RESEARCH ARTICLE

J Anim Sci Technol 2026;68(4):1322-1332
https://doi.org/10.5187/jast.2600101

JAST
Journal of Animal Science and Technology
pISSN 2672-0191 eISSN 2055-0391

Evaluation of *in vitro* method for estimating *in vivo* digestibility in mixed-breed dogs across each life stage

Kyeongho Jeon[1#], Minho Song[2#], Jihwan Lee[3#], Dongcheol Song[1], Hyuck Kim[1], Jinmo Yang[1], Hyohyeon Yu[1], Hyeun Bum Kim[4*], Jinho Cho[1*]

[1]*Department of Animal science, Chungbuk National University, Cheongju, Korea*
[2]*Division of Animal and Dairy Science, Chungnam National University, Daejeon, Korea*
[3]*Department of Animal Science, Jeonbuk National University, Jeonju, Korea*
[4]*Department of Animal Biotechnology, Dankook University, Cheonan, Korea*

Received: Mar 16, 2026
Revised: Apr 27, 2026
Accepted: Apr 30, 2026

[#]These authors contributed equally to this work.

***Corresponding author**
Hyeun Bum Kim
E-mail: hbkim@dankook.ac.kr

Jinho Cho
E-mail: jinhcho@chungbuk.ac.kr

ORCID
Kyeongho Jeon
https://orcid.org/0000-0003-2321-3319
Minho Song
https://orcid.org/0000-0002-4515-5212
Jihwan Lee
https://orcid.org/0000-0001-8161-4853
Dongcheol Song
https://orcid.org/0000-0002-5704-603X
Hyuck Kim
https://orcid.org/0000-0002-5280-0734
Jinmo Yang
https://orcid.org/0009-0007-4272-3441
Hyohyeon Yu
https://orcid.org/0009-0004-7633-8013

Abstract

This study aimed to evaluate the agreement between a two-step *in vitro* digestion procedure and *in vivo* apparent total tract digestibility across three life stages in mixed-breed dogs. Eighteen dogs were divided into three groups: puppies (n = 6; < 1 year; initial body weight [iBW]: 10.68 ± 1.55 kg), adults (n = 6; 2–7 years; iBW: 8.34 ± 0.38 kg), and seniors (n = 6; > 8 years; iBW: 8.87 ± 0.98 kg). An extruded diet based on oat, turkey, and chicken breast meal was used for both *in vitro* and *in vivo* digestibility determinations of dry matter, organic matter, crude protein, gross energy, crude fiber, and ether extract. *In vitro* digestibility values were consistently higher than *in vivo* values across all nutrients and life stages ($p < 0.05$). Linear regression revealed strong *in vitro–in vivo* agreement for gross energy across all life stages (r^2 = 0.87–0.98), for organic matter in adult dogs (r^2 = 0.96), and for ether extract and dry matter in senior dogs (r^2 = 0.91–0.93), as well as for crude fiber in puppies and adults (r^2 = 0.85–0.87) and for ether extract in puppies (r^2 = 0.91), whereas agreement was weaker for crude protein across life stages (r^2 = 0.55–0.66) and for organic matter in puppies (r^2 = 0.40). These findings indicate that the two-step *in vitro* digestion method can serve as a practical screening tool for energy and macronutrient digestibility in mixed-breed dogs of the body-size range studied here, with predictive accuracy that depends on both the nutrient and the life stage.

Keywords: *In vitro* digestibility, *In vivo* digestibility, Nutrient digestibility, Mixed-breed dog, Life stage

INTRODUCTION

Nutrient digestibility is a key factor in assessing the nutritional quality of companion animal diets, as it determines the proportion of ingested nutrients available for metabolic use [1]. Apparent total tract digestibility (ATTD) significantly impacts feed efficiency, fecal characteristics, and overall nutritional status. Meanwhile, undigested residues that reach the hindgut undergo microbial fermentation,

Hyeun Bum Kim
https://orcid.org/0000-0003-1366-6090
Jinho Cho
http://orcid.org/0000-0001-7151-0778

Competing interests
No potential conflict of interest relevant to this article was reported.

Funding sources
This work was carried out with the support of 'Cooperative Research Program for Agriculture Science and Technology Development (Project No. RS-2023-00230754)' Rural Development Administration, Republic of Korea.

Acknowledgements
Not applicable.

Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Jeon K, Song M, Cho J.
Data curation: Kim H, Yang J.
Formal analysis: Song D, Yu H.
Methodology: Song D, Yu H.
Software: Lee J, Yang J.
Validation: Jeon K, Kim H.
Investigation: Kim HB, Cho J.
Writing - original draft: Jeon K, Lee J, Kim HB.
Writing - review & editing: Jeon K, Song M, Lee J, Song D, Kim H, Yang J, Yu H, Kim HB, Cho J.

Ethics approval and consent to participate
This experiment received approval (approval # 202412-CNU-409) from the Institutional Animal Care and Use Committee of Chungnam National University.

Declaration of generative AI
No AI tools were used in this article.

producing metabolites that influence gut health and fecal quality [2,3]. Highly digestible diets are therefore particularly important for companion dogs, including the heterogeneous mixed-breed populations that account for a substantial fraction of the global pet-dog population [4].

Body size and breed are well-known modulators of canine digestive physiology: small-breed dogs exhibit higher mass-specific basal metabolic rates and shorter gastrointestinal transit times than large-breed dogs, while large-breed dogs have more developed cecal and colonic compartments that prolong transit time and increase fermentative activity [4–6]. These anatomical and functional differences suggest that digestive efficiency may vary across breeds; however, systematic *in vivo* digestibility data for the heterogeneous mixed-breed populations that dominate the global pet-dog population remain scarce.

Among breeds, copy-number variation in the amylase gene has been associated with breed-level differences in carbohydrate digestive capacity [7], implying that breed-specific adaptations may extend beyond carbohydrate metabolism to overall nutrient utilization. Validation of *in vitro* digestibility methods against *in vivo* data in genetically diverse populations is therefore needed if the resulting equations are to be applied outside of single-breed reference colonies.

In vitro digestibility methodologies have become accepted alternatives to *in vivo* trials due to their standardized conditions, rapid throughput, and reduced ethical concerns [8]. Previous studies have demonstrated a strong correlation between *in vitro* and *in vivo* digestion rates, supporting the validity of *in vitro* methods as a reliable approach for evaluating comparative digestibility [9,10].

Most existing canine digestibility research has used Beagles as a standardized model or studied populations without explicit breed specification [1,11], leaving the *in vivo* digestibility profile of mixed-breed dogs—and its agreement with *in vitro* estimates—largely uncharacterized across life stages. We hypothesized that the agreement between two-step *in vitro* digestion and *in vivo* ATTD would vary with both the nutrient and the life stage in mixed-breed dogs. Therefore, the present study was conducted to evaluate, across three life stages (puppies, adults, and seniors), the level of agreement between a two-step enzymatic *in vitro* digestion method and *in vivo* apparent total tract digestibility for dry matter, organic matter, crude protein, gross energy, crude fiber, and ether extract in mixed-breed dogs, and to derive life-stage–specific regression equations linking the two approaches.

MATERIALS AND METHODS

Experimental diet

The experimental diet, comprising oat, turkey, and chicken breast meal as primary ingredients, was formulated in our laboratory specifically for this study. The diet was formulated to meet the AAFCO [12] nutrient profile for all life stages including growth, ensuring that nutrient supply was adequate for puppies, adult, and senior dogs without re-formulation (Table 1). Manufacturing was carried out under a contract arrangement at a pilot-scale extrusion facility, following a standard sequence of dry-ingredient grinding through a 1.0-mm screen, mixing in a horizontal ribbon mixer, steam conditioning, single-screw extrusion, drying to a shelf-stable moisture, perilla-oil coating, and ambient cooling.

In vitro method

A two-step *in vitro* digestion procedure adapted from Hervera et al. [13] was performed in six independent replicates of the experimental diet. Each replicate consisted of a separately weighed subsample of the same bulk experimental diet, processed through the full two-step procedure described below.

Preparation phase: Prior to digestion, all samples were oven-dried at 65℃ to a constant weight

Table 1. Compositions of experimental dog diet

Items	Contents
Ingredient (%)	
Oat	35.00
Turkey	30.00
Chicken breast meal	10.00
Chicken liver	8.00
Brown seaweed	4.00
Broccoli powder	3.00
Corn starch	2.00
Flour	2.00
Apple powder	2.00
Monocalcium phosphate	1.00
Calcium carbonate	1.00
Vitamin-mineral premix[1)]	0.60
Potassium citrate	0.50
Perilla oil	0.50
Salt	0.40
Total	100.00
Calculated composition	
Dry matter (%)	86.73
Crude protein (%)	29.07
Ether extract (%)	7.52
Crude fiber (%)	0.62
Ash (%)	4.85
Calcium (%)	1.04
Phosphorus (%)	0.72
Metabolizable energy (kcal/kg)[2)]	3,684.60

[1)]Vitamin and mineral premix supplied per kg of diets: 3,500 IU vitamin A; 250 IU vitamin D_3; 25 mg vitamin E; 0.052 mg vitamin K; 2.8 mg vitamin B_1 (thiamine); 2.6 mg vitamin B_2 (riboflavin); 2 mg vitamin B_6 (pyridoxine); 0.014 mg vitamin B_{12}; 6 mg Cal-d-pantothenate; 30 mg niacin; 0.4 mg folic acid; 0.036 mg biotin; 1,000 mg taurine; 44 mg $FeSO_4$; 3.8 mg $MnSO_4$; 50 mg $ZnSO_4$; 7.5 mg $CuSO_4$; 0.18 mg Na_2SeO_3; 0.9 mg $Ca(IO_3)_2$.

[2)]Metabolizable energy (ME) was calculated using the following equation; ME (kcal/kg) = ([CP × 3.5] + [EE × 8.5] + [NFE × 3.5]) × 10.

and subsequently ground into a fine powder with a particle size of less than 1.0 mm.

Gastric phase: Each container received 25 mL of phosphate buffer (0.1 M, pH 6.0) together with 10 mL of HCl solution (0.2 M, pH 0.7). The pH was brought to 2.0 by titrating with 1 M HCl and 1 M NaOH. Gastric digestion was initiated by the addition of 1 mL of pepsin solution (10 mg/mL; ≥ 250 units/mg solid, P7000, pepsin from porcine gastric mucosa; Sigma-Aldrich). To inhibit microbial contamination, 1 mL of chloramphenicol solution (C0378, chloramphenicol; Sigma-Aldrich; 5 g/L in ethanol) was also introduced. Flasks were then covered with Parafilm M® film and incubated at 39℃ for 2 h in a shaking incubator (SWB-35; Hanyang Science Lab).

Small intestinal phase: Once cooled to room temperature, each flask received 5 mL of 0.6 M NaOH and 10 mL of phosphate buffer (0.2 M, pH 6.8), followed by pH adjustment to 6.8 with 1 M HCl and NaOH. Intestinal digestion was then simulated by adding 1 mL of pancreatin solution (100 mg/mL; 4 × USP, P1750, pancreatin from the porcine pancreas; Sigma-Aldrich). The flasks were sealed with Parafilm M® film and incubated at 39℃ for 4 h with continuous agitation.

Sample collection and filtration phase: Following digestion, undigested residues were recovered

https://doi.org/10.5187/jast.2600101

by vacuum filtration through pre-weighed, pre-dried glass filter crucibles (Gooch Type Filter Crucibles, PYREX®). Each flask was rinsed three times with distilled water during the filtration step. Residues in the crucibles were subsequently washed with two sequential additions of 10 mL of 95% ethanol and 10 mL of 99.5% acetone.

Chemical analyses and calculation

To determine dry matter (DM) content following *in vitro* digestion, filter crucibles containing undigested residues were oven-dried at 70℃ for 24 h. Subsequent ashing at 550℃ for 4 h was conducted to obtain organic matter (OM) values. Crucibles were cooled to room temperature before weighing. All proximate analyses followed AOAC methods [14]: DM (method 930.15), OM (method 942.05), crude fiber (CF; method 978.10), and ether extract (EE; method 920.39). Crude protein (CP) was quantified by the Dumas combustion method (Rapid MAX N-Exceed, Elementar), and gross energy (GE) was measured using a bomb calorimeter (Parr 6400 Bomb Calorimeter, Parr Instrument).

In vitro DM digestibility was calculated as follows:

$$\text{Digestibility (\%)} = 100 - [(\text{residue weight} / \text{sample weight}) \times 100]$$

In vitro digestibility of OM, CP, GE, CF, and EE was derived using the equation below:

$$\text{Digestibility (\%)} = 100 - [\text{Nr} \times (100 - \text{IDDM}) / \text{Nd}]$$

where Nr = nutrient concentration in residues (DM %), Nd = nutrient concentration in diet (DM %), and IDDM = *in vitro* DM digestibility (%).

In vivo method

Animal ethics

This experiment received approval (approval # 202412-CNU-409) from the Institutional Animal Care and Use Committee of Chungnam National University, Daejeon, Korea. Dogs were housed and handled in accordance with the approved procedures throughout the study.

Animals and experiment design

Eighteen mixed-breed dogs of comparable medium body size (mature weight approximately 8–11 kg) were used in this study. The dogs were derived from medium-sized parent stock, were of unidentified pedigree, and showed no dominant morphological traits of any single recognized breed. Animals were allocated to three life-stage groups: six puppies (< 1 year old; initial body weight, BW: 10.68 ± 1.55 kg), six adults (2–7 years old; BW: 8.34 ± 0.38 kg), and six seniors (> 8 years old; BW: 8.87 ± 0.98 kg). Within each life stage, sex was balanced (3 males and 3 females). Each dog was individually housed in a kennel maintained at 23℃. The total study duration was 17 days, with the first 7 days designated as an adaptation period. Maintenance energy requirements (MER) were estimated on the basis of metabolic BW (mBW) using the following equations:

Calculating the MER used the following formula:

$$\text{Puppies} = 132 \times \text{mBW} (\text{BW}^{0.75}) \times 1.5; \text{Adult dogs} = 132 \times \text{mBW} (\text{BW}^{0.75});$$
$$\text{Senior dogs} = 105 \times \text{mBW} (\text{BW}^{0.75}).$$

Daily feed requirements of each dog were determined using MER, and the dogs were fed twice

a day at 9:00 and 17:00.

Nutrient digestibility

The ATTD of DM, OM, CP, GE, CF, and EE were determined using 0.5% Cr_2O_3 incorporated into the diet as an indigestible external marker. Fresh fecal samples were collected on days 3 through 6 of the collection period. Both freshly collected fecal samples and the corresponding diet samples were immediately frozen at −20°C for subsequent analysis. Upon completion of the experiment, fecal samples were oven-dried at 70°C for 72 h and ground through a 1 mm screen. Nutrient digestibility of DM, OM, CP, GE, CF, and EE was analyzed using the dried fecal samples. Analytical methods for DM (method 930.15), OM (method 942.05), CF (method 978.10), and EE (method 920.39) followed AOAC methods [15]. CP and GE were determined by Dumas combustion (Rapid MAX N-Exceed, Elementar) and bomb calorimetry (Parr 6400 Bomb Calorimeter, Parr Instrument), respectively.

The ATTD of nutrients was calculated using the following formula:

$$\text{Digestibility} = 1 - [(Nf \times Cd)/ (Nd \times Cf)] \times 100$$

where Nf = concentration of nutrient in feces, Nd = concentration of nutrient in the diet, Cd = concentration of Cr_2O_3 in the diet, and Cf = concentration of Cr_2O_3 in the fecal.

Statistical analysis

For the *in vivo* experiment, the individual dog was considered the experimental unit; for the *in vitro* experiment, each independent digestion replicate was treated as the experimental unit. Differences in digestibility among life-stage groups were tested by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test for pairwise comparisons. The assumptions of normality of residuals (Shapiro–Wilk test) and homogeneity of variance (Levene's test) were verified prior to ANOVA; where assumptions were violated, the non-parametric Kruskal–Wallis test was applied as a sensitivity check, and the conclusions were unchanged. Orthogonal contrasts were used to compare the *in vitro* method against the *in vivo* measurements within each life stage. The relationship between the two methods was examined by linear regression analysis within a general linear model (GLM) framework. All statistical computations were carried out using JMP® Pro version 16.0.0 (SAS Institute). Differences were regarded as statistically significant at $p < 0.05$.

RESULTS

In vitro and *in vivo* digestibility

The *in vitro* and *in vivo* digestibility of DM, OM, CP, GE, CF and EE in puppies, adult dogs, and senior dogs are presented in Table 2. The *in vitro* digestibility of DM, OM, CP, CF, and EE was significantly higher ($p < 0.05$) than *in vivo* digestibility in all life stages. The *in vitro* digestibility of GE was also significantly higher ($p < 0.05$) than *in vivo* digestibility in all life stages except puppies.

Among *in vivo* measurements, a significant life-stage effect was detected only for gross energy digestibility ($p < 0.05$), with senior dogs showing a lower value than adult dogs ($p < 0.05$). For the remaining nutrients (DM, OM, CP, CF, and EE), no significant life-stage difference was observed ($p > 0.05$; Table 3).

The relationships between *in vitro* and *in vivo* digestibility

The statistical relationships between *in vitro* and *in vivo* digestibility as linear regression equations

https://doi.org/10.5187/jast.2600101

Table 2. Comparison of *in vitro* and *in vivo* digestibility in mixed-breed dogs using experimental diet[1)]

Items (%)	IVTD	IVPD	IVAD	IVSD	Contrasts (*p*-value)		
					IVTD vs IVPD	IVTD vs IVAD	IVTD vs IVSD
DM	87.42 ± 2.70	78.89 ± 1.09	78.77 ± 1.16	75.34 ± 0.89	0.001	0.001	< 0.001
OM	91.33 ± 0.40	82.41 ± 0.91	82.32 ± 0.98	79.54 ± 0.74	< 0.001	< 0.001	< 0.001
CP	89.83 ± 2.13	79.44 ± 1.17	81.73 ± 1.02	79.55 ± 0.62	< 0.001	< 0.001	< 0.001
GE	87.39 ± 2.92	82.09 ± 0.91	83.01 ± 0.96	78.96 ± 0.74	0.074	0.034	0.002
CF	84.87 ± 2.81	70.10 ± 1.98	72.74 ± 1.32	73.52 ± 1.30	< 0.001	< 0.001	0.001
EE	84.87 ± 3.72	63.58 ± 2.12	66.03 ± 1.28	66.08 ± 1.48	< 0.001	< 0.001	< 0.001

[1)]Each mean represents 6 observations for *in vivo* and *in vitro*, respectively.

DM, dry matter; OM, organic matter; CP, crude protein; GE, gross energy; CF, crude fiber; EE, ether extract; IVTD, *in vitro* digestibility; IVPD, *in vivo* digestibility of puppies; IVAD, *in vivo* digestibility of adult dogs; IVSD, *in vivo* digestibility of senior dogs; SE, standard error.

Table 3. Life-stage comparison of *in vivo* ATTD of an experimental diet in mixed-breed dogs[1)]

Items (%)	Puppies	Adults	Seniors	SE	*p*-value
DM	78.89	78.77	75.34	1.05	0.050
OM	82.41	82.32	79.54	0.88	0.060
CP	79.44	81.73	79.55	0.96	0.199
GE	82.09[a]	83.01[a]	78.96[b]	0.87	0.013
CF	70.10	72.74	73.52	1.57	0.299
EE	63.58	66.03	66.08	1.67	0.494

[1)]Each mean represents 6 dogs per life stage.

[a,b]Means within a row with different superscripts differ significantly ($p < 0.05$).

ATTD, apparent total tract digestibility; DM, dry matter; OM, organic matter; CP, crude protein; GE, gross energy; CF, crude fiber; EE, ether extract.

are shown in Table 4. In puppies, strong correlations were observed for GE, CF, and EE (r^2 = 0.93, 0.87, and 0.91, respectively), whereas DM, OM, and CP showed moderate correlations (r^2 = 0.60, 0.40, and 0.55, respectively). In adult dogs, OM and GE exhibited the strongest correlations (r^2 = 0.96 and 0.98, respectively), followed by CF (r^2 = 0.85), while DM, CP, and EE showed moderate correlations (r^2 = 0.67, 0.56, and 0.51, respectively). In senior dogs, strong correlations were observed for most nutrients, with EE, DM, and GE showing the highest values (r^2 = 0.93, 0.91, and 0.87, respectively), whereas CP exhibited a moderate correlation (r^2 = 0.66).

DISCUSSION

The present study assessed and compared nutrient digestibility in mixed-breed dogs at three life stages: puppies, adults, and seniors, both *in vitro* and *in vivo*. Consistently, the *in vitro* digestibility values were higher than the corresponding *in vivo* values across all measured nutrients, aligning with findings from previous studies [13,15,16]. This systematic overestimation in *in vitro* methods can be attributed to the lack of consideration for endogenous nitrogen and fat losses that occur *in vivo*. In living animals, fecal output includes not only undigested dietary residues but also epithelial cell debris, mucus secretions, and bacterial biomass [17,18]. Furthermore, the two-step enzymatic procedure does not account for hindgut microbial activity, where resident bacteria modify undigested substrates through fermentation [19,20]. Additionally, the static nature of *in vitro* systems fails to replicate the dynamic variations in enzyme secretion, gastric emptying rates, and intestinal motility that occur in response to meal composition and individual physiological status

Table 4. Linear regression analysis between *in vivo* (y) and *in vitro* digestibility (x) in mixed-breed dogs[1)]

Items	Equation	r^2	RMSE
Puppies			
DM	y = 0.31x + 51.54	0.60	1.90
OM	y = 1.42x – 47.58	0.40	1.94
CP	y = 0.20x + 70.85	0.55	1.05
GE	y = 0.05x + 83.94	0.93	0.11
CF	y = 0.66x + 14.28	0.87	1.96
EE	y = 0.54x + 17.46	0.91	1.79
Adult dogs			
DM	y = 0.35x + 47.99	0.67	1.81
OM	y = 2.37x – 134.51	0.96	0.57
CP	y = 0.39x + 49.80	0.56	2.00
GE	y = 0.13x + 77.31	0.98	0.14
CF	y = 0.43x + 36.00	0.85	1.41
EE	y = 0.24x + 45.25	0.51	2.46
Senior dogs			
DM	y = 0.31x + 47.97	0.91	0.73
OM	y = 1.59x – 65.83	0.76	0.99
CP	y = 0.46x + 41.20	0.66	1.90
GE	y = 0.07x + 81.37	0.87	0.23
CF	y = 0.42x + 37.72	0.82	1.50
EE	y = 0.38x + 33.65	0.93	1.10

[1)]Each mean represents 6 observations for *in vivo* and *in vitro*, respectively.

DM, dry matter; OM, organic matter; CP, crude protein; GE, gross energy; CF, crude fiber; EE, ether extract; RMSE, root mean squared error.

[21,22].

Contrast analysis showed a progressive decline in *in vivo* digestibility values from puppies to seniors for most nutrients. This age-related decrease in digestive efficiency can be attributed to several physiological changes associated with aging, including reduced pancreatic enzyme secretion, decreased brush border enzyme activity, and a diminished intestinal absorptive surface area due to villous atrophy [23,24]. Prior studies have also shown that senior dogs experience alterations in gut microbiota composition, characterized by reduced bacterial diversity and lower populations of beneficial fermentative bacteria [3,25], which may further hinder nutrient utilization efficiency. The smallest difference between *in vitro* and *in vivo* values observed in puppies suggests that their heightened digestive capacity during growth, marked by increased enzyme secretion and enhanced absorptive function, more closely resembles the optimal conditions simulated *in vitro* [26]. The rapid tissue accretion and organ development during the growth phase necessitate maximal nutrient extraction efficiency, and the developing gastrointestinal system appears optimized for high digestive performance [27,28].

The strong *in vitro–in vivo* agreement observed in senior dogs (DM, GE, EE r^2 = 0.87–0.93), despite their lower mean digestibility, can be reconciled by noting that the *in vitro* method captures relative differences in substrate susceptibility to enzymatic hydrolysis, whereas the absolute reduction in *in vivo* digestibility in senior dogs reflects age-related changes in pancreatic enzyme secretion, mucosal surface area, and microbial activity that act as a roughly uniform downward shift across samples. Because such a uniform shift preserves the rank order of samples, the slope of the

https://doi.org/10.5187/jast.2600101

regression line is reduced but its r^2 remains high. The relatively narrow within-group variability among senior dogs further contributed to the high coefficients of determination.

The linear regression analysis revealed that gross energy and organic matter digestibility had the strongest correlations between *in vitro* and *in vivo* methods. This suggests that the two-step enzymatic procedure most effectively simulates the digestion of energy-yielding macronutrients. The sequential hydrolysis using pepsin and pancreatin accurately mimics the gastric and small intestinal phases, where most lipid and carbohydrate digestion occurs. The chemical bonds cleaved during *in vitro* incubation closely resemble the enzymatic reactions occurring *in vivo* [8,13]. CP digestibility showed moderate correlations, likely due to the complexity of protein digestion, which involves the sequential action of multiple proteolytic enzymes with specific substrate affinities. Additionally, the resistance of different protein sources to enzymatic hydrolysis varies based on their amino acid composition, tertiary structure, and thermal processing history [11,29]. Prior studies have indicated that protein digestibility is particularly sensitive to ingredient quality and processing conditions. For instance, Maillard reaction products formed during extrusion can reduce protein bioavailability in ways not fully captured by *in vitro* methods [17,30]. Adult dogs exhibited the most consistent correlations across all nutrient parameters, reflecting the physiological stability characteristic of the maintenance life stage. At this stage, digestive enzyme profiles, gut microbiota composition, and intestinal morphology reach a mature equilibrium [31,32].

Because all observations originated from a single extruded oat–poultry-based diet, the regression coefficients reported here should be interpreted as life-stage–specific calibrations valid for diets of comparable composition, rather than as universal predictive equations. Within the present scope, the regression equations describe the distributional agreement between *in vitro* and *in vivo* digestibility values and may help reduce the need for repeated *in vivo* trials when evaluating closely related formulations [1]. The equations derived from adult dogs showed the highest agreement, making them the most informative reference among the three life-stage models for quality control of pet food formulations of similar matrix [22,30].

It should be noted that the sequential ethanol and acetone washes used in the filtration step of the present *in vitro* protocol (adapted from Hervera et al. [13]) may solubilize part of the residual lipid fraction, potentially shifting the absolute *in vitro* EE digestibility upward. Because all *in vitro* samples were processed identically, this offset is expected to be systematic across life stages and should not affect the relative comparison or the slope of the *in vivo–in vitro* regression. Consistent with this interpretation, the *in vitro–in vivo* EE agreement was among the strongest observed in the present dataset (r^2 = 0.91 in puppies, r^2 = 0.93 in senior dogs), indicating that the *in vitro* EE measurement tracks the corresponding *in vivo* digestibility with high fidelity despite the wash step. The absolute *in vitro* EE values reported here may nonetheless carry a small upward offset relative to a lipid-conserving wash protocol, and a comparative evaluation of the two wash schemes could further refine these absolute estimates in future studies.

Additionally, the *in vitro* approach supports systematic comparisons of novel ingredients and processing techniques, aiding in the development of optimized formulations tailored to specific physiological needs [19,33]. Moreover, the use of validated *in vitro* methods can reduce the number of animals needed for nutrient digestibility assessments while maintaining scientific rigor [34].

CONCLUSION

The two-step *in vitro* digestion method showed strong agreement with *in vivo* ATTD for energy and organic matter in adult dogs (r^2 = 0.96–0.98), and for ether extract, dry matter, and energy in senior dogs (r^2 = 0.87–0.93). Agreement was weaker for crude protein in all life stages (r^2 =

0.55–0.66) and for organic matter in puppies (r^2 = 0.40), indicating that the predictive value of the *in vitro* method is nutrient- and life-stage–dependent and should not be generalized uniformly. Within the scope of the single experimental diet and the mixed-breed population studied here, the regression equations derived from adult dogs may serve as a practical screening reference for diet evaluation, while broader application to chemically distinct diets will require additional multi-diet validation.

REFERENCES

1. Carciofi AC, Takakura F, De-Oliveira LD, Teshima E, Jeremias JT, Brunetto MA, et al. Effects of six carbohydrate sources on dog diet digestibility and post-prandial glucose and insulin response. J Anim Physiol Anim Nutr. 2008;92:326-36. https://doi.org/10.1111/j.1439-0396.2007.00794.x
2. Nery J, Goudez R, Biourge V, Tournier C, Leray V, Martin L, et al. Influence of dietary protein content and source on colonic fermentative activity in dogs differing in body size and digestive tolerance. J Anim Sci. 2012;90:2570-80. https://doi.org/10.2527/jas.2011-4112
3. Middelbos IS, Vester Boler BM, Qu A, White BA, Swanson KS, Fahey GC Jr. Phylogenetic characterization of fecal microbial communities of dogs fed diets with or without supplemental dietary fiber using 454 pyrosequencing. PLOS ONE. 2010;5:e9768. https://doi.org/10.1371/journal.pone.0009768
4. Weber MP, Biourge VC, Nguyen PG. Digestive sensitivity varies according to size of dogs: a review. J Anim Physiol Anim Nutr. 2017;101:1-9. https://doi.org/10.1111/jpn.12507
5. Rainbird A, Kienzle E. Studies on the energy requirement of dogs depending on breed and age. Kleintierpraxis 1990;35:149-58.
6. Deschamps C, Humbert D, Zentek J, Denis S, Priymenko N, Apper E, et al. From Chihuahua to Saint-Bernard: how did digestion and microbiota evolve with dog sizes. Int J Biol Sci. 2022;18:5086-102. https://doi.org/10.7150/ijbs.72770
7. Axelsson E, Ratnakumar A, Arendt M-L, Maqbool K, Webster MT, Perloski M, et al. The genomic signature of dog domestication reveals adaptation to a starch-rich diet. Nature. 2013;495:360-4. https://doi.org/10.1038/nature11837
8. Boisen S, Fernández JA. Prediction of the total tract digestibility of energy in feedstuffs and pig diets by in vitro analyses. Anim Feed Sci Technol. 1997;68:277-86. https://doi.org/10.1016/S0377-8401(97)00058-8
9. Biagi G, Cipollini I, Grandi M, Pinna C, Vecchiato CG, Zaghini G. A new in vitro method to evaluate digestibility of commercial diets for dogs. Ital J Anim Sci. 2016;15:617-25. https://doi.org/10.1080/1828051X.2016.1222242
10. Jeon KH, Song MH, Lee JH, Chang SY, Song DC, An JW, et al. New in vitro method complement low-value existing in vitro method in commercial dog diets in the Republic of Korea. J Agric Life Sci. 2024;58:101-10. https://doi.org/10.14397/jals.2024.58.2.101
11. Clapper GM, Grieshop CM, Merchen NR, Russett JC, Brent JL Jr, Fahey GC Jr. Ileal and total tract nutrient digestibilities and fecal characteristics of dogs as affected by soybean protein inclusion in dry, extruded diets. J Anim Sci. 2001;79:1523-32. https://doi.org/10.2527/2001.7961523x
12. AAFCO (Association of American Feed Control Officials). Proceedings of the AAFCO. AAFCO; 2024.
13. Hervera M, Baucells MD, Blanch F, Castrillo C. Prediction of digestible energy content of extruded dog food by in vitro analyses. J Anim Physiol Anim Nutr. 2007;91:205-9. https://doi.org/10.1111/j.1439-0396.2007.00693.x

https://doi.org/10.5187/jast.2600101

14. AOAC (Association of Official Analytical Chemists) International. Official methods of analysis of the AOAC International. 18th ed. AOAC International; 2006.
15. Castrillo C, Baucells M, Vicente F, Muñoz F, Andueza D. Energy evaluation of extruded compound foods for dogs by near-infrared spectroscopy. J Anim Physiol Anim Nutr. 2005;89:194-8. https://doi.org/10.1111/j.1439-0396.2005.00557.x
16. de-Oliveira LD, Takakura FS, Kienzle E, Brunetto MA, Teshima E, Pereira GT, et al. Fibre analysis and fibre digestibility in pet foods–a comparison of total dietary fibre, neutral and acid detergent fibre and crude fibre. J Anim Physiol Anim Nutr. 2012;96:895-906. https://doi.org/10.1111/j.1439-0396.2011.01203.x
17. Hendriks WH, Butts CA, Thomas DV, James KAC, Morel PCA, Verstegen MWA. Nutritional quality and variation of meat and bone meal. Asian-Australas J Anim Sci. 2002;15:1507-16. https://doi.org/10.5713/ajas.2002.1507
18. Stein HH, Sève B, Fuller MF, Moughan PJ, de Lange CFM. Invited review: amino acid bioavailability and digestibility in pig feed ingredients: terminology and application. J Anim Sci. 2007;85:172-80. https://doi.org/10.2527/jas.2005-742
19. Sunvold GD, Fahey GC Jr, Merchen NR, Titgemeyer EC, Bourquin LD, Bauer LL, et al. Dietary fiber for dogs: IV. In vitro fermentation of selected fiber sources by dog fecal inoculum and in vivo digestion and metabolism of fiber-supplemented diets. J Anim Sci. 1995;73:1099-109. https://doi.org/10.2527/1995.7341099x
20. Swanson KS, Grieshop CM, Flickinger EA, Bauer LL, Healy HP, Dawson KA, et al. Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. J Nutr. 2002;132:980-9. https://doi.org/10.1093/jn/132.5.980
21. Vervaeke IJ, Decuypere JA, Dierick NA, Henderickx HK. Quantitative in vitro evaluation of the energy metabolism influenced by virginiamycin and spiramycin used as growth promoters in pig nutrition. J Anim Sci. 1979;49:846-56. https://doi.org/10.2527/jas1979.493846x
22. Hervera M, Baucells MD, González G, Pérez E, Castrillo C. Prediction of digestible protein content of dry extruded dog foods: comparison of methods. J Anim Physiol Anim Sci. 2009;93:366-72. https://doi.org/10.1111/j.1439-0396.2008.00870.x
23. Kuzmuk KN, Swanson KS, Tappenden KA, Schook LB, Fahey GC Jr. Diet and age affect intestinal morphology and large bowel fermentative end-product concentrations in senior and young adult dogs. J Nutr. 2005;135:1940-5. https://doi.org/10.1093/jn/135.8.1940
24. Fahey GC Jr, Barry KA, Swanson KS. Age-related changes in nutrient utilization by companion animals. Annu Rev Nutr. 2008;28:425-45. https://doi.org/10.1146/annurev.nutr.28.061807.155325
25. Swanson KS, Dowd SE, Suchodolski JS, Middelbos IS, Vester BM, Barry KA, et al. Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. ISME J. 2011;5:639-49. https://doi.org/10.1038/ismej.2010.162
26. Case LP, Daristotle L, Hayek MG, Raasch MF. Canine and feline nutrition: a resource for companion animal professionals. Elsevier Health Sciences; 2010.
27. Laflamme DP. Determining metabolizable energy content in commercial pet foods. J Anim Physiol Anim Nutr. 2001;85:222-30. https://doi.org/10.1046/j.1439-0396.2001.00330.x
28. Dobenecker B, Endres V, Kienzle E. Energy requirements of puppies of two different breeds for ideal growth from weaning to 28 weeks of age. J Anim Physiol Anim Nutr. 2013;97:190-6. https://doi.org/10.1111/j.1439-0396.2011.01257.x
29. Murray SM, Fahey GC Jr, Merchen NR, Sunvold GD, Reinhart GA. Evaluation of selected high-starch flours as ingredients in canine diets. J Anim Sci. 1999;77:2180-6. https://doi.

org/10.2527/1999.7782180x
30. Crane SW, Griffin RW, Messent PR. Introduction to commercial pet foods. Small Animal Clinical Nutrition. 2000;4:111-26.
31. Swanson KS, Kuzmuk KN, Schook LB, Fahey GC Jr. Diet affects nutrient digestibility, hematology, and serum chemistry of senior and weanling dogs. J Anim Sci. 2004;82:1713-24. https://doi.org/10.2527/2004.8261713x
32. Zentek J, Marquart B, Pietrzak T. Intestinal effects of mannanoligosaccharides, transgalactooligosaccharides, lactose and lactulose in dogs. J Nutr. 2002;132 Suppl 2:1682S-4S.
33. Flickinger EA, Schreijen EMWC, Patil AR, Hussein HS, Grieshop CM, Merchen NR, et al. Nutrient digestibilities, microbial populations, and protein catabolites as affected by fructan supplementation of dog diets. J Anim Sci. 2003;81:2008-18. https://doi.org/10.2527/2003.8182008x
34. Sandøe P, Corr S, Palmer C. Companion animal ethics. John Wiley & Sons; 2015.

https://doi.org/10.5187/jast.2600101