with detection using the P10x transducer (8–4 Hz) to detect cardiac endpoints and the L38xi (10–5 Hz) transducer to measure flow-mediated dilation. After ultrasound was conducted, a 3.0-mL aliquot of plasma was obtained for use in ammonia, urea, and nitrotyrosine assays. 1.0-mL aliquot for blood gases was also collected for methemoglobin analysis using a blood gas electrolyte analyzer (Shinova Medical Co., Shanghai, China).

Nitrogenous Compound and Biomarker Assays

Plasma, urine, feed, and fecal samples were analyzed for nitrite and nitrate (n = 4/diet). Plasma and urine were analyzed directly in the assay, whereas feed and fecal nitrate and nitrite were extracted into solution. Solid feed and fecal samples were ground and diluted using a 1:10 dilution with reagent-grade water. Diluted samples were heated at 60 °C for 3 h to extract nitrogenous compounds. All samples were filtered using a 10 kDa cut-off filter to reduce interference in the colorimetric assays. Nitrite and nitrate were analyzed using a commercially available nitrite/nitrate assay kit based on the Greiss color reaction. Nitrite was measured directly and nitrate was calculated based on subtracting nitrite from the total nitrate/nitrite detected (R&D Systems, Bio-Techne Corporation, Minneapolis, MN). Where sample nitrate or nitrite levels were below detection, a zero value was used in statistical analyses for that sample. Plasma, urine, feed, and fecal samples were analyzed for ammonia and urea. Ammonia and urea were determined in the same sub-sample (n = 8 animals) using a commercially available urea/ammonia (rapid) test kit (Megazyme, Genzyme, Englewood Cliffs, NJ). Plasma samples from the second dog feeding trial were analyzed for nitrotyrosine. Nitrotyrosine was analyzed using a commercially available nitrotyrosine enzyme-linked immunosorbent assay (ELISA) (Hycult Biotech, Uden, The Netherlands). CRP was quantified in plasma samples using a commercially available ELISA kit (United States Biological, Salem, MA).

Statistical Analysis

All data were initially tested for parametric assumptions: a Levene's test was used to test for homogeneity of variance and a KS-test was used to test if data was normally distributed. All data met parametric assumptions. For each feeding trial, endpoints were analyzed independently using 1-way ANOVA followed by Fisher's LSD post hoc tests for pairwise comparisons, with α set at 0.05. Furthermore, linear regressions were used to examine the relationship between the endpoints and crude protein content, with a relationship deemed significant when $r^2 > 0.6$ and $P < 0.1$. A descriptive analysis was used to relate results to price. Data are represented as mean ± SEM. All data analyses were performed using SPSS statistics version 25 (SPSS Chicago, Il, USA, IBM), using linear mixed models.

RESULTS

All diets were enthusiastically consumed by all of the dogs, with no signs of food refusal or alterations in overall health (based on general appearance and behavior). Diets arranged in tables and figures from highest to lowest crude protein content and price per kilogram, with manufacturer 1 being the highest and manufacturer 4 being the lowest. Brand names have been left out of this paper in order to avoid negative bias towards specific manufacturers.

Guaranteed and Proximate Analysis

After a descriptive analysis, this study determined that all commercial diets met and/or exceeded the minimum AAFCO nutritional requirements for dogs (AAFCO, 2013), with crude protein ranging from 18% to 38% in selected diets. As shown

Table 1. Guaranteed and proximate analyses of four commercial and one laboratory-made dry, kibble dog foods

Diet	Price, $/kg	Guaranteed analysis (as fed)					Proximate analysis (% dry matter)
		Calories, kcal/kg	Crude protein, %	Crude fat, %	Crude fiber, %	Moisture, %	Crude protein, %
Manufacturer 1	8.50	3900	38	18	4	12	39
Laboratory-made diet	–	3509	34	15	3.5	10	34
Manufacturer 2	6.61	3627	24	14	5	10	30
Manufacturer 3	3.23	3397	20	9	5	10	23
Manufacturer 4	2.08	3407	18	8.5	6	12	23

Table 2. Ingredient list for four commercial, dry kibble dog foods plus one laboratory-made test diet

Diet	Ingredients
Manufacturer 1	Deboned chicken, Deboned turkey, Atlantic flounder, Whole eggs, Whole Atlantic mackerel, Chicken liver, Turkey liver, Chicken heart, Turkey heart, Whole Atlantic herring, Dehydrated chicken, Dehydrated turkey, Dehydrated mackerel, Dehydrated chicken liver, Whole dehydrated egg, Whole red lentils, Whole pinto beans, Whole green peas, Chicken necks, Chicken kidney, Whole lentils, Whole navy beans, Whole chick peas, lentil fiber, Chicken fat, Natural chicken flavor, Alaskan pollock oil, Ground chicken bone, Chicken cartilage, Turkey cartilage, Mixed tocopherols, Whole pumpkin, Whole butternut squash, Freeze dried chicken liver, Dried kelp, Zinc proteinate, Kale, Spinach, Mustard greens, Collard greens, Turnip greens, Whole carrots, Whole apples, Whole pears, Pumpkin seeds, Sunflower seeds, Thiamine mononitrate, d-calcium pantothenate, Copper proteinate, Chicory root, Turmeric, Sarsaparilla root, Althea root, Rose hips, Juniper berries, Dried *Lactobacillus acidophilus* fermentation product, Dried biffidobacterium animalis fermentation product, Dried lactobacillus casei fermentation product
Laboratory-made diet	Pea starch, Chicken meal, Soy protein concentrate, Chicken fat, Pea fiber, Fish meal, Fish oil, Celite, Potassium chloride, Sodium chloride, Calcium carbonate, Choline chloride, dl-methionine, Mineral premix, Vitamin premix, Taurine, Dicalcium phosphate
Manufacturer 2	Deboned chicken, Chicken meal, Brown rice, Barley, Oatmeal, Pea starch, Flaxseed, Chicken fat, Dried tomato pomace, Natural flavour, Peas, Pea protein, Sodium chloride, Potassium chloride, Dehydrated alfalfa meal, Potatoes, Dried chicory root, Pea fibre, Alfalfa nutrient concentrate, Calcium carbonate, Calcium chloride, dl-methionine, Mixed tocopherols, Dicalcium phosphate, Sweet potatoes, Carrots, Garlic, Zinc amino acid chelate, Zinc sultfate, Vegetable juice, Ferrous sulfate, Vitamin E supplement, Iron amino acid chelate, Blueberries, Cranberries, Barley grass, Parsley, Turmeric, Dried kelp, Yucca extract, Niacin, Glucosamine hydrochloride, Calcium pantothenate, Copper sulfate, Biotin, l-ascorbyl-2-polyphosphate, l-lysine, l-carnitine, Vitamin A supplement, Copper amino acid chelate, Manganese sulfate, Taurine, Manganese amino acid chelate, Thiamine mononitrate, Riboflavin, Vitamin D3 supplement, Vitamin B12 supplement, Pyridoxine hydrochloride, Calcium iodate, Dried yeast, Dried enterococcus faecium fermentation product, Dried lactobacillus acidophilus fermentation product, Dried aspergillus niger fermentation extract, Dried trichoderma longibrachiatum fermentation extract, Dried bacillus subtillis fermentation extract, Folic acid, Calcium iodate, Sodium selenite
Manufacturer 3	Ground whole grain corn, Chicken by-product meal, Ground whole grain sorghum, Chicken, Dried beet pulp, Ground whole grain barley, Chicken flavor, Chicken fat, Dried egg product, Potassium chloride, Brewers dried yeast, Caramel color, Sodium chloride, l-lysine monohydrochloride, Choline chloride, Fish oil, dl-methionine, Carrots, Calcium carbonate, Tomatoes, Flaxseed Fructooligosaccharides, Dicalcium phosphate, Spinach, Green peas, Ferrous sulfate, Zinc oxide, Sodium selenate, Manganese sulfate, Copper sulfate, Manganous oxide, Potassium iodide, Vitamin E supplement, Ascorbic acid, Calcium pantothenate, Vitamin A supplement, Biotin, Thiamine mononitrate, Vitamin B12 supplement, Niacin, Riboflavin, Inositol, Pyridoxine hydrochloride, Vitamin D3 supplement, Folic acid, l-tryptophan, Dried apple pomace, Dried blueberry pomace, l-carnitine, Mixed tocopherols, Rosemary extract, Citric acid
Manufacturer 4	Ground yellow corn, Corn germ meal, Pork and bone meal, Tallow preserved with mixed tocopherols, Poultry by-product meal, Corn gluten meal, Animal digest, Sodium chloride, Calcium carbonate, Peas, Potassium chloride, Natural grill flavour, Choline chloride, Zinc sulfate, Red 40, Ferrous sulfate, dl-methionine, Vitamin E supplement, Manganese sulfate, Yellow 5, Blue 2, Niacin, Vitamin A supplement, Copper sulfate, Calcium pantothenate, Garlic oil Pyridoxine hydrochloride, Vitamin B12 supplement, Thiamine mononitrate, Vitamin D3 supplement, Riboflavin supplement, Calcium iodate, Menadione sodium bisulfite complex, Folic acid, Biotin, Sodium selenite

in Table 1, pet food with a higher price per kilogram also contained higher crude protein, crude fat, and caloric density.

Proximate analysis determined that feed crude protein percentage was higher than what the guaranteed analysis stated on the pet food bags. A descriptive analysis of the diet ingredients (Table 2) revealed that diets with a higher price per kilogram used more expensive ingredient sources and a greater number of ingredients. The more expensive manufacturers also used grain free fiber sources as opposed to corn meal as the primary fiber source, like the less expensive diets.

Protein TTAD and Nitrogen Retention

Protein TTAD differed significantly among diets in dogs (1-way ANOVA; $P < 0.05$) and ranged from 68.6% to 84.2% (Table 3). There was no association between protein TTAD and price, as the highest and lowest priced diets showed the highest TTAD. Nitrogen retention of the diets was high in dogs for all diets, with greater than 90% retention for all of the diets. Only the laboratory-made diet differed significantly from the commercial diets ($P < 0.05$) with lower TTAD (Table 3).

Toxic Nitrogenous Compounds

Table 4 portrays the levels of nitrate and nitrite in feed, plasma, urine, and fecal samples. Nitrate in the feed ranged from 2.2 to 22.8 mg/kg and nitrite ranged from 2.0 to 3.2 mg/kg. There were no manufacturers tested that exceeded the FDA 20 mg/kg limit for sodium nitrite (FDA, 2018). Plasma levels of nitrate were higher than plasma levels of nitrite, following the trend nitrate/nitrite in the feed (Table 4). Only the diet from manufacturer 1 differed significantly from the other diets and produced the highest plasma nitrate levels in dogs ($P < 0.05$; Table 4). Dietary nitrate was primarily excreted in the urine, ranging from 3.4 to 5.5 μM in urine and 0.2 to 1.5 mg/kg in feces. Excretory nitrate and nitrite only differed significantly in the feces ($P < 0.05$), with the laboratory-made diet having the greatest fecal nitrate and nitrite excretion. Dietary nitrate was primarily excreted in the urine in dogs, ranging from 3.4 to 5.5 μM in urine vs. 0.2 to 1.5 mg/kg in dog feces. Excreted nitrate and nitrite only differed significantly in the feces ($P < 0.05$; Table 4), with the laboratory-made diet having significantly greater fecal nitrate and nitrite excretion in dogs compared to all the commercial diets. Moreover, dogs fed the diet from manufacturer 5 had an intermediate level of fecal nitrite, significantly different from the higher laboratory-made diet and all other commercial diets (Table 4).

Table 3. Protein total tract apparent digestibility (TTAD) and nitrogen retention in dogs fed four commercial and one laboratory-made diet

Diet	Protein TTAD, %	Nitrogen retention, %
Manufacturer 1	84.2 ± 1.0^{a}	93.9 ± 1.0^{ab}
Laboratory-made diet	75.3 ± 2.8^{b}	92.7 ± 1.4^{b}
Manufacturer 2	82.0 ± 1.7^{a}	96.1 ± 0.3^{a}
Manufacturer 3	68.6 ± 3.4^{b}	96.3 ± 0.5^{a}
Manufacturer 4	83.7 ± 1.7^{a}	94.9 ± 0.6^{a}

Diets are listed in decreasing level of crude protein inclusion.
Values are shown as mean ± SEM, $n = 4$. Values in a column with superscripts without a common letter differ, $P < 0.05$; 1-way ANOVA with LSD post hoc test.

Table 5 shows the levels of ammonia and urea in dog feed as well as dog plasma, urine, and fecal samples obtained after feeding each diet (2 d for urine and 6 d for plasma and feces). Concentrations of ammonia were higher than urea in all feed samples, but all feeds were low for both compounds. The dog feed concentrations of ammonia ranged from 1.5 to 25.4 mg/kg, whereas urea ranged from 3.2 to 7.2 μg/kg (Table 5). Diets within the mid-range for crude protein content contained the lowest levels of both ammonia and urea in feed (Table 3); thus, protein content did not appear to be the driver for ammonia or urea levels in feed. However, in plasma samples, when these same mid-range diets were fed to dogs, they resulted in significantly higher ammonia and significantly lower urea ($P < 0.05$; Table 5). There did not appear to be a primary route of excretion for ammonia and urea in dogs, as concentrations were similar between urine and feces (Table 5). In urine, ammonia concentrations did not differ significantly with the different diets. In contrast, with urine urea, dogs fed the diet from manufacturer 5 had significantly lower urine urea than all other diets ($P < 0.05$; Table 5). In canine fecal samples, urea levels did not differ significantly among the diets, whereas the diet from manufacturer 1 had significantly higher levels of fecal ammonia than the lower-priced, lower-protein containing dog feeds ($P > 0.05$; Table 5).

Cardiovascular Changes and Biomarkers of Toxicity

After 6 d of feeding each diet to dogs, there were no statistically significant differences in cardiovascular endpoints ($P > 0.05$; Table 6), including blood pressure, heart rate, stroke volume, cardiac output, and flow-mediated dilation. CRP was not detectable in any of the plasma samples.

However, there were significant differences in methemoglobin and nitrotyrosine levels, as shown in Figure 2. Plasma methemoglobin was significantly higher in dogs fed the diets from manufacturer 2 and 4 for 6 d compared to all other diets ($P < 0.05$; Figure 2). Conversely, plasma nitrotyrosine was significantly higher in dogs fed diets from manufacturer 1 compared to when dogs were fed the diets from manufacturer 2 and 4 ($P < 0.05$; Figure 2). Despite significant changes, it is important to note that both methemoglobin and nitrotyrosine

Table 4. Nitrate and nitrite concentrations in dog feed as well as plasma, urine, and feces after being fed commercial diets or a laboratory diet for 6 d (plasma and feces) or 2 d (urine)

Diet	Feed, mg/kg		Plasma, μM		Urine, μM		Feces, mg/kg	
	Nitrate	Nitrite	Nitrate	Nitrite	Nitrate	Nitrite	Nitrate	Nitrite
Manufacturer 1	22.8	2.5	11.1 ± 3.9^{b}	ND[1]	7.9 ± 5.1	4.2 ± 0.7	0.4 ± 0.1^{a}	$1.7 \pm 0.3^{a,b}$
Laboratory-made diet	2.2	2.0	3.1 ± 1.6^{a}	ND	1.3 ± 0.6	3.4 ± 1.4	1.5 ± 0.2^{b}	3.3 ± 0.1^{c}
Manufacturer 2	12.8	3.2	0.7 ± 0.5^{a}	ND	7.7 ± 3.5	3.6 ± 0.7	0.2 ± 0.1^{a}	1.3 ± 0.1^{a}
Manufacturer 3	5.8	2.7	1.9 ± 1.0^{a}	ND	15.7 ± 5.9	3.4 ± 0.5	0.6 ± 0.2^{a}	1.6 ± 0.2^{a}
Manufacturer 4	7.9	2.5	3.8 ± 1.3^{a}	ND	6.6 ± 2.2	5.5 ± 1.2	0.6 ± 0.2^{a}	2.1 ± 0.1^{b}

Diets are listed in decreasing level of crude protein inclusion.
Values are shown as mean ± SEM, $n = 4$ for plasma, urine and fecal samples. Feed samples are shown as averaged values from duplicate determinations of the same sample. Values in a column with superscripts without a common letter differ, $P < 0.05$; one-way ANOVA with LSD post hoc test. Columns without any superscripts showed no significant differences among diets in 1-way ANOVA.
[1] ND, not detectable.

Table 5. Ammonia and urea concentrations in dog feed as well as plasma, urine, and feces after being fed commercial diets or a laboratory diet for 6 d (plasma and feces) or 2 d (urine)

Diet	Feed		Plasma		Urine		Feces	
	Ammonia, mg/kg	Urea, µg/kg	Ammonia, mg/L	Urea, µg/L	Ammonia, mg/L	Urea, µg/L	Ammonia, mg/kg	Urea, µg/kg
Manufacturer 1	19.5	7.2	5.7 ± 2.8^{a,c}	20.7 ± 1.6^{a,c}	59.5 ± 4.3	14.6 ± 1.7^{a}	89.8 ± 3.5^{b}	8.3 ± 0.6
Laboratory-made diet	25.4	4.2	7.7 ± 1.6^{a,b,c}	16.7 ± 1.8^{a,b}	46.7 ± 7.5	17.7 ± 1.7^{a}	61.9 ± 10.7^{a,b}	9.4 ± 0.4
Manufacturer 2	1.8	3.1	11.7 ± 1.8^{a,b}	15.5 ± 1.1^{b}	58.0 ± 13.6	15.2 ± 3.5^{a}	76.0 ± 1.7^{a}	9.5 ± 0.3
Manufacturer 3	5.3	3.8	14.0 ± 3.1^{b}	18.1 ± 1.7^{a,b}	64.1 ± 4.8	14.7 ± 1.4^{a}	59.4 ± 6.7^{a}	10.9 ± 1.0
Manufacturer 4	20.3	4.2	4.2 ± 2.0^{c}	23.6 ± 2.0^{c}	64.8 ± 22.8	5.9 ± 2.9^{b}	59.7 ± 4.4^{a}	10.2 ± 0.5

Diets are listed in decreasing level of crude protein inclusion.
Values are shown as mean ± SEM, $n = 4$ for plasma, urine, and fecal samples. Feed samples are shown as averaged values from duplicate determinations of the same sample. Values in a column with superscripts without a common letter differ, $P < 0.05$; 1-way ANOVA with LSD post hoc test. Columns without any superscripts showed no significant differences among diets in one-way ANOVA.

remained at subclinical levels for all tests, with methemoglobin not exceeding 1.5% and nitrotyrosine remaining below 2 µM.

Regressions

There were significant linear relationships between crude protein percentage in the diets tested and several endpoints measured in the dog study, as illustrated in Figure 3. There was a positive relationship between crude protein in dog diets and crude fat ($P = 0.02$, $r^2 > 0.6$) as well as a weak relationship between price and fecal nitrite ($P = 0.08$, $r^2 > 0.6$). Figure 3 also shows the weak negative relationship between crude protein and either urine nitrate ($P = 0.07$, $r^2 > 0.6$) or plasma ammonia ($P = 0.06$, $r^2 > 0.6$). Finally, a strong positive relationship was found between dietary ammonia concentration and protein TTAD in dogs ($P = 0.01$, $r^2 > 0.6$), as shown in Figure 3.

DISCUSSION

The present study examined differences in protein quality and utilization of commercial pet food in dogs. The major findings of the descriptive analysis of the commercial diets indicated that over a range of price points, protein inclusion differed. As the most expensive ingredient, animal protein inclusion reflected price. Diets at a higher price point contained a greater crude protein content, as well as a greater variety of ingredients and more expensive sources of animal and plant protein (Pitchon et al., 1983). Protein content also increased with fat content and caloric density. Therefore, if daily food portions during feeding are not corrected for caloric density by the pet owner, the higher energy and fat content of more expensive feeds could contribute weight gain and diseases associated with obesity. A study by German et al. (2007) stated that active dogs have greater maintenance energy requirements (MER). These dogs would benefit from a higher protein, higher calorie diet, whereas the average companion canine with a relatively sedentary life would instead benefit from a high fiber diet. In the German et al. study, the dogs who received moderate to low exercise showed weight loss on a high fiber diet. With 34% to 59% of dogs entering veterinary clinics being overweight, high protein, high calorie diets like the high priced diets in the present study should be avoided, unless properly portioned to account for the MER of the dogs to which they are being fed (Switonski and Mankowska, 2013). Thus, owners buy more expensive diets thinking they are healthier may in fact be feeding these diets long-term. This inadvertently promotes weight gain and diseases associated with it like diabetes mellitus, cardiovascular disease, and dystocia, among others (Gossellin et al., 2007).

All diets met and exceeded the AAFCO minimum crude protein inclusion of 18% for adult maintenance in dogs (AAFCO, 2013). With some commercial diets containing up to 38% crude protein in dog diets, certain diets are actually over supplementing with protein to the point where the animals cannot absorb and incorporate all of the included protein into tissues. Nitrogen retention estimates the bioavailability of nitrogen in the diet and how much of that nitrogen is absorbed and utilized by the animal (Ammerman et al., 1995). Ultimately, the excess protein in the high protein diets used in this study would be metabolized for energy or stored as fat, with nitrogen from amino acids wasted through excretion

Table 6. Cardiovascular health in fasted dogs after 6 d of feeding commercial diets or a laboratory-made diet

Diet	Systolic pressure, mmHg	Diastolic pressure, mmHg	Heart rate, bpm	Stroke volume, mL/beat/kg	Cardiac output, L/kg^{-1}min^{-1}	Flow mediated dilation, %
Manufacturer 1	144 ± 3.6	70 ± 2.3	67 ± 4.1	1.2 ± 0.1	10 ± 0.9	3.3 ± 0.6
Laboratory-made diet	145 ± 5.9	73 ± 4.4	76 ± 5.7	1.1 ± 0.1	8 ± 0.9	3.6 ± 0.6
Manufacturer 2	137 ± 2.8	77 ± 2.2	71 ± 5.3	0.9 ± 0.1	8 ± 0.9	5.2 ± 0.8
Manufacturer 3	137 ± 2.7	73 ± 2.2	71 ± 6.9	1.1 ± 0.1	9 ± 0.8	3.5 ± 0.4
Manufacturer 4	134 ± 2.6	73 ± 2.2	67 ± 6.4	1.1 ± 0.04	11 ± 1.1	3.1 ± 0.7

Diets are listed in decreasing level of crude protein inclusion.
Values are shown as mean ± SEM, *n* = 8. No significant differences among diets were found for any of the above end-points, $P > 0.05$; one-way ANOVA.

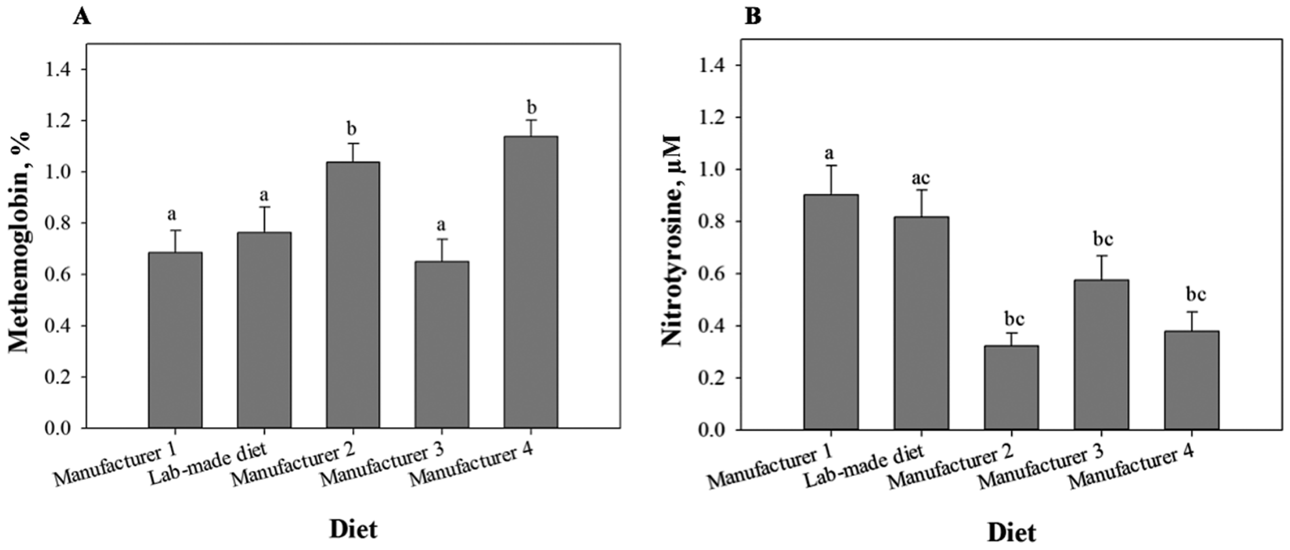

Figure 2. Biomarkers of toxicity in dogs after 6 d of feeding commercial diets or a laboratory-made diet. Methemoglobin (A) and nitrotyrosine (B) analyzed in plasma samples of dogs fasted overnight. Values shown as mean ± SEM, *n* = 8. Values with superscripts without a common letter differ, $P < 0.05$; 1-way ANOVA with LSD post hoc test. Diets are shown in order of decreasing crude protein inclusion from left to right.

(Beynen et al., 2002). The high priced diets maintained a high protein TTAD, indicating greater protein quality and bioavailability in these diets. The high priced diets contained a wider variety of animal protein products; meanwhile, the lower protein diets included one or two animal protein sources, with supplemental plant sources. The high priced diets also contained fish protein sources as opposed to exclusively chicken or pork meal. A study by Dust et al. (2005) examined the ileal digestibility of different protein sources used in pet food. It was determined that of the animal proteins tested, fish proteins were more digestible in cannulated dogs than beef, pork, or chicken. The study noted that while ileal digestibility values were high for all protein sources, protein ingredients containing more fiber, bone, collagen, or connective tissue had lower ileal digestibility. Because this study used total tract apparent, not ileal digestibility, it must also be acknowledged that the contribution of intestinal microbes to metabolism of protein into non-protein nitrogenous products would have not only increased fecal ammonia, nitrate, nitrite, and urea, but also would increase the apparent digestibility.

The concentration of toxic nitrogenous compounds, including nitrate, nitrite, urea, and ammonia, in feed and biological samples was all low and levels of sodium nitrite did not exceed the maximum FDA limit of 20 ppm in the feed (FDA, 2018). The high priced diets from manufacturer 1 did contain the highest concentrations of nitrate, but it is unlikely that this manufacturer was trying to boost the total apparent crude protein with non-nitrogen protein. The high nitrate content is likely due to the manufacturer inclusion of high nitrate-containing plant sources, such as peas and green, leafy vegetables, including kale and spinach (Bondonno et al. 2014). A study by Lehman (1958) tested different inclusions of nitrate in pet products. It was determined that less than 2% inclusion of dietary nitrate per day led to no observable adverse effects in dogs. However, long-term exposure of moderate dietary nitrate could potentially lead to lipid peroxidation and oxidative stress as a result of nitrate cycling and the production of reactive oxygen species (Bruning-Fann and Kaneene, 1993). This coincides with the nitrotyrosine findings of this study, where the diet containing the highest protein and nitrate also produced the highest plasma nitrotyrosine concentrations in dogs after 6 d of feeding. Nitrotyrosine is a biomarker of oxidative stress in the body. Higher levels of nitrotyrosine are indicative of oxidative stress caused by cycling of nitrogenous compounds like nitrate, nitrite, and nitric oxide (Mohiuddin et al., 2006). In contrast, we observed lower methemoglobin levels seen in the dogs fed the diets highest in nitrate. Previous studies have shown that at subclinical levels of nitrate, there may be a reversal of methemoglobin formation, as a result

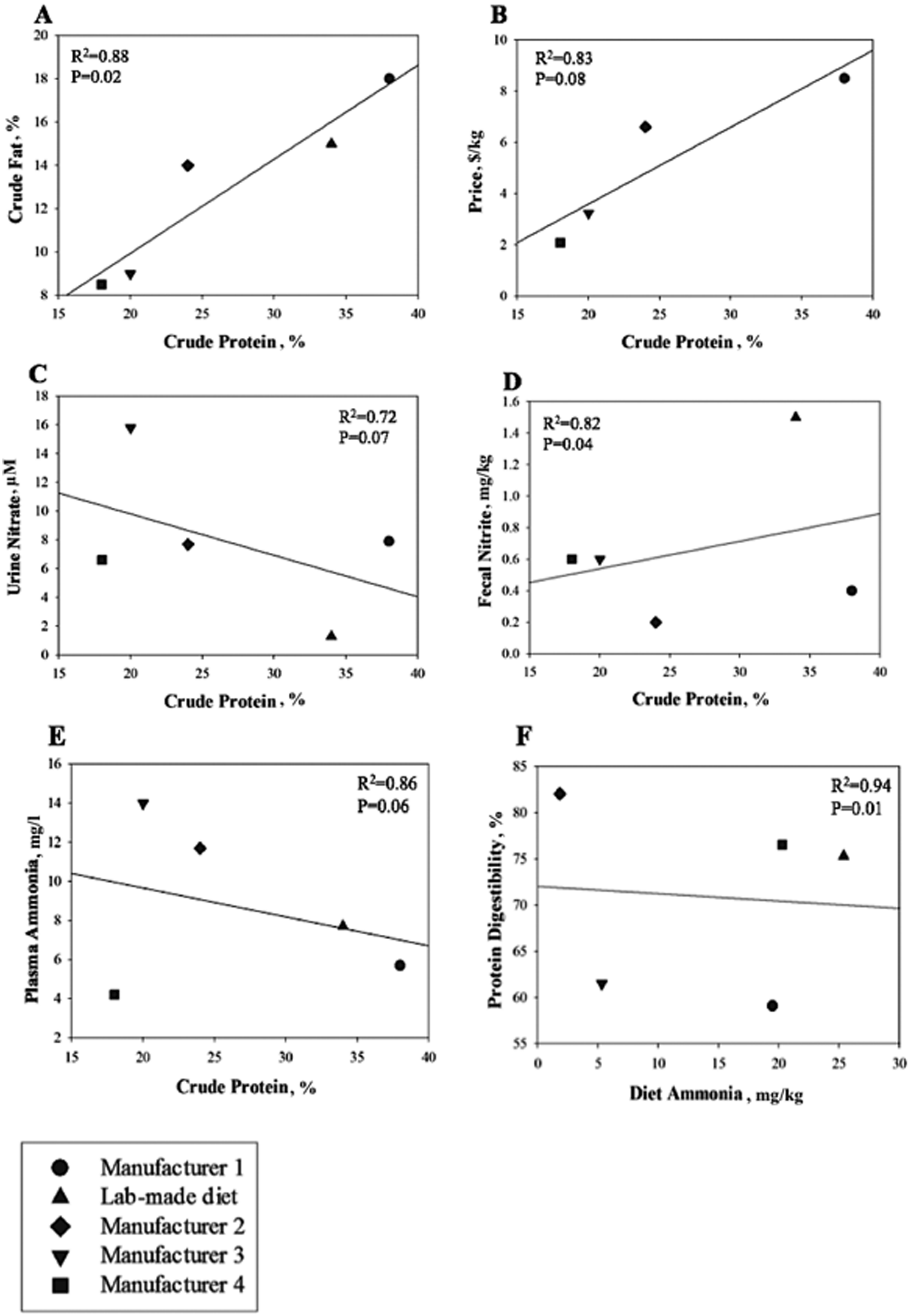

Figure 3. Simple linear regressions showing relationships between crude protein of diets and various end-points from this study: (A) crude fat, (B) price, (C) urine nitrate concentration, (D) fecal nitrite concentration, (E) plasma ammonia concentration, and (F) diet ammonia in dogs after 6 d of feeding commercial diets or a laboratory-made diet and other endpoints. Regression lines shown for relationships where $r^2 > 0.6$.

of increased expression or activity of the methemoglobin-reducing enzyme, methemoglobin reductase (Duncan et al., 1997). Although methemoglobin reductase is not well studied in mammalian animals, a study by Jensen and Nielsen (2018) examined methemoglobinemia recovery in rainbow trout. It was determined that a positive recovery of methemoglobin formation was the result of methemoglobin reductase stimulation as a response to low oxygen saturation.

This study also indicated that as the level of crude protein increases in commercial dog diets, so does the level of dog fecal nitrite. This may indicate that some of the dietary nitrate or other nitrogenous compounds in the diet were converted into nitrite in the gut by intestinal microbes and excreted through the feces. It could also be that a portion of the dietary nitrite was not absorbed and directly excreted. Nitrate is often found at higher concentrations in pet food than nitrite. Nitrate is commonly added as plants sources, whereas nitrite is usually included as a meat preservative (Bahadoran et al., 2016). Upon ingestion, much of the nitrate is converted into nitrite as a result of a combination of low pH, microbial population, and salivary enzymes in the mouth (Sukuroglu et al., 2015). As the nitrite moves through the gastrointestinal system, it can undergo cycling and be converted back into nitrate, converted into other nitrogenous compounds, or be excreted as nitrite (Fritsch et al., 1985). In contrast, there was a negative relationship observed between crude protein and urine nitrate in dogs. This could mean that the sources of nitrate in the high protein diets were more accessible by the animal and more readily converted into nitrite or other nitrogenous compounds in the gut. A study by Van Velzen et al. (2008) examined the oral bioavailability of nitrate in human foods. It was determined that 80% to 85% of dietary nitrate came from fruit and vegetable sources. Of those sources, beet root and leafy vegetables like spinach had the greatest nitrate bioavailability. This supports the findings of the present study, as the high priced diets contained spinach, kale, and beet root and were also associated with the highest fecal nitrite, whereas the lower priced diets did neither.

Ammonia and urea were at low levels in the dog diets and biological samples. However, ammonia increased in concentration between ingestion and excretion, indicated by higher levels in the urine and feces than in the feed and plasma. Ammonia in the urine and feces may arise from a small extent from ammonia added in the diet, but the majority of urinary and fecal ammonia more likely arose when other nitrogenous compounds, including amino acids, from the diet were converted into ammonia in the liver. Ammonia is produced as a by-product of the metabolic process where amino acids are transaminated to pyruvate for energy (Lowenstein, 1972). A review by Huizenga et al. (1996) also stated that a combination of low gastrointestinal pH and presence of microorganisms promoted the deamination of α-amino acids to ammonia in the large and small bowel of dogs. Even compounds like dietary nitrate and nitrite can be reduced into ammonia under a low pH (Becer et al., 2010). Additionally, during the urea cycle, trace amounts of ammonia in food are usually converted by mammals into a non-toxic form (Kung et al., 2000). Ammonia nitrogen only makes up approximately 10% of urea nitrogen excretion, with approximately 50% of ammonia produced during metabolism being excreted directly in the urine (Weiner et al., 2015). Concentrations of urea were highest in plasma and urine, labeling urine as the primary route of excretion for urea in dogs (Bankir and Yang, 2012). Results of the present study also showed that concentrations of ammonia were higher than urea in all samples. Higher levels of ammonia in the urine and feces of all diets indicate that most of the ammonia ingested and produced during metabolism and digestion is not converted into urea during urea cycling. It is unlikely that the ammonia found in the commercial diets was added as non-protein nitrogen to boost apparent protein content and instead results fit with the scenario that diets with high animal protein used ammonia as a preservative. Anhydrous ammonia is specifically used to reduce the incidence of *Escherichia coli* in beef products after processing. Similarly, ammonium hydroxide is used in a variety of non-meat products to reduce incidence of pathogenic microbial species (Tajkarimi et al., 2008). Thus, it is most likely the feed ingredient manufacturers, not the pet food companies themselves that have added ammonia for this purpose. The positive relationship observed between dietary ammonia and protein TTAD in the current study further supports this hypothesis. It is unlikely that the dietary ammonia improved the protein TTAD, but instead the more digestible animal proteins in higher priced dog foods had more ammonia added as a preservative.

There were no differences in any of the cardiovascular parameters after feeding any of the diets for 6 d and all values were within normal ranges for dogs (Hopper, 2009). Although we had hypothesized based on largely human literature that high dietary nitrate would enhance flow-mediated dilation and reduce blood pressure (Jonvik et al., 2016), dietary nitrate appears to lack the same vasodilatory potential in dogs as it does in humans. Companion animals and humans share many of the same cardiovascular traits, which means that they are susceptible to many of the same cardiovascular illnesses (Mubanga et al., 2017). However, there are certain cardiovascular diseases that can affect certain sizes and breeds more than others. Unlike in humans, hypertension is less prevalent in dogs. Approximately 10% of dogs develop hypertension, with most of them being senior animals (Remillard et al., 1991). Canine hypertension is diagnosed when systolic pressure exceeds 160 mmHg. Secondary hypertension is much more prevalent in dogs than primary hypertension, where there is usually an underlying disease, like renal failure, that causes high blood pressure (Serres et al. 2006). Obesity is another major contributor to hypertension in dogs, which can be linked to nutrition (Hall et al. 2000). The beagles used in the present study were in healthy condition and middle aged. Changes in blood pressure as a result of nitrate and nitrite exposure may not have been as evident in healthy animals. Also, the levels of nitrate and nitrite used in the diets tested may not have been high enough, fully bioavailable or fed for long enough to elicit an effect. A 2016 study by Jonvik et al. examined the use of dietary nitrate in the form of spinach, beet pulp, and sodium nitrate, as a vasodilatory agent in humans. The researchers were able to observe changes in blood pressure and flow mediated dilation where the dose of dietary nitrate was 800 mg/kg/day, plasma nitrate ranged from 61 to 69 μM, and plasma nitrite ranged from 115 to 155 μM. These concentrations in both the diet and plasma are much greater than what was found in the present study in dogs. Ultimately, the concentrations of nitrate and nitrite in the commercial canine diets were not high enough to influence vascular distensibility or cause changes in cardiac function.

These findings indicate, at least with the diets tested in this study, that it is not necessary for pet owners to spend money on high priced diets, where protein is concerned. If true for other higher priced diets, it may be most beneficial for pet owners to invest in moderately priced diets in order to avoid health problems with excess calories that would promote weight gain and prolonged exposure to inflammatory proteins, as seen with the high protein, high priced diets. However, although there were differences in detection of non-protein nitrogen in the commercial diets, none of these non-protein nitrogen sources (ammonia, urea, nitrate, or nitrite) were at toxic concentrations. Adult maintenance commercial diets do not possess an observable therapeutic cardiovascular potential in dogs. The concentrations of nitrate and nitrite were likely not at high enough concentrations or fully bioavailable in diet ingredients to produce a vascular effect, but results should be confirmed in a longer-term feeding study.

Conflict of interest statement

The authors have received research funding for other projects than the current study from the Saskatchewan Pulse Growers as well as receiving in-kind donations from Horizon Pet Foods (Rosthern, SK Canada) and Alliance Grain Traders (Saskatoon, SK Canada). These funder/industry partners had no role or influence on the current study.

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