Partial and total substitution of alfalfa hay by pistachio byproduct modulated the counts of selected cellulolytic ruminal bacteria attached to alfalfa hay in sheep

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A B S T R A C T

This study was conducted to evaluate the effect of partial and total substitution of alfalfa hay (AH) by pistachio (Pistacia vera) by-product (PB) on counts of selected cellulolytic ruminal bacteria attached to AH by real-time PCR technique and to determine the effect of this replacement on degradability of AH in Baloochi sheep. Six Baloochi sheep, averaging 40.1 ± 1.77 kg BW, fitted with ruminal cannula were assigned at random to three diets in a double 3 x 3 Latin square design. The dietary treatments were control (basal diet), low PB (LPB) diet (0.50 of AH in basal diet replaced by PB), and high PB (HPB) diet (all of the AH in the basal diet replaced by PB). The daily basal diet was 400 g AH dry matter (DM), 200 g wheat straw DM, 168 g barley grain DM, 24 g cotton seed meal DM, 6.4 g vitamin–mineral supplement DM and 1.6 g salt DM. Alfalfa hay was placed in to the rumen for in situ degradability of DM, organic matter (OM) and microbial attachment. Bacterial populations were assessed by DNA extraction of incubated alfalfa samples followed by real-time polymerase chain reaction analysis. The population of Fibrobacter succinogenes and Ruminococcus albus that were attached to AH decreased with increasing the level of PB in diets (P < 0.05). The effect of dietary treatments on the population of Ruminococcus flavefaciens and total bacteria was not significant. PB replacement for AH decreased ruminal DM and OM degradability of AH (P < 0.05). It was concluded that tannins from PB reduced the attachment of cellulolytic bacteria as well as in situ degradability of AH.

1. Introduction

Pistachio farming is one of main activity in agriculture sector of central Iran and according to the Food and Agriculture Organization (FAO, 2005) Iran is the largest pistachio producer in the world. Pistachio by product (PB) contains 158.2 g/kg crude protein (CP), 69.5 g/kg ether extract (EE), 250 g/kg neutral detergent fiber (NDF) and 207.5 g/kg acid detergent fiber (ADF) (Behgar et al., 2009).

In recent years, the results of some experiments shown that PB can be used as a feedstuff for ruminants (Gholizadeh et al., 2009, 2010; Shakeri and Fazaeli, 2005; Vahmani and Naserian, 2006). However, PB contains high level of phenolic compounds such as tannins, which can affect their nutrient utilization by animals (McSweeney et al., 2001). Phenolic contents of PB include 75–95 g/kg total phenol and 35–45 g/kg tannin (Bohlul et al., 2007).

Microbial adhesion to plant material is the first step in the degradation of insoluble nutrients (Cheng et al., 1990).
The cellulolytic bacteria *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes* adhere to or are found in close proximity to plant cell wall material undergoing degradation in the rumen (Bento et al., 2005).

Tannins may affect the adhesion of rumen microorganisms to the substrate through binding to the substrate (Artz et al., 1987; Asquith and Butler, 1986; Barry et al., 1986; Makkar et al., 1995), or to the bacterial cell surface (Bae et al., 1993; Jones et al., 1994; Molan et al., 2001). It has been suggested that phenolic monomers decrease cellulose and xylan digestion by inhibiting the attachment of ruminal cellulolytic bacteria like *F. succinogenes* to fiber particles (Akin et al., 1988; Varel and Jung, 1986). Also, filter paper digestion and endoglucanase activity by *F. succinogenes*, were inhibited in a dose dependent manner by purified condensed tannin (CT) (Bae et al., 1993).

However, it is notable that the effects of tannins on attachment of bacteria to cell wall vary depending on the tannin type and the plant material (Bento et al., 2005). Very few reports can be found on the effect of tannins on attachment to plant cell wall material under different tannin sources and the concentration at which they affect the attachment of rumen cellulolytic bacteria to plant cell wall.

In previous works, the abilities of rumen cellulolytic bacteria to attach to plant fibers and the mechanisms of this attachment have been studied using pure cultures (Minato and Suto, 1978; Mosoni et al., 1997; Pegden et al., 1998). Although numerous important findings were obtained from these *in vitro* studies, it is difficult to track specific bacterial species quantitatively *in vivo* using traditional enumeration techniques such as culturing. The kinetics of fiber-attachment of ruminal cellulolytic bacteria was studied by different methods, such as PCR based methods (Koike et al., 2003) and isotope labeling methods (Bento et al., 2005). Authors could not found any information about the effects of PB tannin on the attachment of rumen cellulolytic bacteria to plant cell wall.

The objective of the present study was to investigate the effect of partial and total substitution of AH by PB on counts of selected cellulolytic ruminal bacteria attached to AH by real-time PCR technique and to determine the effect of this replacement on degradability of AH in Baloochi sheep.

### 2. Material and methods

#### 2.1. Animals and diet

Six Baloochi sheep (BW 40.1 ± 1.77 kg), fitted with ruminal cannulae were housed indoors in individual metabolism cages in a temperature controlled building (approximately 22 °C) with constant lighting. All diets were supplied as TMR, and offered at maintenance level once daily at 08:00. Clean water was freely available at all times. Sheep were randomly assigned in a double 3 × 3 Latin square design to dietary treatments. Dietary treatments consisted of control diet (basal diets), LPB diet (0.5 of AH in basal diet replaced by PB) and HPB diet (whole of AH in basal diet replaced by PB). The diets were formulated for maintenance requirements according to AFRC (1993). Basal diet consisted of 400 g AH DM + 200 g wheat straw DM + 200 g barley based concentrate DM.

The ingredients and chemical composition of the experimental diets are shown in Table 1. PB was the current year’s annual growth, hand-harvested near Bardaskan (Iran) during summer 2009. Harvested PBHH was sun cured before use. Chemical composition of PB is shown in Table 2.

Trial consisted of three periods and each experimental period consisted of 19d adaptation and 7d data collection including *in sacco* incubation of AH and measurement of rumen cellulolytic bacteria attachment to AH.

#### 2.2. Sample preparation and *in sacco* incubation of AH

Alfalfa hay as a source of fiber was used for nylon bag degradability of DM and OM. Samples were milled (2 mm) and then stored at 4 °C until ready for incubation. *In sacco* degradability was determined according to the method of Orskov (1984) and it was not effective because the rate of passage was not measured. During the experimental periods, about 5 g (DM basis) of AH sample was placed in a 12 cm × 8.5 cm, 50 µm pore size polyester bag.

### Table 1

Ingredients and chemical composition of diets used to evaluate the effect of including pistachio by-product (PB) in the diet of sheep.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g/kg DM)</th>
<th>Control</th>
<th>LPB</th>
<th>HPB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa hay</td>
<td>400.0</td>
<td>200.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Wheat straw</td>
<td>200.0</td>
<td>200.0</td>
<td>200.0</td>
<td></td>
</tr>
<tr>
<td>Pistachio by-product</td>
<td>0.0</td>
<td>200.0</td>
<td>400.0</td>
<td></td>
</tr>
<tr>
<td>Barley grain</td>
<td>168.0</td>
<td>166.0</td>
<td>164.0</td>
<td></td>
</tr>
<tr>
<td>Cotten seed meal</td>
<td>24.0</td>
<td>25.6</td>
<td>27.2</td>
<td></td>
</tr>
<tr>
<td>Vitamin–mineral premix</td>
<td>6.4</td>
<td>6.4</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>Salt</td>
<td>1.6</td>
<td>1.6</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Lime</td>
<td>0.0</td>
<td>0.4</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>918.1</td>
<td>915.6</td>
<td>909.5</td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>904.7</td>
<td>902.3</td>
<td>905.4</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>118.0</td>
<td>118.0</td>
<td>117.4</td>
<td></td>
</tr>
<tr>
<td>EE</td>
<td>14.4</td>
<td>24.8</td>
<td>35.8</td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>504.7</td>
<td>454.9</td>
<td>415.7</td>
<td></td>
</tr>
<tr>
<td>Phenolic compound</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total phenolics</td>
<td>9.1</td>
<td>29.5</td>
<td>42.5</td>
<td></td>
</tr>
<tr>
<td>Tannin</td>
<td>4.3</td>
<td>19.2</td>
<td>30.7</td>
<td></td>
</tr>
<tr>
<td>Condensed tannin</td>
<td>1.0</td>
<td>3.5</td>
<td>6.5</td>
<td></td>
</tr>
</tbody>
</table>

1 LPB (low pistachio by product), HPB (high pistachio by product).

### Table 2

Chemical composition of pistachio by-product (PB) (g/kg DM).

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount (g/kg DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td>900.0</td>
</tr>
<tr>
<td>OM</td>
<td>755.3</td>
</tr>
<tr>
<td>EE</td>
<td>58.0</td>
</tr>
<tr>
<td>CP</td>
<td>153.1</td>
</tr>
<tr>
<td>NDF</td>
<td>259.4</td>
</tr>
<tr>
<td>Total phenolic</td>
<td>78.5</td>
</tr>
<tr>
<td>Tannin</td>
<td>31.6</td>
</tr>
<tr>
<td>Condensed tannin</td>
<td>8.5</td>
</tr>
</tbody>
</table>
The incubations were performed in triplicate (three bags per time) in the rumen of each sheep immediately prior to feeding.

Bags were removed from the rumen at 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h. For time 0, nylon bags were rinsed thoroughly under tap water until the rinsed water is cleared. After removal from the rumen, bags were thoroughly washed under running cold water until it ran clear, and dried in an air-forced oven at 65 °C for 48 h to determine DM losses. Residues were analyzed for OM by standard procedures (AOAC, 1998) to determine OM losses. The percentage disappearance of DM and OM from the bags at each incubation time was calculated from the proportion remaining after incubation in the rumen.

2.3. Attachment of rumen cellulolytic bacteria and total bacteria to AH

Attachment of total and rumen cellulolytic bacteria to AH was determined according to the method of Koike et al. (2003). Using PCR based method for measuring attachment of ruminal cellulolytic bacteria, a new method. On last day of each period, four nylon bags containing chopped AH were placed into the rumen of each sheep immediately prior to feeding. Bags were removed from the rumen at 24 h after incubation. The bags were rinsed thoroughly in water (38 °C) until it ran clear and then squeezed by hand to remove excess water, and then stored at −20 °C until analysis for microbial attachment.

2.4. Chemical analyses

Samples of feed were analysed for DM, OM, EE and nitrogen by standard procedures (AOAC, 1998). NDF was determined according to Van Soest et al. (1991) without amylase application. Total phenols, tannins and CT (butanol procedure) in PB and dietary treatments were determined in aqueous acetone (70:30, acetone:distilled water) extracts, as described by Makkar (2003).

2.5. DNA extraction

The incubated bags were freeze dried. Then the samples (20–50 mg) were homogenized with a mortar and pestle and transferred to 1.5 ml tubes containing glass beads and 200 μl tissue lysis buffer and vortexed twice for 2 min with incubation on ice between shakings. This work allowed disruption of bacterial cell wall and detached bacteria from feed particles. Tubes were centrifuged at 200 × g for 5 min at 4 °C for the sedimentation of feed particles. The supernatants (200 μl) were transferred to fresh 1.5 ml tubes and DNA extraction was performed using a genomic DNA extraction kit (AccuPrepTM, Bioneer Corporation, South Korea) equipped with spin columns.

2.6. Real-time polymerase chain reaction design and assay conditions

Total bacterial, F. succinogens, R. flavefaciens and R. 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 4. 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 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 3 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 ruminicola 23, Butyrivibrio fibrisolvens SH13, Ruminococcus albus SY23, Prevotella albensis M384, Clostridium sticklandii 12 662, Peptostreptococcus anaerobius 27 337, Ruminococcus falvelfaciens Fd1, Mitsuokella multiacidus D15d, Veillonella parvula L59, Prevotella bryantii B14, Prevotella brevis GA33, Lactobacillus casei LB17, Clostridium aminophilum

<table>
<thead>
<tr>
<th>Target species</th>
<th>Forward/reverse</th>
<th>Primer sequence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacteria</td>
<td>F</td>
<td>GTGCGAGGCGGCTGCTCA</td>
<td>Maeda et al. (2003)</td>
</tr>
<tr>
<td>F. succinogens</td>
<td>R</td>
<td>ACGTCCTCCACCTTCCTC</td>
<td>Zhang et al. (2008)</td>
</tr>
<tr>
<td>R. flavefaciens</td>
<td>R</td>
<td>CGGCTCTCTATCTAGTAGTACCG</td>
<td>Zhang et al. (2008)</td>
</tr>
<tr>
<td>R. albus</td>
<td>F</td>
<td>CCTCAAAGAGCTCTAGGGCG</td>
<td>Koike and Kobayashi (2001)</td>
</tr>
</tbody>
</table>

Table 4

PCR primers utilized for amplifying the target bacteria.
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 of each standard dilution was determined during the exponential phase of amplification and regressed against the logarithm of 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.

Count of cellulolytic species attached to AH incubated in sheep fed PB containing diets were compared with those fed control diet using the methods of relative quantification (Relative fold change in genomic DNA=2^{ΔCt}, where: ΔCt=Ct treated—Ct untreated, and Ct is the cycle number at which the fluorescence generated within a reaction crosses the threshold). To achieve optimal relative expression results, all the relative comparisons were made on a constant basis of extracted DNA. Change in cellulolytic species reported as fold change in genomic DNA per μl of extracted DNA. All post-run data analyses were performed using SDS Software (Sequence Detector Software, V1.4).

2.7. Statistical analyses

Dry matter and OM disappearances were fitted to the model described by Orskov and McDonald (1979).

\[ P = a + b(1 - e^{-ct}) \]

where \( P \) represents the loss from the bag after \( t \) h, \( a \) the fraction that immediately disappears from the bag (intercept), \( b \) the fraction that is potentially degraded over time and \( c \) the rate of degradation of fraction \( b \). The equation was fitted to in situ degradation profiles using the NLIN procedure of the SAS (V. 9.0) package.

The statistical model was:

\[ Y_{ijkl} = \mu + T_i + SQ_j + Period (SQ)_k + Sheep (SQ)_l + \epsilon_{ijkl}, \]

where \( Y_{ijkl} \) = observation \( ijkl \); \( \mu \) = the overall mean; \( T_i \) = the effect of treatment; \( \epsilon_{ijkl} \) = random error with mean 0 and variance \( \sigma^2 \). All data were analyzed using the GLM procedure of SAS (V. 9.0). Before statistical analyzing data were tested for normality using Proc UNIVARIATE in SAS (V. 9.0). The tukey test was used to assess the significance of differences between treatment means where the overall treatment effect was significant \( (P < 0.05) \).

3. Results

3.1. Attachment of total and cellulolytic bacteria to AH cell wall

Data generated from real-time PCR assays for total bacteria are expressed as ng per μl of extracted DNA, while quantity of cellulolytic bacteria \( (F. succinogenes, R. flavefaciens and R. albus) \) are expressed as fold changes in genomic DNA compared to control. Population of \( F. succinogenes \) and \( R. albus \) that were attached to AH cell wall decreased with increasing the level of PB in diets \( (P < 0.05) \) (Table 4). The effect of dietary treatments on the population of \( R. flavefaciens \) and total bacteria was not significant.

3.2. Dry matter and organic matter degradability of AH

In situ ruminal DM and OM degradability and the rumen degradation parameters \( (a, b \) and \( c \) values) for DM and OM of AH are given in Table 5. The degradability was not effective. The results indicate that ruminal DM and OM degradability, immediately degradable fraction \( (a) \), potential degradable fraction \( (b) \) and rate of degradation\( (c) \) of AH decreased as the level of PB in the diet increased \( (P < 0.05) \).

4. Discussion

Population of \( F. succinogenes \) and \( R. albus \) that were attached to AH decreased with increasing the level of PB in diets of sheep. PB replacement for AH increased the levels of tannin in dietary treatments (9.1 g/kg, 29.5 g/kg and 42.5 g/kg for control, LPB and HPB, respectively) which may affect the attachment of cellulolytic bacteria to AH cell wall. Similar to our finding, Bento et al. (2005) observed that the addition of mimosa tannin to cellulose reduced microbial attachment compared with cellulose alone in \textit{in vitro} trials. Previous studies demonstrated that

### Table 4

Population of attached bacteria to alfalfa hay (AH) cell wall.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Control</th>
<th>LPB(^{t})</th>
<th>HPB(^{t})</th>
<th>SEM</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total bacteria(^{a})</strong></td>
<td>6373</td>
<td>3341</td>
<td>6235</td>
<td>1076.58</td>
<td>0.13</td>
</tr>
<tr>
<td>( F. succinogenes(^{a}) )</td>
<td>1.635(^{a})</td>
<td>1.047(^{b})</td>
<td>0.955(^{c})</td>
<td>0.019</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>( R. flavefaciens(^{a}) )</td>
<td>1.000</td>
<td>1.127</td>
<td>1.057</td>
<td>0.103</td>
<td>0.70</td>
</tr>
<tr>
<td>( R. albus(^{a}) )</td>
<td>1.437(^{a})</td>
<td>0.860(^{b})</td>
<td>0.322(^{c})</td>
<td>0.087</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

\( ^{a} \) Within rows, means with different letters are significantly different \( (P < 0.05) \).

\( ^{b} \) Within rows, means with different letters are significantly different \( (P < 0.05) \).

\( ^{c} \) Within rows, means with different letters are significantly different \( (P < 0.05) \).

\( ^{d} \) ng per μl of extracted DNA.

\( ^{e} \) Fold change compared to control.

\( ^{f} \) LPB (low pistachio by product), HPB (high pistachio by product).
the presence of tannin in pure cultures in vitro resulted in the formation of tannin–protein complexes on the cell surface of *F. succinogenes*, suggesting that the tannin may interfere with the adhesion process (Bae et al., 1993). Therefore, it is likely that the reduction in microbial attachment is related to binding of tannin to bacterial cell surface (Molan et al., 2001). Moreover, Makkar et al. (1995) showed that tannin may affect the adhesion of rumen microorganisms to the substrate through binding to it.

In the present study, PB tannin may have bound to AH cell wall resulting in a reduction in the availability of binding sites on cell wall for microorganisms. The results presented here are in agreement with previous findings, suggesting that tannins may form strong complexes with substrate and reduce adhesion of microbes (Makkar et al., 1995). Moreover, tannins are known for their antimicrobial activity (Goel et al., 2005; Guimaraes-Beelen 1 et al., 2006) either on cellulosic or proteolytic bacteria and previous studies showed that tannin reduced cellulosic bacteria population in *in vivo* (Newbold et al., 1997) and *in vitro* trials by inhibiting microbial growth (Bhatta et al., 2009; Min et al., 2005). Our previous study showed that PB tannin reduced cellulosic bacteria population in the rumen of sheep (Ghasemi et al., 2012). So, the reduced microbial attachment caused by tannin, is supported by the reduction in microbial population in the rumen (Ghasemi et al., 2012). In the present study, *R. flavefaciens* counts were not affected by PB tannin. This different susceptibility of bacteria to tannins may have resulted from the different mechanisms of attachment to substrate (Bento et al., 2005).

In the present study partial and total substitution of AH by PB increased EE (control 14.4 g/kg DM versus HPB 35.8 g/kg) and decreased NDF (control 504.7 g/kg DM versus HPB 415.7 g/kg) content of experimental diets (Table 1). It is well known that these 2 parameters affect microbial activity and their attachment to substrate. However, the level of EE in HPB diet was below the general recommendation (60–70 g/kg DM) that affect microbial activity (Jenkins, 1993; NRC, 2001). Wiemer et al. (1999) reported that decrease dietary NDF to 22.9% of DM did not affect cellulosic bacteria in the rumen of lactating dairy cows. They suggest that shift in ruminal microbial population is resulted from interaction between many factors that diet is one of these components. In the present study the level of NDF in HPB was 41.57% of DM that was higher than this amount.

Ruminal DM and OM degradability, immediately degradable fraction (*a*), potential degradable fraction (*b*) and rate of degradation (*c*) of AH decreased with inclusion of more PB in diets. Similar observations were obtained by Balogun et al. (1988), Hervas et al. (2000), Makkar et al. (1995), and Martinez et al. (2004) when studying different doses of tannins. The reduction in attachment of bacteria to AH cell wall supports these findings. The reduction of the extent of degradation of the AH by the treatments was basically due to a marked reduction of the immediately degradable fraction (*a*) and lower rate of degradation (*c*) which was observed in HPB treatment. Kumar and Vaithiyanathan (1990) have proposed that tannins reduced the rate of plant fiber degradation in the rumen by reducing the availability of protein nitrogen and sulphur for microbial use. As mentioned before, PB tannin may have bound to substrate cell wall and resulting in a reduction in degradability of them. Similar to our finding, Bohluli and Naserian (2007) showed that PB could limit digestibility of AH and soybean meal in *in vitro* condition. Moreover, Bae et al. (1993) reported that digestion of filter paper by *F. succinogenes* was reduced in the presence of tannin.

### Table 5

**Ruminal degradation profiles of alfalfa hay (AH).**

<table>
<thead>
<tr>
<th>Diets</th>
<th>Control</th>
<th>LPB&lt;sup&gt;1&lt;/sup&gt;</th>
<th>HPB&lt;sup&gt;1&lt;/sup&gt;</th>
<th>SEM</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>a</em>-value (g/g incubated)</td>
<td>0.4023&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3787&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.00031</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><em>b</em>-value (g/g incubated)</td>
<td>0.3754&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3698&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3427&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.00089</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><em>c</em> (per hour)</td>
<td>0.094&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.089&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.076&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0007</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><em>a</em> + <em>b</em> (g/g incubated)</td>
<td>0.7774&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7699&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7214&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.00098</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>OM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>a</em>-value (g/g incubated)</td>
<td>0.4085&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.4021&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3838&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.00047</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><em>b</em>-value (g/g incubated)</td>
<td>0.3624&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3598&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.3477&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.00088</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><em>c</em> (per hour)</td>
<td>0.124&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.111&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.101&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0015</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><em>a</em> + <em>b</em> (g/g incubated)</td>
<td>0.7709&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7619&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7315&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.00133</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Within rows, means with different letters are significantly different (*P* < 0.05).
<sup>b</sup> Within rows, means with different letters are significantly different (*P* < 0.05).
<sup>1</sup> LPB (low pistachio by product), HPB (high pistachio by product).
of tannins on attachment of ruminal microorganisms and degradability of different sources of feed in in sacco incubation.

Conflict of Interest

None of the authors of the manuscript entitled ‘Partial and total substitution of alfalfa hay by pistachio byproduct modulated the counts of selected cellulolytic ruminal bacteria attached to alfalfa hay in sheep’ has declared any conflict of interest within the last years which may arise from being named as an author on the manuscript.

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References


