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Research Article

Quality of alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) mixture silages depending on the share in the mixture and additives

Đorđe LAZAREVIĆ[1]*, Vladeta STEVOVIĆ[2], Zoran LUGIĆ[1], Dalibor TOMIĆ[2], Jordan MARKOVIĆ[1], Vladimir ZORNIĆ[1], Mladen PRIJOVIĆ[1]

[1]*Institute for Forage Crops, 37251 Globoder, Kruševac, Serbia; djordje.lazarevic@ikbks.com (*corresponding author); zoran.lugic@ikbks.com; jordan.markovic@ikbks.com; vladimir.zornic@ikbks.com; mladen.prijovic@ikbks.com*
[2]*University of Kragujevac, Faculty of Agronomy, Cara Dušana 34, 32000 Čačak, Serbia; vladeta@kg.ac.rs; dalibort@kg.ac.rs*

Abstract

Alfalfa silage has a high potential for improving ruminant nutrition. A problem that frequently occurs during its preparation is the process of proteolysis, which could partially be avoided with the use of certain techniques and materials. Red clover, often used in form of silage, expresses weaker proteolysis due to the possession of certain chemical compounds. This research was conducted to study the effects of ensiled alfalfa and red clover mixtures, mixed at different ratios (100:0, 90:10, 70:30, 50:50, 30:70, and 0:100), as well as the influence of additives – two doses of oak tannin extract (6 g kg^{-1} DM and 12 g kg^{-1} DM) and bacterial inoculant (*Enterococcus faecium, Bacillus plantarum,* and *Bacillus brevis*) on fermentation parameters and protein fractions of the silages. The treatments which contained any of the used percentages of red clover, as well as the ones which received the higher dose of oak tannin (12 g kg^{-1} DM), reduced the ammonia nitrogen content, which is one of the main indicators of proteolysis. On the other hand, considering non-protein nitrogen, as another of the indicators of proteolysis, there was no positive sign of the contribution of red clover to the reduction of proteolysis in alfalfa, and neither there was a significant impact of additives that were applied in this experiment.

Keywords: alfalfa; inoculation; mixture; red clover; silage; tannin

Introduction

Alfalfa and red clover are the main source of protein and are the main constituents of ruminant meals (Marković *et al.*, 2021). In the Republic of Serbia, the main way to conserve these legumes is through hay production. Ensiling is rarely used, only in bad weather conditions with low temperatures and high air moisture (Đorđević and Dinić, 2003). This is understandable since preservation of high moisture legumes is usually limited by a shortage of water-soluble carbohydrates (WSC), high buffering capacity (BC) and clostridia proliferation. Alfalfa is one of the forage plants which are difficult to ensile. Small content of the water-soluble

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carbohydrates, very high value of buffering capacity, as well as the extremely unfavourable ratio of WSC/BC, indicate the unsuitability of alfalfa biomass for ensiling (Dinić *et al.*, 2006). The most efficient way to preserve the proteins while conserving forage is ensiling through the production of haylage and silage. In the case of legumes, it calls for the introduction of certain procedures or materials, such as wilting or different additives which will stimulate the proper fermentation (Đorđević and Dinić, 2003). During ensiling, usually more than half of the crude protein (CP) in alfalfa is broken down to peptides and amino acids by enzymes released from cell rupture in the foliage (McDonald *et al.*, 1991). In alfalfa, 44 – 87% of protein is degraded to non-protein nitrogen (NPN) compounds during silage fermentation, whereas for red clover of similar protein content, only 7-40% of the protein is lost due to plant proteolytic degradation (Jones *et al.*, 1995). The high extent of protein degradation in ensiled alfalfa leads to the low utilization of forage nitrogen (N) by ruminants, thus resulting in economic losses to farmers and adverse environmental impacts (Dong *et al.*, 2019). Inhibited proteolysis in ensiled red clover was connected by various studies with polyphenol oxidase (Mayer, 1986), a copper-containing enzyme naturally present in red clover (Jones *et al.*, 1995; Sullivan and Hatfield, 2006). Recent studies have shown that red clover through co-ensiling could confer alfalfa the same features of reducing proteolysis as red clover (Li *et al.*, 2018; Dong *et al.*, 2019). Bacterial inoculants are the primary additives used for legume silage in the USA because they usually have a positive effect on the silage more often than enzyme additives and are much safer to use than acid or formaldehyde additives (Muck and Bolsen, 1991). Although most legumes are prone to excessive proteolysis during or before ensiling, tannin-containing legumes are unique. It was found that within and among seven legume species, proteolysis was negatively correlated to tannin concentration (Albrecht and Muck, 1991). According to Herremans *et al.* (2019), oak tannin extract as an additive seems a good option in order to improve silage protein used by the animal, particularly when considering silages with more degradable nitrogen, such as alfalfa silage.

One of the objectives of the study was to determine the effect of the species ratio in the mixtures on the content of protein fractions in alfalfa - red clover mixtures. The second one was to investigate the influence of the aforementioned ratios, and the use of additives such as oak tannin and inoculants on the fermentation quality parameters and protein fractions content of the alfalfa – red clover silages.

Materials and Methods

Field experiment

Alfalfa (cv. 'K-42', Institute for Forage Crops Kruševac, Serbia) and red clover (cv. 'K-17', Institute for Forage Crops Kruševac, Serbia) were sown at the experimental field of the Institute for Forage Crops Kruševac (21° 19' 35'' E, 43° 34' 58'' N) in spring in 2019, on April the 25th. The sowing rate was 20 kg ha^{-1} and 18 kg ha^{-1} for alfalfa and red clover respectively. The study area was positioned at altitude of 166 m above sea level in Central Serbia. Soil type was with pH in H_2O 6.87; pH in 1 M KCl 5.85; nitrogen content of 0.176%; Al-soluble P_2O_5 and K_2O of 3.6 and 28.6 mg 100 g^{-1}, respectively; humus content of approximately 3.5%. Legumes were harvested at the start of bloom in the second cutting in summer in 2020, on July the 1st, leaving a 5 cm stubble. Alfalfa was wilted in the field for 4h to reach a dry matter (DM) concentration of 34%, and meanwhile, red clover was wilted to 25% DM. After that, the wilted biomass was chopped to approximately 1 cm.

Laboratory experiment

The experiment, involving treatments as combinations of six different mixtures and four additives, was laid out. The mixture ratios were as follows: alfalfa (M0), alfalfa and red clover at a ratio of 90:10 (M10), 70:30 (M30), 50:50 (M50), 30:70 (M70), and red clover (M100) on a fresh (wilted) matter (FM) basis. The additive factor consisted of: control (without additive), two doses of granulated oak tannin extract (6 g kg^{-1} DM, and 12 g kg^{-1} DM; 'Essedielle SRL', Italia), and bacterial inoculant 'BioStabil Plus' which contained homofermentative lactic acid bacteria (*Enterococcus faecium* and *Bacillus plantarum*), and hetero-fermentative lactic acid bacteria (*Bacillus brevis*) with a concentration of $5x10^{10}$ CFU per gram. The mixtures were ensiled in the lab-scale silos holding 5 dm^3, with three replications. The density of silage used in this experiment was 550 g dm^{-3} FM.

DM content of wilted forage and silage samples was measured by oven drying at 60 °C for 48 h. On the samples obtained from neutral detergent fiber (NDF), and acid detergent fiber (ADF) residues, neutral detergent insoluble crude protein (NDICP) and acid detergent insoluble crude protein (ADICP) was determined. The Kjeldahl method, according to AOAC (1990) was applied to determine crude protein (CP) content of all samples. Non protein nitrogen (NPN) was estimated as the difference between total CP and CP of true protein (TP) origin precipitated with 10% trichloracetic acid (TCA) solution. Similarly, according to the method of Licitra *et al*. (1996), soluble protein (SolP) was calculated as the difference between total CP and buffer insoluble CP (IP) estimated with borate phosphate buffer (pH 6.7-6.8) and freshly prepared (1 g 10 mL^{-1}) sodium azide solution. According to the method of Fox *et al*. (2004), protein fractionation as percentage of total CP was made by the Cornell Net Carbohydrate and Protein System (CNCPS). CNCPS is a convenient method to evaluate the protein quality and predict rates of protein digestibility in the rumen (Higgs *et al*., 2015). Also, it is a mathematical model designed to evaluate the nutrient requirements and supply of cattle over a wide range of environmental, dietary, management and production situations (Van Amburgh *et al*., 2007). According to CNCPS, CP is divided into 3 fractions. Shortly, the PA fraction is NPN, the PB fraction is a degradable protein, while the PC fraction is an undegradable and unavailable protein. The PB fraction is further divided into 3 subfractions depending on solubility and rate of ruminal degradation. The degradation dynamic in the rumen of borate phosphate buffer soluble PB_1 is rapid, neutral detergent soluble PB_2 is intermediate and acid detergent soluble PB_3 fraction is slow. To examine the mutual dependence of ensiling in relation to fresh (wilted) matter, the model of linear trend (Njegić *et al*., 1991) was applied:

$$\hat{y} = a + b \cdot x \qquad (1)$$

where:

$$b = \Sigma x_t \cdot y_t - n \cdot \bar{x} \cdot \bar{y} / \Sigma x_t^2 - n \cdot \bar{x}^2 \qquad (2)$$

$$a = \bar{y} - \bar{x} \cdot b \qquad (3)$$

pH value was determined from the silage extract, using pH-meter ('ISKRA' MA 5740). Content of ammonia nitrogen was calculated by the modified method according to Kjeldahl (Đorđević and Dinić, 2003). Quantity of acetic and butyric acid was determined by the distillation method according to Wiegner (1926). The amount of lactic acid was calculated using the amounts of total acidity, free acetic and butyric acid.

Statistical analysis

The experimental data were analysed by a factorial analysis of variance for samples of forage in a completely randomized design using a model that accounted for the main effects of alfalfa: red clover mixtures, and by a two-way analysis of variance for silage samples using a model that accounted for alfalfa: red clover mixture treatments and treatments with the additives. Effects were considered significant at $p < 0.05$ level. The significance of differences between arithmetic means was analyzed by LSD test ('STATISTICA 8', Stat. Soft. 2007).

Results and Discussion

The analysis of alfalfa, red clover and their mixture biomasses before ensiling show that the effects of the mixture rates were significant for the DM content (Table 1). With regard to the mixture rates, DM content declined with the decreasing rate of alfalfa (from 100% to 0%) in the mixture. It ranged from 250.0 (M100) to 339.0 (M0) g kg^{-1}. These results were consistent with the ones obtained by Li *et al*. (2018), where DM content ranged from 24.9 (pure red clover) to 34.5 (pure alfalfa) %. Similar effect of the mixture rates on DM content was obtained in silages (Table 3). Considering CP content, there were no significant differences recorded between different herbage mixtures, while with increasing proportion of red clover, the contents of NPN and SolP decreased almost linearly, which was similar to the results obtained by Owens *et al.* (1999).

Table 1. Primary protein fractions and protein fractions by CNCPS of alfalfa: red clover mixtures

Protein fractions	Alfalfa: red clover mixture					
	M0	M10	M30	M50	M70	M100
DM (g kg^{-1})	339.0 a	334.8 ab	310.2 bc	301.4 c	297.0 c	250.0 d
CP (g kg^{-1} DM)	204.6	203.2	205.4	196.7	199.7	183.3
NDICP (g kg^{-1} CP)	208.1 bc	193.1 c	201.2 bc	218.2 ab	195.8 c	228.8 a
ADICP (g kg^{-1} CP)	110.5 d	132.7 ab	123.8 bc	114.2 cd	135.1 a	132.9 ab
NPN (g kg^{-1} CP)	492.5 a	486.3 ab	449.4 bcd	421.7 cd	460.5 abc	417.2 d
IP (g kg^{-1} CP)	487.9 c	496.9 bc	531.5 abc	541.5 ab	530.4 abc	561.1 a
SolP (g kg^{-1} CP)	512.1 a	503.1 ab	468.5 bc	458.5 c	469.5 bc	438.9 c
TP (g kg^{-1} CP)	507.5 b	513.7 b	550.6 ab	578.3 a	539.5 ab	582.8 a
PA (g kg^{-1} CP)	492.5 a	486.3 ab	449.4 bcd	421.7 cd	460.5 abc	417.2 d
PB_1 (g kg^{-1} CP)	19.6 c	16.8 d	19.1 c	36.8 a	9.1 e	21.7 b
PB_2 (g kg^{-1} CP)	279.8 c	303.7 bc	330.3 ab	323.2 ab	334.6 a	332.3 a
PB_3 (g kg^{-1} CP)	97.6 ab	60.4 d	77.4 c	104.0 a	60.7 d	95.8 b
PC (g kg^{-1} CP)	110.5 d	132.7 ab	123.8 bc	114.2 cd	135.1 a	132.9 ab

M0, 100% alfalfa + 0% red clover; M10, 90% alfalfa + 10% red clover; M30, 70% alfalfa + 30% red clover; 50% alfalfa + 50% red clover; M70, 30% alfalfa + 70% red clover; M100, 0% alfalfa + 100% red clover; DM, dry matter; CP, crude protein; NDICP, neutral detergent insoluble crude protein; ADICP, acid detergent insoluble crude protein; IP, insoluble protein; SolP, soluble protein; TP, true protein; NPN, non-protein nitrogen; PA, non-protein nitrogen, immediately degraded in the rumen; PB_1, soluble true protein, rapidly degraded in the rumen; PB_2, buffer insoluble protein minus protein insoluble in neutral detergent, some fraction PB_2 is fermented in the rumen and some escapes to the lower gut; PB_3, true protein insoluble in neutral detergent but soluble in acid detergent, slowly degraded in the rumen because it is associated with the cell wall; PC, protein that is insoluble in the acid detergent, unavailable or bound protein; different letters in a row denote significant differences between means ($p < 0.05$)

Among fermentation parameters of silage, only the pH did not show any significant differences between the treatments (Table 2). Generally, in all the cases pH showed values which are rather optimal for the process of conserving, and exceed the critical points, which is often a problem with legume silages, especially when ensiling alfalfa. The pH values remained below 4.2 which were slightly lower than the lowest value recorded by Dong *et al.* (2017). Fermentation quality could affect the process of proteolysis of ensiled alfalfa, as proteases are sensitive to pH. Treatments which contained any of the used percentages of red clover, as well as the ones which received the higher dose of oak tannin, reduced the ammonia nitrogen (NH_3-N) content, which is similar to the results obtained by Herremans *et al.* (2019), and Dong *et al.* (2019). This suggests that the use of oak tannin and red clover in certain proportions had a positive influence on the microbial proteolysis during the process of ensiling. Also, the content of NH_3-N decreased almost linearly with the increase of the proportion of red clover. It ranged from 10.66 (M0) to 9.02 (M100) % of total nitrogen (TN). While plant proteases dominate the process of true protein degradation (i.e., the first phase of hydrolysis), microbial enzymes play a primary role in the conversion of free amino acids into ammonia nitrogen (i.e., the second phase deamination, Ohshima and McDonald, 1978). Also, ammonia content can be used to assess the microbial activities in ensiled forage, because amino acids are broken down to NH_3 by deamination, which is carried out by bacteria producing butyric, acetic, and lactic acid (Fijałkowska *et al.*, 2015). Addition of inoculant reduced the content of NH_3-N, but this reduction was not statistically significant, which is different when compared with the results obtained by Huo *et al.* (2021), where the addition of *Lactobacillus plantarum* reduced the content of NH_3-N from 128.0 to 73.8 g kg^{-1} TN. Lactic acid content, which generally was high, has shown a slight linear decrease with increase of the proportion of red clover. It ranged from 99.4 (M100) to 125.7 (M0) g kg^{-1} DM. Acetic acid content was also on rather high level, with small variations between the treatments. Concerning the mixture treatment, it ranged from 56.2 (M50) to 64.1 (M0) g kg^{-1} DM, and as for the additive treatment, it ranged from 58.6 (control and tannin – 6 g kg^{-1} DM) to 63.6 (tannin – 12 g kg^{-1} DM) g kg^{-1} DM. It could be concluded that in our case dry matter content had high influence on the lactic acid content. Further research could explain the reason for the acetic acid content in our experiment, and bring confirmation of assumption about the lactic acid content. The butyric acid was not detected in any of the silages, which indicates good quality in every of the cases.

Table 2. Fermentation characteristics of alfalfa: red clover mixture silages

	pH	NH_3-N (% TN)	Lactic acid (g kg^{-1} DM)	Acetic acid (g kg^{-1} DM)	Butyric acid (g kg^{-1} DM)
Mixtures (M)					
M0	4.04	10.66 a	125.7 a	64.1 a	0
M10	4.02	9.38 b	112.9 bc	62.1 ab	0
M30	3.83	9.49 b	106.6 d	58.4 bc	0
M50	4.02	8.93 b	118.2 b	56.2 c	0
M70	4.09	9.09 b	109.3 cd	60.7 ab	0
M100	4.07	9.02 b	99.4 e	60.5 ab	0
Additives (A)					
Control	3.98	9.88 a	112.3	58.6 b	0
Tannin 6 g kg^{-1} DM	4.10	9.67 a	109.5	58.6 b	0
Tannin 12 g kg^{-1} DM	3.91	8.51 b	112.1	63.6 a	0
Inoculant	4.06	9.64 a	114.1	60.5 ab	0
Significance					
p M	0.568	0.043	< 0.001	0.005	-
p A	0.376	0.023	0.301	0.011	-
p M*A	0.398	0.593	< 0.001	< 0.001	-

M0, 100% alfalfa + 0% red clover; M10, 90% alfalfa + 10% red clover; M30, 70% alfalfa + 30% red clover; M50, 50% alfalfa + 50% red clover; M70, 30% alfalfa + 70% red clover; M100, 0% alfalfa + 100% red clover; NH_3-N – ammonia nitrogen; TN – total nitrogen; DM – dry matter; different letters in a row denote significant differences between means ($p < 0.05$)

Concerning mixture treatment, dry matter content ranged from 267.8 (M0) to 330.4 (M100) g kg^{-1} (Table 3). As it was the case with the herbage mixtures, DM content of the silages also decreased with the increasing rate of red clover. Crude protein content did not significantly change with the increase of red clover percentage, except in case of the pure red clover, where it was lower than the rest of the treatments. Silage from monoculture red clover had the lowest NPN (483.8 g kg^{-1} CP) and SolP (508.3 g kg^{-1} CP), and the highest values had silage from monoculture alfalfa which were 534.5 and 558.9 g kg^{-1} CP, respectively. According to Licitra *et al*. (1996), NPN is direct indicator of hydrolysis. In our research proportion of NPN of M100 was significantly lower in relation to the other mixture treatments, but they did not have any significant differences between themselves. It means that red clover did not show a significant influence on proteolysis in mixtures with alfalfa, even though monoculture red clover showed weaker proteolysis, which is similar to the Purwin *et al*. (2014). When considering the effect of additives, there were no significant differences between the control and any of the additive treatments. This was different when compared with the results obtained by Tao *et al*. (2016), where addition of inoculant significantly reduced the content of NPN from 128.10 to 108.41 g kg^{-1} DM. The highest content of IP was observed in silage from red clover monoculture (M100), while the lowest content of IP was in alfalfa monoculture (M0) (491.7 and 441.0 g kg^{-1} CP, respectively). The results about NDICP showed that there were significant differences between mixture treatments M0 (189.2 g kg^{-1} CP) and

M100 (202.0 g kg^{-1} CP). Despite the significance of the obtained differences, there were no great discrepancies between silage mixtures concerning Neutral Detergent Insoluble Crude Protein, which is similar to the results obtained by Purwin *et al*. (2014).

Table 3. Primary protein fractions of alfalfa: red clover mixture silages

	DM (g kg^{-1})	CP (g kg^{-1} DM)	NDICP (g kg^{-1} CP)	ADICP (g kg^{-1} CP)	IP (g kg^{-1} CP)	SolP (g kg^{-1} CP)	TP (g kg^{-1}CP)	NPN (g kg^{-1} CP)
Mixtures (M)								
M0	330.4 a	195.9 a	189.2 b	104.8	441.0 b	558.9 a	465.4 b	534.5 a
M10	322.8 b	196.7 a	185.6 b	109.6	444.5 b	555.5 a	469.2 b	530.8 a
M30	309.8 c	199.6 a	185.2 b	110.1	466.7 ab	533.3 ab	491.1 ab	508.9 ab
M50	301.0 d	197.3 a	180.5 b	106.2	447.9 b	552.1 a	470.1 b	529.9 a
M70	285.8 e	197.2 a	190.3 ab	109.3	468.5 ab	531.5 ab	492.3 ab	507.7 ab
M100	267.8 f	187.2 b	202.0 a	115.5	491.7 a	508.3 b	516.2 a	483.8 b
Additives (A)								
Control	307.4 a	193.5	190.3	107.8	453.6	546.4	478.1	521.9
Tannin 6 g kg^{-1} DM	304.9 a	194.4	187.8	111.8	469.2	530.8	492.8	507.2
Tannin 12 g kg^{-1} DM	305.1 a	196.5	189.6	112.1	460.6	539.4	486.6	513.4
Inoculant	294.3 b	198.1	187.4	105.2	456.8	543.2	478.7	521.3
Significance								
p M	< 0.001	0.041	0.019	0.497	0.039	0.039	0.045	0.045
p A	< 0.001	0.465	0.916	0.366	0.707	0.707	0.698	0.698
p M*A	< 0.001	0.659	0.007	0.659	0.308	0.308	0.263	0.263

M0, 100% alfalfa + 0% red clover; M10, 90% alfalfa + 10% red clover; M30, 70% alfalfa + 30% red clover; M50, 50% alfalfa + 50% red clover; M70, 30% alfalfa + 70% red clover; M100, 0% alfalfa + 100% red clover; DM, dry matter; CP, crude protein; NDICP, neutral detergent insoluble crude protein; ADICP, acid detergent insoluble crude protein; IP, insoluble protein; SolP, soluble protein; TP, true protein; NPN, non-protein nitrogen; different letters in a row denote significant differences between means ($p < 0.05$)

In all treatments of the investigation, the soluble fraction PA averaged above 50% of total CP, except the one with the monoculture red clover (M100) (Table 4). Despite the absence of significant differences between the treatments which included alfalfa, there was a linear decrease of the PA fraction with the increase of percentage of red clover in the mixture. The decreasing PA (NPN) content with increasing red clover amount meant that efficient utilization of N by ruminant increased with higher red clover proportion (Broderick, 1995). PB_2 and PB_3 fraction showed almost linear increase when the proportion of red clover in the mixture is rising. They ranged from 251.8 g kg^{-1} CP (M0) to 298 g kg^{-1} CP (M100) for PB_2 and from 67.8 g kg^{-1} CP (M0) to 90.7 g kg^{-1} CP (M100) for PB_3. Fraction PB_2 is fermented in the rumen, and some escapes to the lower gut, whereas a high part of PB_3 avoids degradation in the rumen (Sniffen *et al*., 1992). Based on PB_3 data it can be predicted that true protein escaped outside of the rumen increases with the rise of red clover proportion in the mixture. PC fraction generally showed low values, but there were no significant differences between any of the treatments. There was, though, a slight rise of PC fraction with the increasing level of red clover, which is consistent with Li *et al*. (2018). Opposite to the results of Guo *et al*. (2008), additives did not significantly affect the content of any of the protein fractions, except the fraction PB_3.

Table 4. Protein fractions by CNCPS of alfalfa: red clover mixture silages

	PA ($g\ kg^{-1}$ CP)	PB_1 ($g\ kg^{-1}$ CP)	PB_2 ($g\ kg^{-1}$ CP)	PB_3 ($g\ kg^{-1}$ CP)	PC ($g\ kg^{-1}$ CP)
Mixtures (M)					
M0	534.5 a	32.8 a	251.8 c	67.8 c	104.8
M10	530.8 a	29.8 b	258.9 bc	76.7 b	109.6
M30	508.9 ab	24.1 c	278.2 ab	75.9 b	110.1
M50	529.9 a	21.7 cd	267.4 bc	75.1 bc	106.2
M70	507.7 ab	21.1 d	278.2 ab	85.2 a	109.3
M100	483.8 b	15.5 e	298.0 a	90.7 a	115.5
Additives (A)					
Control	521.9	24.5	263.3	81.4 ab	107.8
Tannin 6 $g\ kg^{-1}$ DM	507.2	24.3	284.7	74.3 c	111.8
Tannin 12 $g\ kg^{-1}$ DM	513.4	23.3	270.9	75.3 bc	112.1
Inoculant	521.3	24.6	269.4	83.3 a	105.2
Significance					
p M	0.045	< 0.001	< 0.001	< 0.001	0.497
p A	0.698	0.629	0.078	0.013	0.366
p M*A	0.263	0.005	0.036	< 0.001	0.659

M0, 100% alfalfa + 0% red clover; M10, 90% alfalfa + 10% red clover; M30, 70% alfalfa + 30% red clover; M50, 50% alfalfa + 50% red clover; M70, 30% alfalfa + 70% red clover; M100, 0% alfalfa + 100% red clover; PA, non-protein nitrogen, immediately degraded in the rumen; PB_1, soluble true protein, rapidly degraded in the rumen; PB2, buffer insoluble protein minus protein insoluble in neutral detergent, some fraction PB_2 is fermented in the rumen and some escapes to the lower gut; PB_3, true protein insoluble in neutral detergent but soluble in acid detergent, slowly degraded in the rumen because it is associated with the cell wall; PC, protein that is insoluble in the acid detergent, unavailable or bound protein; different letters in a row denote significant differences between means ($p < 0.05$)

In all of the mixtures, a higher value of the PA fraction content was achieved compared to the content value of the same in the forages before ensiling (Table 1). This is in accordance with the results of Krawutschke *et al.* (2011), who reported increase of PA fraction in silages of the examined red clover cultivars, when compared with material before ensiling. With an increase in the content of red clover in the mixtures, there is a linear increase in the difference between the value of the PA fraction of the silages and the initial material (Figure 1A). The above results are not consistent with the results of Li *et al.* (2018) who reported a linear increase in the differences between silages and forages before ensiling with decreasing red clover content in the mixture, when considering PA content. The assumption is that this happened because significantly more favorable fermentation conditions were achieved in our test, which is reflected in the lower pH value in all of the treatments, compared to the pH values from the research results of Li *et al.* (2018). In most of the mixtures, a higher value of the PB_1 fraction (quickly degradable in the rumen) was achieved in the silages (Table 4), compared to the PB_1 value of the initial material (Table 1). Considering the trend of the change of PB_1 fraction content in the initial material with altering the share of plant species in the mixture, no dependence of the change tendency was determined (the coefficient of determination is R^2=0). However, in the case of ensiled material, a downward trend was determined in relation to the increasing content of red clover in the silage mixture – coefficient of determination R^2=0.959; (Figure 1B). This phenomenon can be attributed to the influence of additives added during ensiling (oak tannin and microbiological inoculum), but their effects would have to be investigated individually and the mechanism of their action determined. The tendency to change the content of the protein fraction PB_2 (intermediately degradable proteins in the rumen) depended only on the percentage of red clover in the mixture and showed a linear tendency for this parameter to increase depending on the increase in the content of red clover. The same tendency was found in the case of silage, where we can conclude that there was no influence of additives, but only of the herbal mixture. The content of the PB_2 fraction is nominally higher in the initial material compared to silage, for all mixtures of plant material.

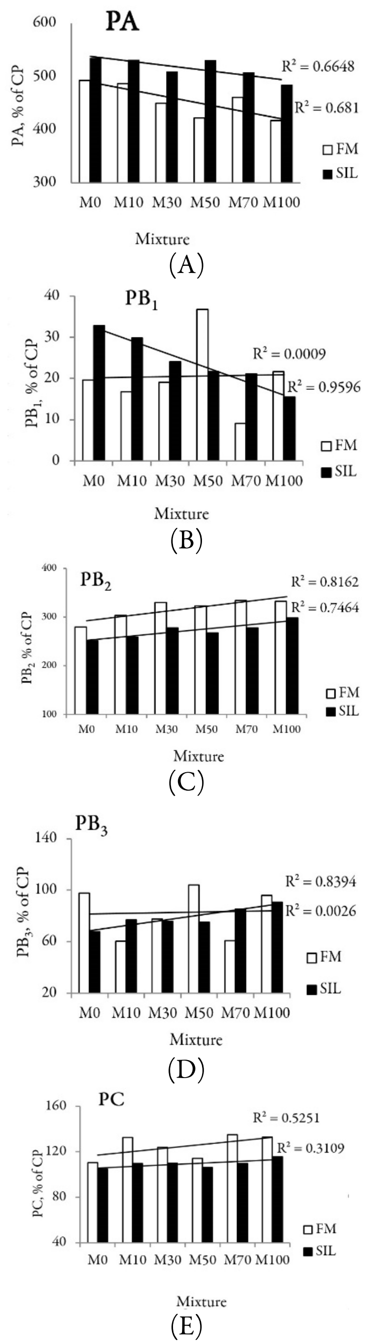

Figure 1. Comparison of the protein fractions by CNCPS of alfalfa: red clover mixture silages and forages before ensiling

M0, 100% alfalfa + 0% red clover; M10, 90% alfalfa + 10% red clover; M30, 70% alfalfa + 30% red clover; M50, 50% alfalfa + 50% red clover; M70, 30% alfalfa + 70% red clover; M100, 0% alfalfa + 100% red clover; PA, non-protein nitrogen, immediately degraded in the rumen; PB_1, soluble true protein, rapidly degraded in the rumen; PB2, buffer insoluble protein minus protein insoluble in neutral detergent, some fraction PB_2 is fermented in the rumen and some escapes to the lower gut; PB_3, true protein insoluble in neutral detergent but soluble in acid detergent, slowly degraded in the rumen because it is associated with the cell wall; PC, protein that is insoluble in the acid detergent, unavailable or bound protein; different letters in a row denote significant differences between means ($p < 0.05$); FM, fresh matter; SIL, silage; R^2, coefficient of determination;

When it comes to the PB_2 fraction, in each of the mixtures, a significantly higher value of the content of the initial material compared to the silage was determined. For both silages and forages before ensiling, there is a significant positive tendency of changes between the content of the PB_2 fraction and the increasing content of red clover in the mixture (Figure 1C). In most of the mixtures, a higher value of the PB_3 (slowly degradable in the rumen) fraction was achieved in the initial material compared to the silage. In the case of forages before ensiling, there is no tendency to change the content of the PB_3 fraction depending on the content of plant species in the mixture, while in the silages there is a tendency to increase the content of the PB_3 fraction under the influence of the increase in the content of red clover in the mixture and probably under the influence of additives (Figure 1D). When it comes to the PC fraction (completely unavailable protein fraction), in most mixtures a higher value was measured in the initial material compared to the silage. Both in the forages before ensiling and in the silages, there is a slight positive tendency to increase the content of the PC fraction with an increase in the content of red clover (Figure 1E).

The results of this experiment open new questions for further research in this field, especially when microbiological proteolysis is taken in consideration.

Conclusions

The study showed that ensiling alfalfa in mixture with red clover together with the application of higher dose of tannin (12 g kg^{-1} DM) had positive effects related to the content of ammonia nitrogen in silages. As for the non-protein nitrogen there was no positive sign of contribution of red clover to the reduction of proteolysis in alfalfa, neither there was significant impact of additives which were applied in this experiment. Considering the field of alfalfa proteolysis, there is a lot of unexplained subjects, and it calls for further investigations in order to preserve the proteins and thus provide a success in livestock production.

Authors' Contributions

Conceptualization: ĐL; Data curation: ĐL, VZ and MP; Formal analysis: ĐL, VS and DT; Investigation: ĐL, VZ and MP; Methodology: JM; Project administration: ZL; Software: VZ and MP; Supervision: VS and ZL; Validation: DT, JM and VZ; Visualization: VZ; Writing – original draft: ĐL; Writing – review and editing: DT, JM and VZ.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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