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Ensiling of *Conocarpus erectus* tree leaves with molasses, exogenous enzyme and *Lactobacillus plantarum* impacts on ruminal sheep biogases production and fermentation

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Abstract This study was performed to evaluate the effect of applying molasses (5% w/w) (M), exogenous enzyme (cellulase; 6 millions U kg^{-1}) (E) and *Lactobacillus plantarum* (3×10^5 cfu g^{-1} of fresh material) (LAB) on the ensiling characteristics of *Conocarpus erectus* leaves. Eight treatments were studied; (1) without additive (conocarpus silage: CS), (2) CS + E (CSE), (3) CS + M (CSM), (4) CS + LAB (CSL), (5) CS + E + M (CSEM), (6) CS + E + LAB (CSEL), (7) CS + M + LAB (CSML) and (8) CS + E + M + LAB (CSEML). The lowest amount ($P < 0.05$) of ash-free neutral detergent fiber (NDF) and ash-free acid detergent fiber were observed for CSE. The pH only affected by addition of molasses ($P < 0.05$), while the concentration of ammonia-N of Conocarpus silage was affected ($P < 0.05$) by molasses, meanwhile it decreased significantly for CSEM and CSM. The results of gas production parameter and in vitro digestibility showed that the use of silage additives leads to increase ($P < 0.05$) the coefficients *b* and *c* for CSML. The cumulative gas production in all hours significantly increased in treatment CSEML, CSML and CSL ($P < 0.05$). The highest amount ($P < 0.05$) of produced gas, organic matter disappearance, apparently degraded substrate (ADS) and microbial crude protein (MCP) were affected by silage additives and increased for CSE (519 and 485 mg g^{-1} of DM, respectively). In addition, ADS and MCP were affected ($P < 0.05$) by silage additives and increased for CSE (519 and 485 mg g^{-1} of DM, respectively). The concentration of short-chain fatty acids (SCFA) increased ($P < 0.05$) significantly for CSML (0.414 mmol l^{-1}). However, the amount of metabolizable energy (ME) and pH were not affected by experimental treatment ($P > 0.05$). In vitro digestibility of dry matter (DMD) and in vitro digestibility of NDF (NDFD) increased ($P < 0.05$) for CSML and the highest concentration of ammonia-N was observed in silage without additive. The result of these experiments showed due to the positive effect of a treatment containing molasses and lactic acid bacteria (CSML), maybe the treatment with it will be effective in improving the nutritional value of conocarpus leaves in ruminants.

Keywords Conocarpus · Silage additives · Gas production · In vitro digestibility

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Introduction

The shortage of feed for the ruminant in arid and semi-arid regions lead to attention to nonconventional fodder sources such as trees leaves as part of the animal feed, in these regions (Razzaque and Al-Nasser 2003). The multi-purpose foliage has the potential to beused in fulfilment of the nutrients requirement of the ruminants (Patra and Saxena 2011). The fodder trees (like subabul, siris and conocarpus) have the tremendous potential in adaptation of the challenges of its ecosystem, ensuring abundant and continuous supply of feedstuffs.

C*onocarpus erectus* is one of these resources, an ornamental shrub of the *Combretaceae* family, native to the coastal areas of Eastern Africa. Analysis of percent secondary metabolites as anti-nutritional factors in leaves of conocarpus showed that there are 3.49% phenols, 0.82% tannins, 0.47% flavonoids, 0.69% nitrates, 2.22% saponins and 0.72% oxalates in leaves of conocarpus (Ehsen et al. 2016). Conocarpus used as an ornamental plant in tropical regions, as its resistance to salinity and dryness is modest (Bhat et al. 2009). It is reported that conocarpus is a non-toxic, tasty plant and attractive for animal feeding, the branches and leaves remaining from the pruning of shrubs used in landscaping can be used as feed for animal feeding (Suleiman et al. 2005). However, conocarpus can be used as an inexpensive source of forage for grazing, storage and silage (Al-Surrayai and Baroon 2005). In a study by Al-Koaik et al. (2014), it was reported that crude protein and fiber of conocarpus leaves were 96.9 and 134.7 g kg^{-1} of DM, respectively. They concluded that the conocarpus leaves could be suitable as a green fodder for ruminants. Moreover, Baroon and Razzaque (2012) reported that the conocarpus silage-containing rations were palatable and could be used as an alternative to alfalfa and straw in the diet of growing heifers.

Conocarpus grows in the countries like Saudi Arabia and Iraq that are hot and dry areas and in Iran, it grows in the provinces of Khuzestan, Bushehr and Bandar Abbas. Its cultivation area has developed a lot in recent years. High vegetative growth, in these regions is the reason for the development of this plant. There is no published information that how much pruned residue of conocarpus produced. However, in pruning season at spring and autumn, every 2 weeks pruning of this tree produce huge amount of leaves and branches that can be used as forage for livestock.

Drying and ensiling are two ways of keeping forage, but the artificial drying process is costly and sun drying is difficult when the weather is often unreliable, so for farmers it is not affordable (Lima et al. 2011). When the sun or artificial drying is not available, the silo is the best method to keep fresh forage with minimal losses. On the other hand, using silage additives can reduce the amount of losses through silage and ensure a good quality silage (Oliveira 1995). It has been also widely accepted that silage additives increase feed intake and improve animal performance through the effect of forage quality (Huuskonen 2016). Silage additives are natural or industrial products that are added in large amounts of fodder or grain. The purpose of using the additive is to control the silage process so that at the time of feeding many of the nutrients of fresh forage are preserved, and ensure that the growth of lactic acid bacteria during the ensiling process as predominantly silage bacteria create good silage (Oliveira 1995). Fermentation stimulants such as molasses, exogenous enzymes (such as cellulases and hemicellulases) and lactic acid bacteria are a group of silage additives that are mainly used. Lactic acid bacteria (LAB) such as *Lactobacillus plantarum*, *L. acidophilus*, *Pediococcus acidilactici*, *P. pentacaceus* and *Enterococcus faecium* stimulate the fermentation rate and mainly produce lactic acid and reduce pH to less than 4, preventing further degradation of sugar and protein in the silage (McDonald et al. 2010). Moreover, the addition of cellulase enzymes has the potential to break down structural carbohydrates during ensiling and improve the digestibility of fiber (Sanchez et al. 1996). On the other hand, the addition of molasses to forage with little water soluble carbohydrate can be beneficial by stimulating lactic acid bacteria (McDonald et al. 1991). So, in this study, the objective is to research the effect of molasses, exogenous enzymes and *Lactobacillus plantarum* on the nutrient composition, in vitro gas production and digestibility of *Conocarpus Erectus* silage.

Materials and methods

Silage production

This experiment was conducted in the campus of Agricultural Sciences and Natural Resources University of Khuzestan in March 2018. Conocarpus leaves were prepared from 8 conocarpus shrubs in the spring from wet pruned leaves and branches, during pruning of trees.

Before ensiling, the leaves and branches were chopped into approximately 20 mm particle length. The content of dry matter (DM), organic matter (OM), ash, crude protein (CP), ash-free neutral detergent fiber (NDF), ash-free acid detergent fiber (ADF) and tannin of leaves before ensiling were 785, 830, 170, 105, 519, 296 and 512 g kg^{-1} of DM, respectively. In this study, it was used *Lactobacillus Plantarum* (3×10^5 cfu g^{-1} of fresh material) (LAB), exogenous enzyme (cellulase; 6 million U kg^{-1}) (E) and molasses (5% w/w) (M) as an additive. The leaves treated with lactic acid bacteria, molasses and the exogenous enzyme (4 replicates per treatment). Therefore, the experimental treatments were: (1) without additive (Conocarpus silage: CS), (2) CS + E (CSE), (3) CS + M (CSM), (4) CS + LAB (CSL), (5) CS + E + M (CSEM), (6) CS + E + LAB (CSEL), (7) CS + M + LAB (CSML) and (8) CS + E + M + LAB (CSEML). Molasses (5% w/w) was added without diluting with water. About 4 h before inoculation, 0.270 g of the *Lactobacillus plantarum* was dissolved in 240 ml of distilled water and then sprayed. Cellulase was used at 0.02% and dissolved in 100 ml of distilled water and then sprayed. Ensiling materials for each treatment were packed into nylon bags (4 silos per treatment) and, after compressing, were covered with tight-fitting lids. The silos were stored in the laboratory for 45 days at room temperature (average 25 °C).

Sampling, pH and ammonia-N measurement

On day 45, the experimental silos were opened. Immediately 20 g samples of each silage were mixed with 130 ml of distilled water and mixed for 60 s. Then, the pH value was measured using a portable pH meter (Metrohm model, Swiss). The extract was filtered and then 9 ml of this extract was mixed with 1 ml of a 7.2 N H_2SO_4 and stored at − 20 °C. The ammonia-N concentration was measured by phenol-hypochlorite assay (Broderick and Kang 1980).

Laboratory analysis

Before the chemical analysis, silage samples were oven-dried at 55 °C for 48 h and then were passed through a 1 mm sieve (Wiley mill, Swedesboro, USA). Dry matter, ash (number 924.05), N content (number 984.13) were analyzed following the AOAC (1990) procedure. Neutral detergent fiber (NDF) was determined without sodium sulfite and expressed exclusive of residual ash (Van Soest et al. 1991). The determination of acid detergent fiber (ADF) was performed and expressed exclusive of residual ash (number 973.18; AOAC 1990). The chemical compositions are shown in Table 1.

In vitro gas production parameters and digestibility

To determine the effect of treatments on gas production and fermentation parameters, rumen fluid was collected from three adult male Arabian sheep before morning feeding that were fed with diet (CP = 10.5%; ME = 2.3 Mcal kg^{-1}) with 60% forage (25% wheat straw, 10% alfalfa hay, 25% corn silage) and 40% concentrate (8% corn, 7% barely, 20% wheat bran, 2% soybean meal, 1% salt, 2% mineral vitamin supplement) for 2 weeks. The rumen fluid was filtered through 4 layers of cheesecloth into the plastic tube and placed in a warm bath at 39 °C. Artificial saliva was prepared according to the Menke and Steingass (1988) (includes distilled water, buffering solution, resazurin solution, macro mineral solution). Silage samples (200 mg) were placed in 100 ml glass vial (four replicate for each silage) at 39 °C in two runs. Then, 30 ml of mixed ruminal fluid and artificial saliva were added to each vialat a ratio of 1:2 and incubated in a warm water bath (with shaker) incubation equipment, at 39 °C for 120 h.

Four vials (only rumen fluid) were considered as blanks and alfalfa hay as standard. Gas volume was recorded at 2, 4, 6, 8, 12, 24, 48, 72, 96 and 120 h after incubation. The gas production data was fitted to the modified model described by Ørskov and McDonald (1979) as follows:

$$y = b\left(1 - e^{-ct}\right) \quad (1)$$

Table 1 Effect of experimental treatments on chemical composition (g kg^{-1} DM), ammonia-N (g kg^{-1} of total N) and pH of conocarpus silage

Treatments[1]	DM	OM	Ash	CP	NDF	ADF	Ammonia-N	pH
CSEML	409^{cd}	861^{a}	139^{d}	11.3	419^{bc}	329^{d}	30.7^{e}	5.16^{b}
CSEL	409^{cd}	842^{bcd}	157^{abc}	11.5	419^{bc}	335^{d}	34.0^{c}	5.73^{a}
CSEM	438^{a}	844^{bcd}	156^{abc}	11.4	421^{bc}	349^{c}	29.0^{f}	5.40^{ab}
CSE	392^{de}	839^{cd}	160^{ab}	11.4	404^{d}	316^{e}	32.0^{d}	5.40^{ab}
CSML	417^{bc}	845^{bc}	154^{bc}	11.4	408^{cd}	329^{d}	31.3^{e}	5.56^{ab}
CSL	382^{e}	848^{b}	152^{c}	11.4	412^{bcd}	336^{d}	37.2^{a}	5.80^{a}
CSM	433^{ab}	838^{d}	162^{a}	11.4	423^{b}	360^{b}	29.2^{f}	5.13^{b}
CS	416^{bc}	856^{a}	144^{d}	11.4	473^{a}	371^{a}	35.8^{b}	5.76^{a}
SEM[2]	2.34	0.41	0.42	0.03	0.456	0.311	0.383	0.169
P value	0.001	0.001	0.001	0.367	0.001	0.001		
E							0.001	0.253
LAB							0.001	0.253
M							0.001	0.008
E × LAB							0.001	0.454
E × M							0.001	0.539
M × LAB							0.001	0.732
E × M × LAB							0.001	0.060

[1] *CS* conocarpus silage without additive, *E* enzyme, *M* molasses, *LAB* lactic acid bacteria, *CSEML* CS + E + M + LAB, *CSEL* CS + E + LAB, *CSEM* CS + E + M, *CSE* CS + E, *CSML* CS + M + LAB, *CSL* CS + LAB, *CSM* CS + M

[2] *SEM* standard error of means; *a-f* Means in the same column with different superscript letters are different ($P < 0.05$)

where y is the volume of gas production at the time, (ml), *b* is gas production from the fermentable fraction (ml 200 mg^{-1} of DM), *c* is the gas production rate constant (ml h^{-1}) and t is the incubation time (h).

Organic matter disappearance (OMD) and metabolizable energy (ME) were calculated using Eqs. 2 and 3, respectively (Menke et al. 1979):

$$\mathrm{OMD}\left(\mathrm{g\ kg^{-1}\ of\ DM}\right) = 148.8 + 8.89\,\mathrm{GP} + 4.5\,\mathrm{CP} + 0.651\,\mathrm{XA} \tag{2}$$

$$\mathrm{ME}\left(\mathrm{MJ\ kg^{-1}\ of\ DM}\right) = 2.2 + 0.01357\,\mathrm{GP} + 0.0057\,\mathrm{CP} + 0.00002859\,\mathrm{CP}^2 \tag{3}$$

where GP is the volume of gas production at the 24 h, (ml), CP is crud protein (g kg^{-1} of DM) and XA is crud Ash (g kg^{-1} of DM).

The apparently degraded substrate (ADS) at 120 h of incubation (mg g^{-1} DM) was calculated as the difference between DM content of substrate and its undegradable DM (Salem et al. 2013). Microbial protein production (MCP) was calculated according to Blümmel et al. (1997):

$$\mathrm{MCP}\left(\mathrm{mg\ g^{-1}\ DM}\right) = \mathrm{ADS} - \left(\mathrm{ml\ gas} \times 2.2\,\mathrm{mg\ ml^{-1}}\right) \tag{4}$$

Also, the short chain fatty acids (SCFA) was calculated according to Getachew et al. (2002):

$$\mathrm{SCFA}\left(\mathrm{mmol\ 200\,mg^{-1}\ DM}\right) = 0.0222\,\mathrm{GP} - 0.00425 \tag{5}$$

where GP is the volume of gas production at the 24 h incubation.

The in vitro digestibility of dry matter (DMD) and NDF (NDFD) of experimental diets were determined using Tilley and Terry method (1963). Rumen fluid was collected and mixed with McDougall buffer in a ratio 1:4. After gasifying with CO_2, tubes were incubated at 39 °C for 48 h incubation, 6 ml of 20% HCl solution and 5 ml pepsin solution were added and then incubated for 48 h simulating post-ruminal degradation. After that, the residual substrates of each tube were filtered and used to determine the digestibility of dry matter (DM) and neutral detergent fiber (NDF).

Statistical analysis

The research was conducted as factorial experiment 2^3 (2 levels and 3 factors) based on a completely randomized design with four replications for calculation of b and c, it is used non-linear procedure of SAS program. The graph plotted using the non-linear regression analysis by excel software.

All data were analyzed by using the mixed procedures of SAS (2008) (version 9.2; SAS Inst. Inc.), based on the statistical model: $y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + e_{ijkl}$. Where y_{ijkl} is observation, μ is the general mean, α_i is the effect of first factor (exogenous enzyme effect at two levels), β_j is the effect of second factor (LAB effect at two levels), γ_k is the effect of third factor effect (effect of molasses at two levels), $\alpha\beta_{ij}$ is the interaction between treatment (exogenous enzyme × LAB), $\alpha\gamma_{ik}$ is the interaction between treatment (exogenous enzyme × molasses), $\beta\gamma_{jk}$ is the interaction between treatment (LAB × molasses), $\alpha\beta\gamma_{ijk}$ is the interaction between treatment (exogenous enzyme × molasses × LAB) and e_{ijkl} is the standard error of term.

Means were compared by the Duncan multiple comparison tests at $P < 0.05$.

Results

Chemical composition

The results of the effect of experimental treatments on chemical composition, ammonia-N and pH of Conocarpus silage are shown in Table 1. As can be seen, the lowest amount of DM was observed in CSL and CSE (382 and 392 g kg^{-1} of DM respectively). The highest amount of DM was observed in CSEM (438 g kg^{-1} of DM) ($P < 0.05$). However, this decrease in DM was not noticeable in CSEML, CSML and CSEL as compared to the control (CS) and CSM. The highest amount of ash was observed in of CSM and CSE (162 and 160 g kg^{-1} of DM, respectively) ($P < 0.05$). Generally compared to the control, the amount of ash increased in all treatments apart from CSEML ($P < 0.05$), where it was similar to the control. Silage additives ($P > 0.05$) did not affect the CP amount. In all treatments, the amount of NDF was lower than CS. The lowest amount of NDF was observed in the CSE (404 g kg^{-1} of DM), that did not have a significant difference with CSML and CSL ($P > 0.05$). Also, in all treatments, the amount of ADF was lower than CS. The use of enzyme in CSE significantly reduced the amount of ADF than other treatments (336 g kg^{-1} of DM) ($P < 0.05$).

The pH in experimental silage was higher than the ideal range of pH (3.8–4.2) for silage. In all treatments except for CSL, the pH was lower than CS. However, the pH of silage in CSEL, CSEM, CSE, CSML and CSL showed no significant difference with CS ($P > 0.05$). The highest and lowest pH was observed in CSM (5.13) and CSL (5.8) ($P < 0.05$). The concentration of ammonia-N significantly increased for CSL (37.2 g kg^{-1} of total N), but reduced for the rest of the treatments.

In vitro gas production kinetics

The results of the effect of experimental treatments on in vitro rumen gas kinetics (*b* and *c*) and cumulative gas production are shown in Table 2. The highest amount of *b* observed in CSML (28.8), but there was no significant difference ($P > 0.05$) with CSEML, CSEM, CSE, CSL and CSM. Also, the high value of *c* were observed in CSM and CSML (0.055) that was not statistically difference ($P > 0.05$) with CSEML, CSE, CSML, CSL and CSM. In addition, the interaction between silage additives showed that for *b* coefficient the effect of LAB and E × LAB and for *c* coefficient the effect of LAB, M and E × M × LAB was meaningful. The cumulative gas production in all hours significantly increased in CSEML, CSML and CSL ($P < 0.05$). Hence, as shown in Fig. 1a (silages containing an additive), the highest amount of gas production was observed in CSL and in Fig. 1b (silages containing several additives), the highest amount of gas production was observed in CSML. The treatments were affected by LAB, M and interaction E × LAB ($P < 0.05$). In addition, the treatments were affected by interaction E × M × LAB (except 120 h) ($P < 0.05$). However, the treatments were not affected by interaction M × LAB (all time), E × M (except 6 h) and E (except 6 and 8 h) ($P > 0.05$).

Table 2 Effect of experimental treatments on in vitro rumen gas kinetics and cumulative gas production (ml 200 mg^{-1} of DM)

Treatment[1]	GPP[3]		Incubation time (h)									
	b	*c*	2	4	6	8	12	24	48	72	96	120
CSEML	27.2^{ab}	0.053^{a}	3.2^{a}	6.2^{a}	7.2^{b}	10.7^{ab}	14.2^{a}	18.5^{a}	22.1^{ab}	25.1^{abc}	27.7^{ab}	30.0^{abc}
CSEL	22.9^{bc}	0.039^{bc}	2.0^{c}	4.2^{c}	5.1^{c}	7.8^{c}	10.6^{c}	13.3^{d}	17.0^{c}	20.2^{de}	22.8^{bc}	25.1^{cd}
CSEM	24.6^{abc}	0.035^{c}	2.1^{bc}	4.3^{c}	4.9^{c}	7.7^{c}	10.6^{c}	13.7^{cd}	17.6^{c}	21.1^{cd}	24.0^{b}	26.5^{c}
CSE	24.1^{abc}	0.047^{ab}	2.2^{bc}	4.6^{bc}	5.0^{c}	8.2^{c}	11.3^{bc}	15.2^{bcd}	18.9^{bc}	22.0^{bcd}	23.8^{b}	25.4^{cd}
CSML	28.8^{a}	0.055^{a}	3.1^{a}	6.3^{a}	9.8^{a}	12.5^{a}	15.5^{a}	18.8^{a}	22.9^{ab}	26.7^{a}	29.8^{a}	32.6^{a}
CSL	28.7^{a}	0.048^{ab}	2.8^{a}	6.2^{a}	8.2^{b}	11.3^{ab}	14.7^{a}	17.8^{ab}	22.0^{ab}	26.0^{ab}	29.4^{a}	32.1^{ab}
CSM	24.2^{abc}	0.055^{a}	2.6^{ab}	5.5^{ab}	7.2^{b}	10.1^{b}	13.2^{ab}	16.6^{abc}	19.5^{abc}	22.5^{bcd}	24.9^{ab}	26.9^{bc}
CS	19.9^{c}	0.032^{c}	1.2^{d}	2.8^{d}	3.0^{d}	5.3^{d}	7.8^{d}	10.6^{e}	13.5^{d}	16.4^{e}	18.6^{c}	20.7^{d}
SEM[2]	1.57	0.003	0.176	0.331	0.428	0.584	0.774	0.929	1.08	1.29	1.52	1.70
P value												
E	0.522	0.167	0.599	0.164	0.001	0.001	0.053	0.281	0.456	0.384	0.300	0.276
LAB	0.004	0.019	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
M	0.560	0.004	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.010	0.014	0.018
E × LAB	0.016	0.574	0.037	0.013	0.001	0.003	0.011	0.023	0.008	0.005	0.008	0.010
E × M	0.943	0.120	0.030	0.321	0.007	0.056	0.159	0.214	0.362	0.433	0.714	0.907
M × LAB	0.902	0.343	0.792	0.806	0.745	0.842	0.988	0.535	0.684	0.914	0.795	0.688
E × M × LAB	0.097	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.025	0.063

[1] *CS* conocarpus silage without additive, *E* enzyme, *M* molasses, *LAB* lactic acid bacteria, *CSEML* CS + E + M + LAB, *CSEL* CS + E + LAB, *CSEM* CS + E + M, *CSE* CS + E, *CSML* CS + M + LAB, *CSL* CS + LAB, *CSM* CS + M

[2] *SEM* standard error of means; *a-d* Means in the same column with different superscript letters are different ($P < 0.05$)

[3] Gas production parameter, *b*: gas production from the fermentable fraction (ml 300 mg^{-1} of DM), *c*: gas production rate constant (ml h^{-1})

In vitro gas production parameter

As shown in Table 3, the highest amount of produced gas, OMD was observed in CSEML and CSML (472 and 468 mg g^{-1} of DM incubated, respectively) ($P < 0.05$) and as can be seen, it was affected by LAB, M, E × LAB and E × M × LAB. In addition, the amount of ADS (519 mg g^{-1} of DM) was affected by silage additives and significantly increased for CSE than CS, CSEL and CSEM ($P < 0.05$). The amount of MCP was only significant between CSE and CSEL ($P < 0.05$). The amount of ADS and MCP only affected by E × M × LAB. Under the influence of silage additives, the concentration of SCFA increased significantly for CSML (0.414 mmol l^{-1}) ($P < 0.05$). The concentration of SCFA was only affected by LAB as a silage additive. The highest concentration of ammonia-N was absorbed in silage without additive ($P < 0.05$). However, the amount of ME and pH were not affected by experimental treatment ($P > 0.05$). However, the highest ME amount was observed in CSML (5.41 MJ kg^{-1} of DM).

In vitro digestibility

The effect of silage additives on the digestibility of IVDMD and NDFD, SCFA, pH and ammonia-N after the incubation time are shown in Table 4. The DMD and NDFD increased significantly for CSML treatment (495 and 481 g kg^{-1} of DM, respectively) ($P < 0.05$). However, the lowest DMD and NDFD was observed in silage without additive (421 and 415 g kg^{-1} of DM, respectively).

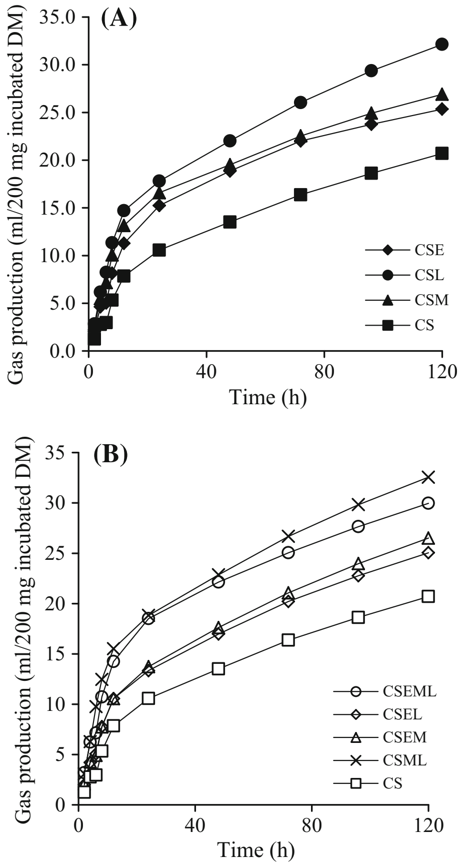

Fig. 1 The cumulative gas production (ml of 200 mg DM) during different incubation times. **a** Silages containing an additive, **b** silages containing several additive. *CS* Conocarpus silage without additive, *E* enzyme, *M* molasses, *LAB* lactic acid bacteria, *CSEML* CS + E + M + LAB, *CSEL* CS + E + LAB, *CSEM* CS + E + M, *CSE* CS + E, *CSML* CS + M + LAB, *CSL* CS + LAB, *CSM* CS + M

Discussion

Chemical compositions

Increasing the amount of DM in the CSEM indicate that fermentation and preserves nutrients in the silage is well (Amanullah et al. 2014). Adding the cellulase to the silage prior to ensiling causes cell wall hydrolysis to be fermentable substrates (Eun and Beauchemin 2007). So, in this study, the addition of cellulase in CSE leads to reduce the content of DM. However, adding molasses to silage increased the amount of DM in CSM and CSEM (433 and 438 g kg^{-1} DM, respectively), and the reason is a high amount of DM in molasses (Baytok et al. 2005). In addition, reducing the DM of the silage containing LAB may be due to the high moisture content of forage prior to ensiling, and also the increase in fermentation by inoculation (Rowghani and Zamiri 2009). In this study similarity with the findings of other researchers (Islam et al. 2001; Mahala and Khalifa 2007; Nkosi et al. 2012), the addition of molasses increased the Ash of silage, since molasses contain 132 g kg^{-1} of DM Ash (Xande et al. 2010). Also, agree with the results of this study, bacterial inoculation did not affect the amount of silage Ash (Jalč et al. 2009; Nkosi et al. 2010; Paviz et al. 2011).

The reason for the reduction of the CP during ensiling was degradation of protein due to microbial activity (McDonald et al. 2010). It has been reported that adding molasses (Mahala and Khalifa 2007) and LAB (Addah et al. 2016) simultaneously causes a rapid decrease in pH of silage. In the present study, the retention of silage crude protein due to the addition of molasses (Migwie et al. 2000; Mahala and Khalifa 2007), LAB (Nkosi et al. 2010) and enzymes (Young et al. 2012) is confirmed by the other researchers.

In this study addition of cellulase leads to reduce the content of NDF and ADF in CSE. Colombatto et al. (2003) reported that the addition of cellulase leads to reduce the content of NDF and ADF in corn silage. Also, Kung et al. (2003) reported by adding the cell wall degrading enzyme to the silage, it increases the degradation of the cell wall plant and improves fermentation conditions by releasing soluble sugars for the LAB in the silage. Therefore, enzyme additives can degrade the plant cell wall and thus improve animal performance (Shepherd and Kung 1996). The addition of molasses to Conocarpus silage reduced the concentration of NDF, because of the low amount of fiber contained in molasses and the dilution effect of molasses. The reason for the decrease in NDF concentration is the increase in fermentation and the acidic pH of the silage, which causes the cell wall to degrade (Rezaei et al. 2009). The reduction in NDF concentration due to the addition of molasses is agreed with the results of other researchers (Islam et al. 2001; Aksu et al. 2006; Lima et al. 2011). The decreasing of the amount of ADF was similar to the decreasing in NDF. In another research, it is reported the cause of reducing ADF content by adding molasses (Rezaei et al. 2009) and LAB to silage (Baytok et al. 2005) was the effect of dilution of molasses and also increased

Table 3 In vitro organic matter disappearance (OMD) (mg g^{-1} of DM incubated), metabolizable energy (ME) (MJ kg^{-1} of DM), apparently degraded substrate (ADS) (mg g^{-1} of DM), microbial CP (MCP) (mg g^{-1} of DM), SCFA (mmol l^{-1}), pH and ammonia-N (mg dl^{-1}) of conocarpus silage

Treatment[1]	Gas production parameters						
	OMD	ME	ADS	MCP	SCFA	Ammonia-N	pH
CSEML	472^{a}	5.36	501^{ab}	460^{ab}	0.407^{ab}	14.2^{c}	6.04
CSEL	422^{d}	4.67	456^{b}	376^{b}	0.292^{bc}	14.4^{b}	5.91
CSEM	426^{d}	4.72	455^{b}	424^{ab}	0.301^{abc}	14.2^{c}	6.14
CSE	439^{c}	4.92	519^{a}	485^{a}	0.334^{abc}	14.1^{c}	6.04
CSML	468^{a}	5.41	496^{ab}	454^{ab}	0.414^{a}	14.2^{c}	5.94
CSL	457^{b}	5.27	489^{ab}	450^{ab}	0.391^{ab}	14.7^{a}	6.04
CSM	453^{b}	5.10	512^{ab}	476^{a}	0.364^{ab}	14.2^{c}	5.94
CS	388^{e}	4.29	455^{b}	432^{ab}	0.230^{c}	14.8^{a}	6.07
SEM[2]	4.78	0.76	18.4	22.5	0.06	0.013	0.44
P value							
E	0.204	0.852	0.669	0.452	0.831	0.001	0.790
LAB	0.001	0.449	0.901	0.362	0.299	0.001	0.431
M	0.001	0.512	0.390	0.394	0.045	0.001	0.430
E × LAB	0.001	0.686	0.523	0.417	0.150	0.030	0.070
E × M	0.012	0.829	0.131	0.508	0.878	0.001	0.790
M × LAB	0.131	0.920	0.276	0.181	0.706	0.001	0.430
E × M × LAB	0.001	0.474	0.007	0.018	0.344	0.013	0.051

[1] *CS* conocarpus silage without additive, *E* enzyme, *M* molasses, *LAB* lactic acid bacteria, *CSEML* CS + E + M + LAB, *CSEL* CS + E + LAB, *CSEM* CS + E + M, *CSE* CS + E, *CSML* CS + M + LAB, *CSL* CS + LAB, *CSM* CS + M

[2] *SEM* standard error of means; *a-d* Means in the same column with different superscript letters are different ($P < 0.05$)

the cell wall plant degradation due to increase in silage fermentation (Rezaei et al. 2009).

Colombatto et al. (2003) reported that the addition of cellulase leads to reduce the pH of corn silage. However, due to the buffering capacity of Conocarpus, the pH was high in experimental treatments. In addition, it has been reported that when the amount of Ash and CP is more than 150 and 230 g kg^{-1} of DM, pH is increased due to the high buffering capacity of silage (Kung and Shaver 2001). However, the addition of molasses compares with other silage additive reduced pH of silage (Bureenok et al. 2011; Cajarville et al. 2012). Similar to our experiment, inoculation of *Lactobacillus buchneri* to silage, pH of silage was increased (Filya 2003), which is due to the presence of compounds such as 1–2 Propanediol (Taylor and Kung 2002; Nishino et al. 2004), Ethanol and Mannitol (Taylor and Kung 2002), Propanol, Propyl acetate, Propylene glycol and 2-Butanol (Kristensen et al. 2010). In addition, the flavonoid and tannin content in the conocarpus leaves confirmed (Abdel-Hameed et al. 2013). If the ammonia-N level is less than 100 g kg^{-1} of total N, fermentation quality is ideal, which in the present study the concentration of ammonia-N is less than this value (McDonald et al. 2010).

In vitro gas production kinetics

Due to the presence of tannin in the conocarpus leaves, the amount of gas produced did not show much increase. It has been reported that the tannins have an inhibitory effect on ruminal microorganisms (Tabacco et al. 2006). However, the highest gas production was observed in CSML, as a result of carbohydrate utilized by LAB. In addition, it has been shown that increased gas production has a positive correlation with DM digestibility (Blümmel et al. 1997). As shown in Tables 4, the highest IVDMD was observed in CSML. There are contradictory reports on the effect of LAB

Table 4 In vitro digestibility of DM and NDF (g kg^{-1} of DM) of conocarpus silage

Treatment[1]	Parameters[3]	
	DMD	NDFD
CSEML	464^{abc}	439^{ab}
CSEL	429^{bc}	417^{b}
CSEM	435^{bc}	420^{b}
CSE	464^{abc}	459^{ab}
CSML	495^{a}	481^{ab}
CSL	476^{ab}	462^{ab}
CSM	481^{ab}	471^{ab}
CS	421^{c}	415^{b}
SEM[2]	16.6	19.4
P value		
E	0.983	0.176
LAB	0.347	0.162
M	0.003	0.472
E × LAB	0.015	0.041
E × M	0.840	0.292
M × LAB	0.083	0.595
E × M × LAB	0.289	0.646

[1] *CS* conocarpus silage without additive, *E* enzyme, *M* molasses, *LAB* lactic acid bacteria, *CSEML* CS + E + M + LAB, *CSEL* CS + E + LAB, *CSEM* CS + E + M, *CSE* CS + E, *CSML* CS + M + LAB, *CSL* CS + LAB, *CSM* CS + M

[2] *SEM* standard error of means; *a-c* Means in the same column with different superscript letters are different ($P < 0.05$)

[3] *DMD* in vitro digestibility of dry matter, *NDFD* in vitro digestibility of NDF, *SCFA* short chain fatty acids

inoculations on gas production; for example, Muck et al. (2007) reported an increase in gas production. However, contrary to Contreras-Govea et al. (2011) reported the ineffectiveness of inoculations on gas production. However, differences in the chemical composition of feeds such as starch, non-structural carbohydrates, OM, CP, NDF and ADF content can lead to differences in the rate of gas production (Getachew et al. 2004; Maheri-Sis et al. 2008). In the treatments containing enzyme except for CSEML, there was no significant increase in gas production, may be factors such as forage maturity effective on enzyme activity (Shepherd and Kung 1996). The molasses and bacteria mixed silages fermented faster (*c*) ($P < 0.05$) and largely (*b*) compared to the control silage. In addition, *c* has a high correlation (R = 0.97) with OMD (Sandoval-Castro et al. 2005).

In vitro gas production parameter and digestibility of silage

Molasses probably increase the OMD by stimulating the microbial activity and thus increasing the digestibility of nutrients (Aksu et al. 2006). In this experiment, the OMD, DMD and NDFD have increased significantly in the treatment containing molasses and LAB ($P < 0.05$) (Tables 3, 4). Khorvash et al. (2006) reported that the reason for improving nutrient digestibility due to the addition of molasses, lack of fermentation of silage carbohydrates during ensiling processes. Also, similar to the results of Shellito et al. (2006) and Sahoo and Walli (2008) use of molasses as a silage additives lead to increase of digestibility of nutrient. As a result of the addition of molasses to forages with low fermentable carbohydrate can be beneficial for lactobacilli (Yitbarek and Tamir 2014). By inoculation LAB, the concentration of cell walls decreased due to the partial degradation of hemicellulose, but it is reported that LAB caused to lack of cellulolytic activity (Charmley 2001). However, similar to our results, Cai et al. (2003) reported the use of LAB increased the IVDMD by decreasing DM. In theory, the enzyme additive to silage can result in the degradation of plant cell wall and lead to increase digestibility nutrient (Dehghani et al. 2012). In addition, treatment of forage with cell walls degradation enzymes such as cellulase and hemicellulase before ensiling can reduce the cell wall content (Dehghani et al. 2012). Although the OMD, DMD and NDFD increased in the treatment-containing enzyme as an additive, but, there was no significant difference between CSE and CS. Nadeau et al. (2000) reported when fibrolytic enzymes were added to lucerne at ensiling, NDF and ADF concentration decreased, but digestibility of NDF unaffected.

The highest amount of ADS was observed in CSE, CSML, CSM, CSL and CSEML, indicating that more DMD. As shown in Table 1, the lowest amount of NDF was decreased in these treatments and there is a negative relationship between digestibility and NDF of feed (Jeon et al. 2003). At the same time, the supply of CP and soluble carbohydrate during fermentation will lead to an increase in MCP (Kaur et al. 2010). In addition, increasing OMD leads to an increase in the MCP (Contreras-Govea et al. 2011). As can be seen in Table 3, the increase of OMD leads to increase substrate for the synthesis of MCP. It has been reported fermentable carbohydrates, such as molasses leads to increase the synthesis of MCP in the rumen (Maiga and Schingoethe 1997).

Rumen microorganisms are usually lead to hydrolysed and deaminated of feeds protein into peptides and free ammonia-N (Reynal et al. 2007). The concentration of ammonia-N in the rumen represents the balance between hydrolysis and utilization of protein in the rumen (Reynal et al. 2007). McDonald et al. (2010) reported that the range of ammonia-N concentration in the rumen was 8.5–30 mg dl^{-1}. In the present study, the ammonia-N concentration was in this range. Similarly, to the previous results, in treatment containing molasses (Babaeinasab et al. 2015) and enzyme (Dönmez et al. 2003) the concentration of ammonia-N was decreased. However, in treatment containing LAB the concentration of ammonia-N was not decreased, that is like the result of Cao et al. (2013). However, Zhang et al. (2017) contrary to our results reported that the use of LAB as a silage additive increased the concentration of ammonia-N in the rumen compared to the control. The low ammonia level was probably due to the presence of tannin in the conocarpus leaves. This can be due to the presence of tannin and reduce protein degradation in the rumen (Patra and Saxena 2011). The VFA is a main product of rumen microbial fermentation (Castillo-González et al. 2014). Carbohydrates are fermented by different ruminal bacteria and converted to VFA by the corresponding enzymes (Wang et al. 2012). Improved digestion in CSML has led to an increase in SCFA compared to other experimental treatments. There is a positive correlation between the production of SCFA and GP (Menke and Steingass 1988). However, the VFA concentration status depends on the amount of water-soluble carbohydrates; crude protein, cellulose and hemicellulose in the concentrate or forage ration (Bureenok et al. 2011).

Conclusions

The results of this study showed that use of silage additives containing molasses or molasses plus lactic acid bacteria improved fermentation parameter and in vitro digestibility of *Conocarpus erectus* leaves. The low enzyme effect can be due to the degree of maturity of the leaves used and the low moisture content. In addition, the results can be affected by the buffering capacity of Conocarpus. So, the use of acidic additives is recommended to compare with our used additives.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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