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ORIGINAL PAPER

In vitro ruminal degradation kinetics of alfalfa silage with the addition of dry ice and different degrees of wilting

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ABSTRACT. The *in vitro* ruminal degradation kinetics of dry matter (DM) and crude protein (CP) were determined in alfalfa silages treated with different doses of dry ice (0, 0.5, 1, and 2 g 750 g^{-1} fresh matter (FM)) and varying wilting time (0, 12, and 24 h). Ruminal fluid was collected from five rumen-cannulated sheep. Feed samples were placed in labelled filter bags and incubated for 0, 2, 4, 8, 24, and 48 h. The lowest ruminal DM degradation was observed in silages subjected to a 12-h wilt with the addition of 0.5 and/or 2 g of dry ice (66 and 65%, respectively). The final CP degradability was the lowest in silages prepared after 12-h and 24-h wilts compared to silages made from unwilted alfalfa. After 48 h of incubation, CP degradability in unwilted silages treated with 0, 0.5, 1, and 2 g of dry ice reached 77, 79, 76, and 70%, respectively. The corresponding CP degradability values in silages subjected to a 12-h wilt were 73, 76, 69, and 67%, and 73, 73, 71, and 68% in silages wilted for 24 h. *In vitro* degradability and degradation kinetics of the analysed nutrients were the highest in silages produced after a 24-h wilt with the addition of 1 and/or 2 g of dry ice. These experimental treatments may potentially improve the efficiency of CP utilisation from alfalfa silage and increase CP availability in subsequent segments of the digestive tract.

Introduction

Alfalfa (*Medicago sativa* L.) is significantly more susceptible to proteolysis than other fodder crops. Alfalfa silage is widely used as a cattle feed component due to its high crude protein (CP) and energy content, as well as its protein that is rapidly degraded in the rumen by proteolytic enzymes (Sırakaya and Büyükkılıç Beyzi, 2022). Proteolysis is a hydrolytic process that leads to the breakdown of peptide bonds and the formation of highly soluble non-protein nitrogen (NPN) compounds. This process is carried out predominantly by plant proteolytic enzymes, which are responsible for most CP conversion processes in the silo, but also enzymes produced by bacteria of the family *Enterobacteriaceae*, which are responsible for the decarboxylation and deamination of amino acids and the degradation of nitrates (Purwin et al., 2014). During ensiling of alfalfa, a large proportion of true protein (TP) is converted to NPN, which accounts for 50–60% or, in some cases, over 80% of total nitrogen (N) in the silage (Broderick et al., 2002).

Extensive research has been conducted to improve the quality of alfalfa silage, primarily by eliminating or limiting anaerobic decomposition (Fijałkowska et al., 2015). However, the overall quality of ruminant feed largely depends on the nutritional value of CP, including its fraction not degraded in the rumen (Purwin et al., 2014). The effective ruminal degradation of CP is influenced by many factors, including feed characteristics, feed intake, the extent of feed processing, and the productive limits of rumen fermentation. These limits are shaped by the fermentation pattern of silage such as its pH, ammonia N, and fatty acid profile (Geron et al., 2007).

Most studies examining various additives in alfalfa silage production focus on improving fermentation and suppressing CP hydrolysis during ensiling (Fijałkowska et al., 2015). However, limited information is available on the ruminal degradability of CP in alfalfa ensiled with additives that reduce rumen proteolysis. It should be noted that proteolysis in the silo increases the ruminal degradability of CP. This occurs because proteolysis raises the proportion of the buffer-soluble CP fraction, which facilitates CP degradation in the rumen. As a result, the efficiency of N utilisation in alfalfa silage decreases, leading to higher urinary N excretion. These processes reduce the effectiveness of microbial protein synthesis in the rumen (Phelan et al., 2015). Therefore, it is necessary to find new additives that would reduce both proteolysis during ensiling and CP degradation in the rumen, thereby increasing the proportion of bypass protein (Broderick et al., 2002).

Herbage is typically wilted or partially dried before ensiling to increase osmotic pressure in plant cells and reduce the rate and extent of proteolysis (Fijałkowska et al., 2015). The degree of proteolysis depends mainly on the rate of pH changes, which can be modified, e.g., by shortening the aerobic phase of fermentation (McDonald et al., 1991). In the present study, dry ice was used as a source of carbon dioxide (CO_2) to rapidly remove oxygen from inter-particle spaces in the ensiled forage mass.

Mentler (1962) performed the first study using dry ice as an additive during silage production. The author only analysed the basic nutrient content and selected fermentation parameters of alfalfa silage and found no effect of dry ice. A more comprehensive study was conducted by Thaysen (1996), who examined the influence of dry ice on the feed value and oxidative stability of maize silage (Thaysen, 1996). Similarly, Nussbaum (1996) investigated changes in temperature, pH, and bacterial and yeast counts at different time intervals in maize ensiled with dry ice. Evidence indicates that CO_2 can be effectively used to inhibit the activity of aerobic microorganisms, minimise oxygen-related sugar losses, and lower the temperature of the ensiled mass. However, for practical reasons, gaseous CO_2 cannot be used in feed production (Mentler, 1962; Nussbaum, 1996; Thaysen, 1996).

It was hypothesised that the addition of dry ice and/or wilting the forage prior to ensiling would reduce the *in vitro* ruminal degradability of CP in alfalfa silage, thereby improving the efficiency of nutrient utilisation by ruminants.

The objective of this study was to evaluate the effect of dry ice application during alfalfa ensiling on the *in vitro* ruminal degradability of silage dry matter (DM) and CP, depending on the quantity of this additive and the period of herbage wilting before ensiling.

Material and methods

The experiment was approved by the Local Ethics Committee (decision No. 73/2021).

Experimental design and treatments

The alfalfa (*Medicago sativa* L.) variety Plato used in the experiment was obtained from a commercial farm in Poland (54°12′03″N, 20°49′33″E) and harvested in the second year of cultivation. The experimental silages varied in the quantity of dry ice added (0, 0.5, 1, and 2 g 750 g^{-1} fresh matter (FM)) and the degree of herbage wilting prior to ensiling (0, 12, and 24 h).

Silage preparation

First-cut herbage was harvested at a height of 5 cm at the early bud stage, between 24:00 and 01:00, using a Krone AM 283 S disc mower (Krone GmbH, Spelle, Germany). Part of the harvested forage was collected directly after cutting (0-h wilt), while the remaining forage was left in the field in swaths with uniform thickness to wilt for 12 and 24 h. The collected forage was mechanically chopped to an average chaff length of 25 mm and ensiled in mini silos with a volume of 1 dm^3 each, prepared in three replicates per experimental treatment. During silage preparation, when 50% of the forage mass was compacted in the silo, 16-mm dry ice pellets were added, after which the remaining forage was included and compacted to a final density of 750 kg FM m^{-3}. The silos were equipped with exhaust pipes and sealed after filling. Herbage samples were collected

at harvest and after 12 and 24 h of wilting. Silage samples were collected after 90 days of fermentation in the mini silos. A portion of herbage and silage samples from each replicate of each experimental treatment were frozen at −25 °C, and the remaining samples were dried at 60 °C for 48 h in a Binder FED 115 dryer (Binder, GmbH, Tuttlingen, Germany) and ground to 1 mm particles in a mill for fibrous materials (ZM 200, Retsch, Haan, Germany). Selected chemical parameters of alfalfa herbage and silage are presented in Table 1.

The pH values in the silage were measured using a HI 8314 pH meter (Hanna Instruments, Woonsocket, Rhode Island, USA). The concentrations of lactic acid, acetic acid and propionic acid were determined as described by Kostulak-Zielińska and Potkański (2001), and Gąsior (2002). Silage samples were homogenised at a 1:5 ratio of sample weight to water volume (w/v) and subsequently filtered through polyamide gauze. The filtrate was passed through a soft filter, deproteinised with a 24% solution of metaphosphoric acid, and centrifuged (13 000 rpm,

Table 1. Selected chemical parameters of alfalfa herbage and silage, g kg^{-1} DM

Item		DM	OM	Haemicellulose	Cellulose	Lignin	pH	$N\text{-}NH_3$	LA	AA+PA
Herbage										
wilting	0 h	232	908	70.0	199	49.3	n/a	n/a	n/a	n/a
	12 h	357	914	75.0	202	60.7	n/a	n/a	n/a	n/a
	24 h	480	909	87.1	213	62.9	n/a	n/a	n/a	n/a
Silage										
wilting	dry ice									
0 h	0 g	230	901	64.2	229	44.4	4.58	106	91.1	7.68
	0.5 g	230	897	64.5	237	50.0	4.71	104	90.0	9.28
	1 g	231	896	66.0	224	49.9	4.74	90.2	86.7	7.08
	2 g	231	897	62.5	230	45.4	4.45	74.8	96.4	6.22
12 h	0 g	351	892	69.8	248	57.1	5.14	134	37.6	6.67
	0.5 g	335	898	53.6	245	57.5	5.11	121	30.2	6.45
	1 g	338	898	64.2	249	61.3	5.16	130	36.7	6.47
	2 g	351	897	58.4	270	62.4	5.25	114	24.6	5.95
24 h	0 g	461	896	64.4	232	52.6	5.16	46.7	16.7	1.15
	0.5 g	468	901	60.0	237	60.2	5.25	50.1	17.0	0.90
	1 g	474	901	67.7	243	58.0	5.23	57.3	14.6	1.17
	2 g	474	900	71.4	233	56.7	5.10	46.1	26.1	1.28

DM – dry matter (g kg^{-1} fresh matter); OM – organic matter; $N\text{-}NH_3$ – ammonia nitrogen (g kg^{-1} total nitrogen); LA – lactic acid; AA – acetic acid; PA – propionic acid; n/a – not applicable

Herbage and silage samples were analysed for DM and crude ash content using standard AOAC International methods (2016). The content of neutral detergent fibre (NDF) was determined using heat-stable amylase and expressed exclusive of residual ash (aNDFom), while the content of acid detergent fibre (ADF) was expressed exclusive of residual ash (ADFom). The acid detergent lignin (ADL) content was measured as described by Van Soest et al. (1991) using an ANKOM 220 fibre analyser (ANKOM Technology Corp., Macedon, NY, USA). The organic matter (OM) content was calculated as the difference between DM and crude ash content. The haemicellulose content was calculated as the difference between NDF and ADF fractions, the cellulose content was derived from the difference between ADF and ADL fractions, and lignin concentration was used to present ADL values.

7 min). Volatile fatty acids were separated by gas chromatography using a Varian 450-GC with a Varian CP-8410 autosampler (Varian Inc., Palo Alto, California, USA), flame-ionisation detector (FID), CP-FFAP capillary column (length – 25 m, inner diameter – 0.53 mm, film thickness – 1.0 µm). The sample size was 1 µl, with a detector temperature of 260 °C, injector temperature of 200 °C, column temperature gradient from 90 to 200 °C, and helium as the carrier gas at a flow rate of 5 ml min^{-1}. The lactic acid content was determined using a high-performance liquid chromatography (HPLC, SHIMADZU, Kyoto, Japan) with isocratic flow. Separation was carried out on a Varian METACARB 67H column (ORGANIC ACIDS COLUMN, Varian Inc., Palo Alto, California, USA), with a mobile phase consisting of 0,002 M solution of sulphuric acid in deionised water, a flow rate of 1 cm^3 min^{-1}, and UV

detector set at 210 nm. External fatty acid standards were supplied by SUPELCO (SIGMA-ALDRICH, Saint Louis, Missouri, United States), and the lactic acid standard was obtained from FLUKA (Chemie GmbH, Buchs, Switzerland). The ammonia N ($N\text{-}NH_3$) content was determined by direct distillation using a 2100 Kjeltec Distillation unit (Foss Analytical A/S, Hilleröd, Denmark).

In vitro ruminal degradability

The ruminal degradation kinetics of DM and CP in alfalfa herbage and silage were determined *in vitro* by the gravimetric method using a Daisy II incubator (ANKOM Technology, Fairport, New York, USA) and ANKOM F57 filter bags (5.0 × 5.5 cm; 25 µm porosity) (ANKOM Technology, Fairport, New York, USA) according to the manufacturer's instructions and the method described by Trujillo et al. (2010). All samples were air-dried before analysis. Filter bags were rinsed with acetone to remove surfactants, completely dried, and weighed to determine the mass of an empty bag. Each bag was labelled, filled with 0.25 g of the appropriate sample and tightly sealed using an ANKOM Heat Sealer #1915 (ANKOM Technology, Fairport, New York, USA). Filled bags were placed in a Daisy II incubator, four bags per digestion jar. The bags were evenly distributed on both sides of the digestion jar divider and immersed in a mixture of ruminal fluid and buffer solutions maintained at a temperature of 39 °C ± 2 °C. Ruminal fluid was collected from five rumen-cannulated Kamieniec sheep (Polish breed of long-wool sheep). The sheep were fed a standard diet according to the INRA feeding system (2016) consisting of meadow hay and barley grain at a ratio of 80:20 (DM basis). Ruminal fluid was collected approximately 1.5 h after the morning feeding and filtered through two layers of cheesecloth into an air-tight container supplied with CO_2. In the laboratory, the fluid was centrifuged at 3000 rpm for 30 s using a MPW-350R laboratory centrifuge (MPW Med. Instruments, Warsaw, Poland). Subsequently, the fluid was filtered through four layers of cheesecloth into a graduated flask supplied with CO_2.

Labelled filter bags containing feed samples were incubated for 0, 2, 4, 8, 24, and 48 h, with different numbers of bags used for each incubation variant: 3 bags for 0 h; 6 bags for 2, 4, 8, and 24 h; and 9 bags for 48. Unincubated (0 h) bags were soaked in distilled water at 39 °C ± 2 °C for 15 min. After incubation, the bags were removed from the digestion jar, rinsed under cold running water, and then rinsed in water in an automatic washing machine in three cycles, without spin washing. The bags with residues were dried to a constant weight in a laboratory dryer at 60 °C for up to 48 h. Subsequently, the dried filter bags were cooled in a desiccator and weighed on an analytical balance to determine the weight of each bag containing feed residues post-incubation. The residual samples were then analysed for CP content following the AOAC International standard procedure (2016).

Calculations and statistical analyses

The ruminal degradation of DM (P_{DM}) for each replicate and incubation time was calculated using the formula proposed by Kowalski et al. (2008):

$$P_{DM} = 100 \times B - \frac{(C - A)}{B},$$

where: P_{DM} – ruminal degradation of DM (%), A – dry weight of an empty filter bag (g), B – dry weight of a sample before incubation (g), C – dry weight of a filter bag with the residues after incubation (g).

The ruminal degradation of CP (P_{CP}) for each replicate and incubation time was calculated using the formula proposed by Kowalski et al. (2008):

$$P_{CP} = 100 \times \frac{(D - E)}{D},$$

where: P_{CP} – ruminal degradation of crude protein (%), D – crude protein content in the dry weight of the sample before incubation (g), E – crude protein content in the dry weight of the sample after incubation (g).

The *in vitro* ruminal degradability of DM and CP was analysed using a degradation kinetics model for experimental herbage and silage. The degradation kinetics of DM and CP was modelled mathematically using the following equation:

$$\frac{dX}{dt} = r \times X \times (1 - \frac{X}{K}),$$

where: K – model constants, X – state variable, r – growth rate constant ($1\ h^{-1}$).

The values of the coefficients *r* and *K* were determined using the knitro_nlp optimisation procedure in the Knitro v 12.4 package (Artelys, Trance) implemented in the MATLAB 201 environment (MathWorks, USA). A binary form of the model was created using IQM ToolPro (IntiQuan GmbH, Switzerland). The objective function (JC) was formulated as follows:

$$J_C\ (model\ coefficient) = \sum_{i=1}^{n} (\frac{X_{mes} - X_{mod}}{X_{mes}})^2,$$

The IQM ToolPro package is an extension to the MATLAB environment and the binary form of the model provides much faster numerical calculation times. The values of the analysed coefficients were determined as the solution to the optimisation problem:

$$\min_{model\ coefficient} J_C\ (model\ coefficient).$$

The ruminal degradability and degradation kinetics of DM and CP in alfalfa herbage and silage were presented graphically, whereas the parameters of the degradation kinetics model were summarised in Table 2.

Table 2. Parameters of the ruminal degradation kinetics model of dry matter and crude protein in alfalfa herbage and silage subjected to varying degrees of wilting and different doses of dry ice

Item		Dry matter		Crude protein	
		r	K	r	K
Herbage					
Wilting	0 h	0.25	73.1	0.35	80.3
	12 h	0.20	69.7	0.37	77.6
	24 h	0.33	65.3	0.65	72.6
Silage					
Wilting	Dry ice				
0 h	0 g	0.15	63.1	0.23	73.9
	0.5 g	0.093	72.8	0.14	76.8
	1 g	0.084	69.2	0.087	74.5
	2 g	0.076	69.2	0.43	65.5
12 h	0 g	0.12	61.8	0.062	72.9
	0.5 g	0.048	72.5	0.033	79.6
	1 g	0.073	69.9	0.095	68.5
	2 g	0.054	68.3	0.51	63.6
24 h	0 g	0.052	72.3	0.062	72.9
	0.5 g	0.061	67.4	0.049	74.6
	1 g	0.10	68.6	0.22	64.9
	2 g	0.11	61.4	0.33	64.0

r – growth rate constant (1 h^{-1}); K – model constants

Results

Selected chemical parameters of the herbage and silage are presented in Table 1. The herbage reached a DM of 357 g kg^{-1} FM, after 12 h of wilting, which increased to 480 g kg^{-1} FM after 24 h. The OM content was similar for all herbage. Prolonged wilting time led to an increase in the concentration of individual structural carbohydrates in the herbage.

Silages prepared from non-wilted (0-h) herbage had the lowest pH, which corresponded to the highest concentration of lactic acid, as well as the combined acetic acid and propionic acid levels (Table 1). With increasing wilting time and the addition of dry ice, the pH of the silages increased, while the content of lactic acid and the sum of acetic acid and propionic acid levels decreased. The highest concentration of ammonia nitrogen was recorded in alfalfa silage after 12 h of wilting, intermediate values were observed in alfalfa silages without wilting (0 h), and the lowest levels in silages wilted for 24 h.

The *in vitro* ruminal degradability of herbage DM tended to decrease with increasing wilting time (Figure 1). Unwilted alfalfa herbage was characterised by the highest amount of rumen-degraded DM across all incubation intervals compared to the wilted herbage samples. Herbage wilted for 12 and 24 h showed significantly different DM degradation rates (Table 2). The lowest ruminal degradation rate of DM was observed in the samples wilted for 12 h, while the highest rate was recorded in the 24-h wilted samples. However, after 48 h of incubation,

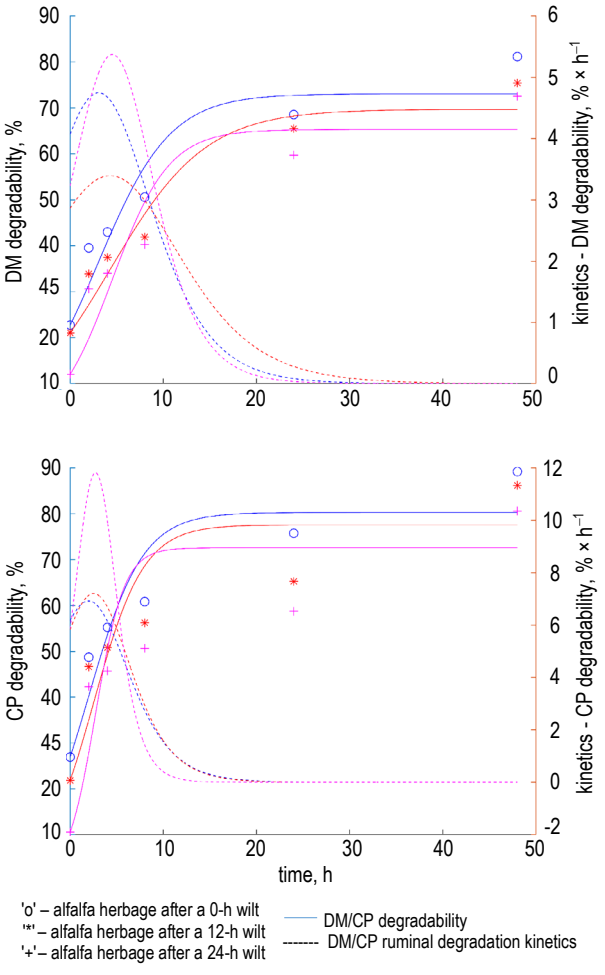

Figure 1. *In vitro* ruminal degradability and degradation kinetics of dry matter (DM) and crude protein (CP) in alfalfa herbage with different degrees of wilting

the amount of DM degraded *in vitro* in the rumen was highest in unwilted herbage, despite its moderate degradation rate, reaching 81%, compared to 75 and 73% in herbage wilted for 12 and 24, respectively.

The *in vitro* ruminal degradability of herbage CP decreased with increasing DM content at all-time intervals (Figure 1). The most pronounced differences in the amount of CP degraded were found between samples wilted for 12 and 24 h during the first 8 h of incubation, followed by 48 h of incubation. The adopted model of CP degradation kinetics demonstrated that the rate of *in vitro* degradation was not affected by a 12-h wilt but increased significantly following a 24-h wilting period (Table 2).

Ensiling increased the amount of DM degraded in the rumen *in vitro* only for non-incubated alfalfa silages, regardless of wilting duration or the quantity of dry ice (Figures 2, 3, and 4), with values ranging from 27 to 41%. However, compared to herbage, the amount of DM degraded *in vitro* was lower after 24 and 48 h of incubation in all silages. In unwilted samples (0 h), the addition of 0.5, 1, or 2 g of dry ice resulted in a higher amount of ruminally degraded DM compared to the control group (without dry ice) during the first 24 h of incubation (Figure 2).

In this group, the DM degradation rate decreased with increasing amounts of dry ice. In silages prepared after a 12-h wilt with the addition of 0.5, 1, and 2 g of dry ice, the quantity of DM degraded in the rumen *in vitro* increased after 0, 2, and 4 h of incubation relative to the control group (without dry ice) (Figure 3). In the subsequent time intervals, the rate of *in vitro* DM degradation was higher in silages with 0.5 and 1 g of dry ice and comparable between silages treated with 2 g of dry ice and the control group (without dry ice). The rate of *in vitro* DM degradation in silages prepared after a 12-h wilt was highest in the control group (without dry ice), while similar degradation rates were recorded in samples with 0.5, 1, and 2 g of dry ice.

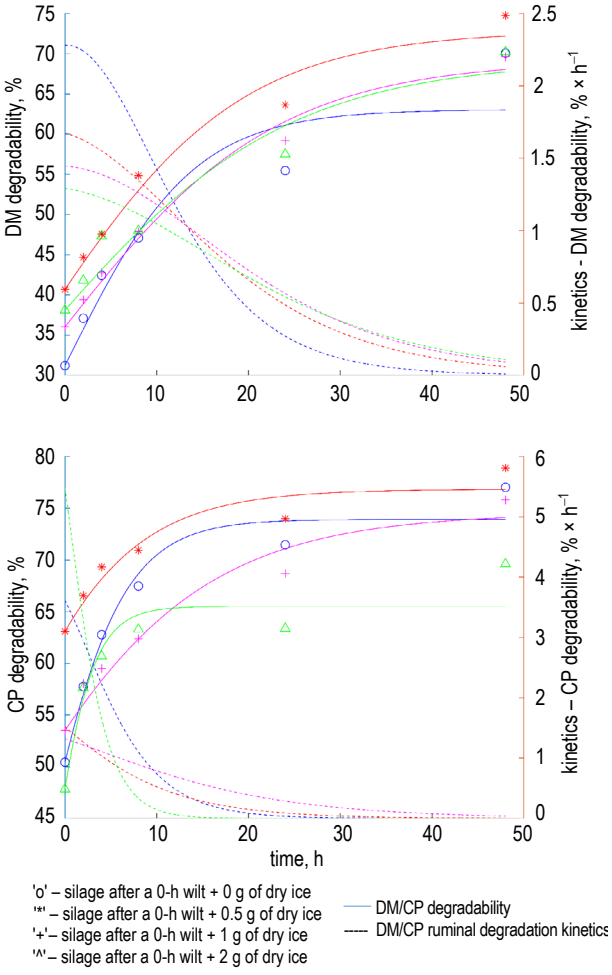

Figure 2. *In vitro* ruminal degradability and degradation kinetics of dry matter (DM) and crude protein (CP) in alfalfa silages after a 0-h wilt with the addition of varying doses of dry ice

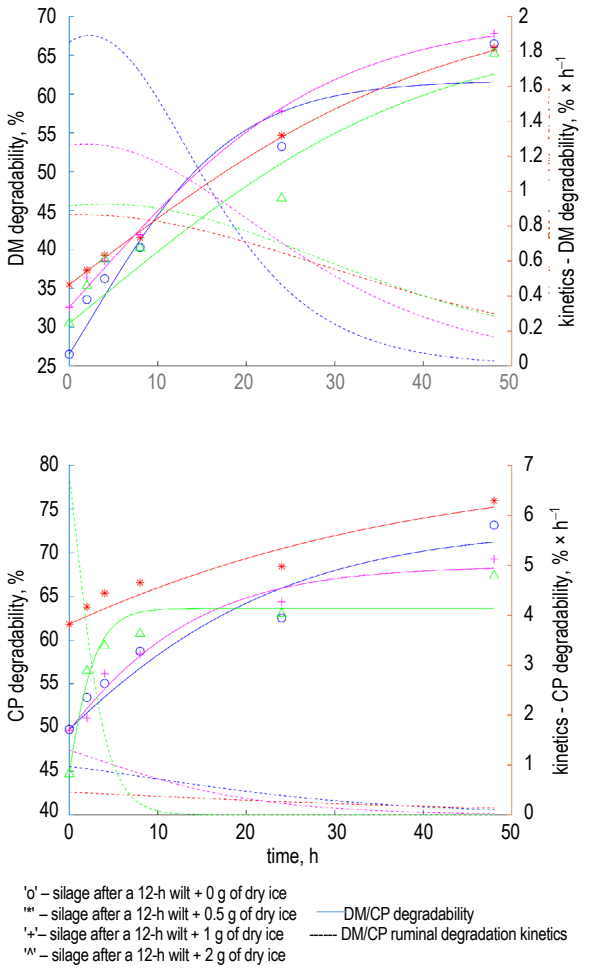

Figure 3. *In vitro* ruminal degradability and degradation kinetics of dry matter (DM) and crude protein (CP) in alfalfa silages after a 12-h wilt with the addition of varying doses of dry ice

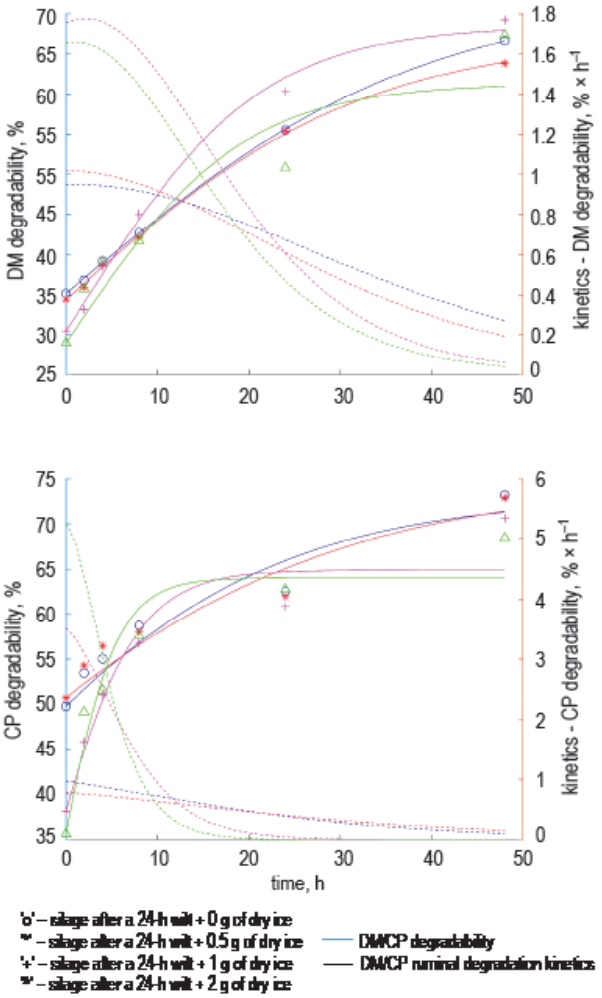

Figure 4. *In vitro* ruminal degradability and degradation kinetics of dry matter (DM) and crude protein (CP) in alfalfa silages after a 12-h wilt with the addition of varying doses of dry ice

In silages prepared after a 24-h wilting period, *in vitro* DM degradability after the addition of 0.5, 1, and 2 g of dry ice was lower in the first 4 h of incubation compared to the control group without dry ice (Figure 4). Silages treated with 1 g of dry ice exhibited a higher amount of ruminally degraded DM after 8 and 24 h of incubation. After 48 h of incubation, the highest and comparable amounts of ruminally degraded DM were found in silages with 1 and 2 g of dry ice. Silages made after a 24-h wilt with 0 and 0.5 g of dry ice showed similar DM degradation rates, while this parameter was higher and similar in silages treated with 1 and 2 g of dry ice.

Irrespective of the wilting degree or the amount of dry ice added, undigested silages had a significantly higher amount of CP degraded in the rumen *in vitro* compared to herbage (Figures 2, 3, and 4). The values exceeded 47% in unwilted silages, 44% in silages prepared after 12 h of wilting, and 35% in silages prepared after a 24-h wilt. The amount of ruminally degraded CP after 0 h of incubation was lowest in silages with the addition of 2 g of dry ice, regardless of the degree of wilting, i.e. silage DM content (Figure 2). In silages prepared from unwilted alfalfa with the addition of 2 g of dry ice, the amount of CP degraded in the rumen *in vitro* was consistently lower between 4 and 48 h of incubation. This trend was not observed in silages prepared after a 12-h wilt (Figure 3), where CP degradation in samples with 2 g of dry ice was lower only during the first 2 h of incubation. In contrast, the addition of 1 and/or 2 g of dry ice to silages prepared after a 24-h wilt resulted in lower *in vitro* degradability of CP during the entire incubation period (Figure 4).

The final ruminal degradability of CP was the lowest in silages prepared after 12 and 24 h wilting compared to silages prepared from unwilted alfalfa, unwilted herbage, and herbage wilted for 12 and 24 h (Figures 1, 2, 3, and 4). After 48 h of incubation, CP degradability in unwilted silages treated with 0, 0.5, 1, and 2 g of dry ice reached 77, 79, 76, and 70%, respectively (Figure 2). The corresponding values for silages made after a 12-h wilt were 73, 76, 69, and 67% (Figure 3), and for silages after a 24-h wilt, they were 73, 73, 71, and 68% (Figure 4). Analysis of the growth rate constant (*r*) revealed that, regardless of the degree of wilting, the CP degradation rate was highest in silages with the addition of 2 g of dry ice (Table 2). Silages prepared after a 12-h and a 24-h wilt without dry ice showed similar CP degradation rates, with greater differences observed when 1 and/or 2 g of dry ice was used.

Discussion

The effect of dry ice as an ensiling additive on the ruminal degradability of silage nutrients has never been investigated in the literature. Therefore, the results of this study cannot be directly compared with findings of other authors. In addition, many studies on rumen degradability of standard feeds have been conducted *in vivo*. Only a limited number of scientific publications utilise *in vitro* methods to evaluate rumen degradability, further complicating comparison of the results.

In the present study, the *in vitro* ruminal degradability of DM and CP was lower in alfalfa silage compared to herbage due to transformations during the ensiling process that reduced the content of easily digestible nutrients. These differences could also be attributed to alterations in the proportions of CP fractions resulting from proteolysis and the potential formation of Maillard reaction products (Damborg et al., 2018).

Differences in the amounts of DM and CP degraded in the rumen *in vitro*, as well as variations in the degradation rates of these nutrients between the experimental silages, may have been influenced by changes in gas formation during fermentation and reduced proteolysis in wilted alfalfa ensiled with a higher dose of dry ice. The addition of dry ice likely shortened the aerobic phase of fermentation, thereby decreasing nutrient availability to microorganisms in silages incubated with a mixture of ruminal fluid (Hashemzadeh-Cigari et al., 2011).

The addition of dry ice influenced the duration of the first fermentation phase by modifying the gas composition during the ensiling process (Nussbaum, 1996), potentially affecting the quantity and availability of protein fractions and their ruminal degradability. As a source of CO_2, dry ice could have inhibited the activity of aerobic microorganisms in the first phase of fermentation, accelerating the acidification of the ensiled forage mass. These processes suppressed the activity of plant proteolytic enzymes, which otherwise increase the proportion of the NPN fraction that is rapidly degraded in the rumen (Fijałkowska et al., 2015) and has the highest ruminal degradation rate reaching 200% h^{-1} (Van Amburgh et al., 2015). It should be noted that dry ice did not significantly affect the content of basic silage nutrients; however, the modified gaseous atmosphere altered fermentation products and probably the composition of the silage microbiome (Hartinger et al., 2020). On the other hand, variations in the rate and extent of DM and CP degradation *in vitro* may have been associated with changes in microbial proliferation rates and activity during fermentation (Hashemzadeh-Cigari et al., 2011) following the addition of dry ice to ensiled alfalfa herbage. The wilting process also modified the proportions of CP fractions during ensiling and, in some cases, may have led to the formation of protein-structural carbohydrate complexes, as previously described in the literature (Sousa et al., 2020).

Nutrient degradation, particularly in the rumen, is a complex process influenced, among other factors, by both the quantity and quality of dietary CP (Purwin et al., 2014). The composition and content of structural carbohydrates in the silage, which varied among the experimental treatments, also played a role in shaping the rate and extent of nutrient degradation. Soluble nutrients in plant cells are almost entirely degraded in the rumen; thus, the quantity and composition of the cell wall, particularly the neutral detergent fibre (NDF) content, are key determinants of the total tract digestibility of feed (Camacho et al., 2010). Nitrogen derived from feed and energy accumulated in the NDF and ADF fractions of structural carbohydrates serve as substrates for microbial fermentation in the rumen. Silages with a higher content of energy and CP are more effectively degraded by ruminal microorganisms (Camacho et al., 2010). Lower CP degradability in the rumen is desirable because it promotes the passage of silage nutrients to distal segments of the digestive tract and increases feed utilisation efficiency in ruminants. Consequently, reduced CP degradation in the rumen leads to improved feed N utilisation and lower N emissions into the environment, addressing a key environmental concern in ruminant nutrition (Repetto et al., 2011; Purwin et al., 2014).

In the work of Zhang et al. (2009), the *in situ* ruminal degradation of alfalfa silage DM was determined to be 63.79% after 48 h of incubation. Comparable results were observed in the present study for silages prepared after 12- and 24-h wilting without dry ice or with the addition of 0.5 or 2 g of dry ice. DM degradability in the experimental silages treated with 1 g of dry ice was higher after 48 h of incubation. Similarly, Purwin et al. (2014) reported that DM degradability in alfalfa silage reached 65.60% after 48 h of *in situ* incubation. These findings align with the results of this experiment for silages prepared after 12- and 24-h wilt. Silages prepared from unwilted alfalfa had higher DM degradability, consistent with the results reported by Xue et al. (2020a,b). In the referenced studies, the *in vitro* DM degradation in silages made from alfalfa and orchard grass ranged from 65 to 71% (Xue et al., 2020a) and from 68 to 74% (Xue et al., 2020b). Similarly, in the work of Purwin et al. (2014), CP degradability after 48 h of incubation was 71.50%, which is comparable to the values observed in the present study for unwilted silages treated with 2 g of dry ice, silages prepared from alfalfa after a 12-h wilt and treated with 1 and/or 2 g of dry ice, as well as silages prepared from alfalfa after a 24-h wilt and treated with 1 and/or 2 g of dry ice. The remaining silages exhibited higher CP degradability compared to the values reported by Purwin et al. (2014). In a study by Mustafa et al. (2000), the *in situ* ruminal degradability of alfalfa silage was evaluated, with DM degradability reaching 66.40%, a value similar to those observed in the present study. However, the effective degradability of CP by Mustafa et al. (2000) was considerably higher at 87.90%. In contrast, the current study found that 68 to 79% of CP was degraded in the rumen *in vitro* after 48 h of incubation.

In alfalfa, wilting increases the proportions of the soluble CP fraction as well as the fraction potentially degradable in the rumen (Fijałkowska et al., 2015). Similarly, Campbell and Buchanan-Smith (1991) observed lower ruminal CP degradability in wilted alfalfa and grass silage compared to unwilted silage. A comparable effect of wilting on CP degradability was also reported by Petit and Tremblay (1992).

Previous studies have analysed the physicochemical properties of legume crops, including alfalfa, that affect nutrient degradation dynamics in the rumen. Fresh alfalfa was characterised by higher rumen passage rates compared to unwilted grass. Alfalfa silage particles are rapidly degraded, and despite high rumen passage rates, a substantial proportion of particles smaller than 2 mm remain in the rumen (Dewhurst et al., 2003). However, high passage rates, combined with the inherently lower digestibility of alfalfa silage, reduce nutrient utilisation efficiency and consequently animal performance. Dewhurst et al. (2003) also reported that microbial energetic efficiency was highest for alfalfa silage, although the significantly lower apparent digestion of organic matter in the rumen limited the overall conversion of rumen-degradable N into microbial N.

Conclusions

The results of the current study demonstrated that the *in vitro* ruminal degradability and degradation kinetics of dry matter (DM) and crude protein (CP) in alfalfa silages were influenced by both experimental factors, i.e. the amount of dry ice added to ensiled herbage (1 and/or 2 g 750 g^{-1} fresh matter) and the degree of wilting (24 h). It was found that alfalfa silage treated with 1 and/or 2 g of dry ice after 24-h wilting period showed the lowest values of rumen CP decomposition throughout the incubation period, which was a desirable outcome. These treatments have the potential to enhance the efficiency of CP utilisation from alfalfa silage and increase CP availability in subsequent segments of the digestive tract. In addition, feeding alfalfa silage with 1 and/or 2 g of dry ice after 24 h of wilting could potentially reduce the emission of unused feed N into the environment. The present study contributes to the current understanding of *in vitro* rumen degradability of DM and CP in alfalfa ensiled with dry ice. It also highlights the potential for further research into the effects of dry ice on microbial fermentation, as well as assessing silage quality on a larger production scale. The use of dry ice as an additive in alfalfa ensiling to improve forage protein quality would allow the reuse of dry ice, which is generated as a by-product in many industries.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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