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NITROGEN AND ORGANIC MATTER RELEASING FROM ALFALFA HAY (*MEDICAGO SATIVA*) TREATED WITH QUEBRACHO TANNIN, *IN VITRO*

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SUMMARY

The objective of the current study was to evaluate the effect of different levels of condensed tannins (quebracho tannins, QT) on degradation kinetics of nitrogen (N) and organic matter (OM) in alfalfa hay (*Medicago sativa*). Also, the ratio between hourly-releasing of N and OM in alfalfa hay under these levels of QT was calculated to determine the synchrony index (SI) as well. Alfalfa hay was treated with quebracho tannin (QT; Unitan, Argentina) at three different levels. Alfalfa hay without treatment (control; QT0), alfalfa hay + quebracho tannin at 1.0% of DM (QT1), alfalfa hay + quebracho tannin at 2.0 % of DM (QT2) and alfalfa hay + quebracho tannin at 3.0% of DM (QT3). Two ruminally cannulated sheep were used to obtain rumen content for *in vitro* study and rumen samples to determine the NH_3-N concentration in rumen liquid.

In vitro study showed that, extend of disappearance of OM was significantly ($P<0.05$) low at QT3 comparing to QT0, and no differences were found among either QT1 or QT2 and QT0. Level of QT had no effect on the degradation rate (*c*) of OM. The *c* of nitrogen was significantly ($P<0.05$) decreased at QT2 and QT3 compared with QT0. Ammonia concentration ranged from 12.6 to 25.5 (mg/dl) for tested treatments, and it was parallel to N disappearance. Synchrony index was improved significantly ($P<0.05$) in QT2 and QT3 than QT0 and no significant differences between QT2 and QT3 treatments, the values were 0.40, 0.40, 0.47 and 0.53 for QT0, QT1, QT2 and QT3, respectively. Results of the current study concluded that the QT could be added to alfalfa hay at 2 % of its DM for that which could enhance efficiency of N utilization.

Keywords: in vitro, quebracho tannins, degradation kinetics, alfalfa hay, synchrony index

INTRODUCTION

Legume forage has a high protein concentration with a fast degradation rate and high portion of soluble protein (Kamel et al., 1995a and El-Waziry et al., 2000). The rate at which dietary intake protein is degraded in the rumen can affect the amount of ammonia-N (NH_3-N) that escapes microbial capture, depending on the availability of readily fermentable carbohydrate sources that

providing ATP to support the microbial protein synthesis (Hoover and Stokes, 1991). If there is insufficient rumen-available energy or the degradation rate of nitrogen (N) and energy are not synchronized, the excess NH_3-N will be absorbed into portal blood and transported to the liver to be converted to urea. Ammonia concentration of rumen liquor was raised up to be 8.28 mM when sheep were fed berseem hay (*Trifolium alexandrinum*) as a sole diet (Kamel et al., 2000), which is higher three times than the minimum level recommended by Satter and Slyter (1974) for microbial protein synthesis. Microbial yield and the efficiency of microbial synthesis are thought to be maximized be synchronizing crude protein and organic matter fermentation in the rumen (Ørskov, 1992 and Chumpawadee et al., 2006). Sinclair et al. (1995) concluded that the optimum microbial protein synthesis was achieved when the ratio between hourly release of N and organic matter (OM) was 25 g N/ kg of ruminally degraded OM. However, Kamel et al. (2004) stated that this ratio found to be 34.5 g N/ kg OM, when sheep were fed berseem hay as a sole diet.

Tannins are polyphenolic substance with various molecular weights and variable complexity. Tannins are tentatively classified into two classes: hydrolysable and condensed tannins (CT). The major anti-nutritive effect of tannins is depend on their ability to combine with dietary protein and forming the protein-tannin complexes, thus reducing ruminal digestion (McNeill et al. 1998, Kakkar et al., 1989, and Makkar, 2003) and degradation. This lead to delay of N release from the dietary protein in the rumen and increasing absorption of amino acids in the small intestine (Aerts et al., 1999; Barry and McNabb, 1999). On this basis, it had been proposed as feed additives to improve digestive utilization of dietary protein (Schwab, 1995). However, tannins also exert some negative effects such as impairing fiber and OM digestion. It is important, therefore, to clarify the extent to which tannins levels are to be applied for protecting alfalfa N from rapidly degradation in the rumen.

Moderate concentrations of CT (2-4.5% DM) can exert beneficial effects on protein metabolism in ruminants, high dietary CT concentrations (>5.5% DM) can depress voluntary intake, digestive efficiency and animal productivity (Aerts et al., 1999; Barry and McNabb, 1999). However, effects are not the same for all CT as they depend upon its chemical structure (Min et al., 2003).

For that reason, four does of quebracho tannins extract, covering a wide range of CT concentrations, were administrated to the sheep in the present work, in order to improve the synchronization between N and OM releasing from alfalfa hay in the rumen.

MATERIALS AND METHODS

1. Animals and feed

Alfalfa hay was treated with quebracho tannin (QT; Unitan, Argentina) at 0, 1, 2 and 3 % of DM, treatments were QT0 (control), QT1, QT2 and QT3, respectively. Quebracho tannin was mixed with hay during steam

binding-procedure for making hay as pellets.

Two ruminally cannulated sheep (Najdi Sheep, native breed) with an average live weight of 55.7 kg were used. Sheep fed alfalfa hay in two equal portions at 2% of their body weight. Experimental period was 28d, with 15 d as adaptation period. On days 16, 21 and 26 of each period, rumen content were collected for *in vitro* study, at the last consecutive three days rumen samples (50 ml) were collected to determined NH_3-N concentration (Chaney and Marbach, 1962). Samples were obtained at time 0h (before feeding), 1, 3 and 6h post feeding. Animals had free access to water and to lick minerals blocks.

2. In vitro procedure

Rumen content (about 4000 ml from both animals) was squeezed through two layers of cheesecloth into pre-warmed flasks to separate the liquid and solid fractions. An automatic incubator (Daisy^{II} incubator, ANKOM Technology) with 4-glass bottles was used. To begin the experiment each glass was filled with 360 ml of strained ruminal fluid and 1440 ml artificial saliva (1:4, v/v; McDougal, 1948); the temperature was adjusted to 39.0 ± 0.1 °C.

Approximately 5.0 g of ground (1-mm screen) alfalfa hay for each treatment were accurately weighed into synthetic bags with a pore size of 45 µm (Swiss Nylon Monofilament, Switzerland). Twenty-four bags were used at each run for all treatments. Six bags (each glass) were incubated and then one bag was removed after intervals of 3, 6, 12, 24, 48 or 72h. After the incubation, bags and hay residuals were washed by running tap water until the water became clear, then they were squeezed gently. Microorganisms attached to the residual samples were eliminated by freezing-rethawing technique as described by Kamel et al. (1995b). During the withdrawal of bags, glasses were flushed with oxygen-free CO_2. Two unincubated bags for each treatment were washed through the same procedure to provide the measure of the washing loss fraction. After washing, the bags contents were dried in an oven at 60 °C for 48h and reweighed. Residuals of N and OM were determined at each bag.

Organic matter and N in alfalfa hay were determined according to the AOAC (1999). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined as described by Goering and Van Soest (1970).

3. Calculation of degradability coefficient and synchronization index

Degradability coefficient was calculated in exponential form by fitting the results obtained for N and OM to the first order model of Ørskov and McDonald (1979) as:

$$P = a + b\,(1\text{-}exp^{-ct}),$$

where P is the cumulative amount degraded at time t, a is the readily degraded fraction, b is the fraction potentially degraded in the rumen, c is the constant rate of degradation of b and t is the incubation time in h. The lag time (L_t) was estimated according to McDonald (1981).

The effective extent of degradation ($'P$) was calculated hourly using the fractional outflow rate (k) of 0.03 per hour as follows:

$'P = a$ up to time L_t

$'P = a + \{(bc)/(c-k)\}\ (1-exp^{-(c-k)(t-Lt)})\ (exp^{-kLt})$, from time L_t onwards

where a, b, c and L_t as described above (Ørskov, 1992).

The quantity degraded per hour was calculated as differences between the cumulative amounts degraded at successive hours and allocated to the appropriate hour of the day. From hourly of OM and N degraded, a synchrony index (SI) of nitrogen to organic matter was then calculated using the following equation as proposed by Sinclair et al. (1993 and 1995):

$$SI = \frac{25 - \sum_{1-24} \sqrt{\left(25 - {}^{hourly}\,N/OM\right)^2}/24}{25}$$

where N represents the amount of N (g) degraded per unit of OM (kg) degraded at a certain time. The value of 25 represents 25 g of N / kg of truly digested OM in the rumen, which is assumed to be the optimal ratio (Czerkawski, 1986). A synchrony index (SI) of 1.0 represents perfect synchrony between nitrogen and energy supply through the day, whilst values < 1.0 refer to the degree of a synchrony (Sinclair et al., 1993). The formulation assumed that the animals were fed in two equal amounts at hour of 0900 and 1600; DM intake was 1 kg/d and ruminal outflow rate was 0.03/h (AFRC, 1993).

4. Statistical analysis

Results were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of SAS/Statview (1999).

RESULTS AND DISCUSSION

1. Chemical composition of alfalfa hay

Chemical composition (%, on dry matter basis) of alfalfa hay was 86.3, 2.8, 43.9 and 25.3% for OM, N, NDF and ADF, respectively.

2. Organic matter and N disappearances

Figure (1-A) illustrates the effect of different levels of QT on *in vitro* OM disappearance in alfalfa hay. No significant differences in OM disappearance were found due to QT1 during all incubation times comparing with QT0, however, values of QT1 tended to be lower than QT0 up to 24 h of incubation. Disappearance of OM was lower ($P<0.05$) for QT2 than QT0 up to 12 h of incubation, however, no effect was observed for the rest of incubation times. The QT3 significantly ($P<0.05$) reduced OM disappearance through all incubation times. Extent of OM disappearance in alfalfa hay was not affected by either QT1or QT2, but the level of QT3 had a significant negative effect on the extent of OM disappearance comparing with QT0. The corresponding values were 80.6, 81.4, 78.8 and 75.0 for QT0, QT1, QT 2 and QT3, respectively.

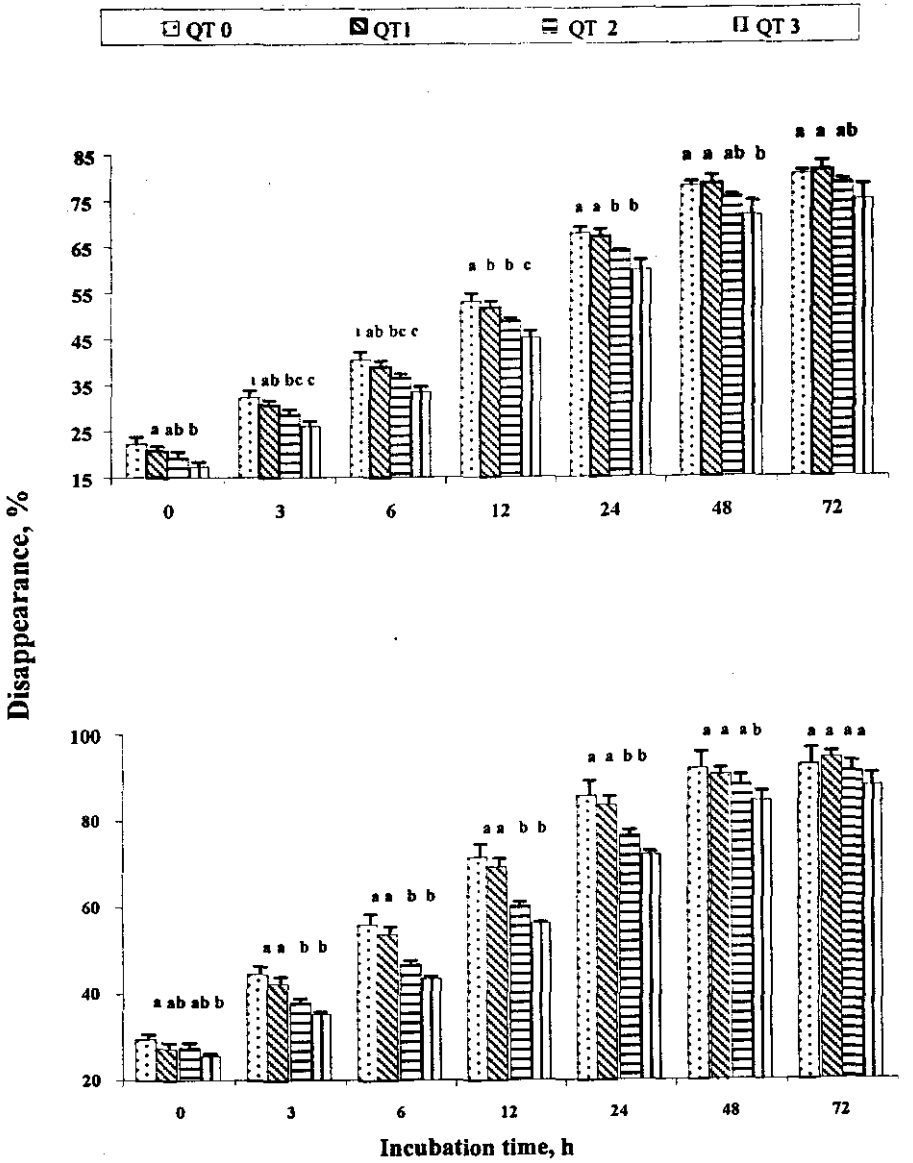

Figure (1). Organic matter (A) and nitrogen (B) disappearances of alfalfa hay treated with different levels of condensed tannins (quebracho tannin, QT; 0, 1, 2 and 3 % of dry matter). Data are means ±SE, bars for each incubation time without a common letter differ (P < 0.05)

The effect of QT on N disappearance of alfalfa hay is presented in Figure 1-B. The QT1 had no effect on N disappearance through all incubation times versus QT0. Treated alfalfa hay with 2% of QT significantly reduced N disappearance at 3 h of incubation and this inhibitory effect was extended up to 24 h of incubation in comparison with QT0 and QT1. Increasing tannin level was associated with extent of inhibitory effect on N disappearance. The QT3 significantly declined the N disappearance comparing with the other treatments at 48 h of incubation. The values of N disappearance at 72 h of incubation were comparable among treatments and no significant differences were detected.

3. Ammonia-N concentration as affected by QT

Table (1) shows the mean value of NH_3-N concentration (mg/dl) measured in rumen fluid of the animals as influence by different levels of QT. In general, treated alfalfa hay with QT reduced the NH_3-N concentration in the rumen liquid. No significant differences among QT1 and the control. At QT2 the NH_3-N concentration was significantly ($P<0.05$) lower than QT0 except at 0h. Animals fed alfalfa hay treated with QT at 3% had lower ($P<0.05$) NH_3-N concentration at all measured times.

4. Degradation kinetics of OM and N of alfalfa hay and SI

Degradation kinetics of OM and N of alfalfa hay are presented in Table (1). Rapidly degraded fraction (*a*) of OM was not affected by either low or moderate levels of QT treatment (i.e. QT1or QT2), however, *a* fraction was significantly ($P<0.05$) declined at QT3. Fraction *b* of OM was significantly ($P<0.05$) depressed when alfalfa hay was treated with QT at the level of 3 %, meanwhile, other treatments had not. Degradation rate (*c*) was numerically declined ($P<0.05$) for all treatments comparing with control. The *c* values were 0.064, 0.061, 0.057 and 0.058 for QT0, QT1, QT2 and QT3, respectively. Time needed to start degradation (L_i) was significantly ($P>0.05$) increased for QT3 in comparison with control.

The inhibitory effect of QT on degradation kinetics of N was obviously noticed. The degradation constants (*a, b* and *c* fractions) were significantly ($P<0.05$) decreased in QT2 and QT3 comparing with QT0, while, treated alfalfa hay with 1% of QT had minor effect on degradation constants. The L_i was increased in QT3 comparing with QT0 and QT1, while QT2 had an intermediate value.

Synchrony index (SI) as the ratio between hourly degraded N and OM during 24 h was significantly ($P<0.05$) improved for QT2 and QT3 compared to QT0, while the difference between QT2 and QT3 was not significant, the QT1 had no significant effect on SI. The values of SI were 0.40, 0.40, 0.47 and 0.53 for QT0, QT1, QT2 and QT3, respectively.

1. Organic matter and N disappearance as affected by different levels of QT

The reduction in OM disappearance in tannin-treated alfalfa hay is in agreement with Khazaal et al. (1993); they found a negative relationship between gas production (an indictor of ruminal degradation) and concentrations of phenolics in assessing phenolics

Table (1): Effect of different levels of condensed tannins (quebracho tannin, QT) on post-feeding changes of ammonia-N concentration (mg/dl) in the rumen liquid of sheep.

Time	Levels of quebracho tannin (QT)			
	QT0*	QT1	QT2	QT3
0 h	17.4±0.53[az]	16.6±0.45[a]	16.2±0.76[a]	14.9±0.39[b]
1 h	21.6±0.64[a]	20.6±0.59[ab]	18.9±0.68[bc]	16.8±0.43[c]
3 h	25.5±0.51[a]	24.2±0.47[a]	21.9±0.53[b]	19.9±0.62[b]
6 h	15.9±0.59[a]	15.5±0.66[a]	14.4±0.28[b]	12.6±0.31[b]

[z] Means ±SE, n=6 *Alfalfa hay + 0% of QT (QT0), alfalfa hay + 1.0 % of QT (QT1), alfalfa hay + 2.0 % of QT (QT2) and alfalfa hay + 3.0% of QT (QT3). [a,b,c] Means in the same row with different letters in their superscripts differ significantly ($P < 0.05$).

Table (2): Effect of different levels of condensed tannins (quebracho tannin, QT) on degradation kinetics of organic matter (OM), nitrogen (N) and synchrony index (SI) of alfalfa hay, *In vitro*

Item	Levels of quebracho tannin (QT)				SEM
	QT0*	QT1	QT2	QT3	
OM					
a	22.17[a]	20.00[ab]	19.40[ab]	17.33[b]	0.678
b	60.40[a]	61.70[a]	58.27[ab]	55.00[b]	1.065
a+b	82.57[a]	81.70[a]	77.67[b]	72.33[c]	0.805
c	0.064	0.061	0.057	0.058	0.002
Lt	1.7[b]	2.0[ab]	2.1[ab]	2.2[a]	0.10
N					
A	29.87[a]	29.10[a]	26.60[b]	25.57[b]	0.419
B	65.93[a]	64.53[a]	57.70[b]	54.70[b]	1.285
a+b	95.80[a]	93.63[a]	84.30[b]	80.26[b]	1.114
C	0.091[a]	0.093[a]	0.082[b]	0.076[b]	0.002
Lt	0.8[b]	0.7[b]	1.0[ab]	1.4[a]	0.13
SI**	0.40[b]	0.40[b]	0.47[a]	0.53[a]	0.014

*Alfalfa hay + 0% of QT (QT0), alfalfa hay + 1.0 % of QT (QT1), alfalfa hay + 2.0 % of QT (QT2) and alfalfa hay + 3.0% of QT (QT3).

a, *b* and *c* are constants predicted by the exponential equation $P = a+b\ (1-exp^{-ct})$ as proposed by Ørskov and McDonald (1979). *Lt*= lag time, calculated as reported by McDonald (1981). **SI= synchrony index calculated as described by Sinclair et al (1993). [a,b,c] Means in the same row with different letters in their superscripts differ significantly ($P < 0.05$).

related anti-nutritive factors in browse species. Similarly, Chiquette et al. (1988); demonstrated lower gas production from high tannin than low tannin containing variety of *Louts corniculatus*. At the same trend, removal of negative impact of tannin when using polyethylene glycol is associated with an increase in gas production in *Acacia* sp. (Hoffmann et al., 2000). A significantly negative correlation was found between the organic matter digestibility and concentration of condensed tannins (CT) in 14 *Acacia* sp. (Kamel, unpublished data). In addition to the interaction between tannins and cell wall of bacteria or extra-cellular enzymes secretion, one of the possible reasons for the negative effect of tannins is that decreasing the attachment of microbes to feed particles (Makkar et al., 1995b). Rumen microorganisms must attach to their insoluble plant substrates in order to effect their digestion; therefore, reduction on bacterial adherence in the presences of tannins would lead to low fermentation of OM. This finding is supported by *in vivo* research, where it was demonstrated that CT from *L. pedunculatus* reduced ruminal digestion of soluble carbohydrate, including pectin and hemicellulose, comparing with tannin-free forage (Barry and Manley, 1984).

Forming hydrogen bonds between the phenolic sub-units of the polymer and carbonyle group of peptides of the protein resulted in a tannin-protein complex which may protect protein from ruminal but not abomasal digestion because their stabilities are depending on the pH (Barry and Manley, 1984). The complex forming ability of tannins means that poly-phenolics are reactive with the bacterial cell wall and the extra-cellular enzymes secreted (McSweeney et al., 2001). The effect of CT from *L. corniculatus* on 11 strains of rumen bacteria was studied by Min et al. (2005) who concluded that CT reduced the rate of proteolysis and inhibited the growth of proteolytic rumen microorganism, and these negative effects were correlated to the level of CT.

The sensitivity of ruminal bacteria to the negative effect of CT are varied as stated by Krause et al. (2005), they demonstrated that population of tannins-sensitive *Streptococcus bovis* was declined in the presence of acacia in the diet to be approximately 0.005 of the total bacterial population. On the other hand, the tannin-tolerant *S. gallolyticus* population increased to be more than 2 % of the total population under the acacia diet. Meanwhile, under feeding in tannin-free diet, the population of *S. gallolyticus* was less than half of the *S. bovis* population. Even though it will take time, the shifting in populations' growth between the tannins-sensitive bacteria and tannin-tolerant bacteria could illustrate an increment in lag time when QT was presented in the diet. Moreover, shifting toward increasing the population of tannin-tolerant bacteria could explain the gradually reduction of the inhibitory effect of QT with increasing incubation time.

2. *Effect of QT on ruminal NH_3-N concentration*

Reduction of ruminal NH_3-N concentration was due to the negative effect of tannins on N utilization. Treated hay with QT inhibited the N released and subsequently ruminal NH_3-

N concentration. Rumen ammonia-N concentrations obtained for QT treated hay (QT1, QT2 and QT3) were similar to values associated with maximum microbial growth (Ørskov, 1992) and well above the 5 mg/ dl likely to limit growth (Satter and Slyter, 1974). This finding suggested that QT impaired wastage of dietary protein from treated alfalfa through microbial degradation in the rumen.

4.3. Condensed tannin as a modulator for ruminal releasing of OM and N

Ratio between the hourly release of N and OM in the current study found to be 39.1g N/ kg OM. This value was higher than that reported by Kamel et al. (2004); he reported 34.5 g N/ kg OM when animals were fed berseem hay (*Trifolium alexandrinum*). A synchronicity between N and OM in the present study comparing to that in pervious report (Kamel et al., 2004) could be attributed to high amount of N released per h in *in vitro* system as a result of higher degradable fractions (i.e. a, b and c) in QT0 than in berseem hay.

The SI (an indicator for the efficiency of microbial protein synthesis, EMPS) was enhanced due to either the moderate or higher level of QT (QT2 and QT3) in the current study. This finding is inagreement with that of using ^{15}N in studying the effect of mimosa tannin on microbial nitrogen synthesis (Bento et al., 2005). They found an increment in EMPS as mimosa tannin was presented in *in vitro* incubation medium. Tannins were shown to alter partitioning of nutrients towards higher microbial yield, and/or efficiency, rather than production of short chain fatty acids (Baba et al., 2002; Makkar et al., 1995a; Blummel, et al., 1997).

Gonzalo et al. (2003) reported that the intra-ruminally administration for QT at the level of 2.8% of DM had no effect on ruminal fermentation activity and CP disappearance of alfalfa hay, meanwhile the dose of QT 8.3 (% of DM) negatively impacted these parameters. In contradicter, results of the current study suggested that QT at level of 2% or 3% of DM could be applied to reduce the N disappearance on alfalfa hay. The disagreement of the results in the present study and the previous report could be attributed to the procedure of mixing the tannin with the diet. Tannin-protein complex may be increased as a result of using steam with high temperature through the manufacturing procedure of alfalfa hay.

CONCLUSION

Overall the results from the present study indicated that extent of inhibitory effect for QT on OM and N disappearances of alfalfa hay is dependent on level of QT. Releasing of OM was not affected by either QT2 or QT3, meanwhile these levels significantly reduced N releasing from alfalfa hay, *in vitro*. Reduction of NH_3-N concentration was observed due to QT treatment (i.e. QT2 and QT3), however, it was in the optimal rang for microbial nitrogen synthesis. Treated alfalfa hay with either 2% or 3% of QT enhanced the efficiency of microbial protein synthesis estimated as SI, moreover, these treatments declined extent of ruminal N degradation.

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انطلاق النيتروجين و المادة العضوية من البرسيم الحجازي المعامل بتانين الكابراتشو، معملياً

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الهدف من هذه الدراسة هو تقيم تاثير مستويات مختلفة من تانين الكابراتشو علي ميكانيكية تحلل النيتروجين و المادة العضوية في دريس البرسيم الحجازي. كذلك النسبة بين انطلاق النيتروجين والمادة العضوية تم حسابه كل ساعة لتقدير دليل التوافق. دريس البرسيم الحجازي تم معاملته باربع مستويات من التانين. المعاملات كانت البرسيم الحجازي+ صفر% تانين من المادة الجافة (كنترول)، البرسيم الحجازي+ ١% تانين من المادة الجافة، البرسيم الحجازي +٢% تانين من المادة الجافة، البرسيم الحجازي+ ٣% تانين من المادة الجافة. تم استخدام عدد ٢ أغنام مزودين بكانيولات مستديمة في الكرش للحصول علي محتويات الكرش لأجراء الدراسة المعملية و تقدير تركيز الامونيا في الكرش.

الدراسة المعملية أوضحت أن مدي تحلل المادة العضوية في دريس البرسيم انخفض معنوياً عند المستوى ٣% من التانين بينما لا يوجد فروق بين الكنترول وأي من المستوى ١% أو ٢% من التانين. مستويات تانين الكابراتشو لم تؤثر علي معدل تحلل المادة العضوية. معدل تحلل بروتين البرسيم الحجازي تناقص معنوياً في المستوي ٢% و ٣% من التانين مقارنة بالكنترول. تركيز الامونيا تراوح مابين ١٢,٦ إلي ٢٥,٥ ملجرام/ ١٠٠ مل من سائل الكرش في المستويات المختبرة وكان متوازي مع اختفاء النيتروجين. دليل التوافق تحسن معنوياً عن الكنترول عند المعاملة ٢% و ٣% تانين ولا يوجد فرق معنوي بينهما و القيم كانت ٤٠, و ٤٠, و٤٧, و ٥٣, في المستوي صفر % و ١% و ٢% و ٣% علي التوالي.

نتائج هذه الدراسة أوضحت أن معامله دريس البرسيم بتانين الكابراتشو عند مستوى ٢% من المادة الجافة خفض من تحلل النيتروجين بينما تحلل المادة العضوية و انطلاق الطاقة كان ثابت و هذا أدي إلي زيادة كفائه تخليق البروتين الميكروبي مقدراً كدليل التوافق، معملياً.