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In Vitro Rumen Fermentation and Furans Degradation Potential of Rock Candy Juice and Sugarcane Molasses

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ABSTRACT

The present study was conducted to examine the rumen fermentation and furans disappearance potential of rock candy juice (RCJ) and sugarcane molasses (SM). In the first experiment, RCJ was compared with SM by *in vitro* gas production technique. Presence and disappearance of furans in the liquid substances were also investigated before and at the end of 24 h incubation. In the second experiment, a multi-phasic model was used for the interpretation of gas production profile from molasses, rock candy juice, sugar, barley and corn grains whereas gas production was measured at 2 min. intervals. There was no significant difference between the two treatments in terms of the molar VFA proportion and acetate to propionate ratio. At the end of fermentation, concentration of ammonia nitrogen was lower ($P<0.05$) in the RCJ compared to SM which supplied more utilizable CP through RCJ fermentation compared to SM. After 24 h of fermentation, concentration of SM and RCJ furans decreased compared to the pre-incubation phase which denoted the microbial degradation of furans during fermentation. In the second experiment, gas production was evaluated after 24 h incubation of SM, RCJ, sugar, barley and corn grains at two-minute intervals using an automated gas measurement system. The gas production was higher ($P<0.001$) in SM compared to the other treatments. On the other hand, rate of gas production from water-soluble carbohydrate sources 'b' was higher compared to the grains with the lowest 'b' value observed in SM. RCJ had relatively slower rumen fermentation rate. As the price of RCJ was lower compared to the sucrose, hence, it could be used for partial replacement of grains in the diet of dairy cattle.

Key words: Furans, Rock candy juice, Rumen fermentation, Sugarcane molasses

INTRODUCTION

Considering the rising demand for cereal grains, increasing grain prices and reduced groundwater and land for plant cultivation, use of by-products for providing livestock feeds is of paramount importance in many countries (Romero-Huelva *et al.,* 2017). Sugar-containing feeds can increase the energy density of the diet, stimulate DMI and change the rumen fermentation pattern, typically decreasing ruminal NH_3 concentration and increasing rumen butyrate concentration (DeFrain *et al.,* 2006). Despite rapid fermentation of sugars relative to starch, rumen pH is expected to be higher in the animals fed on sugar-containing diets instead of starch. Oba (2011) listed following mechanisms: sugar provides less carbon compared to starch for fermentation and acid production per unit of mass, greater dietary sugar supply increases the rate of passage or production of microbial mass so less organic matter would be available for fermentation and acid production, microbial glycogen synthesis from sugars is another possible reason since microbes can convert sucrose to glycogen as a short-term energy storage which temporarily reduces fermentation acid production in the rumen, possibly contributing to higher rumen pH. Furthermore, greater butyrate and valerate production in the rumen on feeding sugar would decrease proton production per unit of ruminally degraded OM compared with acetate or propionate production as 1 mole of hexose ferments to1 mole of butyrate or 2 moles of propionate or acetate. Thus, it was recommended to use 2.5-5% supplemental sugar in the diet of dairy cattle instead of grain portion (Firkins *et al.,* 2008).

Using sugar beet or sugarcane molasses (SM) as sugar containing liquid feeds was recommended for decreasing dust, increasing palatability and serving as carriers for fat, NPN and other ingredients (Emanuele and Sniffen, 2016; Wang *et al.,* 2017). Iran and India

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are leading producers of rock candy (Persian: *Nabat*) in the world and rock candy juice is co-product of the rock candy production. It is separated from the cooled super-saturated sugar solution which has not crystalized during rock candy formation. With an annual production of about 219,000 tones, Mashhad is considered to be the major region of rock candy industry in the world. Because these two liquid feeds have lower price than sucrose and might be alternatives in dairy cattle ration. However, information is scarce regarding the optimum level and nutritional value of rock candy juice (RCJ) for livestock. The current study consisted of two separate experiments to evaluate and compare RCJ as ruminant feedstuff with sugar beet SM (first experiment), as well as sugar, corn and barely grains (second experiment).

MATERIALS AND METHODS

Barley, corn grains and SM were provided by the Dairy Research Farm at the School of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran. In addition, sugar and RCJ were purchased from one of the rock candy producers in the vicinity of Mashhad city.

In vitro gas production was measured (Menke and Steingass, 1988). Two hours before morning feeding, ruminal content was obtained from two ruminally cannulated Holstein steers fed on a diet of alfalfa hay and concentrate mixture (60: 40 at 2.5% BW). The contents were preserved at a temperature of 39°C in water bath under continues CO_2 flushing so as to foster an oxygen-free environment. Incubation was performed in a water bath at the temperature of 39°C for 24 h. Differences among the sets were corrected using four bottles containing standard hay (purchased from the University of Hohenheim). Finally, three buffered rumen fluid samples were considered as zero-time in the beginning, middle and end of the filling bottles for further analysis. Gas produced was measured at 2, 4, 6, 8 and 24 h after the incubation using a pressure transducer (GMS, Radpaya, Iran) equipped with visual display (López *et al.,* 2007). In addition, the gas produced at any time was simultaneously corrected with blank and recorded as the net gas produced from 200 mg of the DM substrate.

In the second experiment, incubation was carried out for 24 h on five substrates (SM, RCJ, sugar, barley and corn grains) in two sets with 2 and 3 replicates per substrate during the first and second set, respectively. At this stage, an automated computerized gas measurement system (designed and developed in Ferdowsi University of Mashhad) was used to determine the pressure of the produced gas from the substrate fermentation at two minute intervals. The system composed of two 16-channel arrays each of which was equipped with an electric valve. The pressure sensor was connected to the bottle via an extension tube that was connected to a needle and inserted into the bottle through a rubber stopper. Based on the system adjustments, when the gas pressure reached 5 mL at any time, the electric valve would open to completely release the accumulated gas in the headspace within five seconds after which the valve would close. The amount of produced gas from the substrates was marked by the number of valve openings.

The bottles containing 30 mLof water were injected with 1-5 mLof air in 1 mL increments and the subsequent pressure changes were recorded. After repeating this process five times, a calibration data set was obtained with 25 points for every channel. Additionally, a linear regression was achieved and the equation was used for the conversion of the pressure into gas volume for each electric valve separately.

After 24 h incubation, fermentation was arrested by chilling the bottles to the temperature of 4°C and pH was immediately measured by a pH meter as well (Metrohm 691). After centrifuging the contents of the bottles (3000×rpm, 15 min.), 10 mL of the obtained supernatants was acidified with an equal amount of HCl (0.2 N) and stored at the temperature of 4°C in sealed serum bottles until ammonia-N measurement by the distillation method (Kjeltec Auto 1030 Analyzer Tecator, Hoganas, Sweden). Following that, 5 mL of the supernatant was processed using the method proposed by Ebrahimi *et al.*(2011) for the estimation of VFAs by a gas chromatography device equipped with a 50-m (0.32 mm ID) silica-fused column (Chrompack, model

CP-9002, Chrompack, EA Middelburg, the Netherlands and CP-Wax Chrompack Capillary Column, Varian). Helium and crotonic acid (trans-2-butenoic acid) were used as the gas carrier and internal standard, respectively. Initial and final temperature of the oven was 55°C and 195°C, respectively and the temperature of the detector and injector was set at 250°C. Before and after incubation, the presence of furans (furfural and D-hydroxymethyl furfural) in the raw SM, RCJ and culture bottles was estimated by scanning the diluted samples using a UV- visible spectrophotometer within a spectral rang of 190-900 nm (Optizen-3220, MECASYS CO LTD, South Korea) (Martinez *et al.,* 2000).

In the second experiment, the amount of gas was expressed as mL/g OM. Furthermore, a multi-phasic model was used to interpret the gas production profile, as proposed by Groot *et al.* (1996).

$$\text{Gas (mL)} = \frac{a}{(1+(\frac{b}{t})^{c})}$$

where *a* denotes the maximal gas production (ml), b is the time at which half the maximal gas production (a) is within reach in h, c represents the determinants of the shape of the curve and *t* shows the time in h. It should be noted that the mentioned gas production curves were fitted using the GraphPad Prism 6 software.

All the samples were assayed for DM, CP and EE (AOAC, 2005). The DM contents of the liquid substrates were verified by freeze-drying (BETA 2-8 LD plus, Martin Christ, Christ Co., Gefriert rocknungsanlagen, GmbH, Germany) and NDF was measured using the procedure of Van Soest *et al.* (1991). In addition, the liquid sample brix was determined using a refractometer (Atago H-80, Rakutenlnc., Japan). Non-fiber carbohydrate (NFC) was calculated (NRC, 2001). In addition, utilizable crude protein (uCP) was estimated based on the procedure proposed by Edmunds *et al.* (2012). Metabolize energy (ME) was calculated, as follows (Menke and Steingass, 1988). Analysis of the data in the first experiment was performed by t-test after fitting the curves were analyzed using the ANOVA procedure of SAS (SAS Institute Inc., 2016). The mean values were separately analyzed by Duncan's Multiple-Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

In comparison with SM, RCJ had higher DM, OM and brix value, however, CP and EE contents were higher in SM compared to RCJ (Table 1). The origin of RCJ is sucrose, however, during boiling, there is possibility of break down and conversion of the sucrose molecule to monosaccharides or other derivatives. Therefore, it was important to assess its rumen fermentability and because of the similar physical form and nature, SM was selected for all comparisons. Principally, gas production is the first sign of fermentation (Menke and Steingass, 1988). Despite the relatively higher amount of gas production from RCJ than from SM, the difference was not significant (Table 2; Fig. 1). SM or RCJ as substrates resulted in the numerically smaller final concentrations of ammonia-N compared to blank with a significantly greater effect on RCJ compared to SM ($P<0.05$). Cone *et al.* (1997) reported that in the

Table 1. Chemical composition (% DM basis) of samples used in experiment

Item	RCJ	SM	Sugar	Barley	Corn
DM (%)	78.61	63.31	99.98	95.21	95.29
Brix (Ú)	70	66	-	-	-
OM	99.42	84.23	99.95	97.22	98.98
Ash	0.58	15.77	0.05	2.78	1.02
CP	0.37	3.57	0	10.73	8.78
EE	0	0.5	0	2.62	4.15
NDF	0	0	0	2.80	1.61
NFC	99.05	80.16	99.95	81.05	81.31

RCJ= Rock candy Juice; SM= Sugarcane molasses

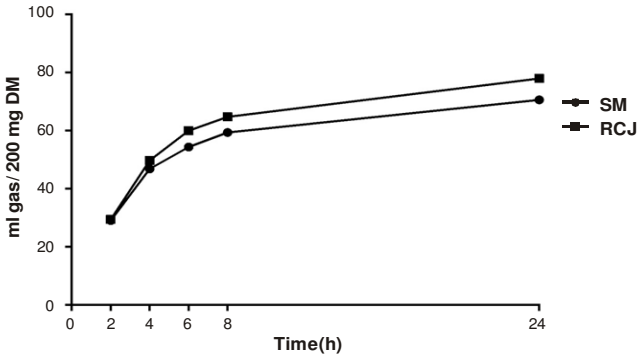

Fig. 1. Cumulative gas production of sugarcane molasses (SM) and rock candy juice (RCJ)

absence of substrates, concentration of ammonia-N increased due to microbial lysis. Ammonia-N in the blank was higher after incubation (37.40 mg/dL) as compared to zero hour (17.38 mg/dL) in the absence of RCJ and SM. Fermentation of SM and RCJ resulted in decrease in the final ammonia-N in the culture media. Similarly, Broderick *et al.* (2008) reported that dietary inclusion of molasses decreased ammonia-N concentration in rumen fluid, however, some workers showed no effect of sucrose supplementation on rumen ammonia concentration (Penner and Oba, 2009; Tuyen *et al.,* 2014).

Fermentation of RCJ provided significantly higher uCP at the duodenum in comparison to SM (P<0.05). Edmunds *et al.* (2012) defined uCP as the sum of microbial protein and rumen un-degradable protein. Since, the CP content of RCJ and SM was significantly lower, the estimated uCP values for the two substrates indicated the utilization of ammonia-N by rumen microorganisms for the production of microbial protein because feeding of rapidly fermentable carbohydrates is expected to capture more ruminally degradable N (Tuyen *et al.,* 2014).

Metabolisable energy content of the two liquid substrates (Table 2) were close to the ME values (2.46-2.87 Mcal/kg) for SM reported by NRC (2001). Metabolisable energy content of SM and RCJ was estimated to be 11.70 and 12.74 MJ/kg, respectively. Total VFA concentrations (Table 2) increased from the baseline (i.e., zero-time, 18.00 mmol/L, from buffered rumen fluid) to 98.89 and 92.54 mmol/L in RCJ and SM, respectively. There was a slight increase in the molar proportion of acetate and propionate and reduction in butyrate as compared to blank. Rumen microorganisms are able to ferment carbohydrates into VFAs (Oba, 2011). *In vitro* rumen fermentation of RCJ and SMT increased both acetate and propionate (Table 2). Therefore, these findings indicated the fermentation of both substances to VFAs. However, no significant differences in individual acids and acetate to propionate ratio were found between two carbohydrate sources. Ferraro *et al.* (2009) reported that fermentation of SM resulted in increase in propionate with a reduction in butyrate. In

Table 2. Effects of SM and RCJ on rumen fermentation parameters

Item	Treatment		p	Zero time	Blank
	RCJ	SM			
Gas production at 24 h (mL/200 mg DM)	78.02	70.64	ns	-	-
Ammonia-N (mg/dL)	26.06[b]	28.24[a]	<0.05	17.38	37.40
Metabolisable energy (MJ/kg)	12.74	11.70	ns	-	-
Utilizable protein (g/kg DM)	146.08	121.68	<0.05	-	-
Total VFA (mmol/L)	98.89	92.54	ns	18	53.20
Individual VFA (mol/100 mol)					
Acetate	60.85	60.61	ns	59.32	60.05
Propionate	26.58	26.94	ns	26.17	24.45
Butyrate	12.57	12.45	ns	14.51	15.50
Acetate: propionate	2.29	2.25	ns	2.27	2.46
pH	6.55	6.60	ns	6.74	6.93

[a,b] Means with different superscripts in row differ significantly (P<0.05); RCJ= Rock candy juice; SM = Sugarcane molasses

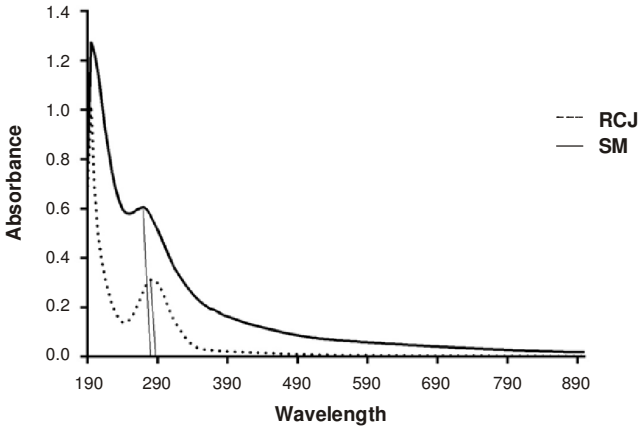

Fig. 2. UV spectra of furfural and HMF in raw sugarcane molasses (SM) and rock candy juice (RCJ) after dilution 1: 1000 in distilled water

contrast, many workers reported that butyrate could increase as a result of ruminal sugars fermentation (DeFrain *et al.,* 2006; Firkins, 2011). Marty and Preston (1970) pointed out that effect of molasses on VFA production was directly correlated with its concentration in the diet. Low dietary levels of molasses (<15% DM) did not modify the fermentation pattern and VFA production was similar to grain diets with proper amounts of propionate as a gluconeogenic substrate for the liver. In contrast, when dietary molasses exceeded 15%, propionate production decreased, alongwith an abnormal increment in butyric acid production. In the present study, total VFAs at zero h was 18.00 mmol/L which reached to 53.20 mmol/L in the blank along with an increase in the butyrate (Table 2). This showed presence of soluble substances in the buffered rumen fluid which could be fermented to provide requirements of mixed rumen microorganisms, therefore, added liquid feeds fermented mainly to acetate and propionate.

Scanning of the pure, diluted SM and RCJ samples showed two peaks at wavelengths 270 and 284 nm, respectively (Fig. 2) which showed presence of furans in two substrates. Peaks in the pre-incubation phase and after terminating the incubation (Fig. 3 and 4) indicated a decrease in the concentration of furans for both substances. Martinez *et al.,* (2000) reported that the maximum absorbance of furfural and HMF was obtained at the wavelength of 270 and 284 nm, respectively. Therefore, furfural was the major furan in SM while HMF was the dominant furan in RCJ. The concentrations of furfural and HMF after 24 h of fermentation were lower compared to the pre-incubation phase suggesting the disappearance or

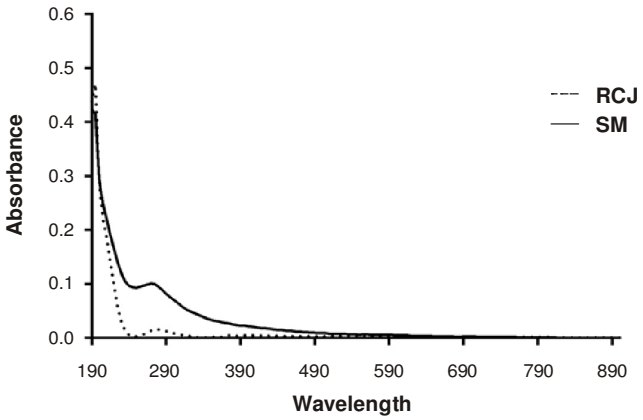

Fig. 3. UV spectra of furfural and HMF in diluted sugarcane molasses (SM) and rock candy juice (RCJ) (1: 1000 in distilled water) before incubation

degradation of furan derivatives. In this regard, Castro *et al.*(1994) claimed that rumen microbes could almost completely degrade both these toxic compounds within 6 h of *in vitro* fermentation. In a similar study, Koopman

Table 3. Calculated values of 'a' 'b','c' and metabolisable energy (MJ/kg)

Parameter	RCJ	SM	Sugar	Barley	Corn	SEM	*p*
a	469.22^{bc}	592.64^{a}	436.78^{c}	517.06^{ab}	570.90^{a}	6.35	<0.001
b	5.75^{ab}	4.85^{b}	5.95^{ab}	6.37^{a}	6.39^{a}	0.80	0.01
c	1.70^{a}	1.22^{b}	1.70^{a}	1.19^{b}	1.20^{b}	0.36	<0.001
ME	11.83	12.19	10.97	11.69	12.89	1.01	0.09

^{a,b,c}Means with different superscripts in a row differ significantly (P<0.01); RCJ= Rock Candy Juice; SM= Sugarcane Molasses; 'a'- asymptotic maximal gas production (mL gas/g OM) 'b'-Time (h) half of maximum production reached; '*c*'- determines the shape of curves

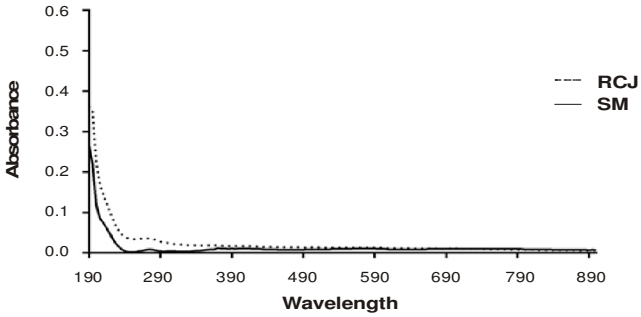

Fig. 4. UV spectra of furfural and HMF in incubated sugarcane molasses (SM) and rock candy juice (RCJ) after dilution 1:1000 in distilled water

et al., (2010) isolated a strain of *Cupriavidus basilensis HMF14* capable of selectively degrading furfural and HMF within a microbial population in the rumen. Liu *et al.* (2015) showed that under anaerobic conditions, over 90% of furan derivatives with an initial concentration below 1 g/L were degraded. There are reports of the toxicity of furfural to yeast and rumen microorganisms (Kyuma *et al.,*1991) and that furan derivatives are regarded as a notorious fermentation inhibitors (Li and Chen., 2008; Liu *et al.,* 2015).

Range of total gas production was 436.78-592.64 mL gas/g OM for sugar and SM, half of it was produced after 5.95 and 4.85 h of starting incubation, respectively. A significant difference was observed between the substrates in terms of parameter *a* (P<0.001). On the other hand, the fermentation rate *'b'* of soluble carbohydrate sources was higher compared to that of the starch-containing sources. In the current study, no significant difference was observed between sugar and RCJ in terms of parameter *b,* however, SM fermentation rate was greater during *in vitro* incubation compared to sugar, RCJ and the two grains (P=0.01).

Rate of the fermentation in barely is generally greater than corn (NRC 2001), however, in the present study similar values for parameter 'b' could be explained because of the fine grinding for *in vitro* incubation. Fermentation of sugars in the rumen begins with the hydrolysis of disaccharides to monosaccharides followed by the fermentation of monosaccharides. Molasses contain 74.7% sugars of which 2/3 is sucrose but glucose, fructose and other monosaccharides are also present in this by-product (Xu *et al.,* 2015; Clemens *et al.,* 2016). Because estimated values of gas production parameters for RCJ were more close to that of sugar (Table 3). So, RCJ may contain a relatively more sucrose than SM.

CONCLUSION

Rock candy juice could be introduced as a readily fermentable ruminant feed and was similar to sugarcane molasses in terms of the metabolisable energy and VFA production with a relatively slower fermentation rate. As RCJ is a by-product and has lower price compared to the sucrose, it could be used for partial replacement of grains in the diet of dairy cattle.

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