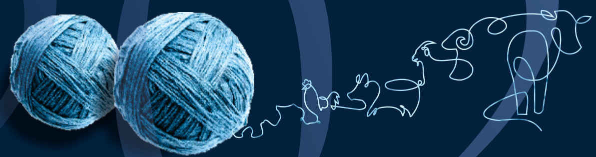
14th INTERNATIONAL SYMPOSIUM

MODERN TRENDS IN LIVESTOCK PRODUCTION

2023



PROCEEDINGS

4 - 6 October 2023, Belgrade, Serbia

Institute for Animal Husbandry
Belgrade - Zemun, SERBIA

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EFFECT OF CONDENSED TANNINS CONCENTRATIONS ON PROTEIN DEGRADABILITY OF RED CLOVER, ITALIAN RYEGRASS AND THEIR MIXTURES

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Invited paper

Abstract: Growing grasses in a mixture with legumes leads to more profitable production, better quality of forages, an increase in soil biogenicity and fixation of a significant amount of nitrogen. The experiment was established as a two factorial trial by the method of randomized complete block design in three replications. Italian ryegrass – monocrop (IR), red clover – monocrop (RC) and their mixtures – IR:RC (15:5 kg ha^{-1}), IR:RC (15:10 kg ha^{-1}), IR:RC (20:5 kg ha^{-1}) and IR:RC (20:10 kg ha^{-1}) were planted in November 2016, with the first cutting in the spring 2017 – on May the 9th, and the second cutting in the early summer – on June the 22nd. The aim of this study was to investigate the concentrations of phenolic compounds such as condensed tannins in Italian ryegrass, red clover and their mixtures, as well as their concentrations impact on protein degradability in the rumen depends on the seeding rate in the mixtures and harvested in the spring and early summer. Results obtained in this study showed that higher concentrations of non-protein nitrogen and soluble protein in forages harvested in the spring influenced higher rapidly degradable protein concentration in investigated mixtures. The highest rumen undegradable protein was determined in Italian ryegrass monocrop harvested in early summer, and we assume that high condensed tannins concentration influenced the slower protein degradability. Our recommendation for plant breeders is that cultivars with higher content of condensed tannins should be created and introduced in animal nutrition.

Key words: protein degradability, condensed tannins, red clover-Italian ryegrass mixtures

Introduction

Red clover is the basic source of protein for ruminants, and it is one of the main constituents of the diet of these animals. *Marshall et al. (2017)* found out that some legumes such as alfalfa, red clover and white clover are important species for sustainable livestock production systems, because in mixture with grasses, they can fix, on average 150 kg N ha^{-1} yr^{-1}, and some of that N subsequently becomes available to the companion grass. Grass-legume mixtures are acknowledged to have higher crude protein concentrations, as well as lower fiber concentrations compared to pure grass stands *(Ball et al., 2001). Brown et al. (2018)* observed that in grass-legume mixtures crude protein content increased, as well as dry matter yield. In the results of the investigation reported by *Albayrak and Ekiz (2005)* the authors indicated that planting legumes with grasses improves forage nutritive value and increases the dry matter yield of pastures compared to pastures with pure grasses.

Italian ryegrass is a forage crop that represents excellent forage quality. In animal nutrition, it can be used fresh, as green forage, for grazing, for preparing hay, and for preparing silage and haylage. According to *Simić et al. (2011)* it is an ideal species for growing in a mixture with red clover. It seems possible that some of the positive effects of Italian ryegrass on ruminant performance may be due not only to high digestibility and high carbohydrate concentrations but also to benefits provided by molecules such as phenolic compound. However, relatively little is known about the soluble phenolic compound composition of Italian ryegrass, making it difficult to determine which compounds might positively influence animal performance. Among the numerous benefits of ruminant nutrition, it is important to mention that some forage species may contain compounds that can reduce protein degradability. Condensed tannins prevent bloat by binding to and precipitating proteins, which reduces protein concentration in the rumen and increases rumen bypass or undegradable protein *(Barry and Manley, 1986)*. The condensed tannins expressed by many forages can bind to salivary proteins, reducing palatability and intake, and they can also form indigestible complexes with rumen microbes and cell wall carbohydrates, reducing the rate of rumen digestion and intake *(Reed, 1995)*.

There is an increased interest in studying those leguminous species that reduce proteolytic processes and reduce protein degradation in the rumen. The aim of this study was to investigate the concentrations of phenolic compounds such as condensed tannins in Italian ryegrass, red clover and their mixtures, as well as their concentration's impact on protein degradability in the rumen depends on the seeding rate in the mixtures and harvested in the spring and early summer.

Materials and Methods

The study was carried out at the experimental field of the Institute for forage crops Kruševac, Serbia. The experiment was established as a two factorial trial by the method of randomized complete block design in three replications. The study area was situated at an altitude of 166 m above sea level in Central Serbia. The mean annual temperature and the total precipitation for the region are 12.6º C and 653.2 mm, respectively. Italian ryegrass – monocrop (IR), red clover – monocrop (RC) and their mixtures – IR:RC (15:5 kg ha^{-1}), IR:RC (15:10 kg ha^{-1}), IR:RC (20:5 kg ha^{-1}) and IR:RC (20:10 kg ha^{-1}) were planted in November 2016, with the first cutting in the spring 2017 – on May the 9th, and the second cutting in the early summer – on June the 22nd. Plants were harvested in full flowering stage.

The dry matter content was determined on a sample weighing 1 kg, by drying at a temperature of 60° C to a constant weight. The samples were ground on a mill with a sieve of 2 mm, and then on a laboratory cyclone mill with a diameter of 1 mm. The samples obtained in this way were dried at 105° C to a constant weight. All results related to chemical composition and amount of nutrients are expressed in absolute dry matter. All chemical analyzes were performed in duplicate.

The CP (Crude Protein) of the samples was determined by Kjeldahl method. The NPN (Non-Protein Nitrogen), NDICP (Neutral detergent Insoluble Crude Protein), ADICP (Acid Detergent Insoluble Crude Protein), SolP (Soluble Protein), TP (True Protein) and IP (Insoluble Protein) were determined by *Licitra et al. (1996)*. The CNCPS (Cornell Net Carbohydrate and Protein System) crude protein fractions of the samples, PA, PB_1, PB_2, PB_3 and PC were calculated based on CP, NPN, SolCP, NDICP, ADICP contents of samples according to *Fox et al. (2004)*: PA = NPN; PB_1 = SolCP – NPN; PB_2 = CP – SolCP – NDICP; PB_3 = NDICP – ADICP; PC = ADICP. Where PA refers to the non-protein nitrogen (g kg^{-1} DM); PB_1 the rapidly degraded crude protein (g kg^{-1} DM); PB_2 the intermediately degraded crude protein (g kg^{-1} DM); PB_3 slowly degraded crude protein (g kg^{-1} DM) and PC the bound crude protein (g kg^{-1} DM).

The CNCPS (Cornell Net Carbohydrate and protein System) 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. Rumen-degradable CP (RDP) was calculated based on CNCPS subfractions using fractional rate of degradation (Kd) values given for legume pasture (*Grabber, 2009*). Rumen degradable protein (RDP) was calculated as follows:

$$RDP = \sum CP \text{ sub-fractions} \times Kd / (Kd + Kp)$$

where Kp is the fractional rate of passage which is assumed to be 0.045 h^{-1}. Fractional degradation rates of CP sub-fractions adapted from legume pasture values reported in the CNCPS v_6.1 feed library (www.cncps.cornell.edu) are as follows: Kd (PA) = 2.00 h^{-1}; Kd (PB_1) = 0.20 h^{-1}; Kd (PB_2) = 0.15 h^{-1}; PB_3 = 0.08 h^{-1}. Rumen-undegradable CP (RUP) was calculated by subtracting RDP from total CP.

The sampled plant material was prepared for the drying process in laboratory conditions for phenolic compound determination. The plant material was dried in a thin layer in a drafty and dark place where the temperature ranged from 18-22° C, and the air humidity ranged from 55-65%. The drying process was regularly controlled, where damaged specimens and those that had changed colour were removed. After a 7-day drying process, the dry plant material was crushed and packed in dark glass containers until the extraction process.

About 2.0000 g of dried homogenized plant material was weighed and 20 ml of methanol:water:HCl (80:19:1) extraction solution was added. After standing for 2 hours at room temperature, the solution was decanted, and the solid residue was extracted 2 more times in the manner already described (20 ml and 10 ml of the extraction solution). The filtrate obtained by squeezing the collected extracts through a Buchner funnel was transferred to a normal 50 ml vessel, and diluted with a solvent to the line. The obtained extract was stored in a dark and cold place.

The determination of total polyphenols (TPP) of sample extracts was performed with the Folin-Ciocalteu reagent according to the method of *Singleton and Rossi (1965)* with some modifications. The absorbance is measured at 765 nm by UV-Vis spectrophotometer (HALO RB-10, Dynamica). The results are expressed in g gallic acid equivalent / 100 g plant dry matter (gE GA / 100 g DM) by reference to the calibration curve of gallic acid. The total flavonoids (TF) determination of sample extracts was performed according to the method described by *Jia et al. (1999)* with some modifications. The absorbance is measured at 415 nm using a UV-Vis spectrophotometer (HALO RB-10, Dynamica). The results are expressed in g quercetin equivalent / 100 g plant dry matter (gE Qu / 100 g DM) with reference to the quercetin calibration curve. The condensed tannins (CT) content of sample extracts were determined using the vanillin assay described by *Makkar and Becker (1993)* with some modifications. The absorbance at 550 nm was measured against a blank using a UV-Vis spectrophotometer (HALO RB-10, Dynamica). The total concentration of condensed tannins was expressed in mg of catechin equivalents / 100 g plant dry matter (mgE C / 100 g DM) with reference to the catechin calibration curve.

The experimental data were analyzed by a two-way analysis of variance for samples using a model that accounted for the main effects of Italian ryegrass – red clover mixtures and cut. Effects were considered significant at $p < 0.05$ level.

The significance of differences between arithmetic means was tested by Tuckey test (STATISTICA 6, Stat. Soft. 2006).

Results and Discussion

Crude protein concentration and primarily protein fractions of red clover, Italian ryegrass and their mixtures harvested in the spring and early summer are presented in Table 1. Crude protein concentration was different between harvest seasons and was lower in the spring (mean value was 146.9 g kg^{-1} DM) and greater in the early summer (155.7 g kg^{-1} DM). Red clover and Italian ryegrass monocrops differed significantly ($p < 0.05$) in CP concentration, whereas similar concentrations between the mixtures were observed with an average of 156.8 to 170.2 g kg^{-1} DM. Red clover monocrop was characterized by the highest CP concentration, followed by the IR:RC (20:5) mixture.

Statistical analysis showed that harvest date and seeding rate of IR and RC in mixtures significantly affected concentrations of primarily protein fractions: TP, NPN, IP, SolP, NDICP and ADICP. Significantly greater NDICP, ADICP, ICP and TP concentrations were recorded in the mixtures harvested in early summer (Table 1), whereas only SolP and NPN concentrations were higher in IR:RC mixtures harvested in the spring than harvested in the early summer (treatments differed by 13.7 and 26%, respectively). The lowest NDICP concentration was recorded in IR:RC (15:5) mixture, whereas the mean values of this protein fraction recorded in other three IR:RC mixtures were similar, and ranged from 20.03 to 21.80 g kg^{-1} DM. On the other hand, ADICP concentration was the highest in IR:RC (20:10) mixture, but ADICP concentrations were similar in the other three mixtures, ranging from 14.73 to 15.15 g kg^{-1} DM and did not differ significantly. The lowest ICP and NPN concentrations were recorded in 20:10 IR:RC mixture, and the mean values were 78.65 and 56.88 g kg^{-1} DM, respectively. On the other hand, this mixture was the highest in SolP and TP (mean values were 85.75 and 102.5 g kg^{-1} DM, respectively). Higher CP concentration in RC monocrop influenced higher ($p < 0.05$) protein fractions content in RC monocrop than in IR monocrop, except NDICP concentration. The concentration of this protein fraction was similar in IR and RC monocrop, and did not differ significantly.

Table 1. Crude protein content and primarily protein fractions of red clover, Italian ryegrass and their mixtures harvested in the spring and early summer

Parameter	Cut	IR	RC	IR:RC 15:5	IR:RC 15:10	IR:RC 20:5	IR:RC 20:10	Mean
CP, g kg^{-1} DM	I	76.36^{e}	179.5^{a}	163.6^{c}	149.4^{d}	170.8^{bc}	141.8^{d}	**146.9^{B}**
	II	81.80^{e}	180.3^{a}	149.9^{d}	175.5^{b}	169.5^{bc}	177.1^{b}	**155.7^{A}**
	Mean	**79.08^{D}**	**179.9^{A}**	**156.8^{C}**	**162.5^{C}**	**170.2^{B}**	**159.4^{C}**	
NDICP, g kg^{-1} DM	I	12.20^{g}	16.00^{ef}	19.80^{bcd}	21.76^{abc}	15.90^{ef}	18.93^{cde}	**17.43^{B}**
	II	16.96^{de}	13.23^{fg}	16.06^{ef}	21.83^{abc}	24.16^{a}	22.76^{ab}	**19.17^{A}**
	Mean	**14.58^{C}**	**14.61^{C}**	**17.93^{B}**	**21.80^{A}**	**20.03^{AB}**	**20.85^{A}**	
ADICP, g kg^{-1} DM	I	9.40^{e}	16.03^{bc}	13.23^{d}	13.63^{cd}	13.20^{d}	18.90^{a}	**14.06^{B}**
	II	12.26^{d}	16.16^{b}	16.23^{b}	16.60^{ab}	17.10^{ab}	16.16^{b}	**15.75^{A}**
	Mean	**10.83^{C}**	**16.10^{AB}**	**14.73^{B}**	**15.11^{B}**	**15.15^{B}**	**17.53^{A}**	
ICP, g kg^{-1} DM	I	30.13^{h}	83.86^{cde}	76.70^{ef}	74.90^{f}	79.56^{def}	70.80^{f}	**69.32^{B}**
	II	56.46^{g}	106.4^{a}	89.23^{bc}	95.90^{b}	90.23^{bc}	86.50^{cd}	**87.45^{A}**
	Mean	**43.30^{D}**	**95.13^{A}**	**82.96^{BC}**	**85.40^{B}**	**84.90^{B}**	**78.65^{C}**	
SolCP, g kg^{-1} DM	I	46.20^{e}	95.66^{a}	86.86^{ab}	74.53^{c}	91.30^{a}	70.93^{cd}	**77.58^{A}**
	II	25.33^{f}	73.90^{c}	60.66^{d}	79.66^{bc}	79.30^{bc}	90.56^{ab}	**68.23^{B}**
	Mean	**35.76^{C}**	**84.78^{A}**	**73.76^{B}**	**77.10^{B}**	**85.30^{A}**	**85.75^{AB}**	
TP, g kg^{-1} DM	I	32.10^{g}	92.80^{d}	85.83^{e}	82.73^{e}	81.70^{e}	91.70^{d}	**77.81^{B}**
	II	55.30^{f}	111.4^{b}	103.6^{c}	119.3^{a}	102.1^{c}	113.3^{b}	**100.8^{A}**
	Mean	**43.70^{C}**	**102.1^{A}**	**94.73^{B}**	**101.0^{A}**	**91.91^{B}**	**102.5^{A}**	
NPN, g kg^{-1} DM	I	44.23^{g}	86.73^{ab}	77.76^{bc}	66.66^{d}	89.20^{a}	50.03^{fg}	**69.10^{A}**
	II	26.50^{h}	68.93^{cd}	46.26^{g}	56.23^{ef}	67.40^{d}	63.73^{de}	**54.84^{B}**
	Mean	**35.36^{C}**	**77.83^{A}**	**62.01^{B}**	**61.45^{B}**	**78.30^{A}**	**56.88^{B}**	

CP – crude protein; NDICP – neutral detergent insoluble crude protein; ADICP – acid detergent insoluble crude protein; ICP – insoluble crude protein; SolCP – soluble crude protein; TP – true protein; NPN – non-protein nitrogen, DM – dry matter; I – plants harvested in the spring 2017 – on May the 9th; II – plants harvested in the early summer – on June the 22nd; IR – Italian Ryegrass; RC – Red Clover; Different letters denote significance different means ($p< 0.05$).

The association of protein solubility with degradability in the rumen and corresponding ruminant performances has been observed by some authors *(Grubić et al., 1996; Grubić and Adamović, 2003; Grubić et al., 2003)*. Solubility of proteins is one of the main factors that determines the protein degradability in the rumen. At the same time, the solubility of proteins indicates their accessibility to the action of proteolytic enzymes. Soluble crude proteins are usually degradable very fast in the rumen, but some part of this fraction can have a significant amount of crude protein whose degradability proceeds at a slower rate.

In addition to the fact that legumes are characterized by high nutritive value, their proteins are subject to rapid degradation in the rumen. Consequently, the rate of protein degradation in the rumen directly affects the efficiency of nitrogen utilization by the animal *(Broderick, 1995)*. *Kingston-Smith et al. (2003)* indicated that both rumen microorganisms and proteases in plant material cause inefficient utilization of nitrogen in animal organisms. Generally, the non-utilization of nitrogen is the result of an imbalance between protein and carbohydrates in the diet, which increases the risk of nitrogen loss and its excretion into the environment *(Tamminga, 1996)*. In order to avoid these losses and environmental contamination, a diet must be formulated to satisfy, but not to exceed the amount of nitrogen that is necessary for the growth of microorganisms, as well as a sufficient amount of amino acids that are necessary for the normal function of the animal organism *(Schwab et al., 2005)*. Modern feeding systems, in order to minimize nitrogen losses imply knowledge of the amount and ratio of degradable and undegradable protein in the rumen, as well as the necessary amounts of nitrogen for sufficient development of microorganisms *(Lanzas et al, 2007; 2008)*.

Protein fractions calculated by the CNCPS system of analysis, RDP, RUP and DMD are presented in Table 2. These protein fractions are mainly affected by the content of primarily protein fractions in the DM of IR, RC monocrop and their mixtures. Results obtained in this study showed that the highest protein fractions were PA and PB_2. The higher content of PA fraction was estimated in the DM of forages harvested in the spring than in the DM of forages harvested in early summer by 36.55% ($p< 0.05$). Concentrations of PB_3 fraction were similar and did not differ between harvest dates (26.21 g kg^{-1} CP in the DM of forages harvested in the spring *vs* 29.60 g kg^{-1} CP in the DM of forages harvested in early summer). Significantly higher concentrations of PB_1, PB_2 and PC were estimated in the DM of forages harvested in early summer than those harvested in spring. The highest value of PB_3 and PC protein fraction was determined in IR monocrop, and influenced the highest RUP concentration. On the other hand, IR:RC 20:5 mixture was characterized by the highest PA and PB_1 protein fractions influenced by the highest RDP concentration (Table 2).

The protein value of feeds for ruminants is based on an estimation of the quantity of dietary and microbial protein absorbed in the small intestine. Dietary nitrogen that escapes degradation in the rumen is, therefore, an important factor in determining the protein value *(Aufreŕe et al., 2002)*. PA fraction served as the main indicator for proteolysis. Usually, extensive protein hydrolysis after harvest and during fermentation is characteristic of forage legumes preserved as silage. Differences in proteolysis among IR:RC mixtures have been observed, but plant characteristics associated with these differences have not been well defined *(Albrecht and Muck, 1991)*. *Coblentz et al. (1998)* suggested that the undegradable fraction of CP was considerably larger in some plants due to the heavy lignification of the stem tissue in plants, especially in the summer.

We noted significant differences ($p< 0.05$) in DMD between the different harvest times of IR and RC monocrops and their mixture, as well as significant differences between IR and RC and their mixtures. DMD was higher in the spring than in early summer. The highest DMD was observed in RC monocrop, higher by 31% than DMD of IR monocrop. DMD in investigated IR-RC mixtures ranged from 657.8 to 686.1 g kg^{-1} DM, and the highest DMD was noted in 15:5 IR:RC mixture, but the lowest DMD noted in 20:10 IR:RC mixture.

Table 2. Protein fractions concentration determined by CNCPS system of analysis or red clover, Italian ryegrass and their mixtures harvested in the spring and early summer

Parameter	Cut	IR	RC	IR:RC 15:5	IR:RC 15:10	IR:RC 20:5	IR:RC 20:10	Mean
PA, g kg^{-1} CP	I	579.3^{a}	482.4^{c}	475.4^{c}	446.3^{c}	521.8^{b}	352.7^{efg}	**476.3^{A}**
	II	323.7^{fgh}	382.3^{de}	308.7^{h}	320.0^{gh}	397.7^{d}	359.9^{ef}	**348.8^{B}**
	Mean	**451.5^{AB}**	**432.4^{B}**	**392.0^{C}**	**383.3^{C}**	**459.8^{A}**	**356.4^{D}**	
PB_1, g kg^{-1} CP	I	26.26^{de}	50.06^{cde}	55.73^{cde}	52.46^{cde}	12.43^{e}	147.4^{a}	**57.38^{B}**
	II	27.20^{de}	27.63^{de}	95.96^{bc}	133.7^{ab}	69.90^{cd}	151.4^{a}	**84.29^{A}**
	Mean	**26.73^{C}**	**38.85^{C}**	**75.85^{B}**	**93.08^{B}**	**41.16^{C}**	**149.4^{A}**	
PB_2, g kg^{-1} CP	I	234.9^{e}	371.2^{cd}	348.1^{d}	355.5^{d}	372.7^{cd}	366.3^{d}	**341.5^{B}**
	II	448.4^{ab}	483.8^{a}	485.9^{a}	421.6^{bc}	389.7^{cd}	359.9^{d}	**431.6^{A}**
	Mean	**341.7^{D}**	**427.5^{A}**	**417.0^{AB}**	**388.6^{BC}**	**381.2^{C}**	**363.1^{CD}**	
PB_3, g kg^{-1} CP	I	36.30^{ab}	10.56^{cd}	39.90^{ab}	54.53^{a}	15.73^{cd}	0.23^{d}	**26.21^{NS}**
	II	50.93^{a}	16.46^{cd}	1.10^{d}	29.80^{bc}	41.90^{ab}	37.43^{ab}	**29.60^{NS}**
	Mean	**43.61^{A}**	**13.51^{C}**	**20.50^{BC}**	**42.16^{A}**	**28.81^{B}**	**18.83^{BC}**	
PC, g kg^{-1} CP	I	123.2^{bc}	89.36^{efg}	80.90^{fg}	91.20^{efg}	77.30^{g}	133.0^{b}	**99.16^{B}**
	II	149.8^{a}	89.80^{efg}	108.3^{cd}	94.63^{def}	100.8^{de}	91.33^{efg}	**105.8^{A}**
	Mean	**136.5^{A}**	**89.58^{C}**	**94.6^{C}**	**92.9^{C}**	**89.1^{C}**	**112.2^{B}**	
RDP, g kg^{-1} CP	I	791.9^{bcd}	804.9^{ab}	803.6^{abc}	787.6^{cde}	817.2^{a}	747.3^{h}	**792.1^{A}**
	II	716.2^{i}	778.9^{def}	754.6^{gh}	765.6^{fg}	772.5^{ef}	776.3^{def}	**760.7^{B}**
	Mean	**754.0^{C}**	**791.9^{A}**	**779.1^{B}**	**776.6^{B}**	**794.8^{A}**	**761.8^{C}**	
RUP, g kg^{-1} CP	I	208.1^{fgh}	195.1^{hi}	196.4^{ghi}	212.4^{efg}	182.8^{j}	252.7^{b}	**207.9^{b}**
	II	283.8^{a}	221.0^{def}	245.4^{bc}	234.4^{cd}	227.5^{de}	223.7^{def}	**239.3^{A}**
	Mean	**245.9^{A}**	**208.1^{C}**	**220.9^{B}**	**223.4^{B}**	**205.2^{C}**	**238.2^{A}**	
DMD, g kg^{-1} DM	I	628.6^{e}	722.1^{a}	692.9^{b}	680.3^{bc}	693.8^{b}	665.3^{cd}	**680.5^{A}**
	II	452.0^{f}	694.2^{b}	679.2^{bc}	653.5^{d}	655.3^{d}	650.2^{d}	**630.7^{B}**
	Mean	**540.3^{E}**	**708.1^{A}**	**686.1^{B}**	**666.9^{CD}**	**674.5^{BC}**	**657.8^{D}**	

PA – instantaneously solubilised protein; PB_1 – rapidly degradable protein; PB_2 – intermediately degradable protein; PB_3 – slowly degradable protein; PC – completely undegradable protein; RDP – Rumen Degradable Protein; RUP – Rumen Undegradable Protein; DMD – Dry Matter Digestibility; I – plants harvested in the spring 2017 – on May the 9th; II – plants harvested in the early summer – on June the 22nd; IR – Italian Ryegrass; RC – Red Clover; Different letters denote significance different means ($p< 0.05$).

Concentrations of total polyphenols, total flavonoids and condensed tannins of IR, RC and their mixtures harvested in spring and early summer are presented in Table 3. Mean values for the concentrations of TPP, TF and CT were significantly higher in samples harvested in spring than in early summer ($p< 0.05$).

Table 3. Concentration of Total Polyphenols, Total Flavonoids and Condensed Tannins in red clover, Italian ryegrass and their mixtures harvested in the spring and early summer

Parameter	Cut	IR	RC	IR:RC 15:5	IR:RC 15:10	IR:RC 20:5	IR:RC 20:10	Mean
TPP, gE GA / 100 g DM	I	10.00[de]	13.01[b]	11.29[c]	13.49[b]	16.20[a]	10.64[cd]	**12.44[A]**
	II	12.50[b]	12.71[b]	9.80[de]	9.22[ef]	10.02[de]	8.48[f]	**10.44[B]**
	Mean	**11.25[B]**	**12.86[A]**	**10.54[C]**	**11.35[B]**	**13.11[A]**	**9.56[D]**	
TF, gE Qu / 100 g DM	I	7.21[d]	10.45[b]	7.29[d]	8.79[c]	14.47[a]	7.30[d]	**9.26[A]**
	II	7.42[d]	8.90[c]	6.93[d]	6.67[de]	8.80[c]	5.69[e]	**7.42[B]**
	Mean	**7.36[C]**	**9.67[B]**	**7.11[CD]**	**7.73[C]**	**11.67[A]**	**6.49[D]**	
CT, mgE C / 100 g DM	I	463.9[c]	490.1[c]	391.6[d]	548.4[b]	542.9[b]	476.8[c]	**485.8[A]**
	II	955.2[a]	406.5[d]	381.0[d]	327.9[e]	382.9[d]	260.5[f]	**452.5[B]**
	Mean	**710.6[A]**	**448.3[BC]**	**386.3[D]**	**438.1[C]**	**462.9[B]**	**368.6[E]**	

TPP – Total Polyphenols; TF – Total Flavonoids, CT – Condensed Tannins; I – plants harvested in the spring 2017 – on May the 9th; II – plants harvested in the early summer – on June the 22nd; IR – Italian Ryegrass; RC – Red Clover; gE GA / 100 g DM - g gallic acid equivalent / 100 g plant dry matter; gE Qu / 100 g DM - g quercetin equivalent / 100 g plant dry matter; mgE C / 100 g DM - mg of catechin equivalents / 100 g plant dry matter; Different letters denote significance different means ($p < 0.05$).

Italian ryegrass monocrop was characterized by the highest content of CT (58.5% higher than in RC monocrop). In investigated IR:RC mixtures, CT concentration ranged from 368.6 mgE C / 100 g DM (IR:RC 20:10) to 462.9 mgE C / 100 g DM (IR:RC 20:5). We assume that the highest CT concentration in IR monocrop harvested in early summer (955.2 mgE C / 100 g DM) influenced the highest RUP concentration (283.8 g kg^{-1} CP). *Waghorn (2008)* indicated that condensed tannins bind to protein in the rumen, reduce protein degradation, and when dietary crude protein concentrations exceed animal requirements for CP, these effects can improve performances. Although IR was superior in CT content related to red clover, this investigation showed that it is not well adapted to being grown in mixed crops with red clover regarding supporting high content of RUP in forages. RUP concentration in mixed IR:RC crops ranged from 182.8 g kg^{-1} CP (IR:RC 20:5) mixture harvested in spring to 252.7 g kg^{-1} CP (IR:RC 20:10) harvested also in spring (Table 2). RC had a slightly higher content of CT than IR harvested in spring, but the difference was not significant. In the summer harvest content of CT in RC was significantly lower than in IR, but it did not affect the high difference between RUP content in these forages. *Albrecht and Muck (1991)* concluded that lower non-protein nitrogen that has been found in red clover results from the action of polyphenol oxidase. This enzyme system reacts with O_2 and phenols normally present in red clover to produce quinones that inhibit the plant proteases that influence the protein degradability of forages *(Broderick et al., 2001; Wilkins and Jones, 2000)*.

Conclusions

This study confirmed that forage legumes provide high protein feed for ruminants, either in single species swards such as red clover or in mixed species swards containing Italian ryegrass and red clover. The lowest Soluble CP and non-protein nitrogen were determined in IR monocrop. As a consequence, the highest RUP content was also determined in IR monocrop. The results of this study indicate the potential importance of using Italian ryegrass forage in order to increase the content of rumen undegradable protein (RUP) in the ration of ruminants. We can conclude that the positive effect of high CT content in IR on reducing the CP degradability compared to RC monocrop and their mixtures may be used for the improvement of N utilization in ruminant nutrition. There are further investigations required in order to determine the optimal seeding rate of IR and RC in the mixtures and to determine the optimal harvest date of these mixtures regarding achieving the optimal RUP and RDP content for ruminant nutrition.

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