Inclusion Levels of Fermented Apple Bagasse on *in Vitro* Rumen Fermentation of Alfalfa Hay

Yamicela Castillo-Castillo¹, Oscar Ruiz-Barrera², Eduviges Burrola-Barraza², Claudio Arzola-Alvarez², Agustin Corral-Luna², Carlos Rodriguez-Muela² and Manuel Murillo-Ortiz³

1. Department of Veterinary Medicine, Multidisciplinary Division, Autonomous University of Juarez City, Nuevo Casas Grandes, Chihuahua 31803, México

2. College of Animal Science and Ecology, Autonomous University of Chihuahua, Chihuahua 31453, México

3. Faculty of Veterinary Medicine and Animal Science, Juarez University of the State of Durango, Durango 34620, Mexico

Abstract: The aim of this study was to assess the effect of inclusion of fermented apple bagasse (FAB) obtained through solid state fermentation on pH, ammonia nitrogen (N-NH₃), volatile fatty acids (VFA) content, *in vitro* dry matter digestibility (IVDMD), lactic acid and microbial counting of alfalfa hay under *in vitro* rumen environment; four levels of FAB were evaluated (0, 0.25, 0.50 and 0.75 g/dry matter of FAB) replacing 1.5 g dry matter (DM) of alfalfa hay and incubated at different fermentation times (0, 4, 8, 12 and 24 h) using a complete random design with repeated measures on time. Counts of live yeast colonies (6.08, 6.33, 6.24 and 6.51 CFU/mL expressed as log 10) was higher when FAB was included in the different levels up to the 12 h of fermentation ($P < 0.0001$); lactic acid content also increased as FAB was included in the different levels (10.61, 13.86, 16.84 and 14.57 µg/mL) up to the 12 h of incubation ($P < 0.001$). Nevertheless, the other variables measured as pH, N-NH₃, VFA, IVDMD, total bacteria and fungi counts, were not affected by the treatments. It is concluded that substitution of FAB by alfalfa hay in an *in vitro* rumen ecosystem positively modified live yeast colonies and lactic acid concentration, without effect on the other fermentative and microbial parameters of the *in vitro* rumen environment, but considering mixes of FAB and alfalfa hay as a quality ingredient for the feeding of ruminants.

Key words: Apple bagasse, alfalfa, microbial, solid state fermentation.

1. Introduction

The search for alternate food sources to feed livestock, to reduce conventional food imports and to reduce production costs has been of prime importance to researchers in recent years. Apple bagasse is a waste product of the juice extraction industry that provides low protein content [1] and is considered a pollutant to the environment. Its use as a food source for ruminants has been improved through a process of solid state fermentation (SSF), which improves the quality and quantity of protein as well as the digestibility of fiber [2, 3]. Many studies show that foods that undergo SSF are processed successfully by ruminants [4, 5]. Ramos [5] found increases in dry

matter (DM) digestibility and improved patterns of rumen fermentation when he supplemented two types of foods produced by SSF in cattle consuming elephant grass (*Pennisetum purpureum*). In the state of Chihuahua, one of the main foods for dairy cattle is alfalfa hay because of its high nutritional value, mainly its high crude protein (CP) content (18%-20%) [6]; however, its market value is too high and out of reach to many low-income producers, therefore cost effective alternatives must be found as partial replacement in the diets of ruminants. The objective of this study was to study the effect of inclusion of fermented apple bagasse (FAB) in SSF in the digestive physiology and microbial quality of fibrous materials, such as, alfalfa hay in an *in vitro* rumen system, as well as its feasibility as an ingredient in feed for ruminants.

Corresponding author: Oscar Ruiz-Barrera, Ph.D., research field: animal nutrition. E-mail: oscarruiz80@gmail.com.

2. Materials and Methods

Apple bagasse were obtained from a local factory of apple processing for elaboration of juice and the work was carried out in the rumen microbiology laboratory of the College of Animal Science and Ecology of the Autonomous University of Chihuahua, located in Chihuahua City, Mexico. Apple bagasse was fermented under SSF process according to the method described by Elias et al. [7]. Apple waste taken from the factory CONFRUTTA, S.A. (Chihuahua, Mexico) was ground with a Wiley mill and mixed with 1.5% urea, 0.2% ammonium sulfate and 0.5% of a mineral salts mixture rich in macro and micro elements. All these ingredients were thoroughly mixed and 340 g were distributed to separate sterile 500 mL Erlenmeyer flasks for SSF. Each flask was plugged with cotton and incubated under static conditions at 32 °C for 0, 24, 48 and 72 h.

2.1 Experimental Procedure

Two ruminally fistulated Holstein cows were used as rumen fluid donor and fed twice daily a total ration mixed of whole grain corn, cottonseed, wheat bran, animal fat, corn gluten meal, cottonseed meal, soybean meal, cane molasses, mineral salts, baking soda, magnesium oxide, salt, urea, bypass fat and silage. Water was provided freely. Rumen fluid was collected before any food consumption in the morning and filtered through several layers of cheesecloth into a pre-warmed Thermos flask. The entire procedure was performed under CO₂ atmosphere in order to ensure anaerobic conditions. The fermentation was performed in 20 serum flasks of 250 mL capacity, and incubated with mechanical agitation at 39 °C. The filtered rumen fluid (FRF) and buffer solution were at a 1:2 ratio (50 mL:100 mL) and then added to a mixed substrate of fermented apple bagasse and alfalfa hay, which were milled and then passed through a 2 mm sieve before being fermented and distributed to the flasks according to the following treatments: (A) 1.5 g of alfalfa; (B) 0.25 g of fermented apple bagasse +

1.25 g of alfalfa; (C) 0.50 g of fermented apple bagasse + 1 g alfalfa; (D) 0.75 g of fermented apple bagasse + 0.75 g of alfalfa. Samplings were recorded at 0, 4, 8, 12 and 24 h. At each sampling time, four flasks were withdrawn from the incubator and the total contents were collected, individually homogenized and frozen for further analysis.

The chemical composition of substrates (dry basis) used were: alfalfa% (DM, 90.87; organic matter (OM), 89.07; ash, 10.93; CP, 18.02; neutral detergent fiber (NDF), 65.6; acid detergent fiber (ADF), 39.0; ether extract (EE), 2.02) and FAB% (DM, 23.71; OM, 89.94; ash, 10.05; CP, 35.05; NDF, 48.31; ADF, 37.54; EE, 5.2).

2.2 Laboratory Analysis

The pH of the filtrate obtained as described in the experimental procedure, was immediately recorded with a table potentiometer (Hanna). For quantification of lactic acid, 0.25 mL from the sample filtrated was diluted in 9.75 mL of distilled water. From this solution, 0.5 mL was used for determination according to the colorimetric method described by Madrid et al. [8]. CFU/mL counts for total bacteria and yeast were determined by Hungate's roll tubes incubation technique [9] under strict anaerobic conditions. Inoculation of total rumen bacteria was performed in culture medium [10] modified by Elias [11], and yeast was inoculated in malt extract agar culture medium with 0.01 g/L chloramphenicol. For inoculations, we used three separate dilutions in anaerobic diluent [10]. For total viable bacteria: 10⁹, 10¹⁰ and 10¹¹ were used and for yeast 10⁴, 10⁵ and 10⁶. The number of colony forming units (CFU) was determined by counting visual appearance of the colonies in roll tubes under the microscope. Counts of protozoa were performed in a Neubauer chamber, pH was measured with a table potentiometer, lactic acid was analyzed by the colorimetric method described by Taylor [12], ammonia nitrogen (N-NH₃) was according to the colorimetric method described by Broderick and Kang

[13], and volatile fatty acids (VFAs) by gas chromatography and *in vitro* disappearance of DM was determined as described by Capetillo et al. [14].

2.3 Statistics

The data were analyzed through Ref. [15] by fitting a model that included the following variables: the level of inclusion of FAB (four levels), the fermentation time and the correlation between the two, and as a random effect, the identification of the flask (experimental unit) nested in the level of bagasse. We performed a trend analysis, where possible, of the variables through different fermentation times in each of the levels of inclusion of bagasse considering the fermentation time as an indicator variable. A completely randomized design with repeated measures over time was used.

3. Results and Discussion

The pH and lactic acid decreased quadratically as fermentation time increased ($P < 0.02$ and $P < 0.0001$, respectively) (Table 1).

The pH values in the control and treatments with increasing levels of bagasse were found in a range from 7.3 to 7.1. These pH results are higher than those reported by Ramos [5] when rumen cannulated cattle were fed with elephant grass with 9.56% CP and supplemented with Sacchasorgo and Sacchapulido, foods produced by SSF, however, the trends over fermentation time of this study were similar to the results obtained by these authors at 12 h. The lack of significant differences between treatments and the high pH values found in this study are possibly due to the high input of nitrogen from alfalfa as well as from

Table 1 pH, lactic acid, N-NH₃ and *in vitro* dry matter digestibility (IVDMD) in rumen fermentation with mixtures of FAB and alfalfa hay.

Estimates	FAB level (g DM)				EE	Tendency		FAB level effect
	0	0.25	0.50	0.75		L	Q	
pH								
0 h	7.32	7.27	7.27	7.31	0.06	0.0003	0.02	0.90
4 h	7.27	7.20	7.33	7.34	0.06			0.90
8 h	7.23	7.20	7.18	7.12	0.06			0.90
12 h	7.23	7.17	7.07	7.10	0.06			0.90
24 h	7.15	7.15	7.10	7.10	0.06			0.90
Lactic acid (µg/mL)								
0 h	16.60 ^a	17.68 ^a	18.29 ^a	24.61 ^b	1.22	0.0001	0.0001	0.001
4 h	16.07	18.66	16.42	17.36	1.22			0.1
8 h	12.51 ^a	16.82 ^b	17.97 ^b	16.73 ^b	1.22			0.001
12 h	10.61 ^a	13.86 ^b	16.84 ^b	14.57 ^b	1.22			0.001
24 h	12.98	15.88	15.51	15.81	1.22			0.1
N-NH ₃ (mg/dL)								
0 h	13.97	14.68	13.58	18.89	0.18	0.16	0.51	0.32
4 h	14.37	15.94	21.68	19.72	0.18			0.32
8 h	18.26	14.72	19.52	18.50	0.18			0.32
12 h	19.17	16.45	16.81	17.95	0.18			0.32
24 h	19.91	18.26	17.91	21.92	0.18			0.32
IVDMD (%)								
0 h	26.18	23.17	20.51	15.76	3.17	0.0001	0.0001	0.29
4 h	37.08	33.81	24.84	24.35	3.17			0.29
8 h	46.23	42.33	39.35	38.25	3.17			0.29
12 h	54.12	52.44	54.24	50.80	3.17			0.29
24 h	60.83	63.01	67.74	67.27	3.17			0.29

^{a,b}Means in the same column with different letters are different; L = linear tendency throughout fermentation time; Q = quadratic tendency throughout fermentation time.

fermented bagasse. The inclusion of alfalfa in the diet produces a constipation effect due to the high protein content inducing high levels of N-NH₃ in the rumen [6]. Lactic acid showed significant differences between treatments at 0, 8 and 12 h of fermentation ($P < 0.0001$). The higher concentrations of lactic acid was found at 0 h when 0.75 g of FAB were included and also at 8 h and 12 h with increasing FAB levels (Table 1) were attributed to FAB having a greater amount of this metabolite, which is produced by the activity of lactic acid bacteria that normally exist in SSF processes. Lactic acid tended to decrease as fermentation time lapsed in all treatments (Table 1), but this difference was increased in the treatment with 0.75 g of FAB, possibly because this treatment had a larger amount of yeast. This stimulates the growth of *Selenomonas ruminantium*, which consumes lactate, leading to stabilization of pH to levels close to neutral (Table 1) [16], thus favoring cellulolysis and DM digestibility.

In vitro dry matter digestibility (IVDMD) showed a quadratic trend ($P < 0.0001$) to increase as fermentation time increased (Table 1), reaching values between 63% and 68% in treatments of inclusion of increasing levels of apple bagasse and 60% in treatment with alfalfa at 24 h, the maximum hours of fermentation. It was also noted that the treatments did not affect IVDMD (Table 1). Results at 24 h where treatments of apple bagasse were included are higher than those reported by Vicente et al. [17] when rumen degradability of apple bagasse silage was measured without the inclusion of a fibrous source as in this case. But results were lower than those reported by Anrique and Viveros [18] who also measured the rumen degradability of silage apple bagasse. The lack of effect of FAB on the IVDMD with respect to the control treatment and the works mentioned above at different time points is possibly due to the fact that pH levels were above 6 in all treatments, the value at which cellulolytic bacteria increase growth and therefore increase fiber digestibility. However, Elias [19] states that the optimum pH for maximum

cellulose breakdown is between 6.6 and 6.8. It can also be attributed to the fact that N-NH₃ concentrations in the four treatments were appropriate and a constant supply of ammonia stimulates growth of rumen bacteria (Table 1) and therefore DM digestibility [20]. In addition to the high content of ADF in the case of alfalfa and fermented apple bagasse, it made the concentration of these structural carbohydrates to remain high in all treatments, and when *in vitro* digestibility was performed, 24 h of fermentation was not enough time to detect significant differences in DM digestibility between treatments. Therefore, future investigations should consider an *in vitro* digestibility time point of 48 h or perform *in vitro* digestibility using the application of effective degradability in time according to the method by Ørskov and McDonald [21].

The concentrations of N-NH₃ found in this study did not show any trend through the different fermentation time points and there were no differences between treatments either (Table 1); however, they were within the appropriate range for efficient microbial protein synthesis in rumen (15-20 mg/dL) according to Ref. [22], which is reflected by the growth of bacteria and total protozoa counts (Table 2).

The behavior in total bacterial growth and protozoa counts across the different fermentation time points was linear ($P < 0.01$) and quadratic ($P < 0.04$), respectively, without differences between treatments (Table 2), probably due to the fact that all treatments had an adequate pH and N-NH₃ for their growth and multiplication.

However, the results in yeast counts (Table 2) showed that they increased as the level of FAB were included at 0, 4, 8 and 12 h ($P < 0.0001$), at which point these organisms stabilized and kept the population constant up to 24 h in all treatments. The highest concentration found of these microorganisms in the first 12 h was possibly due to the fermented apple bagasse used because it had a high concentration of live yeast (2.8×10^7 CFU/mL), which is reflected at

Table 2 Protozoa, total rumen bacteria (TRB) and yeast counts in rumen fermentation with mixtures of FAB and alfalfa hay.

Estimates	FAB level (g DM)				EE	Tendency		FAB level effect
	0	0.25	0.50	0.75		L	Q	
Protozoa (× 10 ⁴ /mL)								
0 h	11.62	16.25	12.87	15.32	3.22	0.57	0.04	0.74
4 h	16.12	16.67	20.12	18.39	3.22			0.74
8 h	14.0	10.37	10.5	17.75	3.22			0.74
12 h	15.75	13.5	12.5	15.12	3.22			0.74
24 h	7.12	9.75	7.25	9.75	3.22			0.74
TRB CFU/mL (log 10)								
0 h	11.88	11.75	11.71	11.82	0.04	0.01		0.83
24 h	11.73	11.85	11.89	11.84	0.04			0.83
Yeast CFU/mL (log 10)								
0 h	6.47 ^a	6.70 ^b	7.07 ^c	7.27 ^d	0.06	0.0001	0.0001	0.0001
4 h	6.26 ^a	6.49 ^b	6.75 ^b	6.92 ^c	0.06			0.0001
8 h	6.05 ^a	6.46 ^b	6.41 ^b	6.68 ^c	0.06			0.0001
12 h	6.08 ^a	6.33 ^b	6.24 ^b	6.51 ^c	0.06			0.0001
24 h	6.10	6.10	6.23	6.15	0.06			0.52

^{a, b, c, d} Means in the same column with different letters are different; L = linear tendency throughout fermentation time; Q = quadratic tendency throughout fermentation time.

Table 3 Concentrations of acetic, propionic and butyric acid in rumen fermentation with mixtures of FAB and alfalfa *in vitro*.

Estimates	FAB level (g DM)				EE	Tendency		FAB level effect
	0	0.25	0.50	0.75		L	Q	
Acetic (mmol)								
0 h	34.10	47.41	37.52	41.08	6.92	0.0001	0.0001	0.26
4 h	48.52	67.17	54.74	48.60	6.92			0.26
8 h	63.95	63.61	70.74	56.37	6.92			0.26
12 h	64.64	66.78	79.89	64.72	6.92			0.26
24 h	66.34	70.85	73.98	70.71	6.92			0.26
Propionic (mmol)								
0 h	12.62	17.63	13.56	15.42	2.45	0.0001	0.003	0.27
4 h	16.85	21.77	17.10	16.35	2.45			0.27
8 h	19.68	19.56	22.64	18.07	2.45			0.27
12 h	21.88	22.28	26.76	21.91	2.45			0.27
24 h	23.35	23.77	24.19	23.27	2.45			0.27
Butyric (mmol)								
0 h	3.18	4.24	3.66	4.08	1.06	0.0001	0.01	0.82
4 h	5.50	6.09	4.89	5.26	1.06			0.82
8 h	5.94	5.32	6.34	5.35	1.06			0.82
12 h	6.42	6.21	7.69	6.64	1.06			0.82
24 h	7.48	6.59	7.39	7.5	1.06			0.82

L = Linear tendency throughout fermentation time; Q = quadratic tendency throughout fermentation time.

the 0 time point. As time of fermentation increased, these tended to decrease quadratically in all treatments ($P < 0.0001$) (Table 2). This may be due to the

presence of yeast in the rumen because it is present in foods consumed by animals. Because they are non-native organism, they take advantage of the

nutrients with which they were deposited [23], such is the case with fermented apple bagasse and the buffer solution containing glucose at low concentrations. Upon reaching the rumen environment, yeasts competes for energy sources with the population of rumen bacteria which are found in high concentrations (10^{11} CFU/mL) and well adapted, which induces yeast to rapidly move into an energy-maintaining state without multiplying and leading to its death phase [24]. So we can infer that yeast can adapt in the rumen environment, but in low concentrations as noted in Table 2.

The concentrations of the acetic, propionic and butyric acids tended to increase quadratically as fermentation time increased (Table 3) and were similar between treatments at different fermentation time points.

The values of acetic acid treatments which included FAB are similar. Propionic and butyric acids increased less than those reported by Ramos [5] after 12 h of fermentation when cattle was supplemented with a *Saccharospora* and *Saccharipulido* feeds.

The similar results in the concentrations of VFA (acetic, propionic, butyric) found in this work at different treatments may be due to the fact that treatment with only alfalfa and treatments which included increased levels of FAB favored an optimal pH and a constant supply of N-NH_3 . This allows for good microbial growth and therefore a better digestibility of DM and adequate rumen fermentation, which is reflected in the VFA concentrations.

4. Conclusions

This study reveals that inclusion of fermented apple bagasse caused important changes in some chemical composition and microbiological characteristics of alfalfa hay. It properly stimulated viable yeast counts in the *in vitro* rumen environment and also increased concentration of lactic acid and decreased pH, which promoted an optimal environment for growth of yeasts. Other variables studied such as rumen pH, $\text{NH}_3\text{-N}$,

VFA, IVDMD, total rumen bacterial count and protozoa were not affected by treatments. It is recommended that such bio product should be part of local rations for domestic animals as an alternative feed source, reducing conventional food imports and production costs.

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