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The Effect of Physically Effective Fiber and Soy Hull on the Ruminal Cellulolytic Bacteria Population and Milk Production of Dairy Cows

R. Valizadeh[1], M. Behgar[2,] *, M. Mirzaee[2], A. A. Naserian[1], A. R. Vakili[1] and S. Ghovvati[1]

[1] Faculty of Agriculture, Excellence Center in Animal Science, Ferdowsi University of Mashhad, P. O. Box: 91775-1163, Mashhad, Iran

ABSTRACT : This study was conducted to evaluate the effects of the particle size (PS) of alfalfa hay (AH) and soybean hull (SH) on milk production of dairy cows and the population of major cellulolytic bacteria in the rumen. Eight lactating Holstein cows, averaging 590±33 kg BW and 47±13 days in milk (DIM), were assigned in a 4×4 Latin square design to a 2×2 factorial arrangement of treatments: alfalfa hay particle size (fine vs. coarse) combined with soy hull (zero or substituted as 50% of AH). The cows were fed diets formulated according to NRC (2001). Physically effective factor (pef) and physically effective fiber (peNDF) contents of diets increased by increasing AH particle size and inclusion of SH in the diets ($p<0.01$). Dry matter intake was not significantly affected by treatments but intake of peNDF was increased marginally by increasing the PS of AH ($p = 0.08$) and by SH inclusion ($p<0.01$) in the diets. Milk production was increased by feeding diets containing SH ($p = 0.04$), but it was not affected by the dietary PS. Milk fat content was increased by increasing AH particle size ($p = 0.03$) and decreased by SH substitution for a portion of AH ($p<0.01$). The numbers of total bacteria and cellulolytic species were not affected by PS of AH or by SH. *F. succinogenes* was the most abundant species in the rumen followed by *R. albus* and *R. flavefaciens* ($p<0.01$). This study showed that SH cannot replace the physically effective fiber in AH having either coarse or fine particle size. In diets containing SH, increasing of diet PS using coarse AH can maintain milk fat content similar to diets without SH. Particle size and peNDF content of diets did not affect the number of total or fibrolytic bacteria in the rumen. (**Key Words :** Physically Effective Fiber, Soy Hull, Cellulolytic Bacteria, Rumen, Real Time PCR, Dairy Cows)

INTRODUCTION

Soybean hull (SH) is a source of non-forage fiber (NFF) that contains a large proportion of digestible NDF and low lignin (Garlebs et al., 1988), and has been considered as a useful replacement for forages. However, NFF sources generally have high density and small particle size (Bhatti and Firkins, 1995) that restrict their utilization by ruminants. Both SH and cottonseed hull are less effective fiber sources (20% and 85%, respectively) compared to hypothetical long grass containing 100% NDF (Mertens, 1986). The average NDF values of NFF sources are lower than those of forages (NRC, 2001).

Substitution of AH with SH has resulted in reduced milk fat content, rumination activity (Weidner and Grant, 1994b) and reduced ruminal mat consistency (Weidner and Grant, 1994a). Long hay has been shown to interact with SH to increase milk fat and ruminal mat consistency, decrease the passage rate of the byproduct, increase rumination activity, and increase extent of ruminal digestion (Weidner and Grant, 1994a,b).

Coarse hay in the diet increases physically effective NDF (peNDF) content (Teimouri Yansari et al., 2004; Yang and Beauchemin, 2005). Increasing dietary peNDF results in increased chewing activity and rumen pH (Beauchemin et al., 2003), as well as improving total tract nutrient digestibility (Yang and Beauchemin, 2005) and microbial protein synthesis in lactating dairy cows (Yang et al., 2002).

Although many data are available on the effects of dietary PS and peNDF on milk production, chewing activity and rumen condition of dairy cows, very few reports can be found on the effect of these physical factors on the rumen bacterial population. The effects of diet PS on rumen

* Corresponding Author: M. Behgar. Tel: +98-261-4464060, Fax: +98-261-4464061, E-mail: mbehgar@nrcam.org

[2] Agricultural, Medical and Industrial Research School, P. O. Box: 31485-498, Karaj, Iran.

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fermentation may be derived from change in the count of major rumen cellulolytic bacteria. Tajima et al. (2001) used a real time PCR method and showed the quantity of cellulolytic bacteria DNA declined in cattle fed more grain instead of hay. Real time PCR, has been successfully used also for enumerating rumen microflora in sheep (Mosoni et al., 2007) and swamp buffalo (Wanapat and Cherdthong, 2009) using primers for 16S rRNA gene. Zebeli et al. (2007) observed that the counts of total eubacterial cells, *Ruminococcus albus* and *R. flavefaciens* were not affected by dietary PS. The main objectives of this study were to evaluate the effects of alfalfa PS and SH on feed intake, milk production, milk composition, and on the count of rumen cellulolytic bacteria in Holstein lactating cows.

MATERIALS AND METHODS

Cows, diets and treatments

Eight lactating Holstein cows, with an initial average body weight of 590±33 kg and 47±13 days in milk (DIM), were housed in individual stalls and milked three times a day at 0600, 1400 and 2200 h. Cows were offered a TMR twice daily at 0800 and 1600 h for *ad-libitum* consumption. The experiment was designed as a 4×4 Latin square with four periods in a 2×2 factorial arrangement of treatments: alfalfa hay PS (fine vs. coarse) combined with SH content (zero or substituted as 50% of AH). The diet was formulated to supply adequate metabolizable energy and protein for a 650 kg cow producing 45 kg/d of milk (NRC, 2001; Table 1). The basal diet contained 60% barley and maize-based concentrate and 40% forage. In the basal diet and in the diets containing SH, 63% and 47% of diet NDF was from forages. Differences in average PS and the pef of the diets were created by altering the PS of AH and by substitution of SH for AH. Alfalfa hay was chopped using a small chopper fitted with either 5 mm or 20 mm sieves to obtain hay with fine and coarse PS. Each experimental period consisted of 14 d adaptation and 5 d data collection including measurement of dietary PS distribution, DMI, ruminal cellulolytic bacteria population, milk production and milk composition.

Feed intake and milk production

Feed intake and milk production were measured daily during the last 5 d of each period. Feed and ort samples were collected daily and composited weekly for DM determination. Composited samples were dried (60°C) and ground through a 2 mm screen (Retsch Cutting Mill Retschmule) for subsequent analysis. NDF was determined using the method of Van Soest et al. (1991) without amylase application.

Daily milk production was recorded. Composites of

Table 1. Ingredients and chemical composition of the total mixed diets (DM basis)

	Diets			
	AH		AH+SH	
	Fine	Coarse	Fine	Coarse
Ingredients (%)				
Alfalfa hay	20.01	20.01	10	10
Soy hull	-	-	10	10
Corn silage	20.01	20.01	20	20
Barley grain	14.01	14.01	13.99	13.99
Corn grain	14.01	14.01	13.99	13.99
Soybean meal	18.99	18.99	17.52	17.52
Sugar beet pulp	4.99	4.99	6.52	6.52
Wheat bran	4.99	4.99	4.98	4.98
Protected fat[1]	2.00	2.00	2.00	2.00
Calcium carbonate	0.30	0.30	0.30	0.30
Salt	0.19	0.19	0.19	0.19
Vitamin and mineral premix	0.50	0.50	0.50	0.50
Chemical (% of DM)				
DM	59.56	60.56	59.02	59.78
OM	92.65	92.65	92.50	92.50
CP	17.10	17.10	17.10	17.10
NDF	31.30	31.30	32.30	32.30
NDF from forage	19.80	19.80	15.30	15.30
NEL (Mcal/kg)	1.53	1.53	1.57	1.57

[1] Energizer RP-10. IFFCO. Malaysia SDN. BHD. Company No 485777-W. PLO 406-Jalan Emas, 81700 Pasir Gudang, Johor Malaysia.

three daily milkings were collected twice in the last five days of each period (days 16 and 19) and analyzed for fat, protein, lactose and solids not fat (SNF) content using a milk analyzer (Astori, Italy).

Particle size distribution

The Penn State Particle Separator (PSPS) was used to measure PS distribution of fiber sources (i.e. SH, AH and corn silage) and the diets as described by Kononoff et al. (2003). Physically effective NDF was estimated by multiplying the NDF concentration on each sieve (19.0, 8.0 and 1.18 mm) by the amount of DM retained on the same sieves (Yang and Beauchemin, 2006).

Rumen sampling and DNA extraction

Samples of ruminal fluid (250 ml) were obtained before (0800 h) feeding using a suction pump with a flexible polyvinyl chloride stomach-tube. Samples were squeezed through cheese cloth layers, then, after pH determination (Metrohm 691), stored at -20°C for later DNA extraction. After thawing, samples were shaken and transferred to 1.5-ml micro tubes containing glass beads and vortexed twice for 2 min with incubation on ice between shakings. Tubes were centrifuged at 200×g for 5 min at 4°C for the sedimentation of feeds particles. The supernatants (200 μl) were transferred to fresh 1.5 ml micro tubes and DNA extraction was performed using a genomic DNA Extraction Kit (AccuPrepTM, Bioneer Corporation) with spin columns.

Real-time polymerase chain reaction design and assay conditions

Total bacterial, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* rDNA concentrations were measured using real time PCR and the SYBR Green PCR Master Mix Kit (SYBR Green I qPCR Master Mix, Syntol, Russia). The 16S rRNA gene-targeted primer sets used in the present study are described in Table 2. Templates (1 μl) were added to amplification reactions (25 μl) containing 0.6 μl of primer mixture containing 10 pmol of each primer, 11.5 μl of SYBR Green I qPCR Master Mix (Syntol) and 12 μl of deionized water. SYBR Green I qPCR Master Mix contained KCl, Tris-HCL (pH 8.8), 6.25 mM MgCl2, dNTP, Taq DNA polymerase, Tween, and SYBR Green I. A no-template (sterile distilled water) negative control was loaded on each plate run to screen for contamination and dimmer formation and to set the background fluorescence for plate normalization. Amplification and detection were performed using an ABI 7300 (Applied Biosystems) sequence detection system under the following conditions: initial denaturation at 95°C for 5 min was followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 61°C for 15 s, extension at 72°C for 30 s, and then by the melting curve program (60-95°C with a heating rate of 0.1°C per second and a continuous fluorescence measurement).

Simultaneously, DNA extracted from each animal was subjected to real-time qPCR for all of the bacteria (total and three cellulolytics). A bacterial rDNA standard curve was generated from DNA extracted from a mix (equal volumes) of 24 cultures of the following rumen bacterial strains grown on Hobson's medium 2 (Stewart et al., 1997): *Prevotella ruminicula* 23, *Butyrivibrio fibrisolvns SH13*, *Ruminococcus albus SY23*, *Prevotella albensis M384*, *Clostridium sticklandii 12 662*, *Peptostreptococcus anaerobius 27 337*, *Ruminococcus falvefaciens Fd1*, *Mitsuokella multiacidus D15d*, *Veillonela parvula L59*, *Prevotella bryantii B14*, *Prevotella brevis GA33*, *Lactobacillus casei LB17*, *Clostridium aminophilum 49 906*, *Streptococcus bovis ES1 and Megasphera elsdenii J1*, all obtained from the Rowett Research Institute (Aberdeen, UK) culture collection.

For total bacteria the threshold cycle of each standard dilution was determined during the exponential phase of amplification and regressed against the logarithm of known total bacterial DNA standards that had been prepared for each animal. Total bacteria population size is reported as nano gram (*ng*) per μl of extracted DNA. The copy number of total bacteria 16S ribosomal RNA gene was determined as: Log10 copy number = Ct-(y-intercept/efficiency) where the formula parameters were derived from a standard curve of total bacteria. The population sizes (copy number) of cellulolytic bacteria were expressed relative to the estimated abundance of total bacterial 16S ribosomal RNA gene. All

Table 2. PCR primers utilized for amplifying the target bacteria

Target species	Forward/reverse	Primer sequence	References
Total bacteria	F	GTGSTGCAYGGYTGTCGTCA	Maeda et al. (2003)
	R	ACGTCRTCCMCACCTTCCTC	
F. succinogenes	F	GTTCGGAATTACTGGGCGTAAA	Zhang et al. (2008)
	R	CGCCTGCCCCTGAACTATC	
R. flavefaciens	F	CGAACGGAGATAATTTGAGTTTACTTAGG	Zhang et al. (2008)
	R	CGGTCTCTGTATGTTATGAGGTATTACC	
R. albus	F	CCCTAAAAGCAGTCTTAGTTCG	Koike and Kobayashi (2001)
	R	CCTCCTTGCGGTTAGAACA	

Table 3. Particle size and physically effective fiber of used hays (% DM)

	Coarse alfalfa	Fine alfalfa	SH	Corn silage	SE
19 mm	1.66[b]	0[b]	0[b]	57.85[a]	1.19
8 mm	28.79[b]	0.95[c]	0[c]	36.21[a]	1.10
1.18 mm	32.98[c]	41.8[b]	77.62[a]	5.41[d]	1.38
pan	36.57[b]	57.26[a]	22.38[c]	0.53[d]	1.17
pef	0.62[c]	0.43[d]	0.78[b]	0.99[a]	0.12
peNDF	30.90[c]	21.16[d]	38.54[b]	66.69[a]	0.57
X_{gm} (mm)[1]	3.12	1.58	0.52	19.04	-
S_{gm} (mm)[2]	3.10	2.13	3.35	1.86	-

[1] Calculated geometric mean length (ASAE, 2001). [2] Calculated standard deviation (ASAE, 2001).
[a, b, c] Means in the same row with no common superscript differ (p<0.01).

post-run data analyses were performed using SDS Software (Sequence Detector Software, V1.4).

Statistical analysis

The statistical model was: $Y_{ij(kl)m} = \mu+SQ_m+Period\ (SQ)_{im}+Cow\ (SQ)_{jm}+A_{(K)}+B_{(l)}+(AB)_{(kl)}+\varepsilon_{ij(kl)m}$, where $Y_{ij(k)m}$ = observation *ij(kl)m*; μ = the overall mean; SQ_m = the effect of square (parity); Period $(SQ)_{im}$ = the effect of period *i* within square m; Cow $(SQ)_{jm}$ = the effect of cow *j* within square m; $A_{(k)}$ = the effect of level *k* of particle size; $B_{(l)}$ = the effect of level *l* of SH; $(AB)_{kl}$ = the effect of interaction of level *k* of particle size with level *l* of SH and $\varepsilon_{ij(kl)m}$ = random error with mean 0 and variance σ^2. Data were analyzed using the GLM procedure of SAS (V 9.0). Data of particle size distribution of fiber sources and pooled data of major cellulolytic bacteria in the rumen of cows were subjected to Student's t-test at p<0.01. All data were tested for normality using Proc UNIVARIATE in SAS (V 9.0).

RESULTS

Particle size distribution and peNDF contents of fiber sources used in the diets are shown in Table 3. The proportion of fine AH and SH DM retained on the medium (8 mm) sieve was significantly (p<0.01) lower than coarse AH and corn silage. Most SH particles were retained on the 1.18 mm sieve (77.62% of DM) compared with other sources. The pef value, as measured by the proportion of particles DM>1.18 mm, was highest for corn silage, followed by SH and alfalfa hays and differences among sources were significant (p<0.01). The same trend was observed for peNDF.

Particle size distributions among diets were significantly affected by PS of AH and SH included in the diets (Table 4). Fine chopping significantly (p<0.01) decreased the dietary DM retained on the medium and 1.18 mm sieves but significantly increased diet DM on the bottom pan. Soy hull significantly increased DM of diets retained on 19 mm (p = 0.01) and 1.18 mm sieves (p = 0.04). Coarse AH and replacement of SH both significantly increased pef and peNDF contents of the diet (p<0.01). Geometric mean length decreased when fine AH was included in diets.

Intake, milk production and milk composition are presented in Table 5. The effect of treatments on DMI was not significant. Cows fed diets containing AH with coarse PS or SH had greater intake of peNDF than cows fed diets containing fine AH or without SH. Although DMI was not different among diets, actual milk yield was higher with SH-containing diets than for those without SH (p = 0.04).

Table 4. Particle size distribution, pef and peNDF contents of diets (% DM)

	Diets				SE	p value		
	AH		AH+SH					
	Fine	Coarse	Fine	Coarse		PS	SH	PS×SH
19 mm	3.92	5.02	5.83	5.52	0.46	0.40	0.01	0.14
8 mm	16.19	23.12	17.81	21.65	0.85	<0.01	0.93	0.08
1.18 mm	44.28	41.24	45.47	43.84	0.88	0.01	0.04	0.43
pan	35.62	30.62	0.31	0.29	1.09	<0.01	<0.01	0.17
pef	0.66	0.69	69.10	71.01	0.11	<0.01	<0.01	0.17
peNDF	26.56	29.51	31.78	31.61	0.54	<0.01	<0.01	0.06
dgm (mm)[1]	2.85	3.42	3.25	3.48	-	-	-	-
Sgm (mm)[2]	2.90	3.04	2.97	2.99	-	-	-	-

[1] Calculated geometric mean length (ASAE, 2001). [2] Calculated standard deviation (ASAE, 2001).

Table 5. Means of intake, rumen pH and milk production and composition of experimental cows

	Diets				SE	p value		
	AH		AH+SH					
	Fine	Coarse	Fine	Coarse		PS	SH	PS×SH
DMI (kg/d)	22.29	21.68	21.99	22.83	0.59	0.84	0.48	0.24
peNDF (kg/d)	5.90	6.25	6.86	7.15	0.17	0.08	<0.01	0.84
Milk production (kg/d)	34.59	34.24	36.26	35.87	0.71	0.60	0.04	0.98
FCM production (kg/d)	31.41	31.54	30.467	32.72	0.97	0.24	0.90	0.29
Milk composition (%)								
Fat	3.55	3.52	2.95	3.47	0.1	0.03	<0.01	0.01
Protein	3.06	3.04	3.05	3.05	0.02	0.51	0.89	0.76
SNF	8.27	8.33	8.33	8.29	<0.01	0.95	0.87	0.50
Lactose	4.58	4.53	4.87	4.43	0.15	0.44	0.20	0.87
Milk composition (kg/d)								
Fat	1.12	1.10	0.91	1.13	0.06	0.09	0.15	0.07
Protein	0.96	0.95	0.93	1.00	0.03	0.28	0.85	0.23
SNF	2.59	2.61	2.53	2.71	0.08	0.21	0.85	0.35
Lactose	1.43	1.42	1.47	1.45	0.06	0.82	0.58	0.95
Milk/DMI	1.57	1.72	1.66	1.58	0.45	0.77	0.77	0.02
FCM/DMI	1.43	1.59	1.40	1.44	0.50	0.06	0.08	0.23
Rumen pH	6.67	6.80	6.80	6.70	0.09	0.84	0.85	0.21

Fat corrected milk (FCM) production was similar among experimental diets. As expected, percentage of milk fat decreased with decreasing PS of AH ($p<0.03$) and SH inclusion ($p<0.01$) in diets. Protein, lactose and SNF percentages were not significantly different among treatments and averaged 3.05, 4.60 and 8.31, respectively. Milk fat yield tended to be lower with fine alfalfa PS compared with coarse alfalfa PS ($p = 0.07$). No effect was noted for yield of milk protein, SNF and lactose which averaged 0.96 kg/d, 2.61 kg/d and 1.44 kg/d, respectively. Efficiency of FCM tended to be increased by coarse alfalfa PS ($p = 0.06$) and tended to be decreased by SH inclusion in diets ($p = 0.08$).

Ruminal pH, which is shown in Table 5, was not significantly different among treatments.

The effects of treatments on ruminal bacteria populations are presented in Table 6. Data generated from real-time PCR assays for total bacteria are expressed as ng per μl of DNA extracted, while quantity of fibrolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus*) are expressed as copy number of 16S ribosomal gene abundance relative to the total bacterial 16S rDNA copy number in each cow. There were no significant differences in total bacteria and cellulolytic bacteria population among treatments. The effect of treatments and cows (not shown) on the bacteria populations were not significant, therefore data on number of each cellulolytic species in all cows were pooled together to examine the abundance of each cellulolytic bacteria in the rumen (Table 7). *F. succinogenes* was the most abundant species in the rumen followed by *R. albus* and *R. flavefaciens* ($p<0.01$).

DISCUSSION

Soy hull used in the present study had high seed content resulting in high CP and fat content (20.5% and 6.9% based on DM, respectively). NDF content of SH was 49.7% and

Table 6. Population of total bacteria[1] and cellulolytic bacteria[2] in the rumen

	Diets				SE	p value		
	AH		AH+SH					
	Fine	Coarse	Fine	Coarse		PS	SH	PS×SH
Total bacteria	20.04	20.25	19.73	20.70	0.44	0.21	0.83	0.40
F. succinogenes	17.80	18.10	17.57	18.43	0.47	0.25	0.85	0.58
R. flavefaciens	15.81	15.79	15.22	16.28	0.43	0.26	0.92	0.24
R. albus	17.18	17.13	16.83	17.78	0.44	0.34	0.74	0.28

[1] *ng* per μl of extracted DNA. [2] Log10 copy number of 16S RNA gene per μl of extracted DNA.

Table 7. The abundance of cellulolytic bacteria in the rumen of dairy cows

Species	Number[1]	SE
Fibrobacter succinogenes	17.98[a]	0.02
Ruminococcus flavefaciens	15.78[b]	0.02
Ruminococcus albus	17.23[c]	0.02

[1] Log10 copy number of 16S RNA gene per µl of extracted DNA.
[a,b,c] Means in the same column with no common superscript differ ($p<0.01$).

was similar to alfalfa hay NDF content (49.5%), therefore the substitution of SH with AH did not alter the NDF content of diets. Although corn silage used in this study had a high percentage of particles on the 19 mm sieve (57.85% of DM), values of pef = 0.99% and peNDF = 66.99% are similar to the silage used in the study of Kononoff et al. (2003). Soy hull had more pef and peNDF compared to coarse and fine AH because of its high content of NDF and high proportion of DM retained on the 1.18 mm sieve. Similar results were noted in diets which contained SH.

Diets containing SH had more particle DM >19 mm. This finding is inconsistent with the fact that SH particles passed through the 19 mm sieve because of their small PS (Table 3). This might be because of entrapping of SH by long and wet particles of the diet, especially corn silage which was the main source of particles on the 19 mm sieve. Diets containing SH had more pef in comparison with diets containing AH, because more DM was retained on the 1.18 mm screen. This finding could be related to the broader shape of SH particles compared with the needle-like shape of AH particles.

The results of this study indicate that PS had no effect on DMI. This result is consistent with some previous studies (Clark and Armentano, 2003; Beauchemin et al., 2003; Yang and Beauchemin, 2005; Yang and Beauchemin, 2006), but others have observed DMI to be increased by reducing PS of diets (Kononoff et al., 2003; Kononoff and Heinrichs, 2003; Teimouri Yansari et al., 2004). Tafaj et al. (2007) quantitatively reviewed 25 published studies and found a weak negative relationship between DMI and forage PS only when the grass silage based TMR was fed to dairy cows. Tafaj et al. (2001) also reported that PS reduction in hay increased DMI when a low concentrate level (~200 g/kg) was offered to either dairy cows or sheep. In the present study the level of concentrate in diets was 60%, and then no effect of diet PS on intake was expected. There was no effect of forage PS on DMI for diets containing 40% forage (Tafaj et al., 2001; Beauchmin and Yang, 2005).

In the present study DMI was not affected when SH replaced AH. In contrast, DMI and NDF intake differed when SH replaced up to 42% of the forage in diets (Weidner and Grant, 1994b). In the present study, only 10% of diet DM was replaced by SH. Similar results were obtained by Halachmi et al. (2004) when SH was substituted for 16% of diet DM. Generally low substitution of diet DM with SH does not affect DMI by dairy cows (Sarwar et al., 1991; Sarwar et al., 1992; Halachmi et al., 2004).

Cows fed diets containing SH tended to consume more peNDF than cows fed diets free of SH. Although in the present study SH had more peNDF than AH, higher intake of peNDF may be due to the supply of NDF from particles with small size which could have limited sorting behavior by cows. Kononoff et al. (2003) also observed highest sorting in cows fed a diet containing corn silage with large particle size. In this study, particles exceeding 19mm were increased from 15.5% in the diet to 60.2% in orts after 24 h.

Milk yield and 4% FCM were not affected by PS of AH (averaging 35.43 and 30.94, respectively, for fine AH; 35.06 and 32.13, respectively, for coarse AH). Generally PS of diets does not affect milk production (Teimouri Yansari et al., 2004; Yang and Beauchemin, 2005; Tafaj et al., 2007). In the present study, cows fed diets containing SH produced more milk compared to cows fed diets without SH (36.07 kg/d versus 34.42 kg/d, respectively). Cows fed SH received more energy than the cows fed AH-containing diets (Table 1). This difference may have influenced milk yield. This observation is consistent with that of Halachmi et al. (2004) who found that cows which received diets containing SH (16.5% of DM) produced more actual milk and 4% FCM.

Cows fed SH produced milk with less fat compared with other treatments, in spite of higher peNDF intake for SH diets. This may be related to the peNDF structure of SH which could not stimulate rumination and digestion as well in comparison with AH (Weidner and Grant, 1994b). Coarse AH particles significantly increased milk fat percentage and also tended to increased fat yield by cows. The positive effects of dietary PS on milk fat reported by some researchers (Soita et al., 2000; Kononoff et al., 2003; Teimouri Yansari et al., 2004) is not supported by others (Yang and Beauchemin, 2006; Tafaj et al., 2007). According to Beauchemin et al. (1994) and Mertens (1997) the effect of PS on milk fat content is likely to be observed when NDF level of diets is below minimum requirements recommended by NRC (2001), i.e. 250 g of NDF and 190 g NDF derived from forage sources per kg of diet. In the present study SH replacement decreased forage NDF from 198 g/kg in the basal diet to 153 g/kg. This is likely to be the reason for increased milk fat yield by coarse particles of AH.

Wiedner and Grant (1994b) showed that addition of AH to a diet with high SH maintained milk fat content at a level similar to that of a control diet, an effect observed in our study. The observed interaction of PS and SH in the present

study confirms the positive effect of coarse alfalfa particles on utilization of SH in diets. Protein, lactose and SNF contents (3.05, 4.60 and 8.31%, respectively) of milk produced by the cows were similar across treatments. Confirmatory results were reported by others (Beauchemin et al., 1994; Clark and Armentano, 2002) that milk composition was not affected by diet particle size. According to Mertens (1997), efficiency of FCM production showing the effectiveness of dietary fiber. In the present study, efficiency of FCM tended to be increased by alfalfa PS and also tended to be decreased by SH inclusion in the diet ($p<0.1$).

The data of ruminal pH in the present study is in agreement with some reports (Kononoff and Heinrichs, 2003a; Yang and Beauchemin, 2006; Tafaj et al., 2007) but in contrast to other reports (Beauchemin et al., 2003; Teimouri Yansari et al., 2004). Tafaj et al. (2007) found a relationship between ruminal pH and PS and NDF contents of diets ($r^2 = 0.55$, n = 76) in early lactating dairy cows fed corn silage- or grass-based diets, but PS explained only 14% of the variation in pH. In this study, acetate to propionate ratio was not affected by PS of forage in diets. In the present study, concentration of VFA was not determined.

Replacing up to 30% of diet ground corn DM with SH produced no change in total and cellulolytic bacteria quantity (Mansfield and Stern, 1994). Arakaki et al. (2007) substituted SH, soybean meal and raw soybean for corn silage in dairy cow diets and found a trend ($p = 0.06$) of increasing total bacteria quantity for diets containing 16.6% of DM substituted by SH. Differences in ruminal cellulolytic and amylolytic bacteria populations were not affected. Zebeli et al. (2007) reported that the counts of total eubacterial, *R. albus* and *R. flavefaciens* in the rumen of dairy cows were not affected by PS of diets but the activity of microbial fibrolytic enzymes in the rumen increased with reducing dietary PS. Therefore, the positive effects of dietary PS on animal responses including digestion, rumen environment, and milk fat production may be simply related to the microbial activities rather than modification of their numbers in the rumen.

The results showed that *F. succinogenes* was the most abundant cellulolytic bacteria in the rumen followed by *R. albus* and *R. flavefaciens* (17.98, 17.23 and 15.78 log10 copy number of 16S RNA gene, respectively). This observation is inconsistent with previous studies showing that *R. albus* is the least abundant species among the three cellulolytic species in the rumen of cattle (Ozutsumi et al., 2006) and sheep (Michalet-Doreau et al., 2002; Koike et al., 2003). However, the data obtained in this experiment are in agreement with those reported by Tajima et al. (2001) who found *F. succinogenes* was the most abundant species in the rumen of cows fed hay.

The copy number of cellulolytic bacteria in the present study was higher than previous reports and this difference as stated by Mosoni et al. (2007) could be related to species variation, environmental factors (diet, age and health of animal, geographical location and season) and to the techniques used (primers, real time PCR versus competitive PCR).

IMPLICATIONS

The results of this study indicated that SH increased milk yield and decreased milk fat content. Supplementation of diets containing SH with coarse AH can maintain milk fat similar to diets without SH. So it can be concluded that in spite of high NDF and peNDF content, SH is not as physically effective as AH in milk fat production. It can be proposed that using only laboratory determination of peNDF by PSPS is not a reliable method of assessment of fiber effectiveness in diets containing SH or other NFF sources. It was also shown that PS and peNDF content of diets did not significantly affect counts of total and fibrolytic bacteria in the rumen of lactating dairy cows.

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