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Effects of Exogenous Fibrolytic Enzyme Derived from *Trichoderma reesei* on Rumen Degradation Characteristics and Degradability of Low-Tannin Whole Plant Faba Bean Silage in Dairy Cows

Jen-Chieh Yang, Victor H. Guevara-Oquendo, Basim Refat and Peiqiang Yu *

Department of Animal and Poultry Science, College of Agriculture and Bioresources, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK S7N 5A8, Canada; jey948@mail.usask.ca (J.-C.Y.); vhg019@mail.usask.ca (V.H.G.-O.); bar163@mail.usask.ca (B.R.)
* Correspondence: peiqiang.yu@usask.ca; Tel.: +1-306-966-4132

Abstract: The objectives of this study were to (1) determine the effect of exogenous fibrolytic enzyme derived from *Trichoderma reesei* on dry matter (DM) and neutral detergent fibre (NDF) degradability of whole plant faba bean silage (Snowbird), (2) evaluate the effects of fibrolytic enzyme (FETR) on DM and NDF degradation kinetics of whole plant faba bean silage, and (3) compare the difference between in the vitro approach (Daisy^{II} incubation method) and the in situ assay-biological approach (nylon bag technique) in the determination of degradability of dry matter (DMD) and neutral detergent fibre (NDFD). The fibrolytic enzyme from *Trichoderma reesei* was a mixture of xylanase and cellulase. The whole plant faba bean silage was treated with seven doses of fibrolytic enzyme, with 0 as a control and 0.25, 0.5, 0.75, 1, 1.25 and 1.5 mL of FETR/kg DM of silage. The results obtained from the in situ method show that fibrolytic enzyme cubically ($p < 0.05$) affected DMD and quadratically ($p < 0.01$) affected NDFD with increasing level of enzyme application. In vitro DM and NDF degradability were quadratically and cubically ($p < 0.01$) affected by the increasing dosage of enzyme. Correlation analysis between the in situ assay-biological approach and the In vitro Daisy^{II} approach showed a strong correlation (r = 0.98, $p < 0.01$) on overall DMD and also a satisfactory relationship (r = 0.84, $p < 0.01$) was found on overall NDFD. The enzyme application showed a great impact on NDF rumen degradation kinetics by decreasing the undegradable fraction and increasing the potential degradable fraction and the effective degradable content of fiber. The washable (W) and potential degradation fraction (D) were linearly ($p = 0.05$) increased by the enzyme treatments. Therefore, the undegradable fraction was linearly decreased ($p = 0.05$) with increasing dosage of enzyme. Both bypass (BNDF) and effective degradable NDF (EDNDF) were cubically ($p = 0.05$) affected by fibrolytic enzyme. In conclusion, the exogenous fibrolytic enzyme derived from *Trichoderma reesei* highly impacted rumen degradation characteristics and degradability of whole plant faba bean silage and could be used to improve fibre digestion of whole plant faba silage in dairy cows.

Keywords: exogenous fibrolytic enzyme; whole plant faba bean silage; rumen degradation kinetics; nutrient degradability; dairy cows

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1. Introduction

The cell wall of plants is a complex matrix composed of polysaccharides, proteins, phenolics, water and minerals, but mainly composed of carbohydrates which consist of cellulose and hemicellulose [1]. Forage fibre is indigestible to most of animals but can be hydrolyzed and fermented by a number of microorganisms in the rumen and therefore utilized by ruminants as a volatile fatty acid. Fibre content and digestibility are important in ruminants because they are the primary factors that determine feed intake and animal performance. Moreover, digestibility of NDF can be used as an important parameter to

identify forage quality because of its variability among forages and consistent effect on animal performance [2]. The NDF degradability of forage is not only closely related to feed intake and lactational performance but can also be used for estimation of physical rumen fill and energy prediction of forage [3,4]. According to NRC (2001), ruminal digestibility of forage NDF can range from less than 25% to over 75% for different forage types. However, the degradation of fibre in rumen is not optimal, as is supported by the fact that fibre recovered from feces is fermentable [5].

Exogenous fibrolytic enzymes have been used in dairy industries in order to improve feed efficiency and animal performance. There are numerous studies that have demonstrated the effect of fibrolytic enzymes on total tract digestibility in dairy cows; most studies have reported a positive response in DM, OM, NDF and/or ADF [6–8]. Moreover, the positive response of fibrolytic enzymes in either in vitro or in situ experiments was also examined [9–11]. While these results were shown with variance, they generally showed an increase in digestibility of nutrients. The inconsistent responses of fibrolytic enzyme among the studies may be due to the difference in enzyme activity, rumen condition, mode of enzyme application and substrate specificity. In this study, it was hypothesized that whole plant faba bean silage treated with fibrolytic enzymes would improve fibre availability and the degradability could be predicted by both in vitro and in situ techniques without significant difference. The objective of this study was to determine the effects of fibrolytic enzymes on rumen DM and NDF degradation characteristics and degradability of whole plant faba bean silage by using both in situ and in vitro techniques.

2. Materials and Methods

2.1. Silage Sample and Chemical Analyses

The variety of faba bean used in this study is Snowbird, which has low or zero tannin content. It was seeded on 12 May 2018 and harvested on 19 August 2018 at late pod stage (97 days old) in Melfort, SK, Canada (crop location NW 16 44 21 W2). Fresh material (226 tonnes) was wilted to a targeted 45% DM and chopped into 1-inch length on 20 August and 21 August 2018. Silage piles were constructed and covered with plastic on 22 August and the fermentation process took 150 days for completion.

The chemical composition of faba bean silage is presented in Table 1. The samples of whole plant faba bean were oven dried at 55 °C for 48 h and ground through a 1 mm screen (Retsch ZM 200, Retsch Inc., Haan, Germany) for chemical analyses. Dry matter (method 930.15) was analyzed according to the AOAC standard [12]. Neutral detergent fibers (NDFs) were analyzed using ANKOM F57 filter bags (ANKOM Technology Corp., Fairport, NY, USA) according to Van Soest et al. [13].

Table 1. Chemical composition of whole plant faba bean silage.

Items [1]	Whole Plant Faba Bean Silage
DM, %	45.7
OM, % DM	92.19
Ash, % DM	7.81
Ether Extract, % DM	1.09
Protein Profile	
CP, % DM	21.9
SCP, % DM	7.5
SCP, % CP	34.1
ADICP, % DM	1.50
ADICP, % CP	6.9
NDICP, % DM	2.17
NDICP, % CP	9.9
Carbohydrate profile	
Starch, % DM	23.7
aNDF, % DM	39.2
ADF, % DM	34.7
Lignin, % DM	5.2
NFC, % DM	32.11
NSC, % DM	24.3

[1] DM: dry matter; OM: organic matter; EE: ether extract (crude fat); CP: crude protein; SCP: soluble crude protein; ADICP: acid detergent insoluble crude protein; NDICP: neutral detergent insoluble crude protein; aNDF: neutral detergent fiber analyzed with amylase; ADF: acid detergent fiber; NFC: non-fiber carbohydrate; NSC: non-soluble carbohydrate.

2.2. Enzyme Preparation

The enzymatic solution used in this study was fibrolytic enzyme derived from *Trichoderma reesei* (a mixture of xylanase and cellulase; AB Vista, UK). The product is a mixture of xylanase (EC 3.2.1.8; xylanase activity = 350,000 BXU/g, where one BXU is the amount of enzyme that is able to release 0.06 micromole of xylose equivalent from birchwood xylan per minute) and cellulase (EC 3.2.1.8; endoglucanase activity = 10,000 ECU/g, where one ECU is the amount of enzyme that is able to release per 0.06 micromole of glucose from medium-viscosity carboxymethylcellulose per minute).

2.3. In Situ Study

The in situ study was conducted with two parts. The first section determined DM and NDF degradability and contained a comparison of the in vitro method. Four rumen cannulated Holstein dairy cows were used for the in situ study at Rayner Dairy Teaching and Research Facility (University of Saskatchewan, Saskatoon, SK, Canada). The cows were kept in tie stalls during the period of sampling and were milked three times a day. Cows were fed a total mixed ration (TMR) with 47.2% of barley silage, 19.4% of barley/corn grain and 9.9% of grass. The cows used for this study were cared for in accordance with the guidelines of the Canadian Council on Animal Care [14] and the protocols were approved by the Animal Research 125 Ethics Board (AREB) at the University of Saskatchewan, Canada, with Animal Use Approval Protocol #19910012. The samples of whole plant faba bean silage, Snowbird, were oven dried at 55 °C and ground through a 3 mm screen using the 8-inch Laboratory Mill (Christy & Norris LTD, Ipswich, UK). The whole faba bean silage samples were treated with seven doses of fibrolytic enzyme, with 0 as control and 0.25, 0.5, 0.75, 1, 1.25 and 1.5 mL of FETR/kg DM of silage. Approximately 7.5 g of whole plant faba bean silage sample was weighed and placed into 10 × 20 cm nylon bags with a size pore of 41 µm. Incubation time in the rumen was completed at 0, 6, 24 and 48 h. After 48 h incubation, bags were removed and washed, and later analyzed for DM and NDF degradability.

The second part of the in situ study was conducted with two rumen cannulated Holstein dairy cows at Rayner Dairy Teaching and Research Facility (University of Saskatchewan, Saskatoon, SK, Canada). The cows were kept in tie stalls during the period of sampling and were milked three times a day. Cows were fed a same total mixed ration (TMR) with the first part. Whole plant faba bean silages, CDC Snowbird, were oven dried at 55 °C and ground through a 1 mm screen using the Retsch ZM 200 grinder (Retsch ZM 200, Retsch Inc., Haan, Germany). The whole faba bean silage samples were treated with seven doses of fibrolytic enzyme, with 0 as control and 0.25, 0.5, 0.75, 1, 1.25 and 1.5 mL of FETR/kg DM of silage. Therefore, in each bag, approximately 0.5 g of faba bean sample was weighed and placed into 5 × 10 cm Ankom bags with 6 µm pore size. Incubation time in the rumen was completed at 0, 3, 6, 9, 12, 24 and 48 h. After 72 h incubation, all bags were removed and washed, and later analyzed for DM and NDF degradability.

2.4. In Vitro Study

An in vitro study was performed using Daisy^{II} Incubators (Ankom®, Tech. Co., Fairport, NY, USA). Two Daisy^{II} incubators were used in this study with two experimental runs. Whole plant faba bean silage samples used for in vitro incubation were oven dried at 55 °C for 48 h and finely ground through a 1 mm screen (Retsch ZM 200, Retsch Inc., Haan, Germany). The whole plant faba bean silage samples were treated with seven doses of fibrolytic enzyme, with 0 as control and 0.25, 0.5, 0.75, 1, 1.25 and 1.5 mL of FETR/kg DM of silage. Therefore, approximately 0.5 g was weighed, placed into each filter bag (F57, Ankom Technology, Macedon, NY, USA) and sealed. Each incubator contained three digestion jars, each jar signified at 0, 6, 24 and 48 h and heated at 39 °C. Each digestion jar was filled with pre-warmed (39 °C) 1500 mL of McDougall's buffer solution (described in detail by Goering and Soest [15] and 500 mL of strained ruminal fluid, and flushed with CO_2. Rumen fluid was collected from two rumen-cannulated Holstein cows fed the same TMR with 47.2%

of barley silage, 19.4% of barley grain, 9.9% of grass hay and 20% of lactation concentrate. After 48 h of incubation, bags were removed and rinsed thoroughly with cold tap water, dried at 105 °C overnight and later analyzed for DM and NDF degradability.

2.5. Rumen Degradation Characteristics and Statistical Analyses

In situ and in vitro degradability of DM and NDF data were analyzed using a randomized complete block design. The model used for this design was as follows: $Y_{ijk} = \mu + T_i + B_j + e_{ijk}$, where Y_{ijk} was an observation of the dependent variable ij; μ was the population mean for the variable; T_i was the treatment effect, as a fixed effect, B_j was a block effect with in situ animals, as a random effect, and e_{ijk} was the random error associated with the observation ij. The difference among treatments was evaluated with a multiple comparison analysis using the Tukey method. Orthogonal polynomial contrast was used to determine the linear, quadratic and cubic effect of increasing enzyme application. The model assumptions were checked using Residual Analysis in SAS. The normality test was preformed using Proc Univariate. Comparison of in vitro Daisy^{II} with the in situ biological approach in DM and NDF degradability of faba bean silage, the paired t test procedure of SAS and Pearson correlation analysis were performed to establish the relationship between the in vitro Daisy^{II} procedure and the in situ biological approach. For all statistical analyses, significance was declared at $p \leq 0.05$ and a trend at $0.05 < p < 0.10$ unless otherwise stated.

The rumen degradation characteristics were performed using the first-order degradation kinetics model described by Ørskov and McDonald [16] and Tamminga et al. [17]. Degradation parameters for dry matter and NDF were calculated using the following formula: $R(t) = U + D \times e - Kd \times (t - T0)$, where R(t) = residue percentage at t hours of incubation in the rumen (%), U = undegradable fraction (%), D = potentially degradable fraction (%), Kd = degradation rate (%/h), and T0 = lag time (h). The effectively degradable fractions (ED) or extent of degradation in the rumen, as well as the ruminally undegradable fractions (RU) of the nutrients were determined with equations described in NRC (2001) and Yu et al. [18]; $ED = S + D \times Kd/(Kp + Kd)$; $RU = U + D \times Kp/(Kp + Kd)$, where S represents the soluble fraction (%), Kp is the flow of degraded feed from the rumen, which was assumed to be equal to 6%/h [17].

3. Results and Discussion

Table 2 shows the effect of adding different enzyme levels on in situ DM and NDF degradability of whole plant faba bean silage. The in situ DM degradability was improved cubically ($p < 0.05$) with increasing FETR level application. There is a quadratic effect ($p < 0.01$) on in situ NDF degradability. These results are aligned with previous studies which reported that fibrolytic enzyme improved DM degradability in in vitro or in situ experiments [6,19,20]. However, some other studies did not observe a significant response of the fibrolytic enzyme on DM degradability [21]. This discrepancy may be due to several factors such as composition of enzyme, substrate and/or interactions of the enzyme with substrates and environment conditions. The highest degradability (56.54%) of DM was observed at the intermediate levels of FETR, which was the groups treated with 1.00 mL of FETR/kg DM of silage. The DMD was decreased at the groups of 0.25 and 0.50 mL of FETR, and then increased and reached a plateau at the groups of 0.75 and 1 mL of FETR/kg DM of silage. Additionally, the highest NDF degradability was also observed at the group treated with 0.75 mL of FE/kg DM of silage and increased from 13% (control) to 27% (0.75 mL of FETR/kg DM of silage). The control (untreated) and the lowest FETR level group (0.25 mL of FETR/kg DM) show the lowest NDF degradability. The most effective enzyme dosage for whole plant faba bean silage, based on both in situ DM and NDF degradability was 0.75 mL of FETR/kg DM with the maximum effect of NDF on the group treated with 0.75 mL of FETR/kg DM. Table 3 shows the in vitro DM and NDF degradability obtained from Daisy^{II} incubators. The DM degradability was quadratically affected ($p < 0.01$) and NDF degradability tended to be cubically ($p < 0.10$) affected with

the increasing dosage of enzymes. The in vitro DM degradability ranged from 52 to 58% and in vitro NDF degradability ranged from 16 to 30% with different levels of enzyme application. The highest in vitro DMD was observed at 1.5 mL of FETR, which is confirmed by other studies which claimed fibrolytic enzyme tended to increase microbial colonization of feed particles and assumed that the exogenous enzyme may act similarly to primary bacterial colonization [6,22]. The highest in vitro NDFD was at the group treated with 0.5 mL of FETR which was up to 30%. Both results from in situ and in vitro techniques suggest that FETR has positive impacts on DM and NDF degradability of pre-treated faba bean silage.

Table 2. Effect of different dosage of fibrolytic enzymes [1] derived from *Trichoderma reesei* on in situ NDF and DM degradability of whole plant faba bean silages at different incubation times.

Item	DM Degradability of Whole Plant Faba Bean Silage (%)	NDF Degradability of Whole Plant Faba Bean Silage (%)
Dose level of fibrolytic enzymes (mL/kg)		
Control (0)	53.49	12.59
0.25	51.83	13.98
0.50	53.37	18.85
0.75	56.45	27.16
1.00	56.54	23.78
1.25	55.97	20.15
1.50	56.47	20.84
SEM	0.797	2.000
In situ incubation time (Time)		
0 h	30.23	5.45
6 h	43.58	9.63
24 h	67.45	19.89
48 h	78.24	43.52
SEM	0.631	1.632
Statistical Analysis	*p* value	*p* value
Dose level	0.0002	<0.0001
Time	<0.0001	<0.0001
Dose level × Time Interaction	0.1033	0.3905
Orthogonal Polynomial Contrast for FETR dose level	*p* value	*p* value
Linear	<0.0001	0.0001
Quadratic	0.4049	0.0002
Cubic	0.0245	0.5151
Orthogonal Polynomial Contrast for incubation time	*p* value	*p* value
Linear	<0.0001	<0.0001
Quadratic	<0.0001	0.0107
Cubic	0.0382	0.4071

SEM: Standard Error of Mean. [1] Fibrolytic enzyme derived from *Trichoderma reesei* (FETR, mixture of xylanase and cellulase.

Table 3. Effect of different dosage of fibrolytic enzymes [1] derived from *Trichoderma reesei* on in vitro NDF and DM degradability of whole plant faba bean silage at different incubation times.

Item	DM Degradability of Whole Plant Faba Bean Silage (%)	NDF Degradability of Whole Plant Faba Bean Silage (%)
Dose level of fibrolytic enzymes (mL/kg)		
Control	55.52	16.75
0.25	52.79	17.05
0.50	53.12	30.26
0.75	55.50	16.75
1.00	56.02	22.48
1.25	54.62	17.05
1.50	57.69	21.33
SEM [2]	0.768	1.583

Table 3. *Cont.*

Item	DM Degradability of Whole Plant Faba Bean Silage (%)	NDF Degradability of Whole Plant Faba Bean Silage (%)
In situ incubation time (Time)		
0 h	32.71	3.81
6 h	44.23	5.82
24 h	68.00	28.31
48 h	75.22	43.90
SEM [2]	0.664	1.296
Statistical Analysis	*p* value	*p* value
Dose level	<0.0001	0.6695
Time	<0.0001	0.0058
Dose level × Time Interaction	0.9429	<0.0001
Orthogonal Polynomial Contrast for dose level	*p* value	*p* value
Linear	<0.0001	0.6695
Quadratic	0.0029	0.0058
Cubic	0.0799	<0.0001
Orthogonal Polynomial Contrast for incubation time	*p* value	*p* value
Linear	<0.0001	<0.0001
Quadratic	<0.0001	0.0031
Cubic	0.9133	0.0005

[1] Fibrolytic enzyme derived from *Trichoderma reesei* (FETR, mixture of xylanase and cellulase); [2] SEM: Standard Error of Mean.

A comparison and correlation analysis between the in vitro approach (Daisy-II incubation method) and the in situ assay approach (nylon bag technique) is shown in Table 4. The difference between in situ and in vitro DM degradability was significant ($p < 0.01$) at 0 and 48 h incubation time. The correlation of overall DM degradability between the two approaches was strong with r = 0.98 ($p < 0.01$). This is in an agreement with the study that was performed with the same comparison (in situ versus Daisy^{II}). Trujillo et al. [23] reported a good correlation on in situ and in vitro disappearance of DM and NDF that exceeded 0.80. On the other hand, the greatest difference (8.42%, $p < 0.01$) of NDF degradability was found at the 24 h incubation time between the two approaches. The correlation of overall NDF degradability between in situ and in vitro was high (r = 0.84, $p < 0.01$). This relationship is in line with a previous study which reported a high correlation (r = 0.94, $p < 0.01$) between in situ and in vitro Daisy^{II} techniques concerning NDF degradability [24]. The overall DM and NDF degradability between the two approaches are non-statistically significant. However, the results obtained from in vitro Daisy^{II} incubators were slightly higher than the in situ method and also had a higher variability. This may be attributed to overestimation of the in vitro procedure because ruminal motility may involve a greater pressure in bags than Daisy^{II} incubators. This finding is in agreement with other studies which used the same methods (in situ versus in vitro Daisy^{II}) to compare the NDF degradability of different cutting frequencies of hay [24]. Robinson et al. [25] reported that the in vitro digestion of NDF at 48 h was higher than the in situ method because of higher continuous fluid flow in in vitro bags. Another reason for this could be attributed to the growth and death of the microorganisms in in situ bags that affect nutrient disappearance [26]. The result of the conventional in situ nylon bag method shows a more consistent result with less variation when compared with the in vitro Daisy^{II} incubator. While in vitro Daisy^{II} may underestimate the nutrient disappearance, the two procedures have showed a good correlation. To conclude, in vitro Daisy^{II} still appears to be a useful tool because of its advantages such as rapidity, simplicity and efficiency.

Table 4. Comparison and correlation analysis between in vitro approach (DaisyII incubation method) and in situ assay-biological approach (nylon bag technique) in the determination of degradability of dry matter (DMD) and neutral detergent fibre (NDFD) at different incubation times for whole plant faba bean silage pre-treated fibrolytic enzyme [1] derived from *Trichoderma reesei* at different dose levels [2].

	Comparison In Vitro (Daisy-II) vs. In Situ Assay-Biological Approach					**Correlation Analysis In Vitro (Daisy-II) vs. In Situ Biological Approach**	
Items	**Mean** $^{\text{In vitro}}$	**Mean** $^{\text{biological}}$	**Difference**	**SED**	***p* Value**	**r**	***p* Value**
Degradability of dry matter (DMD)							
Individual incubation time							
DMD at 0 h incubation (%, *n* = 14)	32.71	30.23	2.48	0.632	0.0017	0.31	0.2762
DMD at 6 h incubation (%, *n* = 14)	44.23	43.58	0.65	1.071	0.5538	0.20	0.5013
DMD at 24 h incubation (%, *n* = 14)	68.00	67.45	0.55	0.858	0.5322	0.28	0.3381
DMD at 48 h incubation (%, *n* = 14)	75.22	78.24	−3.02	0.986	0.0077	0.15	0.6055
Overall (*n* = 56)							
DMD (%)	55.04	54.87	0.17	0.511	0.7476	0.98	<0.0001
Degradability of neutral detergent fibre (NDFD)							
Individual incubation time							
NDFD at 0 h incubation (%, *n* = 14)	3.81	5.45	−1.64	2.415	0.5093	0.06	0.8343
NDFD at 6 h incubation (%, *n* = 14)	5.82	9.63	−3.82	2.757	0.1896	−0.24	0.4166
NDFD at 24 h incubation (%, *n* = 14)	28.31	19.89	8.42	2.155	0.0018	0.14	0.6250
NDFD at 48 h incubation (%, *n* = 14)	43.90	43.52	0.38	2.503	0.8808	0.12	0.6912
Overall (*n* = 56)							
DNDF (%)	20.46	19.62	0.84	1.352	0.5382	0.82	<0.0001

SED = standard error of the difference. R = Pearson correlation coefficient. [1] Fibrolytic enzyme derived from *Trichoderma reesei* (FETR, mixture of xylanase and cellulase. [2] Dose level of fibrolytic enzymes (mL/kg): Control (0), 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50.

The effect of fibrolytic enzyme on in situ DM degradation kinetics of whole plant faba bean silage is presented in Table 5. The degradation rate (Kd), time lag (T0) and degradable and undegradable fractions of DM were not significantly affected ($p > 0.05$) by the enzyme application. Numerically, the control group showed a higher Kd and undegradable fraction than treatments, and the lowest was in degradable fractions. However, the soluble fraction of faba bean silage was increased linearly ($p < 0.05$) as the dose of enzyme increased, and the highest values were obtained with the highest dosage (1.50 mL of FETR/kg DM of silage). This finding is agreed with a previous study which reported that a higher dosage of enzyme had a higher soluble fraction of DM degradation for alfalfa cubes [6]. However, another study claimed that there was no effect of fibrolytic enzymes on the soluble fraction of DM in alfalfa hay [27]. These inconsistent results may be due to different enzyme composition, activity and specificity of substrates that were used in experiments.

When applying firbrolytic enzyme solutions on whole faba bean silage, there were no differences found in rumen degradation characteristics of BDM and EDDM. This may be explained by the specificity of substrates. Van Straalen [28] has indicated that different chemical compositions of forage could influence enzyme efficiency, which may be related to several factors such as cell wall structures, complexity and components [29].

The effect of fibrolytic enzymes on in situ NDF degradation kinetics of faba bean silage is presented in Table 6. In this study, the application of fibrolytic enzymes showed a great impact on the NDF degradation kinetic. The W+D fraction, which refers to washable plus potential degradable fractions, was linearly ($p = 0.05$) increased by the enzyme treatments. In contrast, undegradable fraction was linearly decreased ($p = 0.05$) with increasing dosage of enzymes. These results are in line with the published study, which reported a higher potential degradable fraction and degradation rate on alfalfa hay when treated with enzymes [27]; however, in that study, corn silage, corn stover, elephant grass, Guinea grass and oat straw were not affected by the exogenous fibrolytic enzyme. The bypass (B) and effective degradable (ED) NDF were both cubically ($p = 0.05$) affected by fibrolytic enzymes. The BNDF in the control group was much higher than treatments (54.3 vs. 37.3–42.8% of NDF). In contrast, EDNDF in the control group showed the lowest value (45.7% of NDF or 127 g/kg DM of silage), whereas treatment groups ranged from 57 to 63% of NDF or 160 to 175 g/kg DM of silage). These positive results of enzyme application on in situ rumen degradation characteristics of NDF were in agreement with other studies [20,30]. In this study, exogenous fibrolytic enzymes increased by 26% the washable and potential degradable fraction of NDF and 14% of effective degradable NDF when comparing the untreated control group with the treated intermediate group (0.75 mL of FETR/kg DM). These improvements on the NDF degradation kinetic could result in a potentially greater DM intake by reducing physical rumen fill and therefore increase energy density from diets [31,32]. While the mode of action by the fibre degrading enzyme is still unclear, it would be related to the enhancement of rumen enzyme activity caused by the fibrolytic enzyme [6]. Moreover, the fibrolytic enzyme could also induce the release of soluble carbohydrates from feed particles [33], thus providing additional energy for microbial growth and shortening lag time for microbial colonization [34].

In conclusion, the fibrolytic enzyme showed its highly positive impact on NDF rumen degradation kinetics/characteristics by decreasing the undegradable fraction and increasing the potential degradable fraction and the effective degradable content of fiber. There was less impact on DM rumen degradation characteristics. The overall DM and NDF degradability between the two approaches (in situ versus Daisy^{II}) are not significantly different. However, the results obtained from in vitro Daisy^{II} incubators were slightly higher than the in situ method and also have a higher variability. In this study, the low to medium dosage range (e.g., 0.50 to 1.00 mL of FETR/kg DM) of fibolytic enzyme was selected for further in vivo study to determine the effects of fibrolytic enzyme on lactational performance, rumen fermentation parameters and nutrient digestibility in lactating dairy cows fed with whole plant faba bean silage as a main source of forage in TMR ration.

Table 5. Effect of dose level of fibrolytic enzymes[1] derived from *Trichoderma reesei* on in situ rumen DM degradation kinetics of whole plant faba bean silage using both in situ nylon bag and in situ ANKOM methods.

Item	K_d_DM (%/h)	T0_DM (h)	S_DM (%)	D_DM (%)	U_DM (%)	BDM (g/kg DM)	EDDM (g/kg DM)
Dose level of fibrolytic enzymes (mL/kg)							
Control	6.99	1.89	25.10	49.11	25.80	545.1	454.9
0.25	4.69	1.08	25.32	56.26	18.42	602.1	397.9
0.50	5.58	2.20	28.80	49.28	21.93	531.7	468.4
0.75	6.23	1.42	28.14	48.90	22.97	516.4	483.6
1.00	4.94	0.98	28.15	50.90	20.95	552.7	447.3
1.25	4.93	1.33	28.19	50.79	21.03	562.1	437.9
1.50	5.35	2.14	29.24	50.74	22.03	538.1	461.9
SEM	0.895	0.796	1.414	2.688	2.753	24.28	24.28
In situ methods							
In situ Nylon bag	6.19	2.09	26.55 [b]	55.21 [a]	18.24 [b]	528.7 [b]	471.3 [a]
In situ ANKOM	4.87	1.06	28.57 [a]	46.50 [b]	24.93 [a]	570.7 [a]	429.2 [b]
Orthogonal Polynomial Contrast for dose level (*p* value)							
Linear	0.258	0.990	0.017	0.759	0.380	0.542	0.542
Quadratic	0.497	0.505	0.326	0.901	0.737	0.666	0.666
Cubic	0.548	0.539	0.543	0.415	0.286	0.843	0.843
Statistical Analysis (*p* value)							
Dose level	0.454	0.875	0.172	0.520	0.636	0.313	0.314
In situ methods (trial)	0.047	0.101	0.049	0.0004	0.0043	0.033	0.033

SEM: standard error of mean; [a,b] Means with the different letters in the same row are significantly different ($p < 0.05$); Multi-treatment comparison; Kd: the degradation rate of D fraction (%/h); T0: lag time; S: soluble fraction in the in situ incubation; D: degradable fraction; U: rumen undegradable fraction; BDM: rumen bypass or undegraded feed dry matter; EDDM: effective degraded dry matter. [1] Fibrolytic enzyme derived from *Trichoderma reesei* (FETR, mixture of xylanase and cellulase).

Table 6. Effect of dose level of fibrolytic enzymes [1] derived from *Trichoderma reesei* on in situ rumen NDF degradation kinetic of whole plant faba bean silage using both in situ nylon bag and in situ ANKOM methods.

Item	K_d_NDF (%/h)	T0_NDF (h)	W+D (%)	U_NDF (%)	BNDF (%)	BNDF (g/kg DM)	EDNDF (%)	EDNDF (g/kg DM)
Dose level of fibrolytic enzymes (mL/kg)								
Control	3.58	1.55	67.52	32.48	54.3	169.1	45.67	127.3
0.25	3.03	5.09	94.40	5.60	40.0	124.2	60.02	167.7
0.50	3.65	4.49	87.40	12.60	37.3	115.7	62.76	175.3
0.75	2.99	3.08	94.49	5.52	39.5	122.6	60.54	169.1
1.00	2.56	2.15	95.16	4.84	40.0	124.1	60.04	167.8
1.25	2.50	0.88	93.40	6.60	42.8	132.9	57.22	159.8
1.50	3.62	6.86	94.55	5.45	40.2	125.1	59.77	166.9
SEM	0.689	1.966	7.870	7.870	3.88	12.11	3.884	10.91
In situ methods								
In situ Nylon bag	1.79 b	4.49	88.33	11.67	51.94 [a]	162.2 [a]	48.06 [b]	135.2 [b]
In situ ANKOM	4.47 a	2.39	90.80	9.21	32.06 [b]	98.9 [b]	67.94 [a]	188.8 [a]
Orthogonal Polynomial Contrast for dose level (*p* value)								
Linear	0.485	0.625	0.050	0.050	0.109	0.109	0.109	0.109
Quadratic	0.285	0.593	0.126	0.126	0.028	0.028	0.028	0.028
Cubic	0.221	0.023	0.306	0.306	0.050	0.050	0.050	0.050
Statistical Analysis (*p* value)								
Dose level	0.547	0.356	0.188	0.188	0.088	0.088	0.088	0.088
In situ methods (trial)	<0.001	0.172	0.683	0.683	<0.001	<0.001	<0.001	<0.001

SEM: standard error of mean; [a,b] Means with the different letters in the same row are significantly different ($p < 0.05$); Multi-treatment comparison; Kd: the degradation rate of D fraction (%/h); T0: lag time; W+D: washable and potential degradable fractions; U: rumen undegradable fraction; BNDF: rumen bypass or undegraded feed neutral detergent fiber; EDNDF: effective degraded neutral detergent fiber. [1] Fibrolytic enzyme derived from *Trichoderma reesei* (FETR, mixture of xylanase and cellulase).

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