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Effect of mixture of herbal plants on ruminal fermentation, degradability and gas production

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ABSTRACT. Reducing livestock negative environmental impacts get great interest in last years. So, present study was carried out to determine the effect of adding different levels of mixture of thyme and celery versus salinomycin on ruminal fermentation, gas production, dry, organic matter and fiber degradation. Four experimental treatments were used by *in-vitro* batch culture technique, as follow: 60% CFM, 40% clover hay (control), control diet + 2.5 gm thyme + 2.5 gm celery kg^{-1} DM (T1), control diet + 5 gm thyme + 5 gm celery kg^{-1} DM (T2), control diet + 10 gm thyme + 10 gm celery kg^{-1} DM (T3), control diet + 0.4 gm Salinomycin kg^{-1} DM (T4). Ruminal pH value was significantly increased ($p < 0.05$) with T4 compared with other treatments. While, the T4 recorded the lowest value ($p < 0.05$) for microbial protein, short chain fatty acids concentrations (SCFA), total gas production, dry matter and organic matter degradability (DMd and OMd) compared with other treatments. Fiber fraction degradability (NDFd and ADFd) appeared no significant variance ($p > 0.05$) between control and other treatments except for T1 that recorded the lowest value ($p < 0.05$). It is concluded that mixture of thyme plus celery could be alternate for ionophores in the ruminant diets to enhance ruminal fermentation, reducing gas production without any negative effect on nutrients degradability.

Keywords: thyme; celery; ionophores; gas production; rumen fermentation; nutrients digestibility.

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Introduction

A major goal of livestock production system today is restriction of using antibiotics and other synthetic medicinal chemistry as feed additives and growth promoters, versus natural growth promoters (NGPs) as effective alternatives to those products. Using antibiotic in ruminant nutrition as a supplement are widely applied to alter rumen fermentation and methane emission but due to the extreme of using antibiotic led to be resistance against different drugs by animals in addition antibiotics transfer to ruminant product (milk or meat) which could threaten human health (Zhan et al., 2017; Bayat et al., 2018; Khattab, Abd El Tawab, Hadhoud, & Shaaban, 2020). So, European Union (EU) banned using antibiotics as feed additive, this radical change in laws resulted in an intensive development of research that relates to find effective natural compounds that could inhibit GHG and modulate the rumen fermentation (Matloup et al., 2017)

Many studies have focused on the alternative strategies development to maintain animal productive performance and health (Khattab, Ebeid, Abd El Tawab, Abo El-Nor, & Aboamer, 2016; Khattab et al., 2017; Khattab & Tawab, 2018; Matloup et al., 2017). Phytogenic feed additives commonly known as phytobiotic, or herbal plants can be defined as source of plant derived products supplemented to animal feeds in order to increase production and enhance performance. They originate from tubers, roots, fruits or leaves of herbs, spices and other plants (Alam et al., 2013).

Phytogenic feed additives mainly meant plant secondary metabolites such as tannins, saponins and essential oils which investigated as a natural rumen modifier (Knapp, Laur, Vadas, Weiss, & Tricarico, 2014; Ishlak et al., 2015; Cobellis, Trabalza-Marinucci, Marcotullio, & Yu, 2016; Ali, Mohamed, Sameeh, Darwesh & Abd El-Razik, 2016). It is used in animal nutrition due to their effects of rumen microbial populations by improving ruminal fermentation efficiency and mitigate methane emissions (Khiaosa-ard & Zebeli, 2013). Methane is one of ruminal digestion secondary metabolites which released due to inability of the animal to benefit from H2 and CO2 production during fermentation, methanogens bacteria can be utilized hydrogen and their carbon dioxide to produce methane (Hobson & Stewart, 1997; Martin, Morgavi, & Doreau, 2009). It was estimated that dairy farms contribute more than 3% of the total greenhouse gasses (Knapp et al., 2014) that is in addition to be considered a loss in feed energy by up to 12% in the form of emitted methane (Arndt, Powell, Aguerre, Crump, & Wattiaux, 2015; Li et al., 2018).

Also, several researchers found that phytobiotic have anti-inflammatory activities and strong antioxidant. it has been reported that many of herbal plants contain essential oils and / or active compounds such as lemongrass, turmeric, galangal, rosemary, clove and cinnamomum, etc. on modifying rumen fermentation and positively affect methane emission, volatile fatty acids (VFA's), protein, carbohydrates degradation and reduce ruminal bio-hydrogenation (Khattab et al., 2016; Khattab et al., 2017). While, these positive effect in the ruminal fermentation accompanied with negative effect on fiber degradation (Khattab et al., 2017). Supplementing diets with celery or thyme showed no significant pH, microbial protein, ammonia-N concentrations and improved short chain fatty acids concentrations and significantly lowered total gas production (Khattab et al., 2020).

Ionophores are feed additives inhibiting growth of gram-positive bacteria that produce hydrogen gas for alter rumen microbial populations through ion transfer cross cell membranes which used by *archaea bacteria* to produce methane (Ishlak et al., 2015). Therefore, the objective of the present *in vitro* study to evaluate the effect of using different levels of thyme plus celery mix (natural feed additive) as an alternative to antibiotic feed additives on ruminal fermentation, ammonia nitrogen concentration and gas emission, dry matter and organic matter and fiber degradation.

Material and methods

Experimental treatments

In-vitro incubation procedures were carried out as described by Khattab, Azzaz, Abd El Tawab, and Murad (2019), rumen fluid was collected before morning feeding from 3 ruminally cannulated Holstein dairy cows (mean weight 680 ± 30 kg), mixed and squeezed through 4-layers cheesecloth under continuous flushing with CO_2 and immediately transported to laboratory at 39°C (used as a source of inoculum). Treatments were: 60% CFM, 40% clover hay (control), control diet + mix of (2.5 gm thyme and 2.5 gm celery kg^{-1} DM) (T1), control diet + mix of (5 gm thyme and 5 gm celery kg^{-1} DM) (T2), control diet + mix of (10 gm thyme and 10 gm celery kg^{-1} DM) (T3), control diet + 0.4 gm Salinomycin kg^{-1} DM (T4) (Table, 1). Each treatment was tested in eight replicates accompanied by blank bottles (no substrate). the experiment run were repelicated twice in different weeks. Substrate (400 mg) was added to the incubation bottles of 100 mL capacity. Each bottle was filled with 40 mL of the incubation medium (292 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4.7H_2O$, 64 mg $CaCl_2.{}_2H_2O$, 4 mg Na_2CO_3 and 600 mg cysteine hydrochloride) per 1 liter of double distilled water (ddH_2O) and dispensed anaerobically in the 1:4 (v/v) ratio. Then the bootles were incubated at 39°C for 48h.

Table 1. chemical composition of feed ingredients (%).

item	DM	OM	CP	EE	NDF	ADF	Ash
CFM	93.09	93.98	12.73	6.23	49.98	18.09	6.02
Clover hay	93.86	88.31	11.97	6.1	43.49	33.06	11.69
Experimental diet composition	93.40	91.71	12.43	6.18	47.38	24.08	8.29

CFM: concentrate feed mixture; each Kg DM consisted of 20% yellow corn, 20% wheat bran, 32% sugar beet pulp, 5% soybean meal, 20% cottonseed meal, 0.1% sodium bicarbonate, 1.5% limestone, 1% NaCl, 0.1% vitamins and 0.3% minerals.

Substrates sampling and gas production recording

After 48h of incubation, gas production (GP) was recorded using the pressure reading technique according to Khattab and Tawab (2018) bottles were uncapped, pH was determined using Hanna digital pH meter, 0.8 mL of strained ruminal fluid was mixed with 0.2 mL of a solution containing 250 g of metaphosphoric acid L^{-1} for SCFA analysis by titration, after steam distillation of a 4 mL sample, by the method of Annison (1954). Contents were filtrated of each bottle to obtain the non-digested residue for determination of degradation percent.

Chemical analysis

The non-fermented residues were dried, weighed and digestibility calculated using the equations as described by (Khattab et al., 2016) as follows:

$$In - vitro \text{ dry matter disappearance } (\%) = \frac{(\text{intial DM input} - \text{DM residue} - \text{Blank})}{\text{intial DM input}} \text{ X } 100$$

$$In - vitro \text{ organic matter disappearance } (\%) = \frac{(\text{intial OM input} - \text{OM residue} - \text{Blank})}{\text{intial OM input}} \text{ X } 100$$

Dry matter of TMR was determined by drying at 105°C for 48h (Association of Official Analytical Chemists [AOAC], 1990; method 930.15). Samples were analyzed for CP (method 976.05), ether extract (method 920.39), and ash (method 942.05) according to AOAC (2000). The neutral detergent fiber (NDF) was determined using the (Van Soest, Robertson, & Lewis, 1991) procedure. The heat stable amylase and sodium sulphite were used to determine NDF. The acid detergent fiber (ADF) content was determined according to AOAC (1990) method 973.18. The neutral detergent fiber and ADF values were measured on organic matter (OM) basis**.** Microbial protein production was calculated as 19.3 g microbial nitrogen per kg OMD according to Khattab el al. (2016). The NH_3-N concentration was determined as described by Khattab, Abd-El-Gawad, Abo El-Nor, and El-Sherbiny (2015).

Statistical analysis

Data were statistically analysed using GLM procedure of Statistical Analysis System (SAS, 2009), version 9.2. Significant differences between means of treatments were carried out by the Duncan's test, and the significance threshold was set at $p < 0.05$.

Results and discussion

Ruminal fermentation parameters:

No differences *(*$p > 0.05$) were observed between herbal plant (Mix of Thyme plus Celery) at all different levels compared with control treatments. while, it was significantly decreased ($p < 0.05$) versus the salinomycin treatment in pH values. Also, Ammonia-N concentrations showed no significant ($p > 0.05$) changes between control and salinomycin compared with T2 that recorded the lowest value ($p < 0.05$) (31.00 mg 100 mL^{-1}). Many studies stated the effect of herbal plants, spices or essential oils on ruminal pH. The data of the current study for ruminal pH were in line with that of Cardozo, Calsamiglia, Ferret, and Kamel, (2006); Yang et al. (2007); Kung Junior, Williams, Schmidt, and Hu (2008); Chaves et al. (2008); Chaves, Stanford, Gibson, McAllister, and Benchaar (2008); Fandino, Calsamiglia, Ferret, and Blanch (2008); Meyer et al. (2009); Yang, Benchaar, Ametaj, and Beauchemin (2010); Tager and Krause (2011), who found no effect of essential oils on ruminal pH. Levels used of essential oils or herbal plants could be influenced on ruminal pH (Evans & Martin, 2000) who suggested that supplementation of 400 mg L^{-1} of thymol oil increased ruminal pH while, it was not affected at lower dose (50, 100, 200) (*in vitro).* Increased in pH at a high level of Thyme could be due to inhibition of rumen microbial fermentation, and a reduction in acetate, lactate, propionate and methane concentrations. Also, Castillejos, Calsamiglia, and Ferret (2006) observed the same results when using different levels of some EO, because it has including thymol, limonene, eugenol, guaiacol, vanillin and *Y*-terpinene and the increased ruminal pH consisted with a decrease in rumen fermentation and depression in total VFAs concentration.

Table 2. ruminal parameters of experimental diets supplemented with different levels of mix of Thyme plus Celery powder or Salinomycin.

Item	Control	Mix of Thyme and Celery			Salinomycin	Pr >F
		T1	T2	T3	T4	
pH	6.92b ±0.035	6.86b ±0.034	6.88b ±0.009	6.90b ±0.025	7.00a ±0.034	0.0135
Ammonia-N (mg 100 mL^{-1})	37.06a ±2.056	33.46ab ±2.145	31.00b ±0.781	32.84ab ±1.487	36.98a ±1.511	0.0816
SCFA (mmol)	6.97a ±0.071	6.81a ±0.028	6.81a ±0.028	6.83a ±0.076	6.07b ±0.040	0.0001
Microbial Protein (mg gm^{-1} DM)	215.79a ±1.921	211.29a ±0.774	213.48a ±0.782	211.89a ±2.079	191.48b ±1.095	0.0001

SCFA; short chain fatty acids. Different superscript a, b, c, d in the same row differ significantly ($p < 0.05$).

Herbal plants powder or essential oils had variable influence of ruminal ammonia nitrogen concentration in the many studies. Reduction in NH3-N concentration is suggesting the potentiality of mix of celery plus thymol for inhibiting deamination. These results are in agreement with Wanapat, Cherdthong, Pakdee, and Wanapat (2008); Macheboeuf, Morgavi, Papon, Mousset, and Arturo-Schaan (2008); Cobellis et al. (2016) who found that EOs especially cinnamaldehyde and cinnamon reduced NH3-N concentrations in the rumen. Also, McIntosh, Newbold, Losa, Williams, and Wallace (2000) observed that EO inhibited deamination of amino acids measured *in vitro* by 25%, these potential related to inhibiting bacterial attachment to feed particles (Wallace, McEwan, McIntosh, Teferedegne, & Newbold, 2002). Castillejos, Calsamiglia, Ferret, and Losa (2005) notified that the microbial species affected by antibiotic were the same as those affected by essential oils. The hyper-ammonia producing bacteria (*Clostridium sticklandii* and *Peptostreptecoccus anaerobius*) were associated with a reduction of ammonia production which inhibited by EO added. While the other hyper-ammonia producing species (*Clostridium aminophilum*) were not affected (McIntosh et al., 2003). In addition, the differences in effects of Herbal plants powder or essential oils supplementation to diets on ammonia nitrogen between *in vitro* or *in vivo* studies might be qualified in part by the capacity of rumen microbial populations to adapt and/or degrade EO or powder components (Benchaar & Greathead, 2011).

While, microbial protein and short chain fatty acids concentrations (SCFA) showed no significantly decreased ($p > 0.05$) between herbal plant treatments compared with control treatments while, the salinomycin treatment recorded the lowest value ($p < 0.05$) (6.07 mmol and 191.48 mg gm^{-1} DM) respectively. Reduction of short chain fatty acids (SCFAs) in herbal plants treatments could be a good indicator of simultaneous with methane emission reduction in the rumen tract (Busquet, Calsamiglia, Ferret, & Kamel, 2006), these results are in agreement with Evans and Martin (2000) who observed that at high level of thymol (400 mg L^{-1}) reduced the short chain fatty acids concentration and the proportion of propionate and acetate, while acetate to propionate ratio was increased. Similar results findings were noted by Castillejos et al. (2006) for thymol and eugenol. While, Castillejos, Calsamiglia, Martín-Ereso, and Ter Wijlen (2008) found that at all level of thymol (i.e., 5, 50 and 500 mg L^{-1}) increased short chain fatty acids concentration, but did not affect the proportions of propionate, acetate, valerate, acetate to propionate ratio and branched-chain fatty acids concentration.

Gas production

Ruminal gas production of experimental diets supplemented with different levels of mix of Thyme plus Celery powder or Salinomycin are listed in Table (3). The values cleared that herbal plant addition to diet (T1 to T3) insignificantly lowered ($p > 0.05$) in total gas production compared with control diet (153.5, 153.5, 154 vs. 15725 mL, respectively). While, the salinomycin treatment recorded the lowest value ($p < 0.05$) being (137 ml) for total gas production compared with other treatments. Also, these results showed that gas production per each gram of DM, NDF or ADF recorded the same trend of total gas production.

Table 3. Ruminal gas production of experimental diets supplemented with different levels of mix of Thyme plus Celery powder or Salinomycin.

Item	Control	Mix of Thyme and Celery			salinomycin	Pr >F
		T1	T2	T3	T4	
Total GP (mL)	157.25a ±1.600	153.50a ±0.645	153.50a ±0.645	154.00a ±1.732	137.00b ±0.912	0.0001
GP gm^{-1} DM (mL)	344.28a ±3.956	337.41a ±1.326	338.47a ±2.106	339.78a ±2.699	301.72b ±2.342	0.0001
GP gm^{-1} NDF (mL)	720.70a ±8.282	706.31a ±2.777	708.55a ±4.410	711.29a ±5.650	631.61b ±4.904	0.0001
GP gm^{-1} ADF (mL)	1485.23a ±17.068	1455.59a ±5.724	1460.20a ±9.089	1465.85a ±11.644	1301.64b ±10.107	0.0001

Different superscript a, b, c, d in the same row differ significantly ($p < 0.05$).

Addition of medical plants or EO have caused either a modifying rumen fermentation and positively affected gas emission in several of *in vivo* or *in vitro* studies (Macheboeuf et al., 2008; Patra & Yu, 2012; Lin, Lu, Wang, Liang, & Liu, 2012; Cobellis et al., 2016). The same results were found by Rezaei and Pour (2012) who noted that the gas emission was reduced on addition thyme methanolic extracts also, Chaudhry and Khan (2012) using *in vitro* gas production technique, five curry spices such as turmeric, cinnamon, clove, cumin and coriander as a natural antibiotics killing *methanogenic bacteria* which lead to reduction in methane production by 40%. While, Mariam, El-Zarkouny, El-Shazly, and Sallam (2014) reported that the potential impacts of different levels of EOs mix such as thyme, eucalyptus, peppermint, cinnamon and lemon on ruminal fermentation and nutrient digestibility in Barki sheep, they found that there were no significant differences among treatments of essential oil mix on *in vitro* gas production and methane production, short chain fatty acids (SCFA), NH3-N concentration and protozoa count. While, the result of the study showed that the combination of the five EOs had an additive effect on lower response on rumen microbial fermentation and methane emission. Kim, Adesogan, and Shin (2012) suggested that the herbal plant *extracts* (Wormwood, *Allium sativum for. Pekinense; Artemisia princeps var. Orientalis*; Garlic, *Allium cepa*; Ginger, *Citrus unshiu*; Onion, *Zingiber officinale*; Honeysuckle; Mandarin orange, *Lonicera japonica*) were shown to have properties to reduce acetate to propionate ratio and methane production, increase fibrolytic bacteria species and decrease methanogen population. It well-known that the cell wall contents (NDF and ADF) have negatively affected on gas production which tends to reduce the microbial activity (Khattab et al., 2015).

Nutrients digestibility.

Nutrients digestibility are listed in table (4). The results of dry matter and organic matter digestibilities (DMd and OMd) cleared that there were no significant differences *(*$p > 0.05$*)* between herbal plant groups and control but, salinomycin group actually recorded the lowest value ($p < 0.05$) being 42.18 and 49.61 % respectively.

The data of fiber fraction degradability (NDFd and ADFd) appeared that there were no significant variance ($p > 0.05$) between control and other treatments except T1 there was recorded the lowest value ($p < 0.05$) being 28.81 and 19.57 % respectively.

Table 4. Ruminal DM, OM, NDF and ADF degradability (%) of experimental diets supplemented with different levels of mix of Thyme plus Celery powder or Salinomycin.

Item	Control	Mix of Thyme and Celery			salinomycin	Pr >F
		T1	T2	T3	T4	
DMd	54.71a ±0.880	53.33a ±0.996	54.35a ±1.070	53.48a ±1.466	42.18b ±0.442	0.0001
OMd	55.90a ±0.497	54.74a ±0.200	54.74a ±0.200	54.89a ±0.538	49.61b ±0.283	0.0001
NDFd	30.39ab ±0.578	28.81b ±0.312	30.06ab ±0.727	30.86a ±0.499	30.00ab ±0.220	0.1062
ADFd	20.32ab ±0.530	19.57b ±0.608	20.52ab ±0.363	20.99ab ±0.476	21.19a ±0.409	0.2015

DMd: dry matter degradability.; OMd: organic matter degradability.; NDFd: natural detergent fiber degradability.; ADFd: acid detergent fiber degradability. Different superscript a, b, c, d in the same row differ significantly ($p < 0.05$).

Dry matter and organic matter digestibilities were not affected by addition of herbal plants these results are in agreement with previous studies (Castillejos et al., 2005, Wanapat et al., 2008, Wanapat, Kang, Khejornsart, & Wanapat, 2013; Nanon, Suksombat, Beauchemin, & Yang, 2014; Patra and Yu (2014); Ishlak et al., 2015 and Khattab et al., 2016). Also, Patra (2011) found that EOs have no negative effect on fiber degradation. Using medical plants in animal diets had no negative effect on activity and growth of the major cellulolytic bacterial population (Cobellis et al., 2016). While, Fraser et al. (2007) using *in vitro* two continuous culture systems were evaluated for its study the effect of cinnamon leaf oils on ruminal fermentation and observed significant reduction on microbial activity and decreased in digestibility. Rezaei and Pour (2012) how observed that the degradability of soybean meal using in vitro gas production technique was decreased on addition thyme methanolic extracts.

Conclusion

Under the conditions of the present study, it could be concluded that adding Thyme plus Celery mix (a natural feed additive) to ruminant diets had insignificant effect on ruminal fermentation parameters (reduction total gas production, SCFA and microbial protein), Also, there were no negative effect on DM, OM, NDF and ADF degradability at 2.5 or 5 g celery and thyme kg^{-1} DMd. Recently, the *in vivo* study is carrying out to illustrate more topics such as the effect of those herbal plants on feed intake, rumen fermentation, productive performance, and milk production and compassion.

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