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Nutritional and Microbiological Evaluation and *in vitro* Digestibility of Two In-House Formulated Feed for Adult Dogs

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ABSTRACT

This study was conducted to develop an alternative feed option, addressing the demand for traditional extruded adult dog feed and exploring the possibilities of the development of vegetarian dog feed. Two types of extruded dog feed were formulated, namely a vegetarian variant (T1) and a non-vegetarian variant (T2). The formulations were further subjected to a comparative study along with commercially available dog feed T3. On microbiological evaluation, both formulations revealed a total viable count of 2030 CFU at 10^{-1} dilution, which became negligible in further dilutions, indicating minimal bacterial contamination. The results of *in vitro* digestibility study demonstrated that the digestibility of dry matter and crude protein was significantly (P e"0.05) higher in T1 and T2 group in which in house prepared vegetarian and non-vegetarian food was fed as compared to T3 group in which commercial food was fed, however, the digestibility of ether extract was significantly higher in T1 and T3 group as compared to T2. Moreover, the digestibility of organic matter was significantly lower in T2 group than T1 group but the digestibility of organic matter remains comparable in commercial food with vegetarian (T1) and non-vegetarian (T2) food. These findings highlight that in housed prepared vegetarian and non-vegetarian were equal good to commercial dog food in term of nutritional quality and safety.

KEY WORDS: Dog feed, Feed formulation, In *vitro* digestibility, Microbiological evaluation

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INTRODUCTION

Dogs (*Canis familiaris*) have long been valued companions and serve various roles in human society, including defence and biomedical research (Suchodolski et al., 2004). With a rising number of nuclear families, approximately six lakh dogs are adopted annually in India, coinciding with a projected 4.7% Compound Annual Growth Rate (CAGR) in the pet feed market from 2022 to 2027 (IPFM, 2022).

Studies by highlight prevalent feeding practices among pet owners, with a significant majority preferring homemade diets (HMD), often lacking adequate protein, fiber, calcium, and phosphorus (Sethi et al., 2019; Singh et al., 2022; Kumar et al., 2024) A substantial portion of dogs also receive vegetarian diets, underscoring the need for improved nutritional formulations.

In response, this study explores the use of *in-vitro* digestibility methods to develop nutritionally balanced vegetarian and non-vegetarian adult dog feeds. These methods simulate gastrointestinal processes effectively and economically, potentially reducing reliance on animal trials for feed evaluation (Biagi et al., 2016). By assessing both the nutritional composition and microbial safety of formulated feeds and comparing them with commercial options, this research aims to provide insights into enhancing pet nutrition. Ultimately, it seeks to guide improved feeding practices that promote the overall health and well-being of pets, addressing current nutritional deficiencies prevalent in homemade diets.

MATERIALS AND METHODS

Proximate analysis of feed ingredients

This study was conducted in the Department of Animal Nutrition, Guru Angad Dev Veterinary and

Animal Sciences University, Ludhiana. The diet formulation was carried out using ingredients such as rice, rice gluten, soybean meal, wheat bran, Bengal gram, skim milk powder, meat cum bone meal, limestone powder, dicalcium phosphate, oil and various feed additives. Two distinct types of dog feeds were developed, one being vegetarian and the other non-vegetarian. The formulation of these diets adhered to the guidelines provided by AAFCO (2014) and NRC (2006) for dog feed standards.

The procured samples of feed ingredients underwent analysis for proximate composition *viz*. dry matter (DM), crude protein (CP), ether extract (EE), total ash (TA), acid insoluble ash (AIA), crude fiber (CF) calcium and phosphorus. The analytical methods followed AOAC (2005) and Talapatra et al. (1940) for calcium.

Formulation and preparation of diet

Two different dog feeds i.e. vegetarian (T1) and non-vegetarian (T2) formulations were prepared using different feed ingredients. All ingredients were ground in the mill, converting them into fine flour. These finely ground feed ingredients were precisely measured according to the specified quantities for the formulation of complete dog feed and thoroughly mixed. The mixture was extruded using the twin-screw extruder and the final product was subjected to overnight drying in an oven set at 45°C. The extruded feeds were meticulously analyzed for their dry matter, crude protein, ether extract, crude fiber, Ca, P and aflatoxin content, ensuring a comprehensive assessment of the nutritional and safety aspects of the final products.

Microbiological analysis of the adult dog feed

Total viable count

For analyzing the total viable count, 1 g of the feed formulation was added to 9 ml of 0.1% Buffered Peptone Water (BPW) and further diluted at a ratio of 10^{-1} up to 10^{-5}. 100 ml of each dilution was inoculated into the corresponding labelled nutrient agar plates (Sigma-Aldrich #70148) using a spreader and the plates were incubated at 37ºC for 24 hours. For statistical accuracy, plates with colony counts between 30 and 300 were considered in the calculation. To determine the number of CFUs in the original sample, the count on a dilution plate was multiplied by the corresponding dilution factor.

Detection of *Salmonella spp.*

The procedure described in the OIE Terrestrial Manual (2008) and was employed for the isolation and identification of *Salmonella* species in the dog feed sample. The isolation process involved pre-enrichment of the sample in buffered peptone water, enrichment in Rappaport Vassiliadis (RV) medium and selective plating in Xylose Lysine Deoxycholate (XLD) agar plates. Characteristic red translucent colonies with a typical black centre were presumptively considered as *Salmonella* colonies (Annex, 2010). The selected colonies were further subjected to biochemical characterization

Detection of *Escherichia coli*

The dog food samples were evaluated for the presence of *E. coli* through a standard method with necessary modifications (Lupindu, 2017). The feed samples were enriched using Brain Heart Infusion (BHI) broth followed by inoculation in Eosin Methylene Blue (EMB) agar plates. Colonies with characteristic metallic sheen were presumptively identified as *E. coli*.

Detection of Aflatoxin

Column chromatography (VICAM part #34000) was used to detect the presence of aflatoxins in the dog foods. Briefly, 50g of ground sample along with 5g of salt was blended at high speed for 1 minute in the presence of 100 ml methanol. Ten ml of the filtered extracts was diluted with 40ml purified water. The diluted extract was filtered through 1.5µm glass microfiber and 2ml of filtered extract was passed through the AflaTest column at the rate of about 1 drop/second until air came out through the column. 5mL of purified water was passed through the column at the rate of about 1-2 drops/second twice. Cuvette (VICAM part #34000) was placed under the column and 1 ml of HPLC grade methanol was added into a glass syringe barrel and was eluted at the rate of 1 drop/second and collected in a cuvette.

1 ml of AflaTest developer solution was added to the cuvette and mixed properly. The cuvette was placed in a calibrated fluorometer and readings of aflatoxin (ppb) were taken.

In-vitro digestibility

In-vitro digestibility of the feed formulations was assessed based on the protocol developed by Biagi et al. (2016) with minor modifications. At the end of two digestion phases, the undigested samples were filtered using a nylon bag and washed with cold water instead of centrifugation. For gastric digestion stimulation, 10g of dried and powdered feed samples were treated with 400 mL of pepsin-HCl solution (HCl 0.075N; pepsin 2 g/l) containing gastric lipase (1 g/l) and incubated in a shaking water bath at 39°C for 2 h. For small intestine digestion stimulation, the powdered feed formulations were mixed bile salts (Cholic acid-deoxycholic acid sodium salt mixture) at a final concentration of 25 g/l followed by the addition of with 400 ml of a pancreatin solution. The samples were incubated in a shaking water bath at 39°C for 4 hours. The undigested residue was filtered through nylon bags.

In order to determine the dry matter digestibility of the feed samples, the residue obtained after the *in-vitro* digestion was weighed and digestibility was calculated with the following formula:

Dry matter digestibility = (100-([residue weight-100]/sample weight)

The undigested fraction was analysed for crude protein, ether extract, and ash according to AOAC standard methods (AOAC, 2005). Nutrient digestibility was calculated with the following equation:

100-{[nutrient% in residue= (100-diet digestibility)]/ nutrient % in diet}

Statistical analysis

The data were presented as Standard Error of the Mean (SEM) and the significant mean difference was determined using one-way Analysis of Variance (ANOVA). Subsequently, treatment means were compared utilizing Duncan's Multiple Ranged Test (Duncan, 1995). The statistical analyses were carried out with the aid of the Statistical Package for the Social Sciences (SPSS, version 16.0) provided by SPSS Inc., Chicago, USA.

RESULTS AND DISCUSSION

Nutritional quality

The ingredients used to formulate the two in-house dog feed formulations are mentioned in Table 1. The proximate composition analysis of the formulated vegetarian (T1), non-vegetarian (T2), and commercial dog feed (T3) are mentioned in Table 2. AAFCO Dog Feed Nutrient Profile, 2014 for adult maintenance suggests a minimum crude protein concentration of 18.0% in dry matter, while for growth and reproduction, the recommended level is 22.5%. In this study, crude protein content of T1 and T2 was 19.25%. Hence, both the dog feed displayed crude protein values within the specified range and notably, both the formulated feed compositions fulfilled the essential nutrient requirements set for adult dogs. However, the proximate values of the commercial feed surpassed those of both the vegetarian and non-vegetarian formulations.

Table 1. Ingredients used to formulate the two in-house dog feed formulations

Ingredient	DM (%)	CP (%)	EE (%)	CF (%)	ME (Kcal/kg)	Ca (%)	P (%)
Rice	89.4	7.87	0.78	0.78	3630	0.04	0.06
Wheat bran	89.6	8.75	2.10	15.1	2100	0.12	1.38
Bengal gram	89.4	24.5	4.95	4.40	3120	0.08	0.13
Soybean meal	87.1	42.0	1.50	9.10	2876	0.32	0.28
Skim milk powder	95.8	34.0	0.28	0.22	3590	1.20	1.00
Rice gluten	89.4	47.2	3.90	5.02	3150	0.62	0.78
De-oiled rice polish	88.7	15.7	1.50	23.7	2100	0.07	1.33
Meat cum bone meal	90.0	44.6	5.90	5.99	2500	13.8	6.78
Limestone powder	-	-	-	-	-	35	-
Dicalcium phosphate	-	-	-	-	-	23	18
Oil	-	-	98	-	8500	-	-

Table 2. Percentage nutrient composition of different dog foods

Treatments	DM	CP	EE	CF	Ash	Ca	Phosphorus
T1	90.5	19.2	5.5	3.00	4.18	0.62	0.52
T2	90.3	19.2	5.6	3.23	4.00	0.65	0.54
T3	92.1	21.0	8.32	3.30	6.64	0.80	0.78

Microbiological analysis of different dog feeds

Microbial contamination associated with feed poses a significant risk to animal health (Tessari et al., 2014; Leiva et al., 2019). The raw materials of both plant and animal origin can serve as the starting point for microbial contamination in the feed production chain (Ruzauskas et al., 2005). This contamination can occur during various stages, including storage, transportation, feed production, packaging and final product storage (Girio et al., 2012). Evaluating the microbial load of the feed is crucial for ensuring the safety of animals. Additionally, there have been documented cases of pet owners resulting from contact with contaminated products (Imanishi et al., 2014). It is important to highlight that feed can be an unnoticed source of infection, particularly in vulnerable populations such as young children and the elderly (Behravesh et al., 2010; Stull et al., 2013; Imanishi et al., 2014). The likelihood of pathogenic microorganisms and their harmful by-products appearing in feed can rise in correlation with the overall quantity of microorganisms found in a specific product.

While there is no specific threshold established for the presence of *Escherichia coli* in animal feeds, it is generally recommended that the bacterial count should not surpass 3×10^2 cfu/g (ISO 4832 2006). In this study, there was no evidence of *E. coli* contamination in any of the analyzed dog feed samples. Nonetheless, Holda et al. (2017) reported *E. coli* isolation in the range of 10 to 50 cfu/g in four out of twenty dog feed samples, representing 20% of the tested products.

Salmonellosis ranks as the second most frequently reported zoonotic infection among humans in the European Union (Jansen et al., 2019). The genus *Salmonella* is recognized as the primary microbiological threat in pet feeds (Behravesh et al., 2010). Behravesh et al. (2010) highlighted the initial case of human salmonellosis linked to the handling of dry dog feed. This bacterium can be found in both

dry and wet pet feed, posing a unique challenge for human and pet feed producers due to its ability to survive in high-fat, low-moisture environments. In this study, *Salmonella* spp. was not detected in any of the dog feed formulations. A similar outcome was observed by Holda et al. (2017). Conversely, Wojdat et al. (2004) discovered *Salmonella* spp. in 22 out of 2271 (1%) dry feed samples tested, while Wojdat et al. (2005) isolated *Salmonella* spp. from 10 out of 169 (5.9%) feed samples tested (6.7% from compound feeds and 4.6% from raw materials).

The feed formulations were also subjected to Aflatoxin detection, revealing varying concentrations of occurrence. Among the feeds analyzed, the lowest Aflatoxin content was observed in T1 at 5.6 ppb. T2 exhibited a concentration of 6.6 ppb, while the highest Aflatoxin content was found in T3 at 8.9 ppb. In comparison to these findings, Gazzoti et al. (2015) reported the presence of deoxynivalenol, fumonisins and ochratoxin A in 100%, 88% and 81% of extruded commercial dog feed samples, respectively. These mycotoxins were found at levels exceeding the permissible limit of 5g/kg. Aflatoxins and zearalenone were present in moderate levels, with concentrations below the respective limit of quantification (5g/kg for aflatoxins and 10g/kg for zearalenone) in 88% and 75% of the samples, respectively. According to FDA (2019) guidelines. Aflatoxin levels in whole pet feed for all ages should not exceed 20 parts per billion (ppb). Hence the Aflatoxin concentrations in all prepared and commercial diets in this study were found to be below the permitted limits, ensuring the safety of the pet feed.

In vitro digestibility study

The results for *in vitro* digestibility of the formulated diets are represented in Table 3. The findings revealed that dry matter digestibility and crude protein digestibility were significantly higher ($P<0.05$) in the vegetarian diet (93.48%) and non-vegetarian diet (94.72%) compared to the commercial diet (90.90%). While the ether extract digestibility of the non-vegetarian diet (90.31%) was lower ($P<0.05$) than that of the vegetarian diet (94.62%), and commercial diet (95.10%). Organic matter digestibility exhibited a significant difference, being the lowest ($P<0.05$) in the non-vegetarian diet (93.2%) and the highest ($P<0.05$) in the vegetarian diet (93.2%). The organic matter digestibility of the commercial diet (90.42%) was found to be comparable with both non-vegetarian and vegetarian diets. Similar study was also conducted on nutritional assessment of dal churi (Kaur et al., 2021a), soy nugget (Kaur et al., 2021b), rice gluten and maize fibre (Kaur et al., 2021) based dog food. Singh et al. (2020) also assessed nutritional aspects of tomato pomace in dog food during *in vitro* study.

Table 3. Percentage *in vitro* nutrient digestibility of different dog feed

Treatments	DMD	CPD	EED	OMD
T1	93.1^{b}	93.5^{b}	94.6^{b}	93.2^{b}
T2	93.1^{b}	94.7^{b}	90.3^{a}	89.69^{a}
T3	89.08^{a}	90.9^{a}	95.1^{b}	90.42^{ab}
SEM	0.884	0.734	1.02	0.735
P-value	0.016	0.015	0.04	0.060

^{ab}Values with different superscripts in each column differ significantly ($P<0.05$)

CONCLUSION

In conclusion, this study underscores the potential benefits of formulating nutritionally balanced dog feeds, both vegetarian and non-vegetarian, with a focus on enhancing microbial safety and addressing common deficiencies observed in homemade diets. The findings support the hypothesis that these formulated feeds can improve the health outcomes of domesticated dogs by rectifying deficiencies in essential nutrients like protein, fiber, calcium, and phosphorus. Moreover, the utilization of in-vitro digestibility methods offers a practical approach to evaluating nutritional quality, potentially reducing the need for extensive animal trials and facilitating

quicker advancements in pet nutrition. By providing evidence-based insights into superior dietary choices for dogs, this research aims to guide improved feeding practices and contribute to the development of safer and more effective pet feeds, thereby promoting the overall well-being of pets globally.

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