Original Article

In vitro AND *in situ* DIGESTIBILITY OF ANNUAL RYEGRASS SILAGE FROM DISTINCT PHENOLOGICAL GROWTH STAGES AND PRE-DEHYDRATION TIMES

DIGESTIBILIDADE IN VITRO *E* IN SITU *DE SILAGEM DE AZEVÉM ANUAL ORIUNDA DE DIFERENTES ESTÁDIOS FENOLÓGICOS E TEMPOS DE PRÉ-SECAGEM*

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ABSTRACT: The aim was to evaluate how the fodder pre- dehydration time and its phenological stages influence on ruminal degradability and digestibility of ryegrass silage. The evaluated samples consisted of treatments: Vegetative: Cut and ensiled; cut + 4 hours pre-drying and ensiled and; cut + 7 hours pre-drying and ensiled; Pre-flowering: Silage cutting and ensiled and 4 hours pre-drying and ensiled; Flowering: cut and ensiled, no pre-drying, with four replications each treatment. Silage was storage for four months. It was carried out three *in situ* digestibility assays to determine the curve of ruminal degradability at different times (3, 6, 9, 12, 24, 48 and 72 hours), like *in situ* digestibility and other three assays of 24 hours and 48 hours to determinate *in vitro* digestibility of silage. The digestibility of ryegrass silage was influenced by both the pre-dehydration times and phenological stage, and the dehydration time of cut and silage had a high proportion of degradable components, with higher degradation rate by 24 hours of incubation. The *in vitro* digestibility of the ryegrass silage was greater than that *in situ* for up to 48 hours. The digestibility of the ensiled ryegrass biomass was influenced by the dehydration times, as well as the plant growth stage, regardless of the methodology used, being higher values for the treatment without previous drying of the vegetative stage.

KEYWORDS: *Lolium multiflorum*. Pre-dried silage. Ruminal degradation. Ruminant Nutrition. Winter fodder.

INTRODUCTION

An increasing advance of agricultural frontiers and the competition for agricultural areas has required the intensification of livestock production systems (ANTLE et al., 2017), mostly in developing countries. Into this backdrop, in order to keep beef and dairy farming competitive, it is essential to increase the profitability of these systems. For it, one of the main determinants of a great productive performance is related to the quantity and quality of bulky feed offered.

In temperate and subtropical regions, even in tropical regions of altitude, ryegrass can show an important alternative for maintaining ruminants throughput, mostly due to its nutritional quality (WILKINS; HUMPREY, 2003; ROMAN et al., 2010).

Due to its proven nutritional quality (SKONIESKI et al., 2011; MARCHESAN et al., 2015), ryegrass emerges as an alternative to be used in ruminant nutrition as well as preserved fodder. One of the main tools used to keep the forage productive and qualitative constancy used in ruminant nutrition is the silage process. Firstly, described as an alternative for the conservation of forages for use in times of shortage, it is straightaway part of the food base of more modernized herds, being used without interruption.

The production of silage with winter grasses is a challenge that has been studied in temperate countries, especially in ratio to the physiological stage most suitable for silage. Several studies have been carried out demonstrating the efficacy of ryegrass, both as a feed base in the winter period of livestock systems, as its applicability and characteristics, however, there are few studies that characterize the quality and efficiency of conservation methods such as silage. Conversely, Schmidt et al. (2014) reported that the ensiling of forage with a moisture content of 70% or more will cause qualitative losses of the material. Because of these high moisture contents in their vegetative stage, it is crucial to know the best time to ensilage without losing its nutritional characteristics, avoiding loss of quality (KUNG, 2002) and how it will impact on ruminal degradation.

Usually, *in vivo* assays are carried out to predict feed digestibility for ruminants, however, it requires a long time to obtain results, besides

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relatively high costs with animals, feeding, housing, laboratory analysis and allows the evaluation of a limited number of feed in each assay (SILVEIRA et al., 2009). By contrast, laboratorial methodologies, with significantly lower costs and time, were developed to estimate the digestibility of food at ruminal level, *in situ* and *in vitro* digestibility. Thus, the aim was to determine the ruminal degradability curve, by *in situ* technique, of ryegrass silage at different phenological stages and different times of dehydration of biomass, as its ruminal *in vitro* and *in situ* digestibility at predetermined distinct pre-drying times.

MATERIAL AND METHODS

Local description:

All the assays (field and laboratory evaluation) from this study was conducted at Embrapa Clima Temperado-Estação Terras Baixas, located in Capão do Leão (31◦52’ 20’’ S and 52°21’ 24’’ W), Rio Grande do Sul, Brazil., RS; from April 2013 to March 2015.

Treatments and chemical characterization:

For both assays, we used samples of annual ryegrass silage cv. BRS Ponteio, obtained at different phenological stages and submitted or not to the previous drying. After the establishment of pasture, it were made 7 cm cutting height, into the three plant phenological phases: vegetative, three different drying periods where determined (cut and ensile as such, cut and pre-drying of 4 hours and ensile, cut and a pre drying of 7 hours for ensiling); for the pre flowering phase to drying times were determined (ensiling just after cutting and a pre drying of 4 hours) and at full flowering, due to its low humidity content, there was no pre drying. Experimental design was a randomized blocks with six treatments and four repetitions. To fill up the micro-silos, ryegrass was cut to 5 cm long stubs and ensiled in PVC experimental micro-silos with dry matter density of 650 kg m^{-3}, at all stages, and 4 months stored. When opened, sub samples were taken for the chemical compositions and digestibility evaluations. Samples were dried at a forced-air dryer at 55 °C, for 72 hours and were ground, in Wiley mill type, to pass a 1-mm sieve for *in vitro* and 2-mm sieve for *in situ.*

Chemical characterization (Table 1) included: dry matter (DM; Method 967.03; AOAC, 1998); ash content by combustion in muffle furnace (550°C, 4 hs); organic matter (OM) , estimated by equation 100 – ash (Method 942.05; AOAC, 1998); crude protein (CP; Method 984.13; AOAC, 1997); neutral detergent fiber (NDF) and acid detergent fiber (ADF) determination as describe for Van Soest et al. (1991), adjusted to Senger et al. (2008), using 16 µm polyester bags and 110°C autoclave during 40 min and acid detergent lignin (ADL), by 12 M sulfuric acid treatment on ADF residue (Method 973.18; AOAC, 1998). Hemicellulose was estimated as the difference between NDF and ADF, and cellulose as the difference between ADF and ADL (VAN SOEST et al., 1991).

Table 1. Chemical composition of annual ryegrass silage* originated from different times of dehydration and different phenological stages.

Phenological stage	Vegetative			Pre-Flowering		Full Flowering
Dehydration times	0	4	7	0	4	0
DM (g kg^{-1} wet)	138± 4.8	251±3.5	293±14.2	233±4.3	292±10.2	445±9.7
Composition (g kg^{-1}DM)						
OM	891±2.1	899±5.9	899±3.9	893±7.9	901±7.1	924±8.8
CP	178±6.3	162±8.2	152±10.7	113±3.9	110±6.7	780±2.1
NDF	367±6.9	369±9.7	382±11.7	398±15.2	425±4.5	535±21.1
Hemicellulose	135±9.1	148±8.9	159±2.6	159±6.3	177±9.4	185±8.05
ADF	233±2.4	218±4.9	236±12.5	238±15.8	248±12.1	349±18.4
Cellulose	193±2.2	179±4.9	181±11.7	184±13.2	191±13.1	267±20.4
ADL	40±0.7	43±4.74	43±1.2	53±3.4	57±1.3	82±3.7

* Ryegrass was cut at 5 cm residue when the swards reached a height of 30 cm at each phenological stage. Values are means ±S.e. of samples taken throughout experimental micro-silos (n=4).

In vitro and in situ evaluations

All surgical and animal care protocols were approved by the Ethics Committee on Animal Research from Universidade Federal de Pelotas (CEEA n°5076-2013). Three *in vitro* digestibility assays were carried out for 48 or 24 h, using the method adapted from Goering and Van Soest (1970). For the incubation medium, aliquots of ruminal fluid were collected from four cannulated Jersey cows, fed a diet comprised by *Axonopus affinis* and *Paspalum notatum* and 1 kg day^{-1} of ground corn.

After gathering, ruminal liquid was filtered through two layers cheesecloth, keeping up 39°C, while constantly purged CO_2. A total of 400 mL of the filtered rumen fluid was poured into the incubation jar that contained 1600 mL of buffer (KH_2PO_4, 8.3 g L^{-1}, $MgSO_4*7H_2O$, 0.41 g L^{-1}, NaCl, 0.41 g L^{-1}, $CaCl_2*2H_2O$, 0.08 g L^{-1}, urea 0.41 g L^{-1}, Na_2CO_3 , 2.5 g L^{-1} and Na_2S*9H_2O, 0.16 g L^{-1}), sustained 1:4 rumen fluid:buffer ratio. Samples were alocated into polyester 40 µm bags (i.e. equal an 1 g sample 100 mL^{-1} incubation medium) and the digestibility technique applied for the estimation of *in vitro* true digestibility (IVTD) was the ruminal fermentor methodology for 48 or 24 hours unremitting at *in vitro* fermentor TE-150 Tecnal®, controlled by a thermostat. DM IVTD was estimate by calculation NDF residue content post incubation and *in vitro* DM (IVDM) and *in vitro* OM (IVOM) disappearance was estimate from the incubation residue.

Three *in situ* degradability were also carried out on the same four Jersey cows used for *in vitro* assay ruminal fluid collections (4585 ± 34.2 kg live weight) grazed on grass (*Axonopus affinis* and *Paspalum notatum*). Samples of approximately 1 g DM were placed in polyester bags (5 × 5 cm; pore size of 40 µm; sample:bag as ±15 mg cm^{-2}) and sealed were rumen incubated for 3; 6; 9; 12; 24; 48 and 72 hours using a gradual in/all out sequence, incubated into duplicate in each animal. Polyester bags were allocated into laundry bags (25 x 40 cm) and after were placed within three polyester laundry bags (30 × 40 cm) to ensure similar location within the rumen and to facilitate location for removal (VANZANT et al., 1996) and placed in ventral sac of the rumen. All treatments were incubated in each animal (6 treatments x 7 incubation times x 4 replicates x 4 animals) and the assay three times replicated.

Subsequently removal, all bags were washed in flowing water until the water pour clear. Then, samples were submerged for 15 minutes in a bacterial dissociation solution to eliminate any microbial contamination, washed, oven-dried at 105 °C for 8 hours, and weighed. The 24, 48 and 72 hours residue was treated at same process with neutral detergent previously describe. *In situ* digestibility was estimate by difference between means of sample disappearance for each treatment (with or no NDS procedure) in its respective incubation times and values of the DM incubated.

Statistical procedures

Data were analyzed using mixed procedure of SAS 9.0 (SAS Inst., Inc., Cary, NC) by analysis of variance and means compared by *DMS* Fisher's Test (P<0.05). When interaction observed for total digestibility, means were compared by Student *t* Test. Ruminal degradability data, after analysis of variance, were submitted to polynomial regression analysis.

RESULTS AND DISCUSSION

For *in vitro* assay (Table 2), we observed negative effects (P<0.05) both related to the phenological stage and pre-dehydration time, independent of incubation time (24; 48 h) for all parameters evaluated. Velásquez et al. (2010) discussed that minimization on digestibility with the plant development growth cycle is widely discussed, due to the accretion of structural carbohydrates and cellular content decreased. Another factor to be considered is that this process also contributed to the rate of cell wall degradation, as long as a much larger fraction of the compounds are degraded in the first 24 hours on silage of young plants, howsoever, this is not repeated in plants at the end of productive cycle, substantiate what was found in this study.

Also, was verified negative correlation between DM IVTD and ryegrass silage contents of: NDF (P<0.0001, R=-0.92), ADF (P<0.0001, R=-0.87) and ADL (P<0.0001, R=-0.94) Additionally, CP showed high positive correlation (P<0.0001, R=0.92).

Distinct authors (MARTINS et al., 2006; MACEDO JÚNIOR et al., 2007; SANTOS et al., 2011) reported that the increase of structural carbohydrates occurs parallel to the increase in lignin contents, phenolic component that limits and extension of the fibrous digestion in the rumen, being consistent with those reported in the present research. Lignification restricts the performance of digestive enzymes produced by rumen microorganisms and, hence, decreases digestibility. Additionally, Balsalobre et al. (2003) emphasize that the variation in the fibrous fraction quality interferes directly in energy availability for

ruminant, thereby, progress in plant growth cycle causes increase in the constituents of the cell wall, Thus, reducing the levels of non-fibrous carbohydrates hence lower energy of rapid degradation for ruminal microorganisms, reducing the capacity to be digested.

Table 2. *In vitro* dry matter and organic matter disappearance and *in vitro* dry matter true digestibility at 24 and 48 hours of incubation of annual ryegrass at distinct phenological growth stages and pre- dehydration times.

	Vegetative			Pre-Flowering		Flowering	SEM	R^2	P value
	0	4	7	0	4	0			
24 h incubation									
DM disappearance	43.44	42.03	41.02	32.73	31.33	24.58	2.11	0.92	<0.0001
OM disappearance	48.75	46.76	45.63	36.65	34.78	26.60	2.43	0.92	<0.0001
DM IVTD	50.22	48.16	47.00	37.75	35.82	27.40	2.41	0.92	<0.0001
48 h incubation									
DM disappearance	66.62	63.78	60.82	58.76	53.62	44.68	0.88	0.98	<0.0001
OM disappearance	72.53	68.81	65.63	63.83	57.73	46.9	0.95	0.98	<0.0001
DM IVTD	74.78	70.94	67.66	65.80	59.51	48.35	0.98	0.98	<0.0001

SEM: Standard error mean; R^2:coefficient of determination. *P* value: 0.05

We observed a linear effect ($P<0.05$) under the influence of dehydration for *in situ* (table 3), as like was found for *in vitro,* where the digestibility was negatively affected as the time, as well the phonological stage. According to this evidence, we concluded that after the plant harvest, the process of degradation of the soluble carbohydrates begins with the cellular respiration. This decreases the faster degradable fraction of plant, directly influencing the digestibility of the material, more evidenced in the vegetative stage.

Table 3. I*n situ* digestibility of annual ryegrass silage at distinct phenological growth stages and pre-dehydration times.

Phenological Stage	**Pre- dehydration time**	**Incubation time (hours)**			Regression equation	P value
		24	**48**	**72**		
Vegetative	0	55.97	64.57	70.68	Y=0.306x+49.034 R^2= 0.9231	<0.0001
	4	53.11	62.83	68.61	Y=0.322x+46.02 R^2=0.9493	<0.0001
	7	51.49	60.74	66.26	Y=0.308x+44.728 R^2=0.9271	<0.0001
Pre-Flowering	0	39	52.65	60.29	Y=0.443x+29.363 R^2=0.9516	<0.0001
	4	37.81	49.96	58.72	Y=0.436x+27.916 R^2=0.9675	<0.0001
Flowering	0	25.49	34.1	41.42	Y=0.331x+17.738 R^2=0.947	<0.0001

R^2= Determination coefficient.

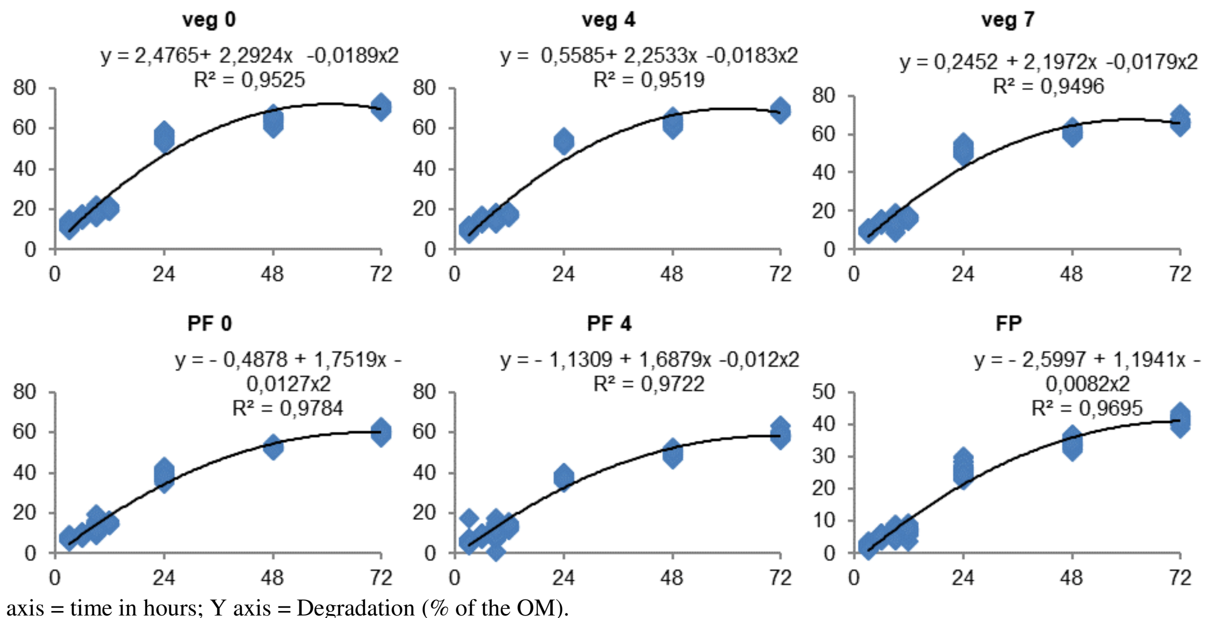
It should be considered the lignin content (Table 1) for the digestibility of NDF be mainly regulated by the content and type of lignin in the forage (OLIVEIRA et al., 2011; RICACHESKI et al., 2017), remaining explicit this rating in both vegetative and pre-flowering. Mutually, plants in full flowering also have low protein content, and this protein is less available because it is bound to lignin. By the progress on plant physiological cycle, there is a reduction in the crude protein content of

forages, resulting in an increase in the proportion of stems and fiber contents as a reduction in the proportion of leaves (BRITO et al., 2003).

Along with the fibrous portion, the proportion of protein available to the microorganisms is a limiting factor for their proliferation and, therefore, bulk digestion. Protein digestion results largely in the ammonia production used by rumen microbiota as the main source for the microbial protein synthesis (COSGROVE et al., 2007; CRUZ et al., 2010; DIJKSTRA et al., 2012), and the speed of its use will be greater when soluble carbohydrates are available for the multiplication of these microorganisms.

The prolonged ryegrass dehydration process also affects the protein and soluble carbohydrate contents (Table 2), infering in less substrates for the entire digestion of silage. However, our results are contraditor by that found for Zamarchi et al. (2014), evaluating oats silage at final growth cycle, not reporting differences in IVTD for pre-drying technique, only between maturation stage.

The ruminal degradation curve presented a unequal behavior with the rising of productive cycle of ryegrass and also with the use of dehydration (Figure 1). The amount of DM disappeared per unit of time was decreasing from vegetative time 0 until flowering (2.29% DM. h^{-1} for vegetative and 1.19 % DM. h^{-1} at flowering). We observed that the amount of DM degraded in the first incubation times has also variated. In the vegetative stage, most of the DM was degraded until the 48 h, above all occurred until the 24 h, when it usually occurs the lack of microbial adherence. So, in this stage, great part of these compounds is degraded faster.



X axis = time in hours; Y axis = Degradation (% of the OM).

Figure 1. *In situ* degradation curve (3 to 72 hours) of the ensiled biomass of annual ryegrass at distinct phenological growth stages and pre- dehydration times.

In the remaining stages, there is a higher concentration of structural carbohydrates and lignin, complicating factors of bulky, mainly because its comprise high variation in their nutritional composition as like as its rate of degradation of its components, varying according to forage species, plant age, time of year, fertilization and management of soil (KASUIYA et al., 2008; LIMA et al., 2012).

It can be observed in figure 2 that after 48 hours the degradability of all treatments tended to stabilize, however, the percentage of degraded material was dependent on the stage of plant development in which the ryegrass was ensiled. With the increase of the NDF content, there is a decrease in the rate of degradation of DM (Figure 1) due to the difficulty of the rupture of these molecules by the bacteria, which affects, mainly, the rate of degradation. In general, this also affects the rate of passage of the feed because, the lower the rate of degradation, the longer the dwell feed retention time in the rumen, (MUNIZ et al., 2012; TOSTO et al., 2015).

The samples of the vegetative stage presented values superior to the others, mostly in the first three hours of incubation, agreeing to several authors report that younger plants have higher soluble carbohydrate contents such as starch and

pectin, both rapidly digested in the rumen, even though pectin is part of the cell wall (PATTON, 1994; OLIVEIRA et al., 2013). This fact contributes to a better perspective for the use of temperate grasses in the feeding of the animals, demonstrating that the process is effective to maintain the fodder quality, a result that can be observed by the satisfactory results of the digestibility of the evaluated silage.

CONCLUSIONS

Vegetative stage with no pre-dehydration has a higher digestibility.

The digestibility of the ensiled ryegrass biomass was influenced by the dehydration times, just as plant growth stage, regardless of the methodology used.

Fiber and lignin contents were shown to be a limiting factor for ryegrass silage digestibility.

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RESUMO: O objetivo foi determinar como o tempo de emurchecimento e o ciclo da planta influenciam na digestibilidade *in vitro* e degradabilidade *in situ* da silagem de azevém. As amostras foram provenientes de experimento a campo, com delineamento experimental de blocos ao acaso, com seis tratamentos (Vegetativo: cortar e ensilar; cortar + pré-secagem de 4 horas e ensilar e; cortar + pré-secagem de 7 horas e ensilar. Pré-florescimento: cortar e ensilar e pré-secagem de 4 horas. Florescimento: cortar e ensilar) e quatro repetições. A armazenagem foi de quatro meses. Foram realizados três ensaios de digestibilidade *in situ* para determinar a degradabilidade ruminal em diferentes tempos (3, 6, 9, 12, 24, 48 e 72 horas) e três ensaios de *in vitro*. A digestibilidade da silagem de azevém foi influenciada, tanto pelos tempos de desidratação, quanto pela idade da planta, sendo que, o tempo 0 do vegetativo apresentou alta proporção de componentes degradáveis, apresentando maior taxa de degradação até as 24 horas de incubação. A digestibilidade *in vitro* da silagem foi maior que degradabilidade *in situ* até às 48 horas. Maior digestibilidade da biomassa foi apresentada pelo tempo 0 do estádio vegetativo, para ambas as técnicas empregadas.

PALAVRAS-CHAVE: Degradação ruminal. Forragem hibernal. *Lolium multiflorum*. Nutrição de ruminantes. Silagem de pré-secado.

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