RESEARCH ARTICLE

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Effects of supplementation alfalfa silage with molasses, orange pulp and *Lactobacillus buchneri* on *in vitro* dry matter digestibility and gas production

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ABSTRACT
This study was conducted to document the effects of supplementation alfalfa silage with molasses, orange pulp and *Lactobacillus buchneri* on *in vitro* dry matter digestibility and gas production. The treatments included: 1) alfalfa hay (control); 2) alfalfa hay with bacterial additive 3×10^8 cfu/g; 3) alfalfa hay with orange pomace; 4) alfalfa hay with orange pomace and bacterial additive 3×10^8 cfu/g; 5) alfalfa hay with 5% molasses; 6) alfalfa hay with 5% molasses and bacterial additive 3×10^8 cfu/g. Alfalfa hay harvested at flowering stage and after 24 hours wilted and mixed orange pomace with a ratio of 2100 g and 760 g, respectively, and was ensiled for 90 days. The data were analyzed in a completely randomized design with three replications. After 24 h incubation, treatments AO (alfalfa + orange pulp) and CON (without additive) had the highest and lowest *in vitro* gas production ($p<0.05$) and adding orange pulp and molasses increased gas production. Adding inoculant decreased *in vitro* DM digestibility. Results showed that ensiling alfalfa with orange pulp and molasses can improve silage quality and increased gas production and *in vitro* DM digestibility.

Key words: alfalfa silage, *Lactobacillus buchneri,* orange pulp

Introduction

Ensiling is an important technique not only for the winter season in cold and temperate zones, but also for dry season in the tropical zone to make good use of different biological materials with the highest nutritive value. Alfalfa is a forage crop with high nutritive value and is often a major component of diets for high-producing dairy cows (Albrecht & Beauchemin, 2003; Schmidt et al., 2009). It has a high buffering capacity, low water soluble carbohydrates (WSC) content and is rich in highly degradable crude protein (CP) (NRC, 2001; Buxton et al., 2003). As a result, it is more difficult to quickly reduce the silage pH, minimize clostridia growth, proteolysis and heterolytic fermentation, and to improve silage palatability compared to maize silage (McDonald et al., 1991).

Growing up feeds, cost values in many parts of the world have increased attending in the utilization of citrus by-product feedstuffs as specific feeds for ruminants. One of the citrus by-products that produced exceedingly is orange pulp and its cost is partly low compared to its nutritive value. According to the FAO (2001), the annual rate of world production of citrus fruits is about 106 million tons that the orange fruits represented the 63% of the world citrus production. Due to the perishable property of these products, it would be convenient to develop methods of preservation that would enable these by-products to be utilized for longer periods of time (Aguilera et al., 1997). According to statistics, Iran is one of 10 countries in the world's main producer of citrus pulp (Spreen, 2000; Revuelta et al., 2008) that if the waste is not properly disposed of in the environment, can in the long term become an environmental problem.

Using of bacterial inoculants as starters for silages have been recommended to ensure rapid fermentation during the early stages of ensiling, to minimize the loss of nutrients, dry matter and to accelerate the decline of pH by promoting homo-fermentation of major water soluble carbohydrates (WSC) to lactate. Rapidly decreasing pH conserves WSC and declining proteolysis and deamination by inhibiting prolonged fermentation (Muck, 1993). Microbial additives based on classical homolactic acid bacteria have been used to improve the efficiency of silage fermentations (Kung et al., 2003). However, using these types of organisms has sometimes made the silages less stable when they are exposed to air (Muck & Kung, 1997) because there is less production of organic acids with strong antifungal characteristics as a result of the actions of the additive. In

contrast, the addition of *Lactobacillus buchneri* (a heterolactic acid bacterium) to silage improves aerobic stability via anaerobic production of acetic acid (Oude Elferink et al., 2001). *Lactobacillus buchneri*, a heterofermentative LAB, has been shown to convert lactic to acetic acid under anaerobic conditions (Oude Elferink et al., 2001).

This study was conducted to document the effects of supplementation alfalfa silage with molasses, orange pulp and *Lactobacillus buchneri* on *in vitro* dry matter digestibility and gas production.

Materials and Methods

The chemical composition of wilted Alfalfa and orange pulp before ensiling is given in Table 1. The chemical compositions feeds were determined using the methods recommended by AOAC (2000). Determinations of N were conducted using the Kjeldahl method in an automated Kjelfoss apparatus (Foss Electric, Copenhagen, Denmark). Neutral-detergent fiber (NDF) and Acid-detergent fiber (ADF) were determined by the detergent procedures of Van Soest et al. (1991).

Table 1. *Chemical composition of alfalfa and orange pomace and before ensiling.*

TRT	DM (%)	pH	NDF	ADF	WCS	CP
Alfalfa	32	6.02	30.9	29.91	3.8	18.02
Orang pomace	25	4.85	24.5	22.8	5.2	6.12

Preparation of treatments

Whole fourth cut Alfalfa was harvested at 35 dry matter and wilted for 24 h at room temperature. The wilted Alfalfa and fresh Orange pulp was chopped manually to an approximately 2 cm theoretical length of cut. The treatments included: 1) alfalfa hay (control); 2) alfalfa hay with bacterial additive 3×10^8 cfu/g; 3) alfalfa hay with orange pulp; 4) alfalfa hay with orange pulp and bacterial additive 3×10^8 cfu/g; 5) alfalfa hay with 5% molasses; 6) alfalfa hay with 5% molasses and bacterial additive 3×10^8 cfu/g. Inoculant was dissolved in distilled water (recommended by a factory) and sprayed uniformly onto the treatment and for control treatment sprayed of distilled water. Experimental treatments were ensiled in triplicate laboratory mini silos for 90d at ambient temperature (15 to 18°C) in a closed barn.

In vitro trial

The amount of *in vitro* DM digestibility and gas production of treatments was measured in serum bottles according to the method of Fedorak & Hurdy (1983). Firstly, 300 mg of finely-ground silage (1 mm screen size) were weighed into 50 mL sterile serum bottles. A 20 mL mixture of rumen fluid and artificial buffer at a ratio of 1:2 (McDougall, 1948), was added to each bottle and kept under continuous CO_2 flow. The rumen fluid was obtained 2 h after the morning feeding from two rumen fistulated sheep fed a total mixed ration of 600 g concentrate and 400 g lucerne hay/kg DM. The rumen content was filtered through four layers of cheesecloth to extract the filtrate to a warm flask containing CO_2, before being transfer to the laboratory. To avoid microbial heat shock, the bottles were warmed up to 39°C for 30 min before and while adding the mixture of rumen fluid and buffer to the sample under CO_2. The bottles were tightly capped and placed in an incubator at 39°C, shaking at 120 rounds per min. For each batch in the *in vitro* study three blank bottles, containing only the rumen fluid preparations without any sample were used to adjust the results for DM originating from the rumen fluid. The amount of DM digestibility of treatments was recorded at 2, 4, 8, 12 and 24 h post-incubation and Gas production was measured in each vial after 2, 4, 8, 12, 16, 24, 36, 48, 72, 96 and 120 h of incubation using a water displacement apparatus (Fedorak & Hurdy 1983).

The metabolizable energy, net energy for lactation and digestible organic matter in dry matter content of feeds was calculated using equation of Menke & Steingass (1987) as:

$ME\ (MJ/kg\ DM) = 2.2+0.136GP+0.0057CP+0.000286CF^2$

$NEL\ (MJ/kg\ DM) = 0.54+0.096GP+0.0038CP+0.000173CF^2$

$DOMD\ (\%) = 16.49+0.9042GP+0.0492CP+0.0387CA$

The short chain fatty acid (SCFA) was calculated using equations of Menke et al. (1979):

$SCFA\ (mmol/200\ mg\ DM) = 0.0222\ GP - 0.00425$

where, GP is 24 h net gas production (ml/200 mg DM); CP, CF and CA are crude protein, crude fat and crude ash (%DM), respectively.

Microbial protein was calculated as 19.3 g microbial nitrogen per kg OMD (Czerkawski, 1986).

Analytic method

Data obtained was subjected to analysis of variance as a completely randomized design by the GLM procedure of SAS Institute Inc (2002) and treatment means were compared by the Duncan test.

Results

Effects of treatments on *in vitro* gas production are given in Table 2 and Figure 1. There were significant differences among treatments ($p<0.05$). After 24 h incubation, treatments AO (alfalfa + orange pulp) and CON (without additive) had the highest and lowest *in vitro* gas production ($p<0.05$) and adding orange pulp and molasses increased gas production. Supplementation treatments with inoculant (AB and AMB vs. CON and AM, respectively) had a significant effect on gas production and increased gas production volume ($p<0.05$) but

Table 2. *Effects of treatments on in vitro gas production at various incubation times.*

Treatments	Incubation times (h)										
	2	**4**	**6**	**8**	**12**	**24**	**36**	**48**	**72**	**96**	**120**
CON	0^{e}	17.17^{d}	33^{d}	46.67^{d}	66.17^{d}	91.08^{e}	111^{e}	123^{d}	142.97^{f}	134.41^{e}	134.41^{e}
AB	0^{e}	18.76^{cd}	36.50^{cd}	51^{cd}	71.17^{c}	98.25^{d}	120.50^{d}	133.25^{c}	145.25^{d}	148.75^{c}	148.75^{c}
AO	3.76^{a}	29.91^{a}	53.41^{a}	71.25^{a}	95.41^{a}	124^{a}	146.56^{a}	159.96^{a}	172.87^{a}	175.37^{a}	175.37^{a}
AOB	1^{ab}	23.75^{b}	42.25^{b}	58.41^{b}	82.25^{b}	111.87^{b}	132.46^{b}	142.87^{b}	154.67^{b}	157^{b}	157^{b}
AM	1.76^{ab}	20.17^{cd}	38.67^{c}	55.67^{bc}	80.67^{b}	107.67^{c}	126.91^{c}	135^{c}	140.17^{e}	141.25^{d}	141.25^{d}
AMB	2.33^{ab}	21.67^{bc}	38.83^{bc}	55.33^{bc}	83.33^{b}	113.7^{b}	132.91^{b}	141.91^{b}	149.75^{c}	151.25^{c}	151.25^{c}
SEM	0.89	0.96	1.20	1.50	1.53	1.29	1.04	1.26	1.39	1.83	1.83

Legend: CON: control silage; AB: alfalfa + inoculant; AO: alfalfa + orange pulp; AOB: alfalfa + orange pulp; AM: alfalfa + molasses; AMB: alfalfa + molasses + inoculant.
Within a column, means followed by different letters differ (P<0.05).

Table 3. *Effects of treatments on in vitro gas production estimated parameters.*

Treatments	Estimated parameters					
	GP (ml/0.2 g DM)	**ME** (MJ/kg DM)	**NE_L** (MJ/kg DM)	**DOMD** (g/kg DOM)	**SCFA** (mmol/0.2 g DM)	**MP** (gr/kg DOM)
CON	18.22^{e}	4.79^{e}	2.36^{e}	33.89^{e}	0.40^{e}	6.54^{e}
AB	19.65^{d}	5.00^{d}	2.51^{d}	35.22^{d}	0.43^{d}	6.80^{d}
AO	24.80^{a}	5.67^{a}	2.99^{a}	39.82^{a}	0.55^{a}	7.69^{a}
AOB	22.38^{b}	5.35^{b}	2.75^{b}	37.68^{b}	0.48^{b}	7.27^{b}
AM	21.53^{c}	5.22^{c}	2.66^{c}	36.94^{c}	0.47^{c}	7.13^{c}
AMB	22.63^{b}	5.37^{b}	2.78^{b}	37.88^{b}	0.50^{b}	7.31^{b}
SEM	0.256	0.035	0.024	0.230	0.006	0.044

Legend: CON: control silage; AB: alfalfa + inoculant; AO: alfalfa + orange pulp; AOB: alfalfa + orange pulp; AM: alfalfa + molasses; AMB: alfalfa + molasses + inoculant.
GP: gas production at 24h; ME: metabolizable energy; SCFA: short chain fatty acid; DOMD: digestible organic matter in dry matter; NE_L: net energy lactation; MP: microbial protein.
Within a column, means followed by different letters differ (P<0.05).

in treatment supplemented with orange pulp decreased gas production (AOM vs. AO).

Effects of treatments on *in vitro* gas production estimated parameters are shown in Table 3. Treatments AO (alfalfa + orange pulp) and CON (without additive) had the highest and lowest ME, SCFA, DOMD, NE_L and MP values (p<0.05) and adding orange pulp increased these parameters. These estimated parameters for treatments AOB and AMB were significantly the same.

Treatments had significant effects on *in vitro* DM digestibility (p<0.05; Table 4). After 24 incubation treatment supplemented with orange pulp (AO) had highest *in vitro* DM digestibility (p<0.05). Adding inoculant decreased *in vitro* DM digestibility.

Treatments AOB and CON had the highest rapidly degradation fraction (*a*) and slowly degraded fraction (*b*) among treatments, respectively (p<0.05) (Table 5).

Discussion

Adding orange pulp and molasses increased gas production. Supplementation treatments with inoculant. *In vitro* gas production is highly influenced by the availability of both N and fermentable carbohydrate content (Nagadi et al., 2000; Kondo et al., 2004). Menke and Steingass (1987) reported a strong correlation between *in vitro* gas production and organic matter degradability of feeds. Many researchers have successfully used this technique to assess the impact of digestibility of feeds through this relationship (Muck et al., 2007; Negesse et al., 2009), because gas production rates can indicate the rate of digestion in the rumen and thereby affect the rate of passage and dry matter intake. In the present study, gas production of additives treated silages were increased as

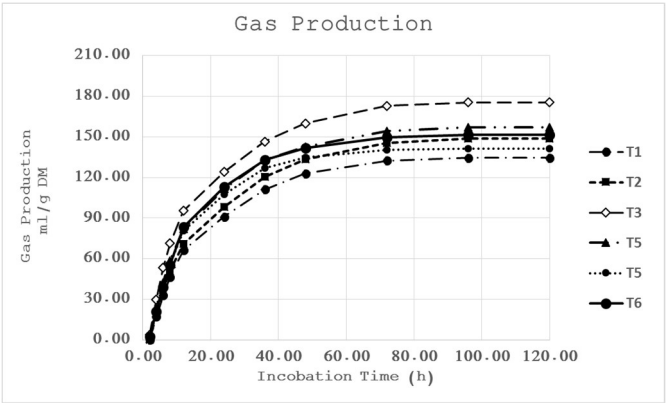

Figure 1. *Effects of treatments on in vitro gas production at various incubation times.*

Table 4. *Effects of treatment on in vitro dray matter digestibility.*

Treatments	Incubation times (h)				
	2	**4**	**8**	**12**	**24**
CON	32.65^{d}	35.80^{d}	39.66^{c}	45.70^{d}	56^{c}
AB	30.98^{e}	33.90^{e}	37.80^{d}	43.93^{e}	52.33^{d}
AO	44.50^{a}	46.50^{a}	48.24^{a}	55.16^{a}	67.06^{a}
AOB	44.52^{a}	46.19^{a}	47.53^{a}	48.50^{c}	54.93^{cd}
AM	40.31^{b}	44.57^{b}	47.82^{a}	53.63^{b}	63.16^{b}
AMB	37.60^{c}	39.10^{c}	43.80^{b}	45.26^{d}	53.50^{cd}
SEM	0.262	0.227	0.242	0.315	0.831

Legend: CON: control silage; AB: alfalfa + inoculant; AO: alfalfa + orange pulp; AOB: alfalfa + orange pulp; AM: alfalfa + molasses; AMB: alfalfa + molasses + inoculant. Within a column, means followed by different letters differ (P<0.05).

Table 5. *In vitro DM degradation characteristics.*

Treatments	Parameters			
	a	**b**	**c**	**RSD**
CON	29.75^{e}	54.29^{a}	0.107	0.82
AB	27.22^{f}	40.83^{b}	0.043	1.05
AO	42.79^{b}	34.48^{b}	0.024	1.39
AOB	44.41^{a}	9.67^{c}	0.035	0.95
AM	37.64^{c}	37.74^{b}	0.044	1.09
AMB	35.75^{d}	29.50^{b}	0.038	0.86
SEM	0.225	3.469	0.032	-

Legend: CON: control silage; AB: alfalfa + inoculant; AO: alfalfa + orange pulp; AOB: alfalfa + orange pulp; AM: alfalfa + molasses; AMB: alfalfa + molasses + inoculant. Within a column, means followed by different letters differ (P<0.05).
a – rapidly degraded fraction (%); *b* – slowly degraded fraction (%); *c* – rate of degradation (/h).

compared with the control silage, probably due to different additive treatments, the reduced loss of nutrients, and then increased gas production. This is consistent with the findings of Kozelov et al. (2008) and Li et al. (2014). An increased gas production might be related to improving the silage quality (Hetta et al., 2007), which would also determine the microbial access to fermentable carbohydrates in the rumen. The increased *in vitro* gas production by the adding of molasses agrees with previous reports on grass and cereal silages (Charmley et al., 1996; Hashemzadeh-Cigari et al., 2011) and can be explained by the higher silage water soluble carbohydrate content and increased carbohydrate fermentation.

Muck et al. (2007) and Hashemzadeh-Cigari et al. (2011) showed that silages treated with inoculants generally produced less gas per unit of incubated DM than the control silages. Blümmel et al. (1997) reported that gas production was positively correlated with DM digestibility, but negatively correlated with microbial biomass yield. Based on these results, they suggested that forages that produce less gas should have better microbial biomass production. Recently, Muck et al. (2007), who conducted an *in vitro* study with alfalfa silage inoculated with one of 14 inoculants plus an uninoculated control, found that some inoculated alfalfa produced less, and some produced more, gas than did uninoculated controls, suggesting that effects of microbial silage inoculants on *in vitro* fermentation of silage are not the same among inoculants. The kinetics of ruminal degradation by *in vitro* gas production technique potentially reflect in vivo digestibility of forages in ruminants (Getachew et al., 2004).

In vitro DM digestibility was lower in silage with inoculant than without inoculant. Adding molasses increased *in vitro* DM digestibility. Furthermore, although there are some reports that adding molasses has no effect on DM digestibility (Wang & Goetsch, 1998; Granzin & Dryden, 2005), further studies (Shellito et al., 2006; Sahoo & Walli, 2008) have reported that diets with molasses have higher ruminal DM digestibility. For AO silage high content of ME, SCFA, DOMD, NE_L and MP can result from its high rate of gas production, the extent of gas production at 24 h and its nutrient composition.

Conclusions

Results showed that ensiling alfalfa with orange pulp and molasses can improve silage quality and increased gas production and *in vitro* DM digestibility.

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