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Research article

Estimating the apparent total tract digestibility at different ages in rabbits by using faecal enzyme activities and *in vitro* digestibility on diets as predictors

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Abstract

The determination of apparent digestibility by non-invasive techniques such as prediction equation is an efficient technique for appraising the gastrointestinal performance and some indication of health status in rabbits. Therefore, faecal enzyme activities and *in vitro* digestibility by using crude enzyme extract from individual (CEi) rabbits at 92-days-old, pooled CEi (CEp), or commercial enzyme (CM) as independent variables was investigated, in order to estimate apparent digestibility in rabbits at different ages. Apparent total tract digestibility and faecal samples for enzyme activities at different age of twelve New Zealand white rabbits was calculated and collected. CEi from different digestive organs were taken from slaughtered 92-day-old rabbits. Based on multiple linear regression models, the percentage of *in vitro* digestibility on dry matter (DM) and organic matter (OM) with CEi fit well relative to using CM to estimate apparent digestibility on DM (DMd; $R^2 = 0.49$ vs 0.17, respectively) and OM (OMd; $R^2 = 0.51$ vs 0.49, respectively). A low determination coefficient for predicting DMd and OMd was found when using faecal enzyme activity with or without *in vitro* digestibility data. However, the use of CEp as independent variables provided a higher determination coefficient than using CM. Moreover, the equation which was run for *in vitro* digestibility on OM using CEp, faecal protease and cellulase activities, was statistically significant in terms of predicting apparent digestibility on crude protein ($R^2 = 0.72$, SEE = 7.05, $P = 0.03$). Therefore, these parameters can be used as independent variables to estimate apparent digestibility to individual rabbit to reveal current digestive efficiency.

Keywords: : Crude enzyme extract, Digestibility, Enzyme activities, Faeces, *In vitro* digestibility

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INTRODUCTION

The determination of apparent digestibility is an efficient technique for appraising the nutritive value of rabbit diets and digestive tract function, which is important information for feed evaluation and gastrointestinal health status. However, the time-consuming and costly aspects of experimental animal facilities are disadvantages of this technique. Nowadays, experiments that use the minimal number of animals, or none at all, is the worldwide regulation. Therefore, several alternative indicator estimations, i.e. chemical composition (Pascual et al., 2000; Villamide et al., 2003), *in vitro* digestibility (Ramos and Carabaño, 1996) and spectroscopy (Xiccato et al., 2003), have been developed and applied to replace apparent total tract digestibility in rabbits.

Dietary chemical composition is a good predictor for estimating *in vivo* digestibility (Pascual et al., 2000; Villamide et al., 2003). The percentage of *in vitro* digestibility by using commercial enzyme (CM), based on *in vitro* digestibility of dry matter using DMdinvitroCM and *in vitro* digestibility of organic matter using OMdinvitroCM, provided superior analytical performance as observed by the equations of diet chemical composition (Ramos and Carabaño, 1996; Pascual et al., 2000; Villamide et al., 2009); this shows adequate repeatability and reproducibility between intra-laboratory analyses and inter-laboratory analyses (Carabaño et al., 2008). Although, DMdinvitroCM and OMdinvitroCM made more accurate when using with chemical composition in diets to estimate dry matter digestibility but the prediction on crude protein apparent digestibility (CPd) is less accurate (Villamide et al., 2009). Near infrared spectrometry can be used to predict DMd, however, a lower determination coefficient than methods using *in vitro* digestibility data as predictors was reported by Xiccato et al. (2003). Since *in vitro* digestibility has been effectively applied to estimate the apparent digestibility, the application and optimization of this technique should be of interest to nutritional studies of rabbits (Ramos and Carabaño, 1996; Pascual et al., 2000; Villamide et al., 2009). CM from swine is generally used for studying *in vitro* digestibility in rabbits (Ramos and Carabaño, 1996; Pascual et al., 2000; Carabaño et al., 2008; Villamide et al., 2009). Therefore, the efficiency of crude enzyme extracts (CE) from rabbits should be compared to a CM.

The current status of apparent digestibility should be considered so as to represent digestive efficiency and health indicator, not only for farm management programs but also for pet animals. Assessing only the chemical components or *in vitro* digestibility of diets without taking relevant samples from rabbits should not be appropriate predictors to estimate individual apparent digestibility. Therefore, the appropriate indicators should be more studied. The correlation between enzyme activity in faeces and digestive organs were observed in white leg shrimp, *Penaeus vannamei* (Córdova-Murueta et al., 2003), green turtles, Chelonia mydas (Kanghae et al., 2017) and hamsters (Huang et al., 2014). In addition, faecal inoculation can be used to estimate apparent digestibility on DM (DMd) and OM (OMd) in rabbits (Pascual et al., 2000). As described above, faecal enzyme activities may be good predictors for revealing the current digestibility status in rabbits.

Comparison between the use of commercial enzyme or crude enzyme extraction from individual rabbits on *in vitro* digestibility to estimate apparent total tract digestibility on dry matter, organic matter and crude protein was the primary aim of this study. The usage of crude enzyme extraction from individual rabbits is not convenient to perform in real-world scenarios, and therefore the crude enzyme extraction from rabbits was pooled (CEp). Hence, predicting apparent total tract digestibility on dry matter, organic matter and crude protein from faecal enzyme activities and *in vitro* digestibility using pooled crude enzyme extraction from rabbits or commercial enzyme was another objective of this study.

MATERIAL AND METHODS

Animals, housing and diets

Twelve weaned 40-day-old New Zealand white rabbits were randomly selected with an equal sex ratio from a rabbitary and collected inside individual cages in a room with controlled temperature (24 ± 1 °C, 12 light: 12 dark cycle) at the Animal Experimental Unit, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand. The experiment was conducted from 40 to 91 days in terms of the rabbits' age. A commercial pelleted diet (Lee Feed Mill, Publ. Co., Ltd., Phetchaburi, Thailand) that was formulated respecting to nutrient requirements, in addition to clean water, were provided *ad libitum*. The DM in experimental diets was 90.7%, whereas OM, ash, ether extract, crude protein, crude fiber, neutral detergent fiber, acid detergent fiber and acid detergent lignin were 94.3, 5.69, 2.42, 16.1, 13.4, 27.7, 17.7 and 4.53% DM, respectively.

This study was carried out following the guidelines of, and achieved allowance from, the Institutional Animal Care and Use Committee of Kasetsart University, Bangkok, Thailand (ACKU60-VET-015).

Apparent digestibility and faecal collection

The apparent digestibility was determined in four-day periods between 45–49, 52–56, 66–70, 73–77, 80–84 and 87–91 days of rabbit age, according to Kovitvadhi et al. (2016a). Feed and hard faeces were weighed and collected daily at 9:00 a.m., and immediately kept at –20°C. The homogenised faecal samples for each period were divided for proximate analysis and enzyme extraction. Both feed and faecal samples were analysed in duplicate for DM, crude protein, ether extract and ash by ignition to 550 °C for OM calculation, according to the Association of Official Analytical Chemists (AOAC, 2000). The apparent digestibility on DM and nutrients was calculated following the European standardised method (Perez et al., 1995).

Faecal enzyme extraction, characteristics and activities

The pooled faeces from 45–49, 52–56, 66–70, 73–77, 80–84 and 87–91 days of age were extracted to analyse both enzyme characteristics in different pH conditions and enzyme activities (protease, amylase and cellulase). The procedures

were the same as described above, however there was some adaptation, as follows. The faecal samples were homogenised with 0.2 M PBS (pH 7, 1:10 w/v) for enzyme extraction. The enzyme characteristics were performed in different pH conditions from pH 2 to 10. The pH conditions at pH 5, 7 and 4 were selected to study amylase, protease and cellulase activities in faecal samples, respectively. Triplicate analyses were performed for each sample, whereas the sample with all solutions without enzyme was used as a blank control.

Enzyme extraction and characteristics from digestive organs

All rabbits were stunned by concussion and slaughtered without fasting at 92 days old, according to the guideline of American Veterinary Medical Association (AVMA, 2005). The stomach, pancreas, duodenum and caecum were immediately separated, stored at 0°C to preserve enzyme activities and sent to the laboratory for enzyme extraction. The stomach mucosa, pancreas, duodenal mucosa and caecal content of each rabbit were collected at 0°C. These samples were subjected for homogenisation with 0.2 M phosphate buffer solution (PBS; pH7, 1:5 w/v; except for caecal content, which used 1:2 w/v) followed by centrifugation at 18,000 g for 30 minutes at 4 ºC. The supernatant containing a CE was stored at –80 ºC for further analysis.

The enzyme characteristics of each organ were studied in triplicate under various pH conditions to identify appropriate pH for a series of *in vitro* digestibility tests. The activities of protease in stomach mucosa, cellulase in caecal content, and amylase in duodenal mucosa and pancreas, were evaluated following Kanghae et al. (2017) with some modifications. For protease activity, a set of equal volumes of substrate (2% casein in 0.1N NaOH) and gastric mucosal CE in different pH buffers of pH 2, 3, 4, 5, 6 and 7 were mixed and then incubated at 40 ºC for 5 minutes. After the incubation, trichloroacetic acid was added to a final concentration of 5% v/v to stop the enzyme reaction before centrifugation at 5,000 g for 20 minutes at 4 ºC. The supernatant was mixed with 0.5N NaOH and Folin-Ciocalteu reagent for further spectrophotometric absorbance measurement of free amino acids at a wavelength of 720 nm. Standard curve of L-tyrosine was used as a reference.

Soluble starch (5% w/v) was used as a substrate for determination of amylase activity in various pH conditions, including pH 5, 6, 7, 8, 9 and 10. After 15 minutes of incubation at 40 ºC, dinitrosalicylic acid (DNS) was added to final concentration of 1% v/v followed by a 5-minute boiling period at 100 ºC before being transferred to room temperature. Distilled water was added and the absorbance was measured at 540 nm. Linear range of standard maltose was used to quantify amount of liberated product after enzymatic hydrolysis.

The cellulase activity was assayed using 2% carboxylmethyl cellulose (CMC; low viscosity). The 75 µl of CMC was mixed with 425 µl of 0.2 M PBS (pH ranging from pH 4 to 10) and 50 µl of CE from caecal contents. The mixture was incubated at 37 ºC for 30 minutes, mixed with 250 µl of 1% DNS, and then boiled at 100 oC for 10 minutes. After adding 2.5 ml of distilled water, the absorbance was measured at 540 nm against linear range of standard glucose. The absorbance in the formulation was deducted by blank, which contained all reagents but without the enzyme.

In vitro digestibility using crude enzyme extracts from rabbits or commercial enzymes

The method of *in vitro* digestibility was adapted from Villamide et al. (2009), which contained three steps of digestion in the stomach, small intestine and caecum. Rabbit commercial pelleted diets (0.5 g) from the experiment were ground into 1 mm and used as substrate. The substrate was mixed with 12 ml of 0.1 M PBS (pH 6), 125 µl of chloramphenicol solution and 5 ml of 0.2M HCl and then adjusted to pH 3 by using 1M HCl or 1N NaOH. Then, CEi from stomach mucosa was added to perform *in vitro* digestibility. The mixtures were incubated at 37 ºC for 90 minutes with continuous shaking at 50 rpm. After incubation, 5 ml of 0.6N NaOH was added to obtain 0.1N NaOH until achieving pH 7. After that, 0.25 ml each of pancreatic and duodenal mucosal CEi was added. After incubation at 37 ºC for 3.5 hours with continuous shaking at 50 rpm, pH was adjusted to pH 5 according to the normal physiology of rabbits by using acetic acid. After that, CEi from caecal content at 0.5 ml was added. The incubation was performed at 37 ºC for 16 hours with continuous shaking at 50 rpm. After the final incubation, all samples were placed at –20 ºC for termination of the enzyme reaction. The sediment was separated by filtered crucible using cold extraction unit of fiber analysis, and was rinsed in distilled water three times. The filtered crucible with sediments was heated at 103 ºC for 2 hours and ignited at 550 ºC for 4 hours for determination of DM and OM, respectively for DMd and OMd calculation. Each sample was performed in triplicate and a blank control containing all reagents without substrate was used to correct the data. Assessing *in vitro* digestibility by using CM was performed according to Villamide et al. (2009) which was the same as previously described but changing CEi to be 1 ml of pepsin solution (25 mg/ml, P-7000, Sigma-Aldrich), 1 ml of porcine pancreatin (100 mg of pancreatin/ml, pancreatin from porcine, grade VI, Sigma-Aldrich n 1750) and 0.5 ml of Viscozyme (120 L, 120FBG/G, Novo Nordisk) for steps one, two and three, respectively. After this experiment, CEi of each rabbit was pooled together to achieve CEp.

Calculation and statistical analysis

All statistical analyses were performed in the statistical software R, using the package Rcmdr in RStudio (R Development Core Team, 2008) and the significance was established at $P < 0.05$. The performance parameters, apparent digestibility and enzyme activities in faeces (the period of rabbit's age as the fixed factor), and characteristic enzyme activities in various pH conditions (fixed factors) from faeces and organ samples were assessed with one-way ANOVA using Duncan's New Multiple Range Test for post hoc analysis. The pH with the highest enzyme activity and/or appropriate levels relative to normal rabbit gastrointestinal tract physiology from the enzyme characteristic study was used to perform *in vitro* digestibility. Pearson's correlation coefficient was determined between apparent digestibility, faecal enzyme activities and *in vitro* digestibility by using CEi, CEp and CM. Multiple linear regression was chosen to estimate the apparent digestibility (dependent variable) of DM and nutrients with the determination coefficient (R^2) and standard error of estimation (SEE), in which the independent variables were *in vitro* digestibility

using CEi or CM in 92-day-old rabbits. For another objective, comparison the predicted equation from *in vitro* digestibility using CEp or CM with or without faecal enzyme activities was then illustrated, in which apparent digestibility of rabbits at different ages were the dependent variables.

RESULTS

The apparent digestibility of DM and nutrients increased with age ($P = 0.01$; Table 1). The digestibility efficiency of rabbits from 66 to 70 days old was fully developed because there was no statistical difference between this period and the older age groups (Table 1), which reflects the normal and expected physiological development of rabbits. There was no difference on faecal amylase and protease activities between the age groups ($P > 0.05$), whereas cellulase activity at 45–49 days old was lower than in other periods ($P < 0.01$; Table 1). The enzyme characteristics from the sample digestive organs and faeces were assessed at different pHs (Table 2). The highest cellulase activities in caecal content were observed at pH 7 and 8, which is the same as in faeces at pH 7 (Table 2). The different peaks of protease activities between stomach mucosa (pH 3) and faeces (pH 7) were presented (Table 2).

The pH with the highest enzyme activities and/or activities appropriate to the animal's physiology were selected to perform in vitro digestibility in the stomach, small intestine and caecum which are at pH 3, 7 and 5, respectively (Table 2). *In vitro* digestibility on DM by using CEi (DMdinvitroCEi) and DMdinvitroCM was correlated with OM digestibility from the same source of enzymes ($P < 0.01$; Table 3). The correlation coefficient between in vitro digestibility by using a commercial enzyme and apparent digestibility was lower than *in vitro* digestibility by using CEi (Table 3).

The equation from multiple linear regression was calculated by using the percentage of *in vitro* digestibility on DM and OM by using CEi or CM as predictors. The use of DMdinvitroCEi as an independent variable was more appropriate than DMdinvitroCM since it provided the lower SEE ($R^2 = 0.49$ vs 0.17; SEE = 6.11 vs 6.91; $P = 0.38$ vs 0.89, respectively) and OM ($R^2 = 0.51$ vs 0.40; SEE = 7.35 vs 7.83; $P = 0.35$ vs 0.55, respectively; Table 4). In Tables 3 and 4, there were no differences between using DMdinvitroCEi or OMdinvitroCEi to estimate DMd, OMd and CPd because the correlation coefficient was not different.

The correlation between apparent digestibility, faecal enzyme activities and *in vitro* digestibility using CEp or CM in different periods of rabbit's age was illustrated in Table 5. A high statistically significant correlation was observed between in vivo digestibility on DM and nutrients. The relationship between faecal enzyme activities and apparent digestibility were low. However, there were statistically significant correlations between faecal cellulase (-0.20, $P<0.01$; Table 5) and protease activities (-0.37, $P<0.01$; Table 5) with CPd. A high correlation was found between DMdinvitro and OMdinvitro when using the same source of enzyme (CEp or CM; $P<0.01$; Table 5), which is similar to previous results with CEi. DMdinvitroCEp, OMdinvitroCEp, DMdinvitroCM and OMdinvitroCM were not correlated with faecal enzyme activities. Using

CM for *in vitro* digestibility was lower coefficient correlation for estimating apparent digestibility than using CEp. DMdinvitroCEp and OMdinvitroCEp were significantly correlated with OMd and CPd ($P < 0.05$; Table 5).

Enzyme activities from faeces were used as independent variables to predict apparent digestibility; however the coefficient of determination (R^2) from equations 7 to 15 was small (Table 6). The DMdCEp and OMdCEp were used as predictors along with faecal enzyme activity to estimate the apparent digestibility, in which the coefficient of determination from equations 16 to 30 with full factorials was 0.44, 0.45 and 0.73 for DMd, OMd and CPd, respectively (Table 6). Predictive equations were also performed by using DMdCM and OMdCM with faecal enzyme activities as independent variables, where the R^2 from equations 31 to 45 with full factorials was 0.38, 0.36 and 0.41 for DMd, OMd and CPd, respectively (Table 6). The R^2 from the equation using CM was lower than using CEp (Table 5). Interestingly, a high coefficient of correlation was observed when using OMdinvitroCEp, faecal cellulase and protease activities to estimate CPd ($R^2 = 0.72$, SEE = 7.05, $P = 0.03$; equation number 28; Table 6).

Table 1 Apparent total tract digestibility and enzyme activities in faecal samples of rabbits in different ages during the experimental period (45-91 days old)

Parameters	Rabbit age (days)							SEM	*P*
	45–49	52–56	59–63	66–70	73–77	80–84	87–91		
Apparent total tract digestibility (%)									
Dry matter	55.7^{b}	55.1^{b}	47.4^{a}	68.2^{c}	68.4^{c}	66.0^{c}	64.8^{c}	1.200	0.01
Organic matter	62.5^{a}	62.1^{a}	58.8^{a}	75.8^{c}	74.5^{c}	72.1^{bc}	68.4^{b}	0.961	0.01
Crude protein	70.5^{b}	70.4^{b}	60.8^{a}	77.8^{c}	77.5^{c}	73.9^{bc}	74.0^{bc}	1.107	0.01
Ether extract	84.5^{bc}	75.3^{a}	79.1^{ab}	89.2^{cd}	93.5^{d}	92.6^{d}	90.7^{cd}	1.175	0.01
Enzyme activities in faeces (U×10^{-3})									
Amylase	4.04	3.31	2.41	3.88	2.90	2.79	3.20	0.256	0.41
Protease	43.4	38.7	30.5	36.9	30.4	27.8	34.2	1.885	0.12
Cellulase	66.6^{a}	86.5^{b}	89.0^{b}	87.8^{b}	83.5^{b}	83.7^{b}	82.5^{b}	1.404	0.001

^{a,b,c,d} Values within a row with different superscripts differ significantly at $P < 0.01$.
SEM = standard error of mean

Table 2 Enzyme characteristics of 92-day-old rabbits from crude enzyme extract from different sources (digestive organs and faeces) and under various pH conditions.

Enzyme activities (U)	pH									SEM	*P*
	2	3	4	5	6	7	8	9	10		
Protease											
Stomach mucosa	68.2^{a}	120^{b}	74.4^{a}	74.9^{a}	71.9^{a}	67.9^{a}	–	–	–	3.46	0.01
Faeces ($\times 10^{-3}$)	39.3^{cd}	26.1^{a}	32.7^{b}	34.1^{b}	38.3^{c}	42.1^{d}	41.4^{cd}	47.4^{e}	49.1^{e}	1.39	0.001
Amylase											
Duodenal mucosa	–	–	–	12.9	12.9	12.9	12.9	12.7	12.5	0.21	0.99
Pancreas	–	–	–	3.63	3.64	3.64	3.64	3.63	3.57	0.07	0.99
Faeces ($\times 10^{-3}$)	4.31^{de}	4.44^{ef}	4.61^{f}	4.65^{f}	4.45^{ef}	4.17^{d}	3.87^{c}	2.87^{b}	0.90^{a}	0.23	0.001
Cellulase											
Caecal content	–	–	23.9^{a}	27.6^{b}	29.6^{bc}	30.0^{c}	30.1^{c}	29.4^{bc}	29.2^{bc}	0.35	0.01
Faeces ($\times 10^{-3}$)	91.5^{e}	16.0^{a}	69.8^{d}	55.1^{bcd}	65.8^{cd}	90.6^{e}	49.8^{bc}	46.0^{b}	49.3^{bc}	0.59	0.001

^{a,b,c,d,e,f}Values within a row with different superscripts differ significantly at $P < 0.05$.
SEM = standard error of mean

Table 3 Correlation matrix (Pearson's coefficient) between apparent total tract digestibility and *in vitro* digestibility using commercial enzyme or individual crude enzyme extract from 92-day-old rabbits.

	OMd	EEd	CPd	$DMd_{invitroCEi}$	$OMd_{invitroCEi}$	$DMd_{invitroCM}$	$OMd_{invitroCM}$
DMd	0.92**	0.30	0.81**	0.53	0.53	–0.23	–0.34
OMd		0.27	0.82**	0.59	0.56	–0.31	–0.43
EEd			0.34	0.45	0.58	–0.27	0.33
CPd				0.55	0.56	0.35	0.37
$DMd_{invitroCEi}$					0.93**	–0.38	–0.31
$OMd_{invitroCEi}$						–0.29	–0.29
$DMd_{invitroCM}$							0.89**

DMd = apparent digestibility on dry matter, OMd = apparent digestibility on organic matter, EEd = apparent digestibility on ether extract, CPd = digestibility on crude protein, $DMd_{invitroCEi}$ = *in vitro* digestibility on dry matter using individual crude enzyme extract from rabbits, $OMd_{invitroCEi}$ = *in vitro* digestibility on organic matter using individual crude enzyme extract from rabbits, $DMd_{invitroCM}$ = *in vitro* digestibility on dry matter using commercial enzyme, OMdinvitroCM = *in vitro* digestibility on organic matter using commercial enzyme.
**Correlation is significant at the level of 0.01 (2-tailed).

Table 4 Prediction equations of the apparent total tract digestibility by using percentage of *in vitro* digestibility from commercial enzyme or individual crude extract enzyme of 92-day-old rabbits as predictors.

Equations	No.	R^2	SEE	*P*
Dry matter digestibility (DMd)				
$DMd = 2.28(DMd_{invitroCEi}) - 1.38(OMd_{invitroCEi}) + 21.0$	1	0.49	6.11	0.38
$DMd = 4.89(DMd_{invitroCM}) - 1.41(OMd_{invitroCM}) - 181$	2	0.17	6.91	0.89
Organic matter digestibility (OMd)				
$OMd = 1.04(DMd_{invitroCEi}) + 0.65(OMd_{invitroCEi}) - 21.4$	3	0.51	7.35	0.35
$OMd = 21.2(DMd_{invitroCM}) - 4.20(OMd_{invitroCM}) - 1154$	4	0.40	7.83	0.55
Crude protein digestibility (CPd)				
$CPd = 0.09(DMd_{invitroCEi}) + 1.29(OMd_{invitroCEi}) + 3.60$	5	0.44	6.79	0.47
$CPd = 17.0(DMd_{invitroCM}) - 2.08(OMd_{invitroCM}) - 986$	6	0.42	6.82	0.49

$DMd_{invitroCEi}$ = *in vitro* digestibility on dry matter using individual crude enzyme extract from rabbits, $OMd_{invitroCEi}$ = *in vitro* digestibility on organic matter using individual crude enzyme extract from rabbits, $DMd_{invitroCM}$ = *in vitro* digestibility on dry matter using commercial enzyme, $OMd_{invitroCM}$ = *in vitro* digestibility on organic matter using commercial enzyme, SEE = standard error of the estimate

Table 5 Correlation matrix (Pearson's coefficient) between apparent digestibility (45–91 days old), *in vitro* digestibility by using pooled crude extract enzyme of rabbits at 92 days old and enzyme activities from rabbit faeces during the experimental period (45–91 days old).

	OMd	EEd	CPd	Amylase	Cellulase	Protease	$DMd_{invitroCEp}$	$OMd_{invitroCEp}$	$DMd_{invitroCM}$	$OMd_{invitroCM}$
DMd	0.94**	0.61**	0.86**	0.10	0.20	–0.19	0.27	0.26	0.19	0.14
OMd		0.59**	0.88**	0.07	0.11	–0.24	0.36**	0.33*	0.11	0.02
EEd			0.54**	–0.02	–0.13	–0.32*	0.07	0.06	0.11	0.10
CPd				0.14	–0.20**	–0.37*	0.37**	0.38**	0.17	0.09
Amylase					–0.19	0.11	0.15	–0.14	–0.19	–0.12
Cellulase						0.10	–0.18	–0.10	–0.13	–0.13
Protease							–0.13	–0.17	0.14	0.11
$DMd_{invitroCEp}$								0.92**	0.11	–0.16
$OMd_{invitroCEp}$									0.13	–0.17
$DMd_{invitroCM}$										0.90**

DMd = apparent digestibility on dry matter, OMd = apparent digestibility on organic matter, EEd = apparent digestibility on ether extract, CPd = digestibility on crude protein, $DMd_{invitroCEp}$ = *in vitro* digestibility on dry matter using pooled crude enzyme extract from rabbits, $OMd_{invitroCEp}$ = *in vitro* digestibility on organic matter using pooled crude enzyme extract from rabbits, $DMd_{invitroCM}$ = *in vitro* digestibility on dry matter using commercial enzyme, $OMd_{invitroCM}$ = *in vitro* digestibility on organic matter using commercial enzyme, * = Correlation is significant at the level of 0.05 (2-tailed), ** = Correlation is significany at the level of 0.01 (2-tailed).

Table 6 Prediction equation of the apparent digestibility using faecal enzyme activities, and *in vitro* digestibility using pooled crude enzyme extract and/or commercial enzyme during the experimental period (45–91 days old) as predictors.

Equations	No.	R^2	SEE	*P*
Faecal enzyme activities as predictors				
Dry matter digestibility (DMd)				
DMd = 0.16(Cell) + 55.1	7	0.20	8.00	0.15
DMd = 0.62(Amy) + 0.18(Cell) + 51.3	8	0.25	8.01	0.22
DMd = –0.14(Pro) + 0.76(Amy) + 0.21(Cell) + 53.7	9	0.34	7.85	0.12
Organic matter digestibility (OMd)				
OMd = –0.18(Pro) + 67.1	10	0.24	9.97	0.09
OMd = 0.55(Amy) – 0.18(Pro) + 65.6	11	0.26	10.0	0.19
OMd = 0.16(Cell) + 0.73(Amy) – 0.20(Pro) + 52.1	12	0.30	10.0	0.20
Crude protein digestibility (CPd)				
CPd = 0.56(Amy) + 69.7				
CPd = –0.05(Pro) + 0.60(Amy) + 71.2				
CPd = –0.01(Cell) – 0.05(Pro) + 0.59(Amy) + 72.1				
In vitro digestibility using pooled crude enzyme extract and faecal enzyme activities as predictors				
DMd				
DMd = 0.93(DMdinvitroCEp) + 19.3	16	0.27	7.86	0.05
DMd = 0.18(Cell) + 0.99(DMdinvitroCEp) + 1.09	17	0.36	7.72	0.04
DMd = –0.11(Pro) + 0.19(Cell) – 0.92(DMdinvitroCEp) + 7.72	18	0.40	7.65	0.04
DMd = 0.70(Amy) – 0.12(Pro) + 0.22(Cell) +0.89($DMd_{invitroCEp}$) + 5.22	19	0.42	7.62	0.05
DMd = 0.85($OMd_{invitroCEp}$) + 0.76(Amy) – 0.13(Pro) + 0.23(Cell) +0.21($DMd_{invitroCEp}$) – 1.94	20	0.44	7.68	0.08
OMd				
OMd = 1.56($DMd_{invitroCEp}$) – 20.7	21	0.36	9.57	0.01
OMd = –0.14(Pro) + 1.45($DMd_{invitroCEp}$) – 10.1	22	0.41	9.46	0.01
OMd = 0.16(Cell) – 0.16(Pro) + 1.50($DMd_{invitroCEp}$) – 25.6	23	0.44	9.41	0.01
OMd = 0.64(Amy) + 0.18(Cell) – 0.17(Pro) + 1.47($DMd_{invitroCEp}$) – 28.1	24	0.45	9.44	0.02
OMd = 0.39($OMd_{invitroCEp}$) + 0.67(Amy) + 0.19(Cell) – 0.17(Pro) + 1.16($DMd_{invitroCEp}$) – 31.4	25	0.45	9.53	0.05

Table 6 Cont.

Equations	No.	R^2	SEE	*P*
CPd				
$CPd = 0.41(OMd_{invitroCM}) + 45.5$	26	0.37	6.99	0.01
$CPd = –0.05(Pro) + 0.61(OMd_{invitroCEp}) + 34.4$	27	0.51	6.99	0.01
$CPd = –0.02(Cell) – 0.04(Pro) + 0.59(OMd_{invitroCEp}) + 37.1$	28	0.72	7.05	0.03
$CPd = 10.49(DMd_{invitroCEp}) – 0.02(Cell) – 0.03(Pro) – 1.29(OMd_{invitroCE}p) – 578$	29	0.72	7.12	0.07
$CPd = 0.65(Amy) + 10.1(DMd_{invitroCEp}) + 0.01(Cell) – 0.04(Pro) – 1.11(OMd_{invitroCEp}) – 569$	30	0.73	7.19	0.12
In vitro digestibility using commercial enzyme and faecal enzyme activities as predictors				
DMd				
$DMd = 0.16(Cell) + 55.1$	31	0.20	8.00	0.15
$DMd = 0.62(Amy) + 0.18(Cell) + 51.3$	32	0.25	8.01	0.22
$DMd = –0.14(Pro) + 0.76(Amy) + 0.21(Cell) + 53.7$	33	0.34	7.85	0.12
$DMd = 4.39(DMd_{invitroCM}) + 0.86(Amy) – 0.15(Pro) + 0.23(Cell) – 255$	34	0.38	7.80	0.12
$DMd = –0.39(OMd_{invitroCM}) + 6.13(DMd_{invitroCM}) + 0.84(Amy) – 0.14(Pro) + 0.22(Cell) – 352$	35	0.38	7.89	0.20
OMd				
$OMd = –0.18(Pro) + 67.1$	36	0.24	9.99	0.09
$OMd = 4.12(DMd_{invitroCM}) – 0.18(Pro) – 221$	37	0.27	9.99	0.16
$OMd = –1.73(OMd_{invitroCM}) + 11.8(DMd_{invitroCM}) – 0.16(Pro) – 650$	38	0.29	10.0	0.22
$OMd = 0.15(Cell) – 1.62(OMd_{invitroCM}) + 11.9(DMd_{invitroCM}) – 0.17(Pro) – 680$	39	0.33	10.0	0.24
$OMd = 0.79(Amy) + 0.18(Cell) – 1.39(OMd_{invitroCM}) + 11.5(DMd_{invitroCM}) – 0.19(Pro) – 669$	40	0.36	10.0	0.26
CPd				
$CPd = 1.39(OMd_{invitroCM}) + 2.17$	41	0.38	7.49	0.45
$CPd = 0.57(Amy) + 1.39(OMd_{invitroCM}) + 0.23$	42	0.40	7.54	0.63
$CPd = – 0.03(Pro) + 0.59(Amy) + 1.37(OMd_{invitroCM}) + 2.05$	43	0.41	4.33	0.04
$CPd = 0.29(DMd_{invitroCM}) – 0.03(Pro) + 0.57(Amy) + 1.06(OMd_{invitroCM}) + 2.22$	44	0.41	2.06	0.01
$CPd = 0.01(Cell) + 0.28(DMd_{invitroCM}) – 0.03(Pro) + 0.59(Amy) + 1.08(OMd_{invitroCM}) + 0.66$	45	0.41	1.17	0.01

SEE = standard error of estimate, Amy = amylase activity in faeces ($U\times10^{-3}$), Pro = protease activity in faeces ($U\times10^{-3}$), Cell = cellulase activity in faeces ($U\times10^{-3}$), $DMd_{invitroCEi}$ = *in vitro* digestibility on dry matter using individual crude enzyme extract from rabbits, $OMd_{invitroCEi}$ = *in vitro* digestibility on organic matter using individual crude enzyme extract from rabbits, $DMd_{invitroCEp}$ = *in vitro* digestibility on dry matter using pooled crude enzyme extract from rabbits, $OMd_{invitroCEp}$ = *in vitro* digestibility on organic matter using pooled crude enzyme extract from rabbits, $DMd_{invitroCM}$ = *in vitro* digestibility on dry matter using commercial enzyme, $OMd_{invitroCM}$ = *in vitro* digestibility on organic matter using commercial enzyme.

DISCUSSION

Enzyme characteristics

Enzymes in faeces were produced from the digestive tract in addition to bacterial production, which are affected by the development of gastrointestinal function and the gut bacterial community (Huang et al., 2014; Kanghae et al., 2017). The faecal enzyme activities were lower than that of the digestive organs at around 1,000 times for protease and cellulase, with amylase activity at around 10,000 times. Most digestive enzymes from animal secretions are digested and absorbed at the ileum. Moreover, the short transit time of hard faeces in the colon results in low bacterial fermentation compared with the caecum (Combes et al., 2013). Therefore, these causes should explain the lower enzyme activities in faeces when compared to digestive organs and caecal contents.

Based on the enzyme characteristic results in Table 2, the bacterial enzyme production could be observed in the hard faeces because there is similar pattern in the bacterial community between caecal content and hard faeces (Kovitvadhi et al., 2016a; 2016b). Furthermore, there is another peak of faecal cellulase activities (pH 4), which was not found in CE from caecal contents. This isoform of cellulase may be produced from bacteria in hard faeces that are not derived from caecal bacteria. However, further studies should be carried out in order to confirm this hypothesis. The present of microorganisms increase markedly with the rabbit's age to response on fiber digestion (Combes et al., 2013; Kovitvadhi et al., 2016a; 2016b). On the one hand, the low cellulase activity in faeces at 45-49 days was observed which this enzyme activity increased and steady after 52-day-old. Therefore, the development of microbial community in caecum should correlate to microbial enzyme activity in faeces which can be used as predictors.

The different peaks of protease activity which were observed in this study, may be from pepsin and trypsin at pH 3 and 7, respectively. On the one hand, the peak of amylase activity in faeces was observed across a wide pH range, from pH 3 to 6, whereas pH did not influence the activities of this enzyme from CE from the pancreas and duodenal mucosa. For these results, most enzyme activity in faeces correlates with enzyme production from microbes than from the digestive tract. However, the cellulase activity in faeces in the first week after weaning (45–49 days old) was lower than after 52 days old, which reflects the uncompleted development of the bacterial community in kittens (Gidenne et al., 2002; Combes et al., 2013). Therefore, this may illustrate how bacterial activities are correlated with digestive efficiency in addition to gut health.

In vitro digestibility using CEi vs CM to predict apparent digestibility

The high coefficient of correlation between DMdinvitroCEi and OMdinvitroCEi were observed. On the one hand, DMdinvitroCM were great correlation to OMdinvitroCM. The results from *in vitro* digestibility on DM can be used to refer to the results from *in vitro* digestibility on OM. However, diets with high proportion of minerals should be considered in future research,

which may provide different results. Correlation coefficients between DMd and DMdinvitroCEi or DMdinvitroCM were low compared with other studies, which were 0.84 (Lee et al., 1985) and 0.91 (Villamide et al., 2009). The differences in experimental design and objectives should be the cause of these diverse outcomes. Diverse chemical composition in the diets of rabbits of the same age was variable in other studies (Lee et al., 1985; Villamide et al., 2009). Conversely, the different ages of rabbits in the current study was the independent variable that affects digestive efficiency, since all observed rabbits were fed with the same diet throughout the experimental period. Different dietary chemical composition as a variable affecting digestive efficiency could be performed further studies.

The coefficient of estimation in this study from using CEi (R^2 =0.49) for DMd was lower than the study of Lee et al., (1985; R^2 = 0.95) and Villamide et al., (2009; R^2 =0.82); the same trend was observed for OMd in the current study (R^2 = 0.51) when compared to Lee et al., (1985; R^2 = 0.95) because of the different experimental designs and study aims, as described above. However, the estimation of CPd using the percentage of *in vitro* digestibility by using CEi (R^2 = 0.44) or CM (R^2 = 0.42) as predictors is similar to the study of Villamide et al., (2009; R^2 = 0.49). From these results, using CEi to perform *in vitro* digestibility was a better indicator to estimate DMd than using CM because using the CEi of one rabbit to predict apparent digestibility should be more specific and obtain a higher precision for estimation than using CM, which come from swine.

Three steps *in vitro* digestibility was performed in this study which simulated a digestive physiology in stomach, small intestine and caecum. There have been no controlled studies which compare differences on digestive enzyme activities between swine and rabbit. It is important to bear in mind the incorrect comparison or discussion on digestive enzyme activities between research articles which using diverse diets because the variation on chemical components in diets greatly influence on these parameters (Varel et al, 1984; Ye et al., 2000). However, studies of Yu et al. (2000) revealed the comparison on digestive enzyme activities between herbivorous (rabbits) and omnivorous (rats) using the same diet formulation. According to our knowledge, swine is considered as omnivorous. Therefore, we can infer the similar digestive enzyme activity between rat and swine. Pepsin is the major enzyme to digest the protein which was found in CE from rabbit's stomach mucosa (Table 2) and in CM (Pepsin solution, P-7000, Sigma-Aldrich). Therefore, the end products after the first step of *in vitro* digestibility should be not large different between two techniques. The higher protease and amylase activity in rat were observed in CE from small intestinal tissue with digesta around 7 and 2 times comparing to rabbits (Yu et al., 2000). Moreover, the mixture of CE from rabbit's pancreas and intestinal mucosa were mixed to perform in vitro digestibility in this study, whereas only CM from porcine pancreas (Pancreatin, grade VI, Sigma-Aldrich n 1750, containing trypsin, amylase and lipase, ribonuclease, and protease) was used in another technique (Ramos and Carabaño, 1996; Pascual et al., 2000; Villamide et al., 2009). From these different, the efficiency of *in vitro* digestibility in small intestine simulation should be different. The last step was represented the digestion from microbial enzyme. The microbial community is unique to animal species respecting to their diets (Varel et al, 1984; Combes et

al., 2013). The fibrolytic activity in rabbit as was greater than rat around two times which involved on several enzymes such as cellulase, endoglucanase, hemicellulase, pectinase, xylanase and others (Yu et al., 2000). The mixture of commercial fibrolytic enzymes (Viscozyme, Novo Nordisk) were used which consisted arabanase, cellulase, β-glucanase, hemicellulase, and xylanase which were different from caecal enzymes in rabbits on type of enzymes and quantity of each enzyme (Yu et al., 2000). The inconsistent between using CM and digestive physiology of rabbits could be the cause of the low coefficient of correlation comparing to using CE from rabbits. However, CEi was not practical in real scenarios, therefore analyses with CEp were performed in order to compare with the usage of CM.

In vitro digestibility using CEp vs CM with or without faecal enzyme activities to predict apparent digestibility

The large variation in digestibility efficiency across rabbits of different ages influences on quality of equation estimation which should be a cause to obtain a low determination coefficient comparing to other studies that using a difference on chemical composition of diets as variables (Lee et al., 1985; Villamide et al., 2009). Therefore, we still need to identify an indicator to illustrate the current digestive function of rabbits. However, obtaining better results when using CEp as a predictor rather than CM may be a consequence of the fact that CEp is extracted from rabbits, whereas CM is extracted from swine which was described above.

The coefficient of determination of equations 7 to 15 were low, therefore faecal enzyme activities should not be used alone for prediction. The lower R^2 was observed for CM compared with CEp, which should be due to the coefficient of correlation and in addition to the difference in species, as described above. The equation number 28 which used compared OMdinvitroCEp, faecal cellulase and protease activities for CPd estimation, was higher R^2 than the study of Villamide et al. (2009), which found $R^2 = 0.49$ using lignin and ether extract from diets as predictors. The higher coefficient of determination in this equation should be due to the high correlation between the predicted and dependent variables. This strong correlation between the chemical composition of diets and digestibility (*in vivo* or *in vitro*) was also observed in several other studies (Lee et al., 1985; Córdova-Murueta et al., 2003; Villamide et al., 2009; Huang et al., 2014; Kanghae et al., 2017). However, the chemical composition of diets cannot currently represent the rabbit's digestive efficiency and health. Therefore, faecal enzyme activities from rabbits along with the chemical composition of the diet in addition to other parameters should be used together as indicators for predicting apparent digestibility for individual rabbits.

CONCLUSION

The percentage of *in vitro* digestibility by using crude enzyme extract from individual rabbits was the best indicator to predict total tract apparent digestibility on dry matter and nutrients. However, this technique cannot evaluate in live rabbits because the digestive organs must be collected for crude enzyme

extraction. Therefore, the *in vitro* digestibility on diets by using the pooled of individual crude enzyme extracts from rabbits and faecal enzyme activity were used as predictors which was not invasive to animals. Based on the results, *in vitro* digestibility on organic matter by using pooled crude enzyme extract from rabbits, faecal protease activities and faecal cellulase activities can be used to estimate apparent total tract digestibility on crude protein, whereas the using of commercial enzyme was not suggested as the low coefficient correlation to predict apparent total tract digestibility.

CONFLICT of INTEREST

All of authors in this article have no conflict of interest to declare.

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