Journal of Veterinary Science & Technology

Hosseini et al., J Vet Sci Technol 2017, 8:4
DOI: 10.4172/2157-7579.1000454

Research Article Open Access

Effect of Additives on *In Vitro* Intestinal Utilizable Crude Protein in Dairy Cows and Mobile Bag Nutrient Digestibility of Corn, Alfalfa and Whole Barley Silages

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Abstract

An experiment was conducted to investigate the effect of additives on intestinal utilizable crude protein in dairy cows, and ruminal and post-ruminal nutrients disappearance of corn, alfalfa, and whole barley silages using *in situ* mobile bag technique. Alfalfa, whole crop corn and barley were harvested and chopped, and then treated with commercial biological and chemical additives; urea at 0.0, 10.8 and 21.6 g/kg DM in corn ($CS_{0.0}$, $CSU_{10.8}$ and $CSU_{21.6}$, respectively) and whole barley ($BS_{0.0}$, $BSU_{10.8}$ and $BSU_{21.6}$, respectively); Biomin® inoculant at 0.0, 800 and 9600 cfu/kg in corn ($CS_{0.0}$, CSB_{800} and CSB_{9600}, respectively) and at 0.0 and 800 cfu/kg in whole barley ($BS_{0.0}$ and BSB_{800}); formic acid at 0.0, 4 and 4.4 ml/kg in alfalfa ($AS_{0.0}$, ASF_4 and $ASF_{4.4}$, respectively) and at 0.0 and 4 ml/kg ($BS_{0.0}$ and BSF_4) in whole barley. Urea caused the enhancement of *in vitro* intestinal utilizable crude protein (uCP) and its effectiveness (EuCP). *In vitro* intestinal utilizable crude protein was not affected by formic acid in whole barley silage, although treated alfalfa silages had higher uCP than $AS_{0.0}$. Ruminal CP disappearance was higher for urea-treated corn and whole barley silages at both levels, although formic acid and Biomin® did not change it. Ruminal NDF disappearance was greater for urea-treated corn silages and $BSU_{21.6}$ than $CS_{0.0}$ and $BS_{0.0}$. Post-ruminal disappearance of protein increased significantly in $AS_{4.4}$ and reduced in urea-treated corn and whole barley silages ($P<0.05$). Utilizable CP at 8 h was higher for dry hays in comparison with those of silages in both alfalfa and whole barley ($P<0.05$). Results of the present study showed that, for the first time, silages treated with urea and formic acid had higher *in vitro* utilizable crude protein in dairy cow intestine compared with the untreated silages.

Keywords: Silage; Hay; Additives; uCP; Mobile nylon bag technique

Introduction

Dairy cow requirements should be met by forage especially silages which have become the chief forage component in the ration of dairy cows over the last few decades [1]. Although protein concentration in silages is lower than that of most concentrates, dry matter intake of silages is high and therefore protein entering into the digestive tract is considerable. On the contrary, nitrogen utilization in dairy cows is inefficient and high N inputs via expensive protein concentrates are not in balance with production outputs; consequently, large losses of N occur via animal excretion leading to environmental pollution [2]. Silages are often a wise choice when selecting forages for providing fiber and energy to dietary component [3]. Nevertheless, nutritional value of silages can vary and depends on stage of maturity and ensiling process [4]. Fermentation in the silo can be an uncontrolled process and some times, they are not optimal which leads to nutrients' loss [5]. Silage additives have been utilized to enhance the ensiling fermentation and prevent the production of undesired acids by limiting the extent of fermentation to produce well-preserved silages with subsequent improvements in animal performance [6]. There are several types of silage additive, that are classified according to their effect on fermentation such as fermentation stimulants [7], fermentation inhibitors [8], and nutrient additives [5]. Urea is a synthetic, non protein nitrogen compound classified as a nutrient silage additive because it is a source of nitrogen for bacteria in the rumen [9]. It seemed that corn silage (a high energy, low protein feed) might be an ideal type of feed to be considered for use with urea as an additive to increase its crude protein content. Although urea on alfalfa silage had been used in some cases, it is not recommended [10]. Formic acid, as an inhibitor of fermentation and for its antibacterial effect cause a reduction in protein degradation and deamination in legume such as alfalfa [11]. Alfalfa silage treated with formic acid reduced soluble protein fraction and increased potentially degradable fraction when compared to untreated silage [12]. These authors noted that alfalfa treated with formic acid could provide more rumen undegradable protein than untreated silages in the diets of cows with slow ruminal turnover. Inoculants are microbial silage additives containing homolactic or heterolactic bacteria that have been selected to grow rapidly and efficiently on crops in a silo [13]. Determination of the *in vitro* intestinal utilizable crude protein (uCP) which includes rumen undegradable crude protein (RUP) and the rumen microbial crude protein (MCP) is an important factor for diet evaluation in many modern protein evaluation system [14,15]. Protein utilization in silages is poor and is related to proteolytic activity by crop enzymes after harvest and further microbial breakdown of protein during ensilage. This process causes a portion of the protein degraded to non-protein nitrogen during ensiling [16]. The poor utilization of silage protein along with lower feed intake of silages can limit milk production. Methods for enhancing silage protein utilization include the use of microbial and chemical additives, methods of conservation (hay vs. silage) or selection of crops having characteristics that decrease protein solubility and alter ruminant digestibility. Therefore, digestibility of nutrients in the rumen and post-rumen is the most critical key to

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Received June 03, 2017; **Accepted** June 21, 2017; **Published** June 23, 2017

Citation: Hosseini SM, Mesgaran MD, Vakili AR, Naserian AA (2017) Effect of Additives on *In Vitro* Intestinal Utilizable Crude Protein in Dairy Cows and Mobile Bag Nutrient Digestibility of Corn, Alfalfa and Whole Barley Silages. J Vet Sci Technol 8: 454. doi: 10.4172/2157-7579.1000454

evaluate silage quality [17]. In addition, voluntary intake of a specific feed depends mostly on the rate of ruminal and post-ruminal digestion of potentially digestible nutrients [18]. Digestibility of silage nutrients in the rumen and post-rumen is also the most critical key for influencing milk production [17]. An increase in silage digestibility will increased milk production by improving voluntary feed intake and utilization of nutrients. One percent increase in silage digestibility appears to be 0.24 - 0.37 kg of milk or increase by 1.1 kg of milk for each 0.5 MJ/kg DM increase in silage metabolizable energy content [19]. Therefore, accurate estimation of ruminal and intestinal protein disappearance of silage is also important for diet formulation. The present study aimed at evaluating the *in vitro* intestinal CP utilization of treated silages with chemical and biological additives, and also assessed additives on rumen and post-rumen disappearance of DM, CP, and NDF for corn, alfalfa and whole barley silages.

Materials and Methods

Ensiling procedure and additives

The whole crop corn (hybrid 700; Mashhad, Iran) in d 17 September 2015 at 2/3 milk line of kernel maturity stage, whole barley forage (*Hordeum vulgare* L.) in d 20 May 2015 at dough stage, and alfalfa forage (*Medicago sativa* L.) at d 15 September 2015 in third cutting at 40% bloom were harvested and chopped at a theoretical length (approximately 30 mm), then ensiled in laboratory silos, 4 L polyvinyl tubes, and were immediately sealed by polypropylene screw cap on top with a rubber seal. Four replicates were made for each treatment, and the silos were opened after 90 days. Before ensiling, 4 samples from any forage were collected for hay samples. Silage additives were urea, formic acid, and Biomin® inoculant (Biomin Gmbh, Industriestrasse 213130 Herzogenburg, Austria). The inoculant Biomin® (BioStabil), containing a blend of Enterococcus faecium (DSM 3530), Lactobacillus brevis (DSM 19456) and Lactobacillus plantarum (DSM 19457), was applied in liquid at least 2×10^5 colony forming units per gram according to the manufacture's recommendations for both corn and whole barley silages. Formic acid was used within the range of four liters per ton of fresh forage, according to founding of Nagel and Broderick [20] and this was 10% higher for alfalfa forage. The controls were sprayed with distilled water for preserved humidity. The experimental treatments comprised: urea at 0.0, 10.8 and 21.6 g/kg DM in corn ($CS_{0.0}$, $CSU_{10.8}$ and $CSU_{21.6,}$ respectively) and whole barley ($BS_{0.0}$, $BSU_{10.8}$ and $BSU_{21.6,}$ respectively); Biomin® inoculant at 0.0, 800 and 9600 cfu/kg in corn ($CS_{0.0}$, CSB_{800} and CSB_{9600}, respectively) and at 0.0 and 800 cfu/kg in whole barley ($BS_{0.0}$ and BSB_{800}); formic acid at 0.0, 4 and 4.4 ml/kg in alfalfa ($AS_{0.0}$, ASF_4 and $ASF_{4.4}$, respectively) and at 0.0 and 4 ml/kg ($BS_{0.0}$ and BSF_4) in whole barley based fresh forage.

Mobile bag technique

Two Holstein steers fitted with ruminal and T-shape duodenal cannulas were used to determine ruminal and post-ruminal dry matter, protein and NDF disappearance of corn, alfalfa and whole barley silages treated with several additives. Steers (310 ± 11 kg BW, 11 ± 0.3 month age) were fed 2.1 kg of dry matter (DM) of alfalfa hay, 3.2 kg of DM corn silage, and 2.5 kg of DM concentrate plus supplemental vitamins and minerals (158 g CP kg^{-1} of DM) and kept in individual pens. Six nylon bags (10 × 19 cm; 47 µm pore size) per treatment (three bags per steer) were filled with 6 g dry matter silage samples, and closed using rubber bands, then incubated in the rumen for 16 h simultaneously just before the animals were offered the first meal of the morning at 7.00 a.m. Bags were then removed from rumen, placed in a conventional washing machine. Washings were repeated until the water remained clear. Samples were then dried in an oven at 65°C until a constant weight was achieved before determination of DM disappearance. One gram of dry samples of the 16 h. ruminal incubation residue was inserted in the nylon bag (3 × 6 cm; 47 µm pore size) for every 30 min. into the duodenum through the T-shaped proximal duodenum cannulas. Duodenal bags were collected from faeces and hand rinsed until the water remained clear. Samples were dried in oven at 65°C for 48 h until a constant weight was obtained before the determination of DM disappearance. Bags not found within 30 h were discarded. Due to the limited amount of sample remaining, replicates within steers were pooled and DM, protein, and NDF disappearance were evaluated (expressed as g/kg DM). Individual bag residue disappearances were subsequently calculated based on the formula:

Digestibility coefficient [21]**=F-f/F;** where, F is the amount of feed component (mg) in the bag and f is the amount of the feed component in the rumen or in the faeces (mg). Correction for bacterial contamination was estimated using exponential equation as described by Krawielitzki et al. [22].

$$MA=A_{max}(1-e^{-Rt});$$ where A_{max} is the maximum extent of bacterial colonization at $t \approx \infty$, R is the rate of colonization (h^{-1}) and t denotes the incubation time (hours). The rate of microbial attachment (R) and maximum extent of bacterial colonization (A_{max}) were calculated:

$$R\ (h^{-1})=(133+0.09\ NDF\ (g/kg\ DM)-0.35\ CP\ (g/kg\ DM))/1000$$

$$A_{max}\ (mg/g\ residue\ CP)=(506+0.48\ NDF\ (g/kg\ DM)-0.77\ CP\ (g/kg\ DM))/10$$

A_{max} was estimated by treating a subsample of the residue with neutral detergent solution with the assumption that the residues only contained cell wall bound CP (estimated from neutral detergent insoluble N; NDIN) and microbial matter was soluble in neutral detergent [23].

In vitro intestinal utilizable crude protein and metabolizable energy

In vitro intestinal utilizable crude protein was determined using the modified Hohenheim gas test (modHGT) based on the method described by Edmunds et al. [2]. The modHGT follows procedures of the HGT [24] with a chemical alteration of 2 g/l increase in NH_4HCO_3 and 2 g/l reduction in $NaHCO_3$ in the buffer solution. Recommended incubation times were 8 and 24 h for concentrates and 8 and 48 h for forages [25]. Rumen fluid was collected from two fistulated Holstein steers, as mentioned. The fluid was obtained before the morning feed at 7:00 a.m. and transported to laboratory in water bath (39°C) under constant free CO_2. Rumen fluid and digesta were mixed with a blender, clarified through four layers of cheesecloth and strained through a nylon material with 46 µm pore size. Then, added to the reduced buffer solution. Approximatley, a 200 mg of ground silage sample was placed into a 120 ml glass serum bottle, then incubated with 30 ml incubation medium in triplicate. The starting time of the incubation was recorded after all bottles had been filled. At the end of each incubation time (8 and 48 h), gas volume was recorded and glass bottles were removed from water bath. Gas production (GP) was also recorded at 24 h for use in the calculation of metabolizable energy (ME). NH_3-N concentration was determined by using a modified phenol-hypochlorite reaction adapted from Broderick and Kang [26], and used in the following calculation (Edmunds et al. [2]):

$$uCP(g/kg\ DM)=((NH_3\text{-}N_{blank}+N_{sample}\text{-}NH_3\text{-}N_{sample})/weight(mg\ DM))\times 6.25\times 1000)$$

where N sample is N added to the bottles from the measured amount of silages (mg), weight is the amount of sample weighed into the glass bottle and calculated to DM and other variables are as previously described. Metabolizable energy was achieved in the following calculation:

ME (MJ/kg DM)=7.81+0.07559 GP-0.00384 Ash+0.00565 CP+0.01898 fat-0.00831 ADF_{OM}

where GP is *in vitro* gas production at 24 hours (ml/200 mg DM), fat is crude lipids (g/kg DM), ADF_{OM} is acid detergent fiber expressed without residual ash (g/kg DM) and Ash and CP are expressed in g/kg DM in accordance with mathematical calculation cited in GfE [27]. For the calculation of effective uCP, two incubation time points of three runs were plotted against a log time (Ln. (t)) scale, where 't' is the time of incubation and assumed passage rates (Kp) of 0.02, 0.04, 0.06, 0.08, and 0.10 h^{-1} through the rumen, using the formula:

effective uCP=y+a × Ln.(1/Kp)

where y is the intercept and a is the slope.

Analytical method

All samples were analyzed for crude protein (CP; N × 6.25; AOAC [28], method 990.03; using Kjeltec 2300 Auto analyzer, Foss Tecator AB, Hoganas, Sweden), Ash (AOAC [28], method 942.05; ignition samples at 550°C for over night), Neutral detergent fiber (NDF; assayed without heat stable amylase and sodium sulphite), and acid detergent fiber (ADF [29]). Water soluble carbohydrate (WSC [30]), and starch [31] contents were measured by an anthrone-sulphuric acid procedure using glucose as standard. Samples of silage extract for pH analysis and NH_3-N concentration were prepared by blending 50 g of fresh silage and 450 mL of doubled distilled water (w/v) to a homogenized state using a blender for 2 min, then pH was determined immediately by a digital portable pH meter (WinLab, portable). A portion of extracts strained through four layers of cheese-cloth and 5 ml of fluid samples were acidified with 5 ml of 0.2 N HCl, then centrifuged at 3500 × g for 10 min. The supernatant was then analysed using a modified phenol-hypochlorite reaction adapted from Broderick and Kang [26], to determine NH_3-N concentration. All chemical analyses were conducted in triplicate on each individual sample.

Statistical analysis

Silage fermentation characteristics, *in vitro* intestinal utilizable crude protein and mobile nylon bag data were analyzed as a randomized complete design and were evaluated by the Generalized Linear model (PROC GLM; version 9.2; SAS Institute, Inc., Cary, NC [32]). Utilizable intestinal CP (independent variable) was regressed against calculated values (dependent variable) with three runs to determine regression coefficients such as slope and intercept. Given that there were significant differences among crops due to characteristic differences of forages; dunnett test was used between a control (untreated) and all other means (treated with additives) to comparison differences within each crop. Differences among means were tested using the LSMEANS test. The PDIFF option in the LSMEANS statement was used to separate means. Standard errors of means were calculated from the residual mean square in analysis of variance. Significance was declared at $P \leq 0.05$. Data of measurements were subjected to SAS [32] (version 9.2) analyses according to the following model:

$$Y_{ij}=\mu+T_i+e_{ij}$$

where, Yij=amount of each observation, μ=general mean, T_i=treatment effect and e_{ij}=standard of error term. Unavailable crude proteins calculated as (1-EuCP) were regressed against indigestible crude protein calculated as (1-total tract CP) to determine the presence or absence of linear bias.

Results and Discussion

Silage fermentation characteristics

Chemical composition and pH value of untreated and treated with various additives of corn, alfalfa and whole barley silages are presented in Table 1. The inclusion of urea as an additive regardless of levels increased pH, CP and NH_3-N concentrations of both corn and whole

	Treatments[6]															
	Corn silage						Alfalfa silage				Whole barley silage					
Items	$CS_{0.0}$	$CSU_{10.8}$	$CSU_{21.6}$	CSB_{800}	CSB_{9600}	SEM	$AS_{0.0}$	ASF_4	$ASF_{4.4}$	SEM	$BS_{0.0}$	$BSU_{10.8}$	$BSU_{21.6}$	BSB_{800}	BSF_4	SEM
DM[1]	29.32	28.76	28.27*	29.09	28.97	0.46	26.48	24.33*	23.81*	0.22	36.32	36.76	36.27	36.97	37.09	0.28
pH	3.76	4.02*	4.08*	3.51	3.67	0.13	4.66	4.38*	4.13*	0.1	4.15	4.63*	4.81*	4.1	3.80*	0.06
NH_3-N	2.08	4.27*	4.77*	1.48*	1.48*	0.01	2.61	2.26*	1.53*	0.004	1.64	2.45*	3.47*	1.38	1.18*	0.006
CP[2]	82.5	123.6*	150.5*	91.5*	87.5*	1.58	176.6	189.3*	186.4*	2.14	117.1	156.5*	183.1*	117.4	117.6	1.33
NDF[3]	556.6	569	574.6	563.1	549.9	7.16	435.9	462.6*	468.3*	5.62	563.2	555.9	557.3	554.7	561.7	6.32
ADF[4]	355.4	360.8	363.8	367.2	377	5.59	365.8	370.8	369.8	4.97	353.6	348	347.2	344.2	351.5	5.67
WSC[5]	19.12	18.38*	16.10*	17.49*	18.02*	0.28	9.50	10.10*	11.39*	0.24	26.41	21.18*	19.77*	19.14*	32.20*	1.57
Starch	296.3	293.9	297.2	291.3	291.1	3.16	214.57	214.91	214.75	1.9	135.3	138.2	130.7	134.9	135.9	3.43

1: Dry matter 2: Crude protein 3: Neutral detergent fiber 4: Acid detergent fiber 5: Water soluble carbohydrate 6: corn silage as untreated (control, $CS_{0.0}$) or treated with 10.8 ($CSU_{10.8}$) and 21.6 ($CSU_{21.6}$) g/kg DM urea, or 800 (CSB_{800}) and 9600 (CSB_{9600}) cfu/kg Biomin® inoculant; Alfalfa silage as untreated (control, $AS_{0.0}$) or treated with 4 ml/kg (ASF_4) and 4.4 ($ASF_{4.4}$) ml/kg formic acid; Whole barley silage as untreated (control, $BS_{0.0}$) or treated with 10.8 ($BSU_{10.8}$) and 21.6 ($BSU_{21.6}$) g/kg DM urea, or 4 ml/kg formic acid (BSF_4), or 800 cfu/kg (BSB_{800}) Biomin® inoculant; *Within a row, means with an asterisk differ significantly from control (P<0.05); SEM=standard error of means

Table 1: Chemical composition (g/kg) and pH value of untreated and treated with various additives of corn, alfalfa and whole barley silages.

barley silages (P<0.05). Nevertheless, inoculation had no effect on pH in corn and whole barley silages throughout the ensiling. These findings are in line with the results of previous studies which reported that the inclusion of urea increases pH [33], insoluble N and true protein [34]. Ariza et al. reported that NH_3-N concentration in the fermenters depends on the extent of CP degradation and N uptake by ruminal bacteria [35]. McDonald et al. reported that lower pH value inhibits protein degradation in silage [6]. So, higher NH_3-N concentration in urea-treated silages might be attributed to higher pH and CP content in these silages. The inoculation decreases pH [36], and ammonia nitrogen concentration in corn silage [10]. However, fermentation efficiency of inoculated forage is still dependent on epiphytic microbial populations and chemical components [37]. No detectable differences was observed in starch, and acid detergent fiber among the three crops with the various additives (P>0.05). Alfalfa and whole barley silage treated with formic acid had higher water soluble carbohydrate (WSC) concentration compared with $AS_{0.0}$ and $BS_{0.0}$, whereas urea and inoculant additives had lower water soluble carbohydrate concentration than those of $CS_{0.0}$ and $BS_{0.0}$. Inclusion of formic acid to alfalfa silage irrespective of level, reduced dry matter content, pH, NH_3-N concentration, and increased CP and NDF contents than $AS_{0.0}$ (P<0.05). This result is in line with Jaakkola et al. [11] which states that addition of acid to silage reduces pH and ammonia-N concentration and increases water soluble carbohydrate concentration.

In vitro intestinal utilizable crude protein and metabolizable energy

In vitro intestinal utilizable crude protein (g/kg DM) after 8 and 48 h of incubation, metabolizable energy and effective uCP (g/kg DM) at different assumed rates of passage (k_p 0.02 through k_p 0.10) for untreated and additive treated of corn, alfalfa and whole barley silages are shown in Table 2. *In vitro* intestinal utilizable crude protein value was approximately 90% higher in alfalfa silage than those of corn and whole barley silages. A higher uCP for alfalfa silage than other silages was expected because crude protein of legumes is consistently reported to be greater [6], which supports the findings of the present study throughout the course of the experiment. Urea regardless of crops (corn or barley) and level (10.8 and 21.6 g/kg DM), increased uCP at 8 h after incubation, and effective uCP (EuCP) at different assumed passage rate. It should be noted that uCP and EuCP increased by urea level elevation in corn and whole barley silages. Nevertheless, the ratios of uCP to CP for $CSU_{21.6}$, CSB_{800} and also $BSU_{10.8}$, $BSU_{21.6}$ were significantly lower than those of $CS_{0.0}$ and $BS_{0.0}$ (P<0.05). This is likely related to the loss of crude protein as ammonia with urea supplementation. On the other hand, due to increase pH when urea is applied to silages (as mentioned in Table 1), nitrogen compound during silage processing would alter non protein nitrogen (NPN). Hence, uCP/CP was different than uCP alone in silages treated with urea. After 48 h of incubation, uCP was higher for both corn and whole barley silages supplemented with urea at both levels ($CSU_{10.8}$, $CSU_{21.6}$, $BSU_{10.8}$, and $BSU_{21.6}$). The ratio of uCP to CP at 48 h unlike 24 h was higher in $CSU_{21.6}$ and $BSU_{21.6}$ than $CS_{0.0}$ and $BS_{0.0}$ (P<0.05). These various results may be explained by variety of microbial activity to protein degradation during incubation. The rate and extent at which protein degradation occurs will depend on proteolysis activity of the ruminal micro-flora and the type of protein. Urea-treated silages had higher potential to convert ammonia during incubation than $CS_{0.0}$ and $BS_{0.0}$. Ammonia-N concentration increased at initial hours of incubation but reduced over time. *In vitro* intestinal utilizable crude protein and uCP/CP at 8 and 48 h were not significantly affected in BSF_4, although treated alfalfa silages had higher uCP and effective uCP value at 48 h after incubation than $AS_{0.0}$ (P<0.05). These conflicts are likely attributed to differences in nitrogen construction between alfalfa and barley silage. These results confirmed reports from researchers who noted that an increase in microbial synthesis in rumen and microbial-N flow at the duodenum with addition of formic acid to alfalfa for silage preparing has occurred [11]. On the other hand, silage treatments with acid might decrease the apparent digestibility of CP in the rumen and elevate the proportion of by-pass protein flowing to the duodenum. Applying inoculant did not result in a consistent change in uCP at 8 and 48 h after incubation in corn and whole barley silages (P>0.05), but effective uCP was influenced in treated whole barley silage. Metabolizable energy was lower in $ASF_{4.4}$, but urea and inoculant supplementation had no effect on metabolizable energy. These results may be explained due to increase NDF content with supplemented additives to silage (as already reported in Table 1). However, the present finding is not in line with Acosta Aragon et al. [38] who noted that whole crop corn silage treated with BSM (blend of homo- and heterofermentative lactic acid bacteria) has higher digestible and metabolizable energy than untreated silage. Our results confirmed the findings of Saricicek and Kilic [39] which showed that alfalfa silage treated with formic acid salts has lower ME than untreated control. According to Figure 2, uCP at 8 h was higher in dried forages compared with those of silages in alfalfa and whole barley silages as well uCP at 48 h in alfalfa hay than alfalfa silage. While there were no detectable differences for uCP at 8 and 48 h in corn silage and corn hay, no significant differences were observed for ME between the type of preservation among the crops. The higher uCP in dried alfalfa and whole barley forages compared with the silages might be attributed to the change of protein fraction during silage fermentation process such that marginal proportion of true proteins in forage are degraded to soluble fractions like NH_3-N [6,40]. Hristov and Sandev compared the chemical composition of alfalfa silage and hay. They noted that NH_3-N concentration is lower in hay compared with silage [41]. These differences regarding protein fraction between hays and silages affect the synthesis of microbial protein. Given that fermentation products provide little energy (ATP) to rumen microbes, it can be concluded that hays promote higher microbial protein synthesis compared with silages [42]. Thomas and Rae [43] reported that animals fed hay-based diets had higher efficiency of microbial protein synthesis than animals fed silage. Moreover, the higher uCP found in dried forage than silage might be attributed to alter protein degradation site from rumen to intestine. Lack of difference for uCP at 8 and 48 h in corn could be due

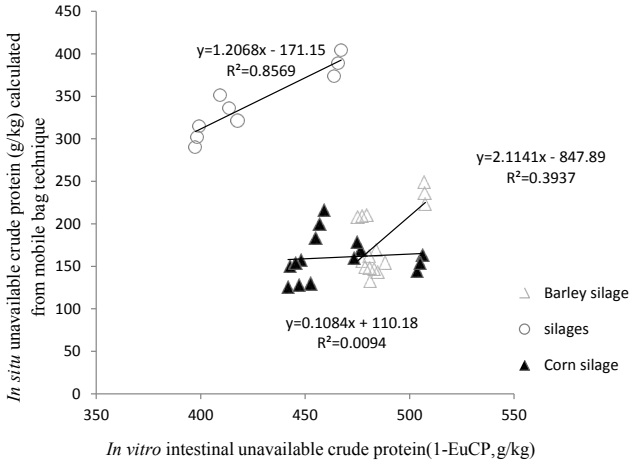

Figure 1: Correlation between *in vitro* intestinal unavailable crude protein at passage rate of 0.06/h (**x**-axis) and *in situ* unavailable crude protein calculated from mobile bag technique (**y**-axis).

	Treatments[1]															
	Corn silage						Alfalfa silage				Whole barley silage					
Items	**$CS_{0.0}$**	**$CSU_{10.8}$**	**$CSU_{21.6}$**	**CSB_{800}**	**CSB_{9600}**	**SEM**	**$AS_{0.0}$**	**ASF_4**	**$ASF_{4.4}$**	**SEM**	**$BS_{0.0}$**	**$BSU_{10.8}$**	**$BSU_{21.6}$**	**BSB_{800}**	**BSF_4**	**SEM**
8 h																
ucp	56.04	80.43*	94.87*	56.71	57.60	1.78	118.96	135.76	140.57	8.55	76.16	90.77*	111.85*	77.71	79.06	1.41
Ucp/cp	67.93	65.10	63.04*	61.98*	65.83	1.23	67.26	71.72	75.41	4.55	65.04	58.00*	61.09*	66.19	67.22	1.16
48 h																
ucp	29.48	42.00*	62.06*	28.58	34.89	1.86	58.23	73.47*	70.77*	1.64	37.72	57.33*	71.53*	34.69	34.47	1.51
Ucp/cp	35.73	33.99	41.24*	31.24	39.89	1.94	32.92	38.81*	37.97*	0.89	32.22	36.63	39.07*	29.55	29.31	1.11
ME(MJ/KgDM)	5.12	5.61	5.78	5.38	5.20	0.52	4.71	4.13	3.65*	0.47	5.30	4.74	4.77	4.66	5.20	0.94
EuCP																
Kp **0.02**	35.30	33.72*	40.83*	30.73*	39.54*	0.28	32.30	38.12*	37.22*	0.09	31.64	36.55*	38.38*	28.41*	28.75*	0.28
Kp **0.04**	48.08	45.63*	49.64	42.55*	49.61	0.40	45.54	50.85*	51.83*	0.14	44.55	44.63	44.73	42.66	43.34	1.08
Kp **0.06**	55.28	52.51*	54.31	49.51*	54.31	0.29	53.44	58.66*	60.16*	0.27	51.94	49.28*	52.28	51.53	51.92	0.26
Kp **0.08**	60.36	57.53*	57.93*	54.86*	59.67	0.38	58.78	64.08*	66.44*	0.38	56.96	52.72*	55.59*	57.41	57.93*	0.18
Kp **0.10**	64.25	61.54*	60.53*	58.30*	62.72*	0.25	63.25	67.73*	70.87*	0.14	60.98	55.50	58.49	61.69	62.32	0.13
slope	-14.82	-21.45	-18.31	-15.70	-12.67		-33.89	-34.77	-38.95		-21.45	-18.66	-22.50	-24.01	-24.88	
intercept	86.87	125.05	132.96	89.35	83.96		189.44	208.05	221.56		120.77	129.58	158.64	127.64	130.80	

1: corn silage as untreated (control, $CS_{0.0}$) or treated with 10.8 ($CSU_{10.8}$) and 21.6 ($CSU_{21.6}$) g/kg DM urea, or 800 (CSB_{800}) and 9600 (CSB_{9600}) cfu/kg Biomin® inoculant; Alfalfa silage
as untreated (control, $AS_{0.0}$) or treated with 4 ml/kg (ASF_4) and 4.4 ($ASF_{4.4}$) ml/kg formic acid; Whole barley silage as untreated (control, $BS_{0.0}$) or treated with 10.8 ($BSU_{10.8}$) and 21.6 ($BSU_{21.6}$) g/kg DM urea, or 4 ml/kg formic acid (BSF_4), or 800 cfu/kg (BSB_{800}) Biomin® inoculant; *Within a row, means with an asterisk differ significantly from control (P<0.05); SEM=standard error of means

Table 2: *In vitro* intestinal utilizable crude protein (g/kg DM) after 8 and 48 h of incubation, metabolizable energy at 24 h, effective uCP (g/kg DM) at different assumed rates of passage and also slope and intercept obtained from regression of uCP against Ln.(time) for untreated and treated with various additives of corn, alfalfa and whole barley silages.

to proper fermentation in corn silage. The fermentation quality of the experimental corn silage than alfalfa silage was relatively good, due to higher water soluble carbohydrate concentration and lower pH, the small differences between corn silage and its hay was not unexpected. Huhtanen and Broderick [42] noted that there is no evidence for greater protein value of grass hay compared with well-fermented grass silage.

Ruminal and post-ruminally digestibility

Ruminal, post-ruminal of ruminal-undegraded and total tract DM, CP and NDF disappearance (g/kg) of untreated and treated with various additives of corn, alfalfa and whole barley silages are presented in Table 3. Throughout the course of the experiment, there were significant additives effects on ruminal and post-ruminal disappearance of the nutrients evaluated (P<0.05). Ruminal CP disappearance was higher for urea-treated corn and whole barley silages at both levels than those of $CS_{0.0}$ and $BS_{0.0}$ (P<0.05). Formic acid and inoculant treated silages did not protect protein from ruminal degradation. The results are in line with Gasior and Brzoska [44] who claimed that no significant effect of formic acid and inoculant is obtained on rumen protein degradability of grass silages. On the contrary, some investigators showed that formic acid supplementation decrease protein [45] and DM degradability [46] in both silage and rumen, suggesting that ruminal protein degradation is associated with the extent of nitrogen compound degradation during ensilage. In addition, no significant effect of silage additives on rumen protein degradation is measured by the *in sacco* method [47,48]. Inoculation and formic acid had no effect on NDF disappearance in the rumen (P>0.05), but ruminal NDF disappearance was greater for $CSU_{10.8}$ and $CSU_{21.6}$ as well as $BSU_{21.6}$ than those of $CS_{0.0}$ and $BS_{0.0}$. Keady and Murphy [49] observed negative response to the inclusion of formic acid to ryegrass silage for NDF digestibility. On the other hand, Cushnahan and Mayne [50] showed no effect of restricting silage fermentation on NDF digestibility. Gasior and Brzoska [44] reported that NDF digestibility by applying inoculant to grass silage cause 5.5% increase. Weimer [51] suggested that the buffering effect of silage inoculant on rumen pH may be a possible explanation for the beneficial effects of inoculated silage on fiber digestibility. Preparation of silages with formic acid increased post-ruminal disappearance of DM in $ASF_{4.4}$ and BSF_4 than $AS_{0.0}$ and $BS_{0.0}$. Nevertheless, it was approximately 15%

	Treatments1															
	Corn silage						Alfalfa silage				Whole barley silage					
Items	$CS_{0.0}$	$CSU_{10.8}$	$CSU_{21.6}$	CSB_{800}	CSB_{9600}	SEM	$AS_{0.0}$	ASF_{4}	$ASF_{4.4}$	SEM	$BS_{0.0}$	$BSU_{10.8}$	$BSU_{21.6}$	BSB_{800}	BSF_{4}	SEM
Ruminal																
DM	568.96	571.48	586.80	539.83	538.86	15.33	696.52	718.80	715.91	17.50	514.50	487.36	530.38	540.75	509.27	10.79
CP	563.36	591.44*	646.66*	579.19	574.11	16.56	807.25	748.53	794.73	29.86	640.71	743.33*	749.72*	628.82	587.96	20.33
NDF	411.29	682.47*	650.55*	500.25	403.19	51.37	300.28	274.41	246.33	29.10	280.9	319.92	520.48*	360.40	240.39	35.97
Post-ruminal of ruminal-undegraded																
DM	434.88	433.49	438.93	438.33	425.16	11.06	333.66	341.50	437*	16.91	351.66	333.66	326.33	305*	390.83*	8.19
CP	872.61	830.19*	800.28*	856.31	856.51	8.53	610.76	660.59	712.13*	23.87	837.67	763.89*	791.08*	856.52	852.12	5.81
NDF	7.20	6.42	5.58	8.81	6.70	0.65	7.88	6.10	6.68	0.57	7.29	9.06	7.92	6.47	7.01	0.83
Total Tract																
DM	773.83	768.10	768.45	734.94	740.99	10.94	760.43	824.36*	839.59*	11.78	679.50	651.21	683.56	680.67	700.75	9.33
CP	938.92	930.13	932.10	935.12	934.51	4.67	925.55	858.53	863.01	46.60	941.97	930.19	933.10	946.55	946.27	7.11
NDF	445.40	696.11*	671.25*	530.04	431.64	32.10	328.02	306.59	270.89	26.08	326.80	360.84	544.12*	419.56	294.89	40.18

1: corn silage as untreated (control, $CS_{0.0}$) or treated with 10.8 ($CSU_{10.8}$) and 21.6 ($CSU_{21.6}$) g/kg DM urea, or 800 (CSB_{800}) and 9600 (CSB_{9600}) cfu/kg Biomin® inoculant; Alfalfa silage
as untreated (control, $AS_{0.0}$) or treated with 4 ml/kg (ASF_{4}) and 4.4 ($ASF_{4.4}$) ml/kg formic acid; Whole barley silage as untreated (control, $BS_{0.0}$) or treated with 10.8 ($BSU_{10.8}$) and 21.6 ($BSU_{21.6}$) g/kg DM urea, or 4 ml/kg formic acid (BSF_{4}), or 800 cfu/kg (BSB_{800}) Biomin® inoculant; *Within a row, means with an asterisk differ significantly from control (P<0.05); SEM=standard error of means

Table 3: Ruminal, post-ruminal of ruminal-undegraded and total tract DM, CP and NDF disappearance (g/kg DM) of untreated and treated with various additives of corn, alfalfa and whole barley silages.

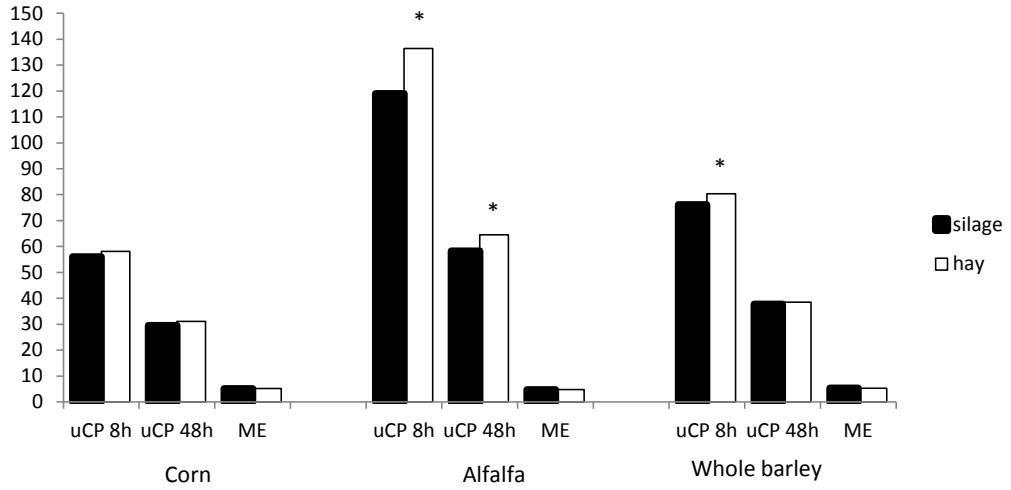

Figure 2: *In vitro* intestinal utilizable crude protein and metabolizable energy of corn, alfalfa and whole barley silages and hays.

lower for whole barley silage treated with Biomin inoculant than $BS_{0.0}$ (305 vs. 351.66). Post-ruminal disappearance of ruminal-undegraded protein increased significantly in $ASF_{4.4}$ and decreased in urea-treated corn and whole barley silages (P<0.05). These results confirmed the findings of Nowak et al. [12] who reported high intestinal protein digestibility when silages are treated with formic acid. The lowest post-ruminal digestibility of ruminal-undegraded protein in silage prepared with urea may have resulted from the greater protein degradability in rumen [46]. Post-ruminal NDF disappearance was similar for three silages than their untreated controls and was approximately 5-9% in the intestine which is fairly similar with the study of Lopes et al. [52] and estimation of Cornell Net Carbohydrate and Protein System Model [53] which reported that hindgut digestion of NDF is assumed to be 10% of total NDF digestion. Throughout the present study, total tract protein disappearance was not significantly affected by the silage additives (P>0.05). This result is in agreement with Nowak et al. [12] who reported that total tract protein digestibility is not affected by inoculation and inclusion of formic acid to grass silage. There were no significant differences in corn and whole barley silages supplemented with the additives for DM disappearance, but treated alfalfa silages had higher dry matter disappearance than $AS_{0.0}$. Ruminal CP disappearance of corn, alfalfa and whole barley was affected by preservation method and was lower in hays compared with silages (Figure 3). The present results were confirmed by Jaakkola and Huhtanen who noted that the ruminal protein degradation in dairy cattle fed hay-based diets is lower than that in cattle fed silage-based diets [54]. A greater difference in ruminal CP degradation of grass silage compared with hay is observed [55]. Ruminal DM disappearance was higher in corn and alfalfa silages compared with their hays (P<0.05). Alfalfa hay had higher post-ruminal CP dissapparance than alfalfa silage (P<0.05).

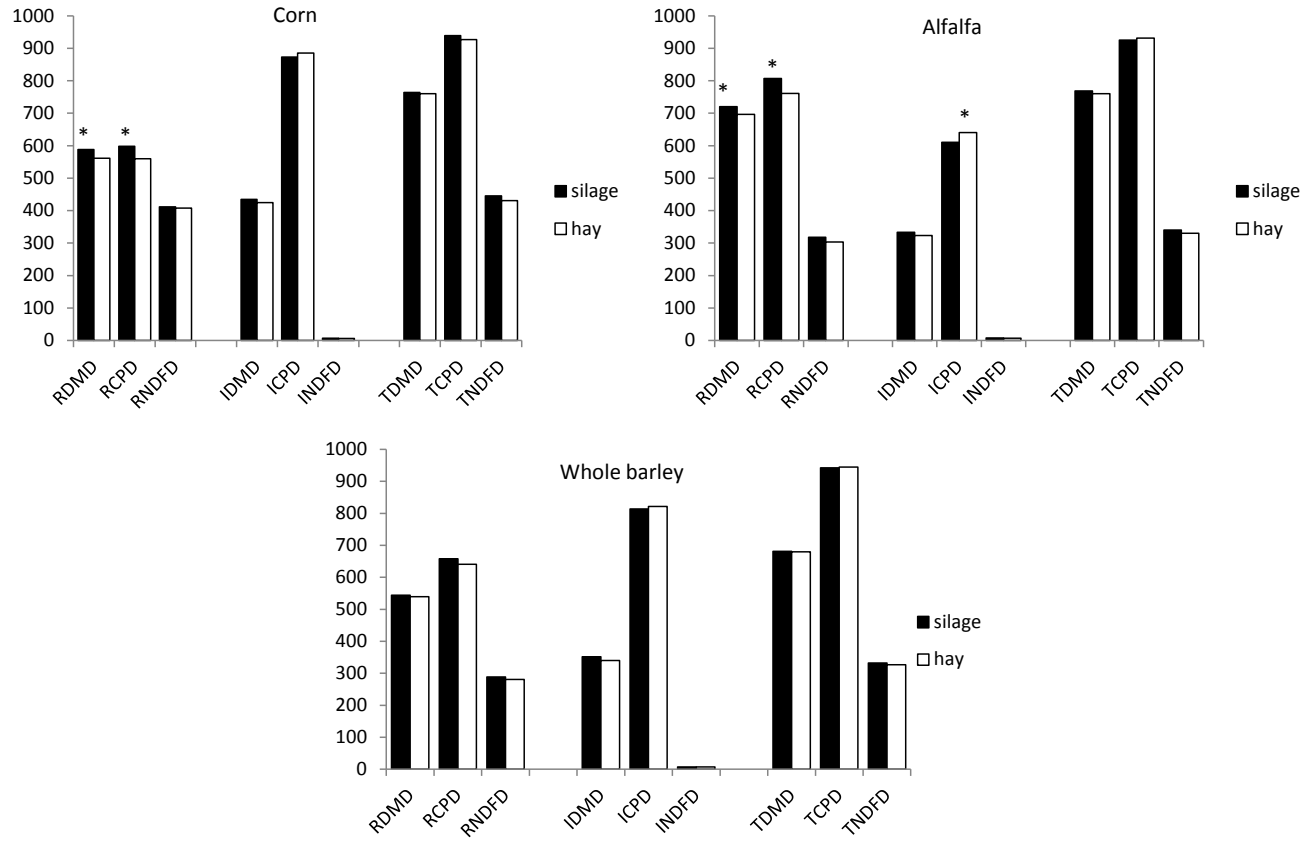

Figure 3: *In situ* mobile bag nutrient disappearance of corn, alfalfa and whole barley silages and hays (RDMD: rumen DM disappearance; RCPD: rumen CP disappearance; RNDFD: rumen NDF disappearance; IDMD: intestinal DM disappearance; ICPD: intestinal CP disappearance; INDFD: intestinal NDF disappearance; TDMD: total tract DM disappearance; TCPD: total tract CP disappearance; TNDFD: total tract NDF disappearance).

Although NDF disappearance was numerically higher in silages than hays, the differences were not significant (P>0.05). Increase of DM and NDF digestibility in silages could be due to the change of chemical composition that occurred in preservation type. Change in cell wall structure during ensiling fermentation and enzyme activity (plant and microorganism) might result in significant changes in structural polysaccharide affecting fiber concentration and digestibility [56].

Total tract unavailable CP

Results of the linear regression analysis between *in vitro* intestinal unavailable crude protein using modified gas test at assumed passage rate of 0.06/h (1- EuCP) and in situ unavailable crude protein calculated from mobile bag technique of corn, alfalfa and whole barley silages are shown in Figure 1. We found a high correlation between modified gas test and mobile bag technique methods for alfalfa silage (r^2=0.93, P=0.0003) and moderate correlation for whole barley silage (r^2=0.39, P=0.012). Nevertheless, correlation between two methods for total tract unavailable CP of corn silage was very weak (r^2=0.009, P=0.73). These results indicated that the correlation between two techniques is dependent on the type of silage. The higher correlation for alfalfa silage suggested that modified gas test provide realistic estimates of indigestible crude protein in intestine for legumes or forages with high protein level and can be used as an alternative method instead of mobile bag method which is more expensive and expend more time than gas method.

Conclusion

The results of the current study indicated that the inclusion of urea to corn and whole barley silages increased *in vitro* utilizable crude protein at duodenum and EuCP as well as alfalfa silage treated with formic acid. Urea also caused increase ruminal CP disappearance and reduce post-ruminal CP disappearance. Formic acid increase post-ruminal CP disappearance in alfalfa silage and led to increase of microbial protein synthesis and consequently uCP in silages treated formic acid. Therefore, urea was the appropriate additive for corn and whole barley silages same as formic acid for alfalfa silage. This study also shows that the method of forage conservation affects dry matter and crude protein disappearance. Forage conserved as hays compared with silages resulted in a greater uCP. It is speculated that hays appears to alter site digestion from rumen to intestine.

Acknowledgements

Researchers wish to acknowledge funding from department of Animal Science, Ferdowsi University of Mashhad, Iran.

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