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RESEARCH ARTICLE

The use of different N sources for the treatment of permanent grassland and effect on forage quality

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ABSTRACT

During an experiment conducted in Serbia, near the city of Šabac over two growing seasons (2012/13 and 2013/14), the yield and quality of grassland treated with an organo-mineral fertilizer (farmyard manure 30 t ha^{-1} + 10 wt.% zeolite) was monitored and compared to fertilization with pure manure (30 t ha^{-1}), spring nitrogen application (50 kg/ha N), treatment with pure zeolite, and treatment without the addition of fertilizers. All fertilizers were spread in the autumn of 2012. Nitrogen was exceptionally applied in the spring of 2013 and 2014. Dry matter (DM) yield and forage quality were evaluated during the growing season, in two forage harvests. In addition to yields, the chemical composition of the biomass, concentrations of total protein fractions and *in vitro* DM digestibility were examined in detail. The biomass yields of both cuts in the first season were considerably higher after the application of manure with zeolite, pure manure and mineral nitrogen, compared to the control and zeolite. No significant differences among the treatments were noted with regard to the chemical composition of the biomass, or the effect of the type of fertilization on DM digestibility of the biomass from the natural grassland. The application of the organo-mineral fertilizer considerably reduced the share of NPN in total protein, compared to pure manure.

Keywords: Manure; Nitrogen; Forage quality; Yield; Zeolite

INTRODUCTION

Nitrogen is a crucial element for improving yields of most crops, including forage plants. Restrictions imposed on the use of nitrogen-based mineral fertilizers on grasslands in European countries, along with high prices and low cost-effectiveness, influence fertilization approaches in the mountainous regions of Serbia (Simić et al., 2015; Vučković et al., 2016). As a result, farmers are increasingly opting for traditional fertilizers (e.g., cattle manure), as a significant source of nitrogen and a low-cost and readily available source of nutrients. Nitrogen is present mainly in the organic matter, the content of the latter in the fresh manure being about 50 wt.%. However, during fermentation, manure loses significant amounts of nitrogen. Thus, the loss in four days may reach up to 90% due to an extensive liberation of ammonia (Huijsmans et al. 2003). Zeolite appears to be an acceptable solution because it is able to bind ammonia ions (Glisic et al., 2009). Moreira and Satter (2006) reported that cattle manure excretion depends on monthly temperatures, where nitrogen loss reached 40% during warm months compared to 16% during the cold months. According to Petersen et al. (1998), due to volatilization and denitrification during six months of storage of farmyard manure (FYM), N content decreased by 20%, compared to fresh FYM. The results of the previous research indicated that the addition of 10 wt.% of the natural microporous aluminosilicate (zeolites), can play a role as binding agents and retain 90% of the ammonia of the applied FYM. (Simić et al., 2013). Another researcher reported better herbage yield and nitrogen uptake by plants with clinoptilolite and NH_4-N (Kavoosia, 2007). Forage palatability is highly dependent on forage quality and the interaction of forage with the microbial population of the rumen (Allen, 1996). Crude protein (CP) concentrations as well as fiber fractions ADF (acid detergent fiber) and NDF (neutral detergent fiber) are the main indicators of forage quality. The crude protein content and herbage yield could be increased by N fertilization (Buxton, 1996).

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As opposed to the effect on the CP content, the influence of N fertilizers on fiber fractions (NDF and ADF) varies (Coleman et al., 2004). Protein degradability of forage can be influenced by growing conditions (where the addition of nitrogen through fertilization has the greatest impact), plant maturity, and growing season (Buxton, 1996). The application of nitrogen-based fertilizers reduces the proportion of non-degradable protein in the green mass of the herbage, which means that the degradability of their protein increases (Peyraud and Astigarraga, 1998). However, as the plants mature, their protein becomes less degradable. The level of maturity of forage is an important factor, given the lignification that comes with maturity; the extent to which the forage protein is integrated into the plant structure affects its availability for action with proteolytic enzymes (Peyraud and Astigarraga, 1998). The season of utilization could play an important role in the chemical composition of grasses, where higher levels of fiber fractions NDF and NDICP and energy level decreases in rainy seasons have been determined (Buxton, 1996). A larger proportion of NDF in grasses invariably means a lower level of energy for livestock. For that reason, livestock performance would be limited in rainy seasons because although feed consumption is not reduced, meals with higher concentrations of structural carbohydrates will not ensure adequate growth of the microbial population in the rumen (Rueda et al., 2003). Even though the availability of nitrogen is seemed to be a limiting factor in plant production, nitrogen-based fertilizers are applied to increase biomass yields (Tilman et al., 2001). The application of nitrogen causes considerable changes to the plant composition and the biochemical processes within the plant (Cooke et al., 2005). Fertilization with nitrogen had no effect on lignin concentrations of *Trifolium subterraneum* (Nori et al., 2006), but did have such an effect in the case of the tall fescue (Wolf and von Boberfeld, 2003). Larger amounts of N could postpone plant developmental stages and, in turn, result in higher plant quality and digestibility (Coleman et al., 2004). Some authors point out that clinoptilolite, or zeolite, has a major impact on the accumulation of nitrogen in the soil, and also claim that this source of nitrogen is more accessible to the plant and easer to uptake. As a result, these authors emphasize the accumulation of large amounts of nitrogen, or protein, in the biomass, and particularly in the grain (Nur Gevrek et al., 2009; Yolcu et al., 2011).

The goal of our two-year research was to examine the effect of applying an organo-mineral fertilizer (a mixture of manure and zeolite) on the quantity and quality of the grassland yield. A hypothesis that the applied zeolite with manure would improve N use efficiency and the impact of the results on the practical use of zeolites in grassland systems are discussed.

MATERIALS AND METHODS

Grassland yield and quality in western Serbia were studied for two years and the comparative treatments included mineral nitrogen in spring or pure manure in autumn, pure zeolite, and control (no fertilization). The experiment involved the establishment of field plots (5 mx2 m) on a permanent pasture in 2012/13 with five treatments: a) pure zeolite (3 t ha^{-1}); b) pure manure (30 t ha^{-1}); c) manure + zeolite (30 t ha^{-1} +10 wt.% zeolite); d) spring nitrogen application (50 kg/ha N), and e) control treatment without the addition of fertilizers. The field trial was conducted on Planosol soil, in the vicinity of Šabac, Serbia (44°40'40"N 19°39'05"E, 123 asl.), with a randomized complete block design and four replications. The main source of zeolite, zeolitic tuff containing 70% of clinoptilolite from Zlatokop deposits, Vranjska Banja, Serbia, was added to the manure. Fresh cattle manure was prepared with clinoptilolite ground to grain size 0.063-0.1 mm, to homogeneous consistency, and fermented for three months prior to application. The plots were treated for two seasons with manure, zeolite, and their mix in autumn, while nitrogen was applied early in spring. The biomass was harvested at the developmental stage - inflorescence formation of main grass species, which was in May. Fresh biomass was weighed, herbage samples were air-dried and the dry matter (DM) was calculated.

Some of the quality parameters (ash, crude protein and ether extract) were determined using standard procedures (AOAC, 2005). Crude fiber was determined according to AOAC 978.10, NDF was tested following the procedures described by Van Soest and Robertson (1980), and the acid detergent fiber (ADF), hemicellulose (HCL) and lignin concentrations were assayed according to Van Soest et al. (1994). A two stage pepsin-cellulase method was used for *in vitro* DM digestibility (De Boevar et al., 1986). Total carbohydrates and non-fiber carbohydrates were calculated according to NRC (2001).

Some crude protein fractions (NPN, NDICP, ADICP, SolP, TP and IP) were assayed according to Licitra et al. (1996), where: NPN is the non-protein nitrogen (g/kg CP); SolCP is the soluble crude protein (g/kg CP); NDICP is the neutral detergent insoluble crude protein (g/kg CP); ADICP is the acid detergent insoluble crude protein (g/kg CP); TP is the true protein (g/kg CP); and IP is the insoluble crude protein (g/kg CP) and NPN_{SolCP} (gNPN/kg SolCP).

The soil was analyzed before the experiment was set up. The grassland in western Serbia is formed at an acidity of 5.07 in $CaCl_2$ and 5.73 in H_2O. The concentration of phosphorus was found to be 19.8 mg/kg, of potassium 115.1 mg/kg, and of total N 0.16%. The pasture yield and quality were assessed during two cuts in May. The data were analyzed using ANOVA with Statistica 10 (version 10) to identify significant effects of the fertilization treatments by variance analysis (ANOVA) and LSD test ($P \leq 0.05$).

Climate data showed differences between the growing seasons; the amount of precipitation was lower in the first than the second growing season (563 mm and 720 mm, respectively). Also, highest and lowest monthly temperatures in average were registered in different months: in August and December in the first growing season, and in July and November in the second growing season (Table 1).

RESULTS AND DISCUSSION

There were two cuts in each study year. A considerable increase in yield was noted with fertilization, particularly in the first year and especially in the first cut – double the control yield (Table 2). The effect of manure was somewhat prolonged for the second cut of the first year, whereas the zeolite treatment had an adverse effect on yield in dry summer conditions. In the second growing season, the prolonged effect of manure resulted in a higher yield than that of the control and pure zeolite treatment. The application of mineral nitrogen improved DM yields. Contrarily, the application of clinoptilolite resulted in the lowest yields, similar to the treatment without fertilization.

With regard to the overall yield of the natural grassland in two growing seasons, there was a considerable increase with the application of manure, the organo-mineral fertilizer and mineral nitrogen, compared to the control and pure clinoptilolite. Even though the organo-mineral fertilizer did not surpass the other two treatments, the results point to the possibility of applying manure with zeolite on grassland, as a substitute for costly and environmentally risky mineral nitrogen fertilizers.

There were significant differences between experimental years and cuts. The first cut provided a higher yield in both years, so its botanical composition was more important for assessing the treatments. Grassland in the second year of the experiment was changed by the removal of biomass in the first year, the prolonged effect of the 1st year treatment, and the application of 30 kg/ha of N. Also, the botanical composition of the grassland was affected by weather conditions.

All the treatments, even with pure zeolite, enhanced the contribution of grasses in both growing seasons (Fig. 1). The largest proportion was recorded in the second year and the organo-mineral treatment (72%), but that treatment had the smallest proportion of legumes in the first year

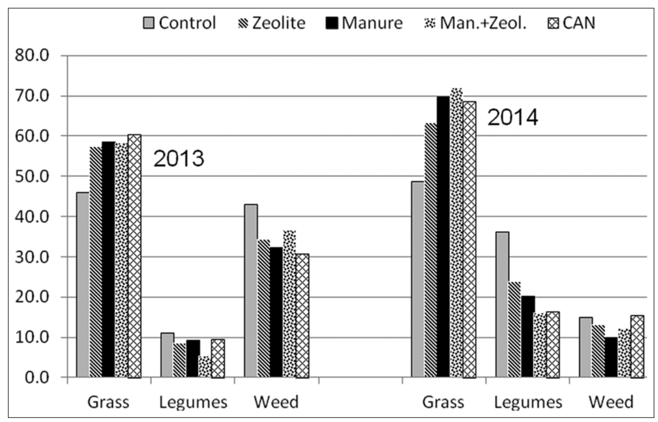

Fig 1. Botanical composition by coverage (%).

Table 1: Mean monthly temperatures (°C) and monthly precipitation during the growing seasons of 2012/13 and 2013/14

Year	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Total
Temperature													
2012/13	23.8	19.3	12.8	9.3	1.1	3.2	3.9	6.3	13.1	17.2	19.9	21.2	12.6
2013/14	15.9	13.7	8.4	1.7	4.2	6.8	9.5	13.3	16.1	19.8	21.9	21.2	12.7
Total precipitation													
2012/13	1.0	17.6	36.2	24.0	57.5	56.2	47.8	65.3	32.0	119	62.0	44.5	563
2013/14	61	71.6	34.1	5.8	51	17	47	76	188	38	75	56	720

Table 2: Dry matter yield (t ha^{-1})

Treatment	1st season 2012-2013			2nd season 2013-2014			2012/14
	1st cut	2nd cut	Total	1st cut	2nd cut	Total	Overall
Control	1.99^{c}	1.02^{ab}	3.01^{c}	1.24^{b}	0.79^{a}	2.03^{b}	5.04
Zeolite	2.38^{c}	0.91^{b}	3.29^{c}	1.31^{b}	0.73^{a}	2.04^{b}	5.33
Manure	4.53^{a}	1.38^{a}	5.91^{a}	2.09^{a}	0.67^{a}	2.76^{ab}	8.67
Manure+Zeolite	4.11^{ab}	0.97^{ab}	5.08^{ab}	1.62^{ab}	0.68^{a}	2.30^{ab}	7.38
CAN	3.31^{b}	0.96^{ab}	4.27^{b}	2.06^{a}	0.72^{a}	2.78^{a}	7.05
$LSD_{0.05}$	0.89	0.32	0.93	0.65	0.24	0.73	
$LSD_{0.01}$	1.24	0.45	1.30	0.91	0.33	1.02	

*Values denoted by the same letter are not significantly different according to Fisher's protected LSD values; $LSD_{0.05}$ – least significant difference at $P \leq 0.05$

(5.3%). This shows that manure with zeolite favors grasses because they are large nitrogen consumers, whereas it is of no significance for legumes which assimilate nitrogen through fixation.

The results of the two-year study show different yield responses in the two growing seasons, attributable to different weather conditions. There was more precipitation in the season of 2013/2014, which favored grasses and white clover, given their high soil moisture demand. The effect of weather conditions and cuts on the yields, depending on the treatment, was found to be predominant in this experiment. Also, the results corroborate previous reports on natural zeolite combined with manure (Glisic et al., 2009), used to rehabilitate soils with unfavorable chemical properties and increase yields.

The results do not show any significant differences in forage quality among the treatments, cuts or study years. Slight but significant differences were noted only in the proportions of ash and nitrogen free extracts (NFE) in the first cut of the second year, and ash in the second cut of the first year (Fig. 2). Efficient N fertilization of grasses generally enhances CP (Puoli et al., 1991), but there appears to be no evident pattern with regard to N fertilization and fiber fractions. Buxton and Fales (1994) reported that in humid years higher nitrogen amounts tended to increase NDF concentrations of the grasses, as a result of higher stem contribution in the leaf:stem ratio.

The fiber fraction analysis (Fig. 3) revealed different proportions of lignin, but these were not consistent because at times the higher proportions were noted with the mineral nitrogen treatment and at other times with the pure zeolite treatment. A higher proportion of grasses in the biomass resulted in high concentrations of structural carbohydrates – NDF and ADF, which was particularly apparent in the first season, with much less precipitation than in the second season. Statistically significant differences were noted only in lignin concentrations and NDF lignification in the first season, which were the highest in the first cut and the mineral nitrogen (CAN) treatment, relative to the second cut and zeolite, where

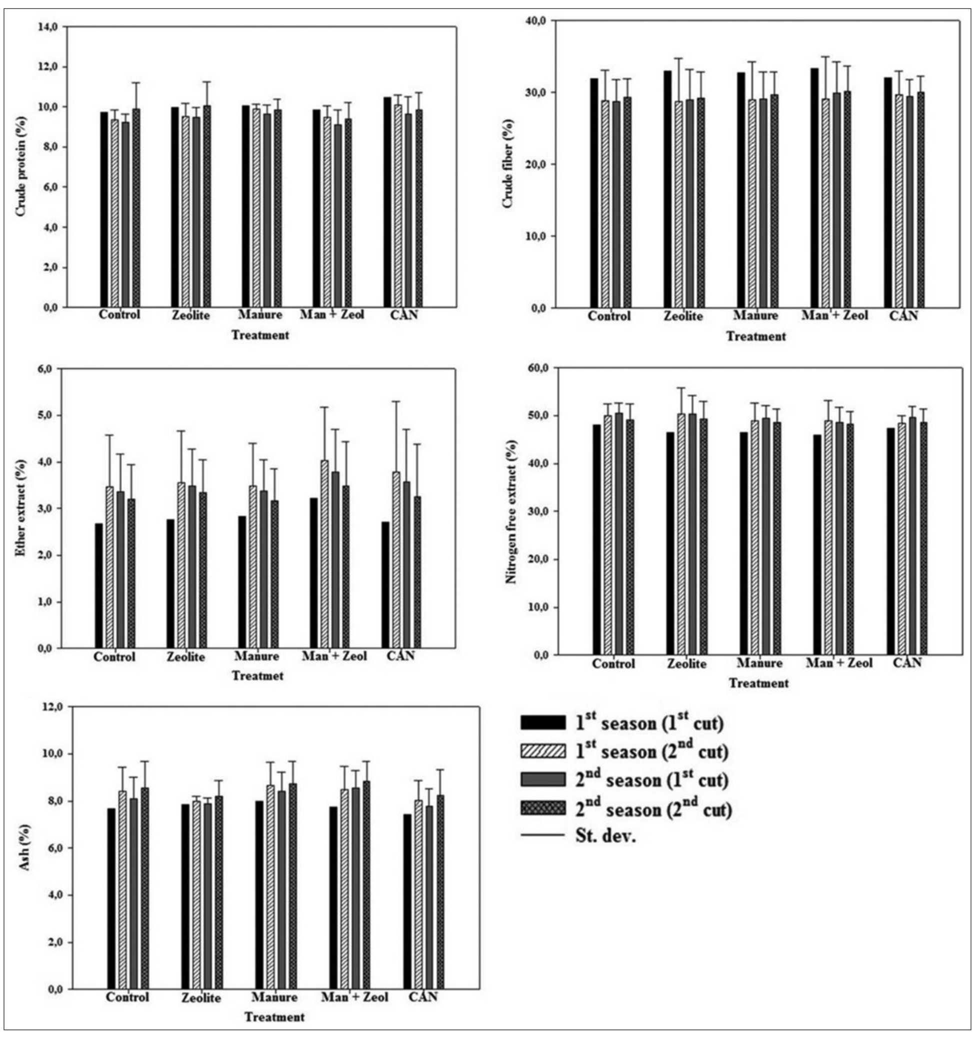

Fig 2. Chemical composition of biomass (% of DM).

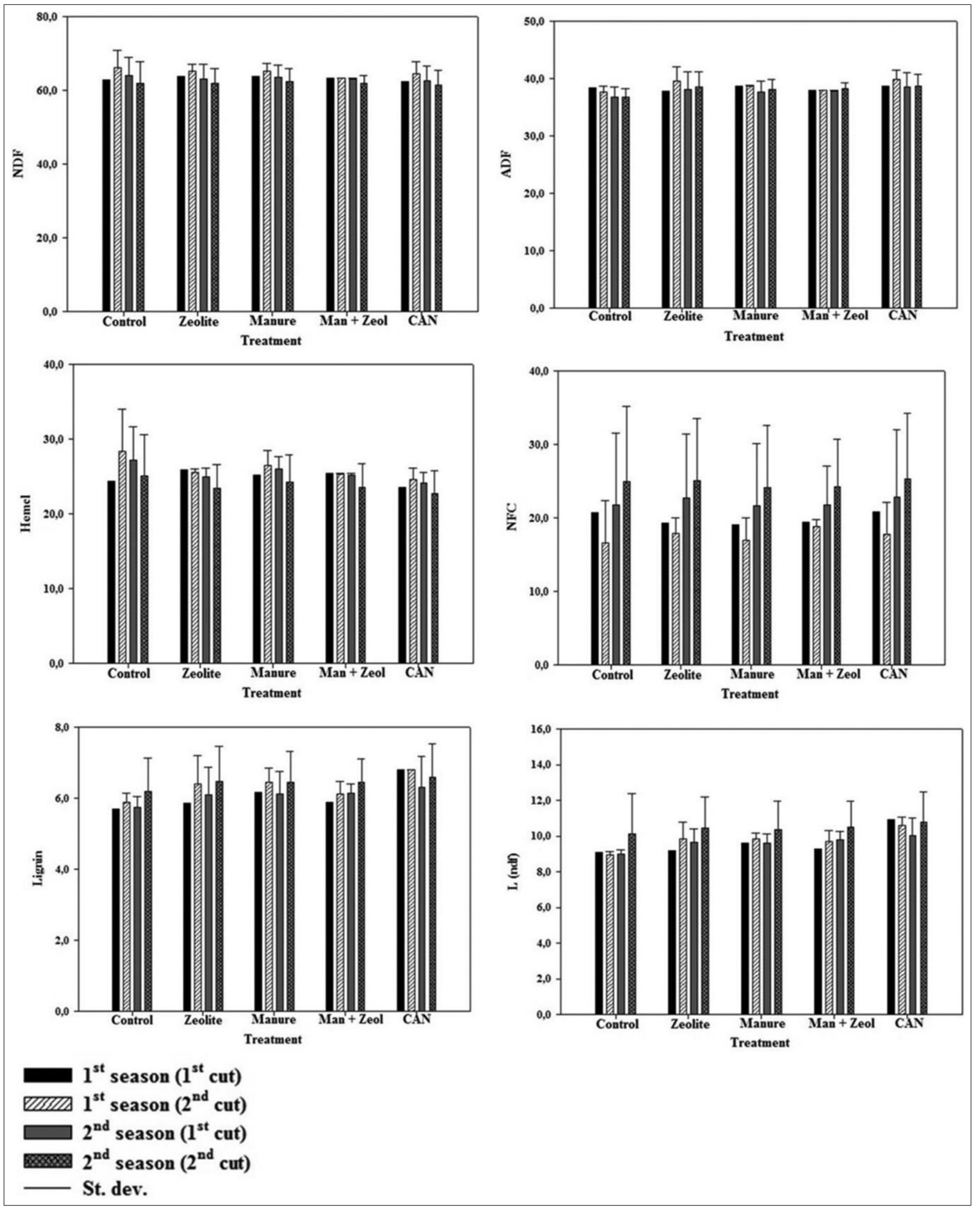

Fig 3. Fiber fractions and non-fiber carbohydrates.

weather conditions were probably one of the reasons. These circumstances also caused lower digestibility in the first season, compared to the second, which was specific due to protracted rainfall events. The concentrations of structural carbohydrates practically did not vary with different levels of the nitrogen fertilizer, although Valk et al. (1996) report an increase in NDF with less nitrogen fertilizer. These authors also state that the differences were more pronounced in the early stages of growth, and that NDF changes were insignificant 6-8 after harvest. Lignification exhibited a mild upward trend with decreasing levels of nitrogen fertilizer.

Along with a lower CP of the first cut in the second season, there was a higher proportion of non-fiber carbohydrates (NFC). In the first season, there was a high NFC concentration in the second cut and the manure + zeolite treatment, whereas in the second cut there were no NFC differences among the treatments or cuts. Valk et al. (1996) point out that an increase in NFC is attributable to a lower level of hydrocarbon chains usage for protein synthesis and the provision of enough energy to first reduce nitrates. These authors also found that NFC of grasses increased with decreasing amounts of nitrogen fertilizer.

The use of different nitrogen sources did not affect digestibility, but it should be noted that DM digestibility was higher in the second season. Consistent with the effect of nitrogen fertilizers on the proteins in grasses, poorly managed grasslands, fertilized with limited rates, decrease cell wall digestibility. A decrease in fiber digestibility by

0.06 units was reported by (Peyraud et al., 1997), while the CP content decreased from 160 to 110 g/kg, although fiber digestibility was stable when CP dropped from 210 to 170 g/kg (Van Vuuren et al., 1992). Some researchers reported a somewhat better cell wall degradability with reduced nitrogen treatment (Messman et al., 1991, Valk et al., 1996). This was attributable to a lower CP, accompanied by higher concentrations of readily utilizable carbohydrates, which are easy to digest.

For this reason, the protein fraction analysis (Fig. 4) clearly indicated consistency in the first season, with a significant difference in the proportion of true protein in the organo-mineral treatment, compared to the smaller proportion in the manure treatment and both cuts of the first year of study, when the effect of manure was more pronounced. In the second season there was a significant difference between the mineral nitrogen treatment and the much smaller proportion of true protein in the zeolite treatment.

Based on the increase in true protein (TP), one can hypothesize that zeolite plays the role of transmitter of important nitrogen compounds from the manure, which become incorporated into TP, and that pure manure loses those compounds through evaporation or leaching during the manure fermentation process. The increase in TP of two cuts after treatment with the organo-mineral fertilizer is not primarily indicative of the potential for increasing grassland yields with the application of this type of fertilizer, but of the improvement in grassland quality and as an alternative and environmentally more valuable fertilizer than manure, produced in a similar manner, with

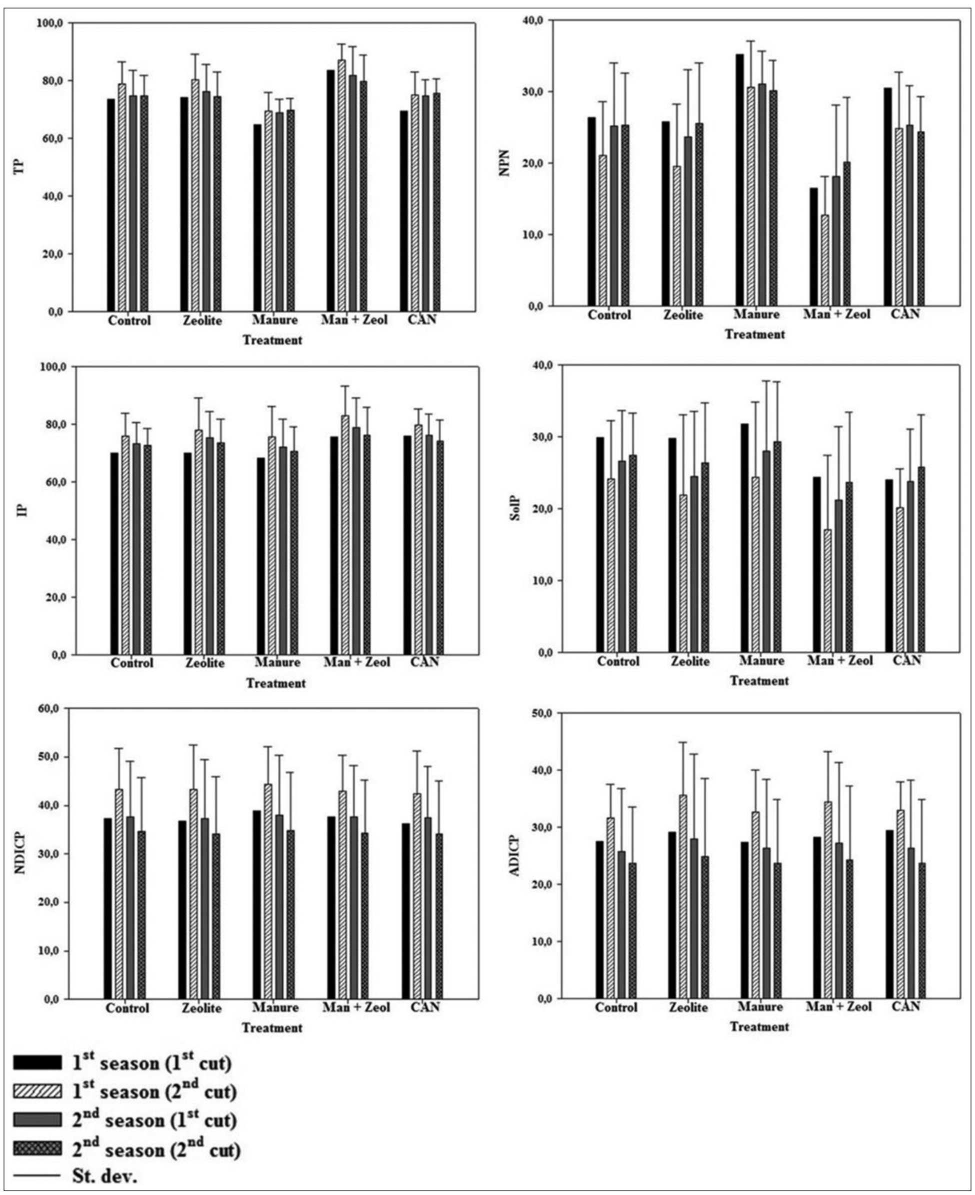

Fig 4. Crude protein fractions in herbage from natural grassland.

the addition of clinoptilolite.

Fertilization with nitrogen had a direct effect on the proportion of TP in the plants. The percentage of protein nitrogen in grasses, relative to total nitrogen, increased from 75 to 90% with the application of the nitrogen fertilizer decreasing from 400 kg/ha to the control level (0 kg/ha), annually (Peyraud and Astigarraga, 1998). Nitrogen uptake rapidly increases with increasing amounts of nitrogen fertilizer, which leads to the accumulation of non-protein nitrogen compounds and a decrease in the proportion of protein nitrogen (i.e. TP). When larger quantities of nitrogen fertilizers (in excess of 400 kg/ha annually) are applied, and because of the higher rate of nitrogen uptake, nitrates are stored and they further reduce the accumulation of protein-based nitrogen – TP.

The conversion of nitrates into ammonia compounds, which could be incorporated into proteins, is somewhat limited due to insufficient activity of nitrate reductase. This enzyme can limit high levels of nitrogen fertilizers, which invariably leads to nitrate nitrogen storage in plants (Gillet, 1980). Gillet reports that the amount of nitrate nitrogen can have DM values of 6-8 g/kg, which is half the lethal dose for ruminants, but that the concentration of nitrogen compounds decreases rapidly after three weeks.

The amounts of nitrogen associated with fiber fractions of carbohydrates did not differ considerably among the treatments, but higher values were noted in the first season than in the second. The proportion of nitrogen associated with the NDF fraction decreases with decreasing application of nitrogen fertilizer, but this decrease can be expressed relative to the decreasing content of total nitrogen. This practically means that the proportion of N not associated with NDF increases with decreasing amounts of applied N fertilizers (Peyraud and Astigarraga, 1998).

Kolver et al. (1998) point out that the CNCPS method is useful for predicting metabolic energy requirements, and the production of milk by grazing cows, where *in vitro* DM digestibility ranges from 65 to 80%. These authors state that the optimal proportions are 460 g/kg, of NDF, 60 g/kg of lignin and 234 g/kg of raw protein, while the content of soluble protein should be 272 g/kg of CP. Lower protein solubility means greater protein availability for microbial growth and, consequently, better fiber degradation (Rueda et al., 2003). Lower lignin concentrations improve milk productivity, mainly because a larger proportion of NDF is digestible.

In addition, higher lignin concentrations, expressed as the proportion of lignin in NDF, are indicative of lower digestibility and faster fermentation of the non-lignified fraction of the forage (Van Soest, 1994). A smaller proportion of insoluble fiber or faster digestion of this forage fraction would contribute to higher yields of microbial protein and amino acids, which are necessary for milk synthesis (Rueda et al., 2003).

CONCLUSIONS

Cattle manure enriched with clinoptilolite can be used as a fertilizer for grasslands, as a slow-releasing and valuable nitrogen source. Grassland fertilization using manure with zeolite can reduce the application of mineral N fertilizers on natural grasslands. The DM yield in the first growing season, after the application of manure and the organo-mineral fertilizer, was the highest and exceeded 5 t ha^{-1} in two cuts. The next was the mineral nitrogen treatment. The control and pure zeolite treatment produced much lower yields. The fertilizer treatments also resulted in the highest yields in the second year, but there were no major differences because of abundant rainfall and reduced manure performance. The fertilizer treatments had a favorable effect on the proportion of grasses, and an adverse impact on the proportion of legumes in the grassland. The largest contribution of zeolite was reflected in a considerably larger proportion of protein in both cuts of the first growing season (83.5 vs. –64.8 and 91.0 vs. 73.9 mg/kg), compared to the manure treatment. Nonprotein nitrogen (of which there was more in pure manure) had the opposite effect, indicative of the role of zeolite in the retention and transport of important nitrogen compounds from the manure for the synthesis of true protein.

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Author's contributions

All the listed authors had participated in the manuscript. Aleksandar Smić, Zorica Bijelić and Violeta Mandić took part in establishing field experiment and collecting the samples. They also wrote the draft of the manuscript. Jordan Marković did part of the lab experiment and helped in the interpretation of data. Bojan Stojanović and Željko Dželetović also helped in interpretation of data and proof reading of the final copy. Savo Vučković helped in designing of study and revising of the manuscript.

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