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ARTICLE INFORMATION	Fill in information in each box below
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Article Title (within 20 words without abbreviations)	Evaluation of the Nutrient Digestibility at Each Age in Dogs Diet by *In vitro* and *In vivo* Methods
Running Title (within 10 words)	Evaluation of nutrient digestibility in dog diets
Author	Kyeongho Jeon[1, #], Jihwan Lee[2, #], Minho Song[3, #], Kihyun Kim[4], Minseok Jo[5], Seyeon Chang[1], Dongcheol Song[1], Sehyun Park[1], Hyuck Kim[1], Hyeunbum Kim[6, *], Jinho Cho[1, *]
Affiliation	[1] Department of animal science, Chungbuk National University, Cheongju 28644, Korea [2] Swine Science Division, National Institute of Animal Science, Rural Development Administration, Cheonan 31000, Korea [3] Division of Animal and Dairy Science, Chungnam National University, Daejeon 34134, Korea [4] National Institute of Animal Science, Rural Development Administration, Wanju 55365, Korea [5] Central Research Institute, Woosung Feed Co., Ltd, Daejeon 34379, Korea [6] Department of Animal Biotechnology, Dankook University, Cheonan 31116, Korea
ORCID (for more information, please visit https://orcid.org)	Kyeongho Jeon / jeonkh1222@gmail.com (https://orcid.org/0000-0003-2321-3319) Jihwan Lee / junenet123@naver.com (https://orcid.org/0000-0001-8161-4853) Minho Song / mhsong@cnu.ac.kr (https://orcid.org/0000-0002-4515-5212) Kihyun Kim / kihyun@korea.kr (https://orcid.org/0000-0002-9834-2126) Minseok Jo/ msjo@woosung.kr (https://orcid.org/0000-0001-6742-4316) Seyeon Chang / angella2425@naver.com (https://orcid.org/0000-0002-5238-2982) Dongcheol Song / paul741@daum.net (https://orcid.org/0000-0002-5704-603X) Sehyun Park / parksae0808@naver.com (https://orcid.org/0000-0002-6253-9496) Hyuck Kim / harrck85@naver.com (https://orcid.org/0000-0002-5280-0734) Hyeunbum Kim / hbkim@dankook.ac.kr (https://orcid.org/0000-0003-1366-6090) Jinho Cho / jinhcho@cbnu.ac.kr (http://orcid.org/0000-0001-7151-0778)
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Authors' contributions Please specify the authors' role using this form.	Conceptualization: Kyeongho Jeon, Jihwan Lee, Minho Song, Hyeunbum Kim, Jinho Cho Data curation: Kyeongho Jeon, Sehyun Park, Hyuck Kim Formal analysis: Dongchoel Song, Seyeon Chang Methodology: Dongcheol Song, Sehyun Park Software: Minseok Jo, Seyeon Chang, Hyuck Kim Validation: Dongchoel Song, Sehyun Park Investigation: Jihwan Lee, Minho Song, Minseok Jo, Hyuck Kim Writing - original draft: Kyeongho Jeon, Jihwan Lee, Minho Song Writing - review & editing: Kyeongho Jeon, Jihwan Lee, Minho Song, Kihyun Kim, Minseok Jo, Seyeon Chang, Dongcheol Song, Sehyun Park, Hyuck Kim, Hyeunbum Kim, Jinho Cho
Ethics approval and consent to participate	This experiment was examined and approved (approval # 202310A-CNU-179) by the Institutional Animal Care and Use Committee of Chungnam National University, Daejeon, Korea. In experiment, dogs were collected and managed by the procedures.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Jinho Cho[1] Hyeunbum Kim[5]
Email address – this is where your proofs will be sent	[1] jinhcho@chungbuk.ac.kr [5] hbkim@dankook.ac.kr
Secondary Email address	
Address	[1] Department of animal science, Chungbuk National University, Cheongju 28644, Korea [6] Department of Animal Biotechnology, Dankook University, Cheonan 31116, Korea
Cell phone number	[1] +82-10-8014-8580 (Jinho Cho)
Office phone number	[1] +82-43-261-2544 (Jinho Cho) [5] +82-41-550-3653 (Hyeunbum Kim)
Fax number	[1] +82-43-273-2240 (Jinho Cho)

Abstract

The objective of this study was to evaluate *in vitro* predictions of digestibility at each age (puppy, adult, and senior) in dogs of dry matter (DM), organic matter (OM), crude protein (CP), gross energy (GE), crude fiber (CF), and ether extract (EE) using dog diets. First, to determine the digestibility of dog diets using pepsin and pancreatin incubations, conduct the *in vitro* method. Later, 18 mixed-sex beagles were used in this experiment to compare *in vivo* digestibility. Beagles are divided into 3 groups according to their age and body weight: six puppies (under 1-year-old; 6.21 ± 0.56 kg), six adult dogs (2 to 7 years old; 8.16 ± 0.64 kg), and six senior dogs (over 8 years old; 6.95 ± 1.39 kg). Except for DM in puppies and adult dogs, in all cases, *in vitro* digestibility values were higher than *in vivo* digestibility values ($p < 0.05$). In puppies, there were strong relationships for DM and GE with r^2 values of 0.95 and 0.84, respectively, between *in vitro* and *in vivo* digestibility. Also, in adult dogs, there were strong relationships for DM and GE with r^2 values of 0.97 and 0.84, respectively, between *in vitro* and *in vivo* digestibility. However, in senior dogs, there was a lower relationship for DM, OM, CP, GE, CF, and EE with r^2 values of 0.18, 0.42, 0.01, 0.02, 0.11, and 0.04, respectively, between *in vitro* and *in vivo* digestibility. In conclusion, *in vitro*, the prediction of nutrient digestibility of DM and GE in puppies and adult dogs seems to have significant potential for practical application. However, additional research is needed to compare senior dogs with the *in vitro* method.

Keywords (3 to 6): *in vitro* digestibility, *in vivo* digestibility, dog, age

Introduction

Pets positively affect people’s physical health and emotional stability [1]. These effects improve their quality of life and increase people's preference for pet ownership [2]. Pets are raised in about 66%, 69%, and 60% of households in the United States, Australia, and the United Kingdom, respectively [3-5]. Interest in pets has increased as the majority of the population is raising them, which raises nutritional and health anxiety about their diets [6]. Because dogs are normally provided nutrients from complete and balanced diets, the nutrient content of diets and nutrient digestibility are important [7]. Pet food companies routinely perform digestibility testing to provide important information on the nutrient content of their diets [8]. Several nations have recognized the importance of the nutrient digestibility of dogs and offered related information [9-11].

In the Republic of Korea, pets have become a fundamental component of daily life, and the number of households with dogs has increased dramatically in recent years [12]. According to Joo et al [13], dogs represent 77.4% of the total household pets. However, research on domestic dog diets is insufficient in the Republic of Korea compared to the increasing number of dogs being raised. Most domestic dog diets developed in the Republic of Korea consult overseas nutritional requirements, such as NRC [9] and AAFCO [10]. Few nutritional studies have been conducted on dog diets, so it is necessary to investigate and establish nutrient digestibility standards.

Both *in vitro* and *in vivo* methods are used to evaluate the nutrient digestibility of diets [14]. Among them, *in vitro* methods have positive features, such as being cheaper, ethical, and more time-saving, and can be utilized as an alternative to *in vivo* methods [15]. Numerous studies have used two-step *in vitro* methods to simulate digestion in the stomach and small intestines of dogs [16,17]. Most *in vitro* studies have compared feedstuff digestibility to *in vivo* studies and generated predictive equations for their relationships [18]. However, few studies based in the Republic of Korea have used dog diets to study *in vitro* digestibility and compared them with *in vivo* digestibility. Therefore, this study was conducted to evaluate *in vitro* prediction of digestibility at each age (puppy, adult, and senior) of dry matter (DM), organic matter (OM), crude protein (CP), gross energy (GE), crude fiber (CF), and ether extract (EE) using dog diets.

Materials and Methods

Experimental diet

The experimental diet using *in vitro* and *in vivo* methods based on hydrolyzed chicken powder, soy protein, and brown rice was manufactured in extruded form. The diet was formulated to meet or exceed the nutrient requirements according to the AAFCO guideline (Table 1).

In vitro method

The *in vitro* method described by Hervera et al. [19] method was conducted in two steps with 6 replicates of dog diet.

Step 1: The samples were prepared in finely ground (< 1.0 mm) form. In stomach simulation, weigh (1.000 ± 0.001 g) of each sample in 250 mL Erlenmeyer flasks, then add 25 mL of phosphate buffer (0.1 M, pH 6.0) and 10 mL of HCl solution (0.2 M, pH 0.7) to each flask. The pH was adjusted to 2.0 using 1 M HCl and 1 M NaOH solution, and 1 mL pepsin solution (10 mg/mL; ≥ 250 units/mg solid, P7000, pepsin from porcine gastric mucosa; Sigma-Aldrich, St. Louis, MO, USA) was added to the flask to simulate stomach digestion in the dog. In addition, 1 mL of chloramphenicol solution (C0378, chloramphenicol; Sigma-Aldrich, St. Louis, MO, USA with 5 g/L ethanol) was also added to avoid bacterial fermentation. The flasks were closed with a Parafilm M® film and incubated in a shaking incubator (SWB-35; Hanyang Science Lab Co., Seoul, Republic of Korea) at 39°C for 2 h.

Step 2: 5 mL of NaOH solution (0.6 M) and 10 mL of phosphate buffer (0.2 M, pH 6.8) were added to the flask after cooling at room temperature. The pH was adjusted to 6.8 using 1 M HCl and 1 M NaOH solution, and 1 mL of pancreatin solution (100 mg/mL; 4 × USP, P1750, pancreatin from the porcine pancreas; Sigma-Aldrich, St. Louis, MO, USA) was added in the flask to simulate digestion conditions in the small intestine of the dog. Then, the flasks were closed with a Parafilm M® film and incubated in a shaking incubator (SWB-35; Hanyang Science Lab Co., Seoul, Republic of Korea) at 39°C for 4 h.

Then, the collected undigested samples were filtered through pre-dried and pre-weighed glass filter crucibles (Gooch Type Filter Crucibles, PYREX®, UK). During filtering, the flasks were rinsed three times with distilled water. Additionally, 10 mL of 95% ethanol and 10 mL of 99.5% acetone were added twice to the glass filter crucibles.

Chemical analyses and calculation

At the end of the *in vitro* procedure, the filter crucibles containing undigested residues were dried at 70°C for 24 h to calculate DM. Then, they were burned at 550°C for 4 h to calculate OM. After being dried and combusted, it was cooled to room temperature and then weighed. The methods utilized for the determination of DM (method 930.15), OM (method 942.05), CF (method 978.10) and EE (method 920.39) were conducted with the methods of AOAC [19]. The CP and GE content were analyzed by using the dumas (Rapid MAX N-Exceed, Elementar, Langenselbold, Germany) and bomb calorimeter (Parr 6400 Bomb Calorimeter, Parr Instrument Co., Moline, IL, USA), respectively.

Calculating the *in vitro* digestibility of DM using the following formula:

"Digestibility (%) = 100 – {(residue weight/sample weight) × 100}

Calculating the *in vitro* digestibility of OM, CP, GE, CF and EE used the following formula:

"Digestibility (%) = 100 – {Nr × (100 – IDDM)/Nd}"

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

***In vivo* method**

Animal ethics

This experiment was examined and approved (approval # 202310A-CNU-179) by the Institutional Animal Care and Use Committee of Chungnam National University, Daejeon, Korea. In experiment, dogs were collected and managed by the procedures.

Animals and experiment design

A total of 18 mixed-sex beagles were used in this experiment. Beagles were divided into 3 groups according to their age: six puppies (under 1 year old), six adult dogs (2 to 7 years old), and six senior dogs (over 8 years old). Total experimental period was 17 days which included 7 days adaptation period. Each dog was managed in individual cage (0.9 m × 0.9 m × 0.9 m), and the temperature was maintained at 23℃. The maintenance energy requirements (MER) for each growth stage were calculated using metabolic body weight (mBW).

Calculating the MER used the following formula:

"Puppies = 132 × mBW ($BW^{0.75}$) × 1.5; Adult dogs = 132 × mBW ($BW^{0.75}$); Senior dogs = 105 × mBW ($BW^{0.75}$)".

Daily feed requirements were calculated in accordance with MER applied to each dog and fed twice a day at 9:00 and 17:00.

Nutrient digestibility

At the bottom of each kennel, dense mesh was attached to separate urine and feces for collecting pure fecal samples. Pee pads absorbed urine through the mesh, and the fecal samples remained on the mesh. Fecal samples for calculating digestibility by the total fecal collection method were collected during 8 days of experimental periods. Fresh fecal and feed samples were stored in a freezer at -20℃ after collection immediately. The stored fecal samples were dried at 103℃ for 12 h and then finely ground (< 1 mm) for chemical analysis at the end of the experiment. The total fecal collection digestibility of DM, OM, CP, GE, CF and EE were analyzed using samples. The methods utilized for the determination of DM (method 930.15), OM (method 942.05), and EE (method 920.39) were conducted with the methods of AOAC [19]. The CP and GE content were analyzed by using the dumas (Rapid MAX N-Exceed, Elementar, Langenselbold, Germany) and bomb calorimeter (Parr 6400 Bomb Calorimeter, Parr Instrument Co., Moline, IL, USA), respectively. The equation for the total fecal collection method described by Renan A Donadelli et at al [20].

Total fecal collection digestibility was determined by the following formula:

“Digestibility (%) = [{%Nutrient in Diet * Feed Intake(g)} – {%Nutrient in Fecal * Fecal Output(g)}] / [(%Nutrient in Diet * Feed Intake)]”

Statistical analysis

Dog means served as the experimental unit. The means of the treatments were also compared by using orthogonal contrasts: *in vitro* digestibility vs. other treatments. Variability in the data was expressed as the SEM. The relationship between *in vitro* and *in vivo* digestibility measured in dogs was determined by regression analyses using a general linear model (GLM) in a JMP (JMP® Pro version 16.0.0, SAS Institute Inc. Cary, NC, USA). The model was y = ax + b, where y = *in vivo* digestibility, a = slope, x = *in vitro* digestibility and b = intercept. Statistical differences were determined to be 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 of puppies, adult dogs, and senior dogs are presented in Table 2. The *in vivo* digestibility of DM in senior dogs was significantly higher ($p = 0.027$) than *in vitro* digestibility. Also, the *in vivo* digestibility of CP, GE, CF, and EE in all ages was significantly higher ($p < 0.001$) than *in vitro* digestibility. However, there was no significant difference in the *in vitro* digestibility compared to the *in vivo* digestibility of DM in adults and senior groups and OM in all age groups, respectively.

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

The statistical relationships between *in vitro* and *in vivo* digestibility as linear regression equations are shown in Table 3. There was a strong relationship between DM and GE (r^2 = 0.95 and 0.84, respectively) in puppies. In adult dogs, there was a strong relationship between DM and GE (r^2 = 0.97 and 0.84, respectively). However, in senior dogs, there was a low relationship between whole contents (DM, r^2 = 0.18; OM, r^2 = 0.42; CP, r^2 = 0.01; GE, r^2 = 0.02; CF, r^2 = 0.11; EE, r^2 = 0.04).

Discussion

This study evaluated the digestibility of a dog diet using *in vivo* and *in vitro* methods and generated predictive equations for the relationships between *in vivo* and *in vitro* digestibility. Previous studies reported that *in vitro* digestibility was higher than *in vivo* digestibility due to endogenous losses in the body [18, 21, 22]. In this study, the *in vitro* digestibility of CP, GE, CF, and EE was higher than the *in vivo* digestibility at all ages. Consistent with our results, Penazzi et al. [23] suggested that *in vitro* digestibility overestimated *in vivo* digestibility. Endogenous losses in the body have a significant influence on *in vivo* digestibility [18]. In the *in vitro* method, chloramphenicol was added to avoid bacterial fermentation, and the method was conducted under strictly controlled temperature, digestion time, pH, and enzyme content conditions [24], which explains why *in vitro* digestibility was higher than *in vivo* digestibility. Le Bon et al. [25] reported that senior dogs had less inflammation and attributed it to gut microbial diversity decreases in aging dogs. Decreases in gut microbial diversity affect gut health, leading to low digestibility [26]. In this study, a significant difference between DM *in vivo* and *in vitro* digestibility was seen due to the low digestibility of senior dogs.

The *in vitro* method can assist in identifying nutritional availability in non-ruminant animals [27]. Prior studies were conducted on the *in vitro* digestibility of dog diets compared to *in vivo* digestibility [17, 28, 29]. This study adopted a modified two-step *in vitro* procedure for dogs, which involved reducing the doses of exogenous digestive enzymes to account for the shorter gastrointestinal tract and faster digestion rate in dogs compared to pigs [17].

The wide range of nutrient contents in dog diets may affect the accuracy of *in vitro* equations for predicting nutrient availability [26]. Endogenous losses, enzymatic secretion, and microbial activity were reported to be other influencing factors [30]. In this study, a predictive equation was generated by comparing *in vivo* and *in vitro* digestibility in each age group. A strong relationship between DM and GE was found in puppy and adult-aged dogs. Satterlee et al. [31] reported that the analysis of animal protein-based diets resulted in lower accuracy, leading to differences in the digestibility relationship. Burrows et al. [32] suggested that the presence of dietary fiber also affects the digestibility of diets. Consistent with previous studies, Biagi et al. [29] assumed that the low relationship between *in vitro* and *in vivo* digestibility could be attributed to the fact that feces include bacteria and other endogenous protein sources, as well as to proteins derived from diets, which causes protein digestibility to be underestimated. In this study, the low relationship between the *in vitro* and *in vivo* digestibility of CP, CF, and EE was assumed to be caused by endogenous losses. In senior dogs, a low relationship between in vitro and in vivo digestibility was found for all dietary components analyzed. The low level of adjustment may have been affected by the limited number of samples and the consistent *in vivo* values recorded across samples [33]. Weber et al. [34] reported that growth affected digestibility by altering the transit time of the digestive system. Consistent with previous studies, our findings were likely due to differences in *in vivo* digestibility due to age differences, resulting in a low correlation with *in vitro* digestibility values.

Based on these results, we can use equations to predict age-specific digestibility through *in vitro* experiments. However, additional research is needed to investigate the relationship between *in vitro* and *in vivo* methods in senior dogs.

Conclusion

There were strong linear relationships between *in vivo* and *in vitro* digestibility (DM and GE) in puppies, (DM and GE) in adult dogs. *In vitro*, prediction of digestibility (DM and GE) in puppies and adult dogs seem to have significant potential for practical application. However, additional research investigating the *in vitro* method in senior dogs is needed.

Disclosure statement

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Table 1. Compositions of experimental dog diet

Items	**Contents**
Ingredient, %	
Hydrolyzed chicken powder	35.00
Brown rice	32.65
Tapioca starch	5.00
Soy protein	15.00
Carrot	1.00
Sweet pumpkin	2.00
Cabbage	2.00
Salt	0.40
Canola oil	3.00
Monocalcium phosphate	1.80
Calcium carbonate	1.60
Vitamin-mineral premix[1]	0.50
Tocopherol	0.05
Total	100
Chemical composition	
Dry matter, %	91.09
Crude protein, %	40.84
Ether extract, %	6.65
Crude fiber, %	0.27
Calcium, %	0.78
Phosphorus, %	0.65
Crude ash, %	6.55
Nitrogen free extract, %	38.81
Metabolic energy[2], kcal/kg	3,707.00

[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 follow equation; ME (kcal/kg) − ([CP × 3.5] + [EE × 8.5] + [NFE × 3.5]) × 10.

Table 2. Comparison of *in vitro* and *in vivo* digestibility using developed dog diet[1]

Items (%)	IVT	IVVP	IVVA	IVVS	SE	Contrasts (*p*-value)		
						IVT vs IVVP	IVT vs IVVA	IVT vs IVVS
DM	95.87	95.92	96.14	95.30	0.17	0.809	0.266	0.027
OM	92.05	92.06	92.88	92.32	0.48	0.983	0.241	0.695
CP	96.10	92.25	92.01	89.65	0.54	<0.001	<0.001	<0.001
GE	95.22	92.99	93.82	92.63	0.28	<0.001	<0.001	<0.001
CF	94.59	79.47	84.11	83.40	0.73	<0.001	<0.001	<0.001
EE	93.60	82.86	86.23	85.63	0.49	<0.001	<0.001	<0.001

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

[2]Abbreviaiton: DM, dry matter; OM, organic matter; CP, crude protein; GE, gross energy; CF, crude fiber; EE, ether extract; IVT, *in vitro* digestibility; IVVP, *in vivo* digestibility of puppies; IVVA, *in vivo* digestibility of adult dogs; IVVS, *in vivo* digestibility of senior dogs; SE, standard error.

Table 3. Linear regression analysis between *in vivo* (y) and *in vitro* digestibility (x) in dog diets[1]

Items	Equation	r^2	RMSE
Puppies			
DM	y = 0.85x+14.11	0.95	0.08
OM	y = -0.19x +109.83	0.43	0.50
CP	y = 0.12x+80.52	0.01	1.03
GE	y = 0.66x+30.47	0.84	0.12
CF	y = 1.48x-60.63	0.20	2.12
EE	y = -0.08x+90.74	0.01	1.43
Adult dogs			
DM	y = 1.17x-16.13	0.97	0.08
OM	y = 0.11x+82.85	0.25	0.43
CP	y = 0.05x+87.14	0.00	1.34
GE	y = 1.07x-7.66	0.84	0.19
CF	y = 1.39x-47.51	0.29	1.57
EE	y = -0.06x+91.54	0.02	0.64
Senior dogs			
DM	y = 0.65x+32.55	0.18	0.54
OM	y = -0.31x+120.63	0.42	0.82
CP	y = 0.30x+61.13	0.01	2.24
GE	y = 0.40x+54.56	0.02	1.34
CF	y = 1.20x-29.68	0.11	2.39
EE	y = 0.18x+69.19	0.04	1.40

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

[2]Abbreviaiton: DM, dry matter; OM, organic matter; CP, crude protein; GE, gross energy; CF, crude fiber; EE, ether extract; IVT, *in vitro* digestibility; IVVP, *in vivo* digestibility of puppies; IVVA, *in vivo* digestibility of adult dogs; IVVS, *in vivo* digestibility of senior dogs; SE, standard error.

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