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Effects of Additives and Mixing Alfalfa (*Medicago sativa* L.) with Red Clover (*Trifolium pratense* L.) at Different Ratios on Proteolysis, Fermentation Quality and Microbiota of Silage

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ABSTRACT

The nutrition of ruminants can be improved by using alfalfa silage, as well as red clover silage, as a low-cost source of proteins. However, proteins from ensiled legumes, especially alfalfa, have poor usability because of the high level of degradation in the rumen. This is a consequence of the intensive proteolytic processes during silage fermentation. In recent decades there were significant investigations of different materials and techniques which limit proteolyisis and contribute to a better usage of proteins from silage. This research was conducted with the aim of determining the effect of additives - two doses of oak tannin extract [6 g kg^{-1} of dry matter (DM) and 12 g kg^{-1} DM] and bacterial inoculant (*Enterococcus faecium, Bacillus plantarum,* and *Bacillus brevis*), as well as the effect of ensiled mixtures of alfalfa and red clover, in different ratios (100 : 0, 90 : 10, 70 : 30, 50 : 50, 30 : 70, and 0 : 100) on the content of protein fractions, the number of microorganisms, as well as fermentation quality parameters. The ammonia nitrogen (NH_3-N) content, which is one of the main indicators of proteolysis, was generally equal at the different mixture and additive treatments as well as the pH value, and below the critical point of 10% which was probably the consequence of favorable conditions at every of the treatments. On the other hand, considering nonprotein nitrogen (NPN), as another of the indicators of proteolysis, there was a positive sign of the contribution of inoculant and red clover to the reduction of proteolysis in alfalfa only in case of the silage mixture with the 70% of red clover share. Increase in the share of red clover in the mixture generally caused growth in the number of lactic acid bacteria, as well as in the number of yeasts and molds.

Keywords: alfalfa, additive, mixture, red clover, silage.

INTRODUCTION

Alfalfa and red clover are common legumes used for ruminant feeding systems (Sousa et al., 2020). One of the basic reasons for this is that forage legumes provide high protein feed, either in single-species swards, or in mixed-species swards (Marković et al., 2022). The main way to conserve these legumes in the Republic of Serbia is through hay production. Ensiling is considered almost only in bad weather conditions with low temperatures and high air moisture. Legumes exhibit high moisture, high buffering capacity, low concentration of soluble carbohydrates, expressive water activity, which together results in a prolonged fermentative process with high consumption of fermentable substrates (Borreani et al., 2018; Castro-Montoya and Dickhoefer, 2020). Alfalfa particularly has a reputation for being difficult to ensile, because when ensiled alone, the fermentation outcomes are often suboptimal due to its high buffering capacity and low sugar and dry matter concentrations (Liu et al., 2018; Wang et al., 2019). 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. Approximately 50-80% of the total nitrogen in alfalfa silages could be converted to NPN (Ohshima and McDonald, 1978), whereas for red clover of similar protein content, only 7-40% of the protein is transformed due to plant proteolytic

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degradation (Jones et al., 1995). The protein utilization efficiency in ruminants fed alfalfa silage was normally low due to extensive proteolysis of alfalfa silage, resulting in an increase in ruminal ammonia nitrogen concentration (Chen et al., 2021). Red clover contains polyphenol oxidase (PPO), which participates in reducing proteolysis during ensiling and can inhibit the activity of proteolytic microbes. Recent studies have shown that alfalfa through co-ensiling with red clover could obtain the same features of reducing proteolysis as red clover (Li et al., 2018; Dong et al., 2019). In order to obtain high-quality silage with strong digestibility, stimulation of the fermentation process is required by adding different biological additives. Possible changes in silage fermentation, when ensiled with lactic acid bacteria (LAB), among other changes include reduced proteolysis, and increased dry matter recovery. Certain research has focused on enhancing alfalfa fermentation indicators by LAB additives, such as reduction of pH and NH_3-N content and the increase in lactic acid (LA) content (Filya et al., 2007). Previous studies have reported that tannins can reduce proteolysis during ensiling and rumen fermentation, due to the antimicrobial and protein-binding features of tannins (Patra et al., 2012; Jayanegara et al., 2019). During ensilage process, LAB produce predominantly lactic acid, which rapidly reduce pH and inhibits the growth of many aerobic bacteria and thus ensures feed preservation. Consequently, number of LAB and their relation to the total number of bacteria in silage is one of the main indicators of fermentation quality. The number of yeasts and molds is also important, because when lactic acid is destroyed by yeasts, the rising pH causes the growth of molds, significantly reducing the quality of silage (McDonald et al., 1991).

The objective 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 herbage mixtures, as well as the influence of the aforementioned ratio, and the use of additives such as oak tannin and inoculants on the fermentation quality parameters, protein fractions content, as well as the number of different groups of microorganisms of the alfalfa-red clover silages.

MATERIAL AND METHODS

Trial design

At the experimental field of the Institute for Forage Crops Kruševac (43°58’31.7”N, 21°20’80.9”E, 166 m a.s.l.) red clover (cv. K-17, Institute for Forage Crops Kruševac, Serbia) and alfalfa (cv. K-42, Institute for Forage Crops Kruševac, Serbia) were sown in spring in 2021, on 25th of April. The sowing rate was 18 kg ha^{-1} and 20 kg ha^{-1} for red clover and alfalfa, respectively. The legumes were harvested in the second cutting in 2022, on July the 1st, at the beginning of flowering, leaving a 5 cm stubble. Red clover was wilted in the field for 4 h to a dry matter content of 25% DM, and meanwhile, alfalfa was wilted to 34% DM. The wilted biomass was then chopped to approximately 1 cm.

The trial with treatments as combinations of four additives and six different mixtures, was laid out. There were six mixtures, 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 matter (FM) basis. The additive variants were: control (without additive), granulated oak tannin extract, which included two doses (6 g kg^{-1} DM, and 12 g kg^{-1} DM; ‘Essedielle SRL’, Italia), and bacterial inoculant ‘BioStabil Plus’ including homofermentative lactic acid bacteria (*Enterococcus faecium* and *Bacillus plantarum*), and hetero-fermentative lactic acid bacteria (*Bacillus brevis*) with a concentration of 5x10^{10} cfu g^{-1}. The degree of compaction of silage used in this experiment was 700 g dm^{-3} FM. It was done using lab-scale silos holding 5 dm^{3}. The experiment was done with three replications, and the silage fermentation lasted 56 days until opening.

Laboratory experiment

The initial material and silage were dried in an oven at 60°C during 48 h and the DM content was determined. The crude protein

(CP) content was determined using the Kjeldahl method, according to AOAC (1990). The proportion of protein fractions within the total CP was analyzed using Cornell Net Carbohydrate and Protein System (CNCPS) (Fox et al., 2004). According to CNCPS, there are three fractions within the total CP, where the PA fraction is NPN, the PB fraction represents degradable protein, while the PC fraction refers to undegradable and unavailable protein. The PB fraction has three subfractions, with different rates of degradability in the rumen (PB_1 fraction - fast degradable; PB_2 fraction - medium degradable; PB_3 fraction - slow degradable). Using pH meter (‘ISKRA’ MA 5740) the pH value was determined from the silage extract. The modified Kjeldahl method (Đorđević and Dinić, 2003) was used to calculate the NH_3-N content. The distillation method according to Wiegner (1926) was used to determine the acetic and butyric acid content. The amount of total acidity, free acetic and butyric acids were used to calculate the amount of lactic acid. Also, the following microbial parameters were analyzed: total bacterial count, lactic acid bacteria count, and yeast and mold count. To examine the interdependence of ensiling in relation to fresh matter, simple linear regression was used, when $R^2 > 0.6$ the coefficient of determination is considered significant.

The experimental data were analyzed by two-factor analysis of variance for forage samples in a completely randomized design using a model that calculated the main effects of the alfalfa-red clover mixture. Also, for silage samples using a model that calculated the treatments of the alfalfa-red clover mixture, as well as the treatments with additives, a two-way analysis of variance was used. Significance for all effects was declared at $P < 0.05$ level. The least significance difference test (LSD) was used for analyzing the significance of differences between arithmetic means. Statistical analysis were conducted in EXCEL (Microsoft Corporation, Redmond, WA, USA) and STATISTICA, version 8.0 (StatSoft Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Analyses showed that the proportion of alfalfa and red clover in the biomass mixture before ensiling significantly affected the dry matter content (Table 1).

Table 1. Dry matter, crude protein, and protein fractions content of alfalfa and red clover mixtures before ensiling

	Alfalfa-red clover mixture						
	M0	M10	M30	M50	M70	M100	*P* M
DM (g kg^{-1})	339 ^{a}	335 ^{ab}	310 ^{bc}	301 ^{c}	297 ^{c}	250 ^{d}	< 0.001
CP (g kg^{-1} DM)	204	203	205	197	200	183	0.141
PA (g kg^{-1} CP)	492 ^{a}	486 ^{ab}	449 ^{bcd}	422 ^{cd}	460 ^{abc}	417 ^{d}	0.006
PB_1 (g kg^{-1} CP)	19.6^{c}	16.8 ^{d}	19.1 ^{c}	36.8 ^{a}	9.1 ^{e}	21.7 ^{b}	< 0.001
PB_2 (g kg^{-1} CP)	280 ^{c}	304 ^{bc}	330 ^{ab}	323 ^{ab}	335 ^{a}	332 ^{a}	0.007
PB_3 (g kg^{-1} CP)	97.6 ^{ab}	60.4 ^{d}	77.4 ^{c}	104.0 ^{a}	60.7 ^{d}	95.8 ^{b}	< 0.001
PC (g kg^{-1} CP)	110 ^{d}	133 ^{ab}	123 ^{bc}	114 ^{cd}	135 ^{a}	133 ^{ab}	0.001

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; PA, nonprotein 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 ($P < 0.05$).

With regard to the mixture rates, DM content increased with the decreasing rate of red clover (from 100% to 0%) in the mixture and it ranged from 250 g kg^{-1} (M100) to 339 g kg^{-1} (M0). Li et al. (2018) presented similar results, where DM content ranged from 24.9% (M100) to 34.5% (M0). Also, in silages was reported a similar effect of mixture rates on DM content (Table 3). Consistent with Owens et al. (1999), there were no significant differences in CP content between different herbage mixtures.

Considering fermentation parameters of silage, pH showed some significant differences between the treatments, but these variations were within a very narrow range (Table 2). On the other side, investigations showed that silage pH tends to decrease with increasing proportion of red clover (Li et al., 2018). Generally, in all of the cases pH showed values which do not exceed the critical points and are rather optimal for the process of conserving (around pH 4), which is often a problem with legume silages, especially when ensiling alfalfa. Fermentation quality could affect the process of proteolysis of ensiled alfalfa, as proteases are sensitive to pH. The content of NH_3-N showed no significant differences between silage mixtures and it ranged from 9.11% (M30) to 8.65% (M100) of total nitrogen (TN). Also, differences between treatments with tannin and inoculant were rather small, which is consistent with the results of Wang et al. (2023). This is probably a consequence of restriction of proteolytic enzymes, caused by low pH values in all of the treatments (Dong et al., 2019). According to Đorđević and Dinić (2003), the acceptable quantity of NH_3-N in these kind of silages is around 10%. Since all of the values were below 10%, these results, together with the ones about pH, confirm the fact that in every treatment were favorable conditions for ensiling. The NH_3-N was created by the synergistic effect of plant enzymes and microbial activity, and the reduction of silage NH_3-N was attributed to the rapid acidification with LAB. Still, NH_3-N concentration in silage is largely a product of microbial, rather than plant enzyme activity (Ohshima and McDonald, 1978). Lactic acid content, which generally was high, has shown a tendency to decrease with increase of the proportion of red clover and it ranged from 112 g kg^{-1} DM (M70) to 128 g kg^{-1} DM (M50). Also, lactic acid content was significantly higher on the variant with inoculant, compared to the control, which was similar to Filya et al. (2007). It could be concluded that in our case dry matter content as well as the inoculant had high influence on the lactic acid content. Acetic acid content was also on rather high level, with small variations between the treatments. Concerning the mixture treatment, acetic acid content showed a slight linear decrease with increase of the proportion of red clover, and ranged from 53.3 g kg^{-1} DM (M100) to 64.0 g kg^{-1} DM (M0) (Table 2). The butyric acid was not detected in any of the silages, which indicates good quality in every of the cases. For all analyzed fermentation quality parameters, a significant interaction between mixture treatment and additives was observed.

Table 2. Fermentation characteristics of alfalfa and red clover silage mixtures

	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	4.06 ^{a}	8.99	120 ^{bc}	64.0 ^{a}	0
M10	4.01 ^{abc}	8.85	116 ^{cd}	58.1 ^{b}	0
M30	3.94 ^{c}	9.11	122 ^{b}	60.8 ^{ab}	0
M50	3.96 ^{bc}	8.86	128 ^{a}	58.2 ^{b}	0
M70	4.04 ^{ab}	9.01	112 ^{d}	59.6 ^{b}	0
M100	4.08 ^{a}	8.65	113 ^{d}	53.3 ^{c}	0
Additives (A)					
Control	3.95 ^{b}	8.59 ^{b}	116 ^{b}	59.3	0
Tannin 6 g kg^{-1} DM	3.97 ^{b}	8.74 ^{b}	116 ^{b}	57.1	0
Tannin 12 g kg^{-1} DM	4.08 ^{a}	8.74 ^{b}	119 ^{ab}	60.0	0
Inoculant	4.05 ^{a}	9.56 ^{a}	123 ^{a}	59.7	0
Significance					
P M	0.005	0.767	<0.001	<0.001	-
P A	<0.001	0.001	0.008	0.185	-
P M*A	<0.001	<0.001	<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 ($P < 0.05$).

Crude protein content significantly increased with the increase of alfalfa percentage, and ranged from 177 g kg^{-1} DM (M100) to 204 g kg^{-1} DM (M0) (Table 3).

Table 3. Dry matter, crude protein, and protein fractions content of alfalfa and red clover mixture silages

	DM (g kg^{-1})	CP (g kg^{-1} DM)	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	332 ^{a}	204 ^{a}	527 ^{a}	20.9	277 ^{ab}	75.2	101 ^{b}
M10	324 ^{a}	197 ^{b}	545 ^{a}	18.4	249 ^{bc}	86.2	101 ^{b}
M30	312 ^{ab}	196 ^{b}	553 ^{a}	23.9	232 ^{c}	89.5	101 ^{b}
M50	259 ^{c}	195 ^{b}	538 ^{a}	26.0	249 ^{bc}	80.0	107 ^{ab}
M70	287 ^{abc}	188 ^{c}	486 ^{b}	35.6	283 ^{ab}	79.2	116 ^{a}
M100	267 ^{bc}	177 ^{d}	459 ^{b}	38.0	300 ^{a}	89.8	115 ^{a}
Additives (A)							
Control	306	193	531	19.8 ^{b}	260	81.6	107.1
Tannin 6 g kg^{-1} DM	305	192	517	20.1 ^{b}	269	83.5	112.2
Tannin 12 g kg^{-1} DM	305	194	520	28.3 ^{b}	265	83.2	103.9
Inoculant	271	193	504	40.4 ^{a}	266	84.9	105
Significance							
P M	0.013	<0.001	<0.001	0.053	0.009	0.253	0.003
P A	0.178	0.957	0.373	0.003	0.948	0.961	0.180
P M*A	0.724	<0.001	0.013	0.539	0.389	0.508	0.709

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; PA, nonprotein 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 ($P < 0.05$).

In addition, a significant interaction was observed between mixture treatment and additive, regarding the CP content. Treatments with tannin and inoculant did not differ significantly from the control in terms of CP content, but their interaction with mixtures was significant. The interaction of pure alfalfa with bacterial inoculant was significant in reducing crude protein content, while in pure red clover, bacterial inoculant caused changes with relative character, which are a consequence of a decrease in the content of fermentable carbohydrates (Figure 1).

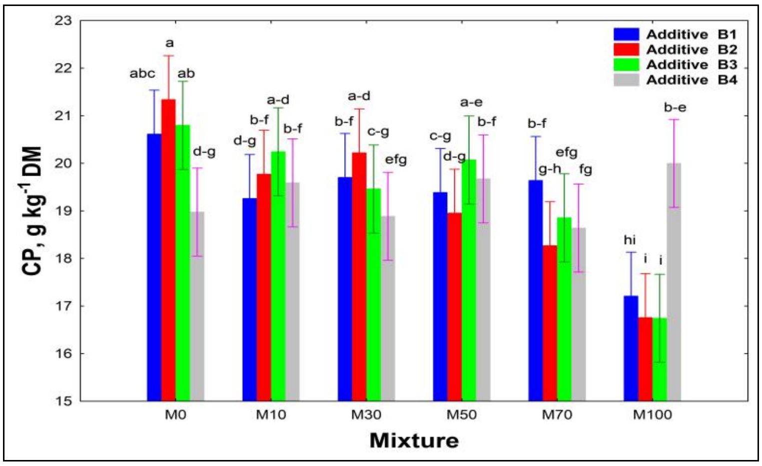

Figure 1. Average crude protein (CP) content (g kg^{-1}) in dry matter (DM) of alfalfa and red clover mixture silages with additives

A suitable method for assessing protein quality and predicting the rate of protein degradability in the rumen is the CNCPS method (Higgs et al., 2015). It is also a mathematical model designed to estimate the nutrient requirements and supply of cattle under a wide range of environmental, dietary, management and production situations (Van Amburgh et al., 2007). Nonprotein nitrogen (PA fraction) is a direct indicator of protein hydrolysis. Silages M100 and M70 had the lowest PA fraction content (459 g kg^{-1} CP and 486 g kg^{-1} CP, respectively), which was significantly lower than the rest of the mixture variants. It means that red clover showed a significant influence on proteolysis during ensiling alfalfa, only in case of M70 mixture, which was similar to the results of Lazarević et al. (2023).

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) and the mixture with 70% of red clover (M70) (Table 3). The results in this study were similar to Nadeau et al. (2016) who reported that NPN increased and indigestible true protein showed slight rise after ensiling. In red clover (M100) and one of the mixed silages (M70), possibly benefited by PPO’s protein protection, as a reflection of PA fraction content, protein breakdown less occurred as opposed to the alfalfa silage, which is consistent with Dong et al. (2019). Also, the PA content on the treatments with additives was slightly lower compared with control, and similar results were recorded by Chen et al. (2021). In addition, a significant interaction was observed between mixture treatment and additive, regarding the PA fraction content. The interaction was significantly established in pure alfalfa where bacterial inoculants significantly reduced the PA fraction content (Figure 2).

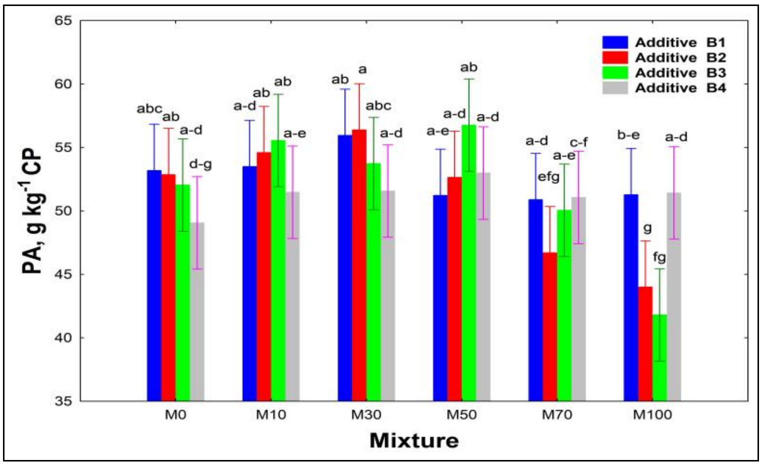

Figure 2. Average content of PA fraction (PA - nonprotein nitrogen, immediately degraded in the rumen) (g kg^{-1}) in crude protein (CP) of alfalfa and red clover mixture silages with additives

Fractions PB_2 and PB_3 showed smaller variations concerning both mixture and additive treatments and almost linear increase when the proportion of red clover in the mixture is rising. They ranged from 232 g kg^{-1} CP (M30) to 300 g kg^{-1} CP (M100) for PB_2 and from 75.2 g kg^{-1} CP (M0) to 89.8 g kg^{-1} CP (M100) for PB_3. Similar to Li et al. (2018), lower PC proportion in M10 and M30 than M100 indicated a positive effect from alfalfa, resulting in higher protein degradability than pure red clover silage. Generally low values were recorded considering PC fraction. Additives significantly affected the content of the fraction PB_2 and PC, which is different when compared with the results of Guo et al. (2008).

A higher value of the PA fraction content was achieved in silages compared to the value of the same in the forages before ensiling, in all of the mixtures (Table 1 and Table 3). This was similar to Li et al. (2021), who reported increase of PA fraction in silages of the examined mixtures of alfalfa with paper mulberry, when compared with material before ensiling. With an increase in the content of red clover in the mixtures, there was a linear decrease in the difference between the value of the PA fraction of the silages and the initial material (Figure 3A).

The above results were consistent with the results of Li et al. (2018) and Dong et al. (2019) who also reported a linear decrease in the differences between silages and forages before ensiling with increasing red clover content in the mixture, when considering PA content. The assumption is that this happened because degradation of plant protein during ensiling is inevitable and results in the transformation of true protein to nonprotein N with poor nutritive value (Dong et al., 2019).

In most of the mixtures, a higher value of the PB_1 fraction (quickly degradable in the rumen) was recorded in the silages, compared to the PB_1 value of the initial material (Table 1 and Table 3). 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 was $R^2 = 0$). However, in the case of ensiled material, a upward trend was determined in relation to the increasing content of red clover in the silage mixture (the coefficient of determination was $R^2 = 0.874$) (Figure 3B).

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 PB_2 fraction (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. 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 was 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 3C).

In half 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 was no tendency to change the content of the PB_3 fraction depending on the content of plant species in the mixture, as well as in the silages (Figure 3D).

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. Unlike the initial material, in silages there was a slight positive tendency for the content of the PC fraction to increase with increasing red clover content (Figure 3E).

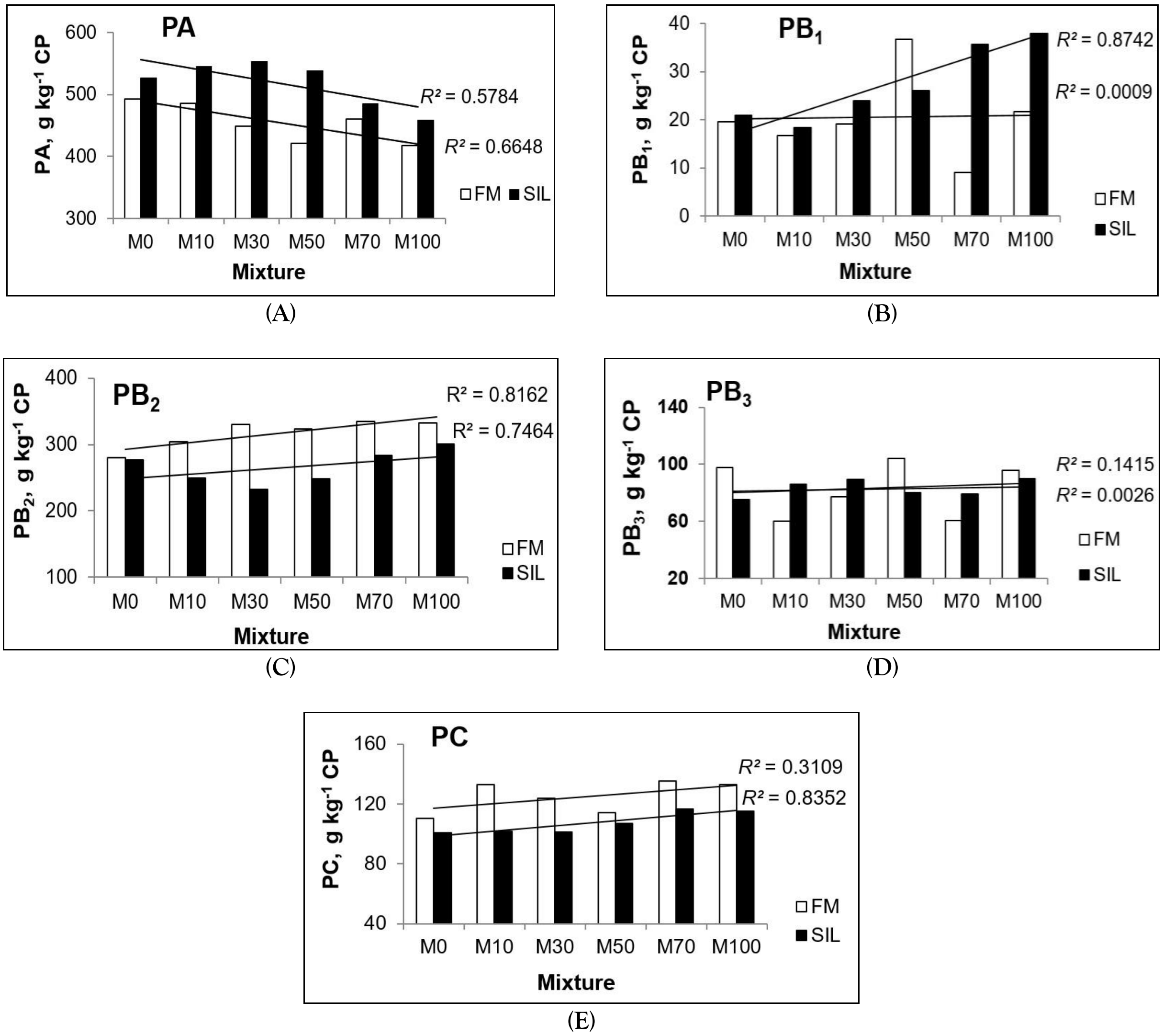

Figure 3. Comparison of the protein fractions by CNCPS of alfalfa-red clover mixture silages and forages before ensiling. M0, 100% alfalfa + 0% red clover; M10, 90% alfalfa + 10% red clover; M30, 70% alfalfa + 30% red clover; M50, 50% alfalfa + 50% red clover; M70, 30% alfalfa + 70% red clover; M100, 0% alfalfa + 100% red clover; PA, non-protein nitrogen, immediately degraded in the rumen; PB_1, soluble true protein, rapidly degraded in the rumen; 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 ($P < 0.05$); FM, fresh matter; SIL, silage; R^2, coefficient of determination.

Concerning the number of lactic acid bacteria, it can be concluded that in the mixture treatments M100 and M70 was a significantly higher quantity, compared with the rest of the treatments. Also, a significantly higher number of lactic acid bacteria was recorded in treatment with inoculant, compared with the control treatment. There was no large variations concerning the influence of tannin, which was consistent with Xie et al. (2022).

With the rise of the red clover share in the mixture, there was an increase in the number of yeasts and molds. Similar to Yuan et al. (2017), in case of any additive used, there was a lower number of yeasts and molds, when compared with the control treatment. In general, in all of the additive or mixture treatments, there was a smaller number of yeasts and molds compared to the case in some other investigations (Yuan et al., 2017; Xie et al., 2022) (Table 4). A significant interaction between mixture treatment and additive was observed for all analyzed microbiological parameters (Figure 4). On the mixture treatments M10, M50 and M100, there was a significantly higher number of lactic acid bacteria on the variant with inoculant, compared with the control variant, which was not consistent with Li et al. (2022).

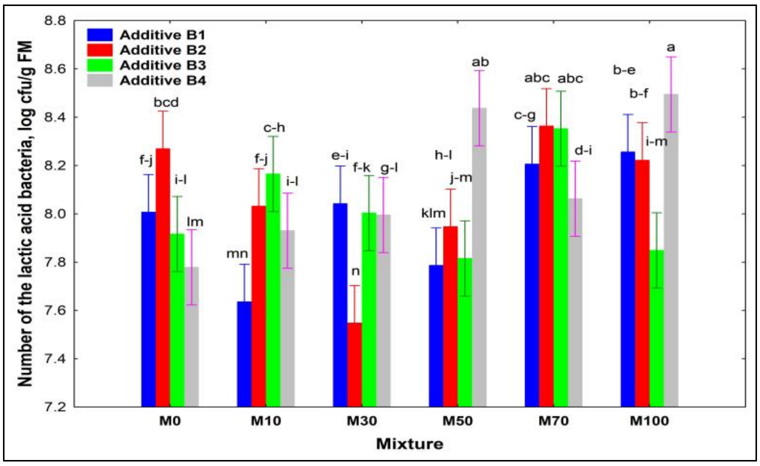

Figure 4. Average number of lactic acid bacteria (log cfu g^{-1}) in fresh matter (FM) of alfalfa and red clover mixture silages with additives

Table 4. Abundance of different groups of microorganisms in alfalfa and red clover mixture silages

	Number of lactic acid bacteria (log cfu g^{-1} FM)	Number of yeasts and molds (log cfu g^{-1} FM)	Total number of bacteria (log cfu g^{-1} FM)
Mixtures (M)			
M0	7.99 ^{b}	2.84 ^{b}	8.66 ^{a}
M10	7.94 ^{b}	2.77 ^{b}	8.52 ^{c}
M30	7.90 ^{b}	2.80 ^{b}	8.59 ^{b}
M50	8.00 ^{b}	2.92 ^{ab}	8.52 ^{c}
M70	8.25 ^{a}	2.93 ^{ab}	8.69 ^{a}
M100	8.20 ^{a}	3.01 ^{a}	8.56 ^{b}
Additives (A)			
Control	7.99 ^{b}	3.00 ^{a}	8.54 ^{c}
Tannin 6 g kg^{-1} DM	8.06 ^{ab}	2.91 ^{a}	8.67 ^{a}
Tannin 12 g kg^{-1} DM	8.02 ^{b}	2.68 ^{b}	8.60 ^{b}
Inoculant	8.12 ^{a}	2.93 ^{a}	8.54 ^{c}
Significance			
P M	<0.001	0.027	<0.001
P A	0.034	<0.001	<0.001
P M*A	<0.001	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; FM - fresh matter; DM - dry matter; different letters in a row denote significant differences between means ($P < 0.05$).

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

CONCLUSIONS

Concerning the nonprotein nitrogen content there was a significant positive sign of contribution of red clover to the reduction of proteolysis in alfalfa. Also, the used additives had a positive influence on pH, fatty acid content, as well as the PA fraction both individually and in interaction with mixtures. As for 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 cattle milk and meat production.

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