1 **JAST (Journal of Animal Science and Technology) TITLE PAGE**

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Abstract

This study evaluated the comparison between *in vitro* and *in vivo* methods for predicting nutrient digestibility across different life stages in Sapsarees. The research performed both *in vitro* methods of dog gastrointestinal conditions such as stomach and small intestine conditions and *in vivo* methods using 18 Sapsaree groups. Sapsarees were categorized into three groups by age and weight: six puppies (under 1 year; 9.94 ± 5.27 kg), six adult dogs (2-7 years; 23.49 ± 3.90 kg), and six senior dogs (over 8 years; 21.57 ± 2.27 kg). The nutrients examined included dry matter, organic matter, crude protein, gross energy, crude fiber, and ether extract. The significant differences were found in the digestibility of OM, CF, and EE between the methods ($p < 0.05$) except the digestibility of DM in puppies and adult dogs and GE digestibility. In puppies, there were strong linear relationships for OM, GE, CF, and EE with r^2 values of 0.85, 0.90, 0.85, and 0.82, respectively, between *in vitro* and *in vivo* digestibility. Also, in adult dogs, there were strong linear relationships for DM, GE, and CF with r^2 values of 0.85, 0.90, and 0.91, respectively, between *in vitro* and *in vivo* digestibility. In the relationship between *in vitro* and *in vivo* digestibility of senior dogs, there were strong linear relationships for OM with r^2 values of 0.87. The *in vitro* method shows a strong correlation with *in vivo* digestibility and is predicted to have significant potential for practical application.

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

Introduction

The population of companion animals has experienced a remarkable surge in recent years, reshaping how we view and interact with them [1]. Although companion animals were once primarily kept for practical purposes such as for protection or work, they have now become integral members of our families, forming deep emotional connections with their human counterparts [2, 3]. This change has brought about a heightened focus on pet health and wellness, highlighting proper nutrition and the need to better understand their digestive processes [4]. Nutritional qualities of companion animal diets depend heavily on their digestibility and how readily available their nutrients are. Moreover, compositions of a dog's diet and accessibility of its nutrients play a crucial role in shaping canine cognition and behavior, with these factors often interacting in complex pathways [5].

Nutrient digestibility in dogs has been recognized as a crucial aspect of canine nutrition across numerous countries, leading to extensive research and information on this subject. These studies have provided rich data to understand canine nutrition requirements and digestive processes [6, 7]. Previous studies have employed *in vivo* and *in vitro* methods to assess nutrient digestibility in animal diets [8]. *In vivo* methods can directly measure digestibility within living organisms, whereas *in vitro* methods typically involve exposing feed samples to enzymes or microbial inocula under controlled conditions that mimic the gastrointestinal environment. Compared to in vivo methods, *in vitro* methods are typically more cost-efficient with fewer ethical issues. In addition, they can be conducted more quickly [9]. Consequently, *in vitro* digestibility assays have become valuable alternatives of *in vivo* experiments [10].

The Republic of Korea hosts several traditional canine breeds, including Cheju, Donggyeongi, Jindo, Pungsan, and Sapsaree [11]. Canine species in Korea comprising approximately 350 breeds show extreme variabilities in body mass and morphology, with weights ranging from 1 kg to 100 kg [12]. Weber et al. [13] have reported that obvious morphological differences between breeds show biological differences that can affect gastrointestinal function and physiological metabolism. However, studies on nutrient digestibility of Republic of Korea breeds have been notably scarce in the existing literature. Therefore, this study aimed to evaluate *in vitro* prediction of digestibility at Sapsaree's each life stage (puppy, adult, and senior) for 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 used in this study was based on hydrolyzed chicken powder, brown rice, and soybean meal, and was manufactured in extruded form. According to the AAFCO guideline [14], the diet was formulated to meet or exceed the nutrient requirements (Table 1).

In vitro method

The *in vitro* method described by Hervera et al. [15] method was conducted in two steps with 6 replicates of dog diet.

Preparation phase: Samples were dried at 65℃ until constant weight was achieved and then pulverized into a fine powder (particle size below 1.0 mm).

Gastric phase: 25 mL of phosphate buffer (0.1 M, pH 6.0) and 10 mL of hydrochloric acid (HCl) solution (0.2 M, pH 0.7) were introduced into each container. The acidity was adjusted to pH 2.0 using HCl and sodium hydroxide (NaOH) solutions (both 1 M). To mimic gastric digestion, 1 mL of pepsin solution (10 mg/mL; ≥ 250 units/mg solid, P7000, pepsin from porcine gastric mucosa; Sigma-Aldrich, St. Louis, MO, USA) was added. Additionally, 1 mL of chloramphenicol solution (C0378, chloramphenicol; Sigma-Aldrich, St. Louis, MO, USA with 5 g/L ethanol) was added to avoid bacterial growth. The flasks were sealed with Parafilm M® film and placed in a shaking incubator (SWB-35; Hanyang Science Lab Co., Seoul, Republic of Korea) at 39°C for 2 hours.

Small intestinal phase: After cooling to room temperature, 5 mL of NaOH solution (0.6 M) and 10 mL of phosphate buffer (0.2 M, pH 6.8) were added to each flask. The pH was then adjusted to 6.8 using HCl and NaOH solutions (both 1 M). To simulate small intestine conditions, 1 mL of pancreatin solution (100 mg/mL; 4 × USP, P1750, pancreatin from the porcine pancreas; Sigma-Aldrich, St. Louis, MO, USA) was added. The flasks were closed with a Parafilm M® film and incubated in a shaking incubator at 39℃ for 4 h under agitation.

Sample collection and filtration phase: The undigested residue was filtered through pre-weighed, pre-dried glass filter crucibles (Gooch Type Filter Crucibles, PYREX®, UK). The flasks were rinsed thrice with distilled water during filtration. The filtration process conducted with two separate additions of 10 mL of 95% ethanol and 10 mL of 99.5% acetone to the crucibles.

Chemical analyses and calculation

The undigested residues in filter crucibles were dried at 70°C for 24 h to quantify DM content after *in vitro* digestion process. Then, dried samples were ashed at 550°C for 4 h to determine OM. After cooling to room temperature, the samples were weighed. Nutrient composition analysis adhered to AOAC method [16], including protocols for DM (method 930.15), OM (method 942.05), CF (method 978.10), and EE (method 920.39). For CP and GE content, the Dumas (Rapid MAX N-Exceed, Elementar, Langenselbold, Germany) and bomb calorimeter (Parr 6400 Bomb Calorimeter, Parr Instrument Co., Moline, IL, USA) utilized, 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}"

where 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 experiments, dogs were collected and managed by the procedures.

Animals and experiment design

A total of 18 mixed-sex Sapsarees were used in this experiment. Six puppies (under 1 year old), six adult dogs (2 to 7 years old), and six senior dogs (over 8 years old) were the three life stage groups of Sapsarees. The 7 days were allotted for adaptation during the 17 days study period. Using metabolic body weight (mBW), the maintenance energy requirements (MER) for every growth stage were determined.

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 of each dog were determined using MER, and the dogs were fed twice a day at 8:00 and 16:00.

Nutrient digestibility

Apparent total tract digestibility (ATTD) of DM, OM, CP, GE, CF and EE were determined using 0.5% chromic oxide (Cr_2O_3) as an indigestible marker in the diet. Fresh fecal samples were collected from 3 to 6 days. Fresh fecal and diet samples were stored in a freezer at -20°C immediately after collection. At the end of the experiment, fecal samples were dried at 70°C for 72 h and then crushed on a 1 mm screen. Nutrient 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), CF (method 978.10) and EE (method 920.39) were conducted with the methods of AOAC [16]. 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 ATTD digestibility of nutrients used the following formula:

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

where Nf = concentration of nutrient in fecal, 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

Individual dogs served as the experimental unit of analysis in this study. Treatment effects were evaluated using orthogonal contrast comparisons. To assess the relationship between in vitro and in vivo digestibility measurements obtained from the dogs, regression analyses were conducted using a general linear model (GLM). These statistical procedures were performed using JMP (JMP® Pro version 16.0.0, SAS Institute Inc. Cary, NC, USA). Statistical significance was defined as $p < 0.05$, with values below this threshold considered to indicate a significant difference between treatments.

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 was significantly higher ($p = 0.026$) than *in vivo* digestibility in senior dogs. Also, the *in vitro* digestibility of OM, CF, and EE was significantly higher ($p < 0.05$) than *in vivo* digestibility in all ages. In the *in vivo* digestibility of CP in adult and senior dogs was significantly lower ($p < 0.05$) than *in vitro* digestibility.

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, OM, GE and EE ($r^2 = 0.70$, 0.90, 0.85 and 0.82, respectively) in puppies. In adult dogs, there was a strong relationship between DM, GE, and CF ($r^2 = 0.85$, 0.90, and 0.91, respectively). Also, in senior dogs, there was a strong relationship between OM ($r^2 = 0.87$).

Discussion

This study employed a modified two-stage *in vitro* procedure adapted to account for distinctive digestive characteristics of dogs, specifically their shorter gastrointestinal tracts and accelerated digestion rates relative to pig models [17]. Utilizing this tailored methodology, we examined the correlation between *in vitro* and *in vivo* digestibility across various age groups of Sapsarees. *In vitro* digestibility consistently demonstrated higher values than *in vivo* digestibility across all analyzed nutrients (DM, OM, CP, GE, CF, and EE). Specifically, the *in vitro* DM digestibility was 94.07%, whereas *in vivo* DM digestibility values were 93.86%, 93.62%, and 92.76% for puppies, adult dogs, and senior dogs, respectively. Statistical analysis revealed significant differences between the *in vitro* and *in vivo* methods, particularly for CF and EE. According to Savoie [18], *in vitro* methods have a tendency to slightly overestimate *in vivo* digestibility. *In vitro* methods provide a highly reproducible environment that optimizes digestion processes by isolating factors, such as enzyme activity and pH levels, while also mimicking the composition and activity of gastrointestinal microbiome [19]. Consistent with current results, differences in the *in vitro* and *in vivo* digestibility might be attributed to endogenous losses and controlled conditions in an *in vitro* system [20]. Interestingly, we observed distinct age-related patterns of nutrient digestibility. The CP digestibility showed a clear declining trend with age, with values of 88.71%, 84.47%, and 82.55% for puppies, adults, and senior dogs, respectively. Dogs experience a decline in digestive function as they age, similar to other mammals, including humans. This is likely attributed to alterations in intestinal structures and functions that occur over time [21, 22]. Especially, protein had a higher digestibility in puppies than in other ages because their body was growing rapidly and a large amount of muscle was being deposited [23]. The results of the present study suggest that age-related physiological changes, particularly a potential decrease in protein requirements with age, could have a significant influence on nutrient utilization in Sapsarees.

Diverse nutrient compositions of diets can significantly impact the accuracy of *in vitro* equations used to predict nutrient digestibility and availability across animals [24]. Age-specific endogenous losses, enzyme secretion, and microbial activity represent additional factors influencing *in vivo* predictions [25]. This study developed age-specific predictive equations by correlating *in vivo* digestibility with *in vitro* results across different life stages. The regression analysis yielded varying degrees of correlation between *in vitro* and *in vivo* digestibility. In puppies, strong correlations were observed for GE ($r^2 = 0.90$), CF ($r^2 = 0.83$), and EE ($r^2 = 0.82$). Adult dogs exhibited robust correlations for CF ($r^2 = 0.91$) and GE ($r^2 = 0.90$), although OM showed an unexpectedly weak correlation ($r^2 = 0.03$). However, senior dogs generally demonstrated weaker correlations, with only OM showing a strong correlation ($r^2 = 0.87$). Protein, fat, and carbohydrate were major energy sources in dog diets [26]. As dogs age,

they typically undergo a reduction in muscle mass and an increase in fat mass, resulting in a lower energy requirement to sustain their body weight and function [27, 28]. The varying RMSE values across nutrients and age groups indicate differential prediction accuracies, suggesting that the reliability of *in vitro* methods might be age and nutrient dependent. These findings provide valuable insights into future research directions, particularly regarding the need for more comprehensive studies of senior dogs. Furthermore, our results suggest that *in vitro* methods can effectively predict nutrient digestibility in Sapsarees. However, age-specific variations must be carefully considered when applying these predictive equations in practical applications. Further research, particularly targeting senior dogs, would be beneficial for validating and improving these predictive equations.

Conclusion

The *in vitro* digestibility showed strong linear relationships with *in vivo* digestibility for puppies (OM, GE, CF, EE), adult dogs (DM, GE, CF), and senior dogs (OM). Therefore, predicting *in vivo* digestibility for Sapsarees of different ages using the *in vitro* digestibility method is expected to have significant potential for practical application.

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 age-based *in vivo* digestibility of Sapsaree diet[1]

Items (%)	*In vitro* digestibility	*In vivo* digestibility of puppies	*In vivo* digestibility of adult dogs	*In vivo* digestibility of senior dogs	SE	Contrasts (*p*-value)		
						In vitro digestibility vs *In vivo* digestibility of puppies	*In vitro* digestibility vs *In vivo* digestibility of adult dogs	*In vitro* digestibility vs *In vivo* digestibility of senior dogs
DM	94.07	93.86	93.62	92.76	0.38	0.704	0.415	0.026
OM	92.60	86.98	86.45	85.86	0.41	<0.001	<0.001	<0.001
CP	90.55	88.71	84.47	82.55	1.45	0.380	0.008	0.001
GE	89.18	88.46	88.57	87.81	1.39	0.718	0.762	0.492
CF	86.10	73.64	74.45	77.22	2.24	0.001	0.002	0.011
EE	85.73	78.72	78.63	80.88	1.62	0.006	0.006	0.047

[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.

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

Items	Equation	r^2	RMSE
Puppies			
DM	y = 0.09x + 85.47	0.70	0.12
OM	y = 0.30x + 65.32	0.85	0.24
CP	y = 0.18x + 72.16	0.56	1.04
GE	y = 0.05x + 83.62	0.90	0.14
CF	y = 0.26x + 50.96	0.85	1.22
EE	y = 0.20x + 61.18	0.82	0.81
Adult dogs			
DM	y = 0.30x + 65.32	0.85	0.24
OM	y = 0.02x + 85.08	0.03	0.20
CP	y = 0.35x + 52.59	0.56	2.00
GE	y = 0.13x + 76.80	0.90	0.33
CF	y = 0.27x + 51.54	0.91	0.93
EE	y = -0.03x + 81.09	0.24	0.43
Senior dogs			
DM	y = 0.07x + 86.43	0.19	0.27
OM	y = 0.10x + 76.84	0.87	0.08
CP	y = 0.42x + 44.93	0.65	1.95
GE	y = 0.02x + 86.42	0.03	0.62
CF	y = 0.20x + 60.07	0.73	1.35
EE	y = -0.10x + 89.17	0.61	0.66

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

IVT, *in vitro* digestibility; IVVP, *in vivo* digestibility of puppies; IVVA, *in vivo* digestibility of adult dogs; IVVS, *in vivo* digestibility of senior dogs; DM, dry matter; OM, organic matter; CP, crude protein; GE, gross energy; CF, crude fiber; EE, ether extract; RMSE, root mean squared error.