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Ruminal fermentation profile, yield milk and chemic and microbiologic quality in dairy cattle feed with nitrogen enriches apple pomace

Perfil de fermentación ruminal, producción y calidad química y microbiológica de la leche de bovinos lecheros alimentados con bagazo de manzana enriquecido con nitrógeno

Alberto Muro Reyes [1] - Francisco Javier Gutiérrez Piña [1] - Carlos Rodríguez Muela [2]
Carlos Abel Amaya Guerra [3] - Héctor Gutiérrez Bañuelos [1] - Luis Humberto Díaz García [1]
Laura Alicia Polín Raygoza [1] - Alejandro Espinoza Canales [1]

[1] Universidad Autónoma de Zacatecas, Unidad Académica de Medicina Veterinaria y Zootecnia
[2] Universidad Autónoma de Chihuahua, Facultad de Zootecnia y Ecología, Chihuahua
[3] Universidad Autónoma de Nuevo León, Departamento de Alimentos de la Facultad de Ciencias Biológicas

Correspondence author: fpina@uaz.edu.mx

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Abstract
Solid-state fermented apple pomace (AP-SSF) enriched with non-nitrogen protein has been studied as an alternative ingredient for feeding dairy cattle. The present study aimed to evaluate the effect of the inclusion of AP-SSF in the feeding of dairy cows in early lactation on the yield and quality of milk, microbial contamination, and ruminal fermentation parameters, as well as the concentration of ammonia and the number of protozoa. Twenty Holstein cows were used, 20 of 660 kg on average, with 2-4 lactations and an average of 89 days in milk. Two groups were formed with ten cows each, randomly distributed to form a 2 x 2 Latin square with standard periods. Experiment diets were formulated with traditional ingredients, and one of them was added AP-SSF. Rations were gradually incorporated, giving ten days for adaptation and another 20 days for sampling. In lactose and production variables, no difference was statistically significant (P> 0.05) in milk fat and protein differences (P < 0.05) in the first component for the control treatment and the second toward treatment, as AP-SSF succeeded in increasing the percentage of milk fat. For *Staphylococcus sp.*, *Streptococcus sp.* and Total coliform differences (P < 0.05) in favor of the treatment were achieved AP-SSF and decreased the CFU / mL for *Salmonella sp.* while there was no difference (P > 0.05). Volatile fatty acids showed statistical difference (P < 0.05) for Acetic, Propionic, and Butyric acids. AP-SSF treatment for the rest of the parameters ruminal pH, ammonia, and protozoa did not show any significant difference (P > 0.05) between treatments. Concluded that it is possible to incorporate AP-SSF as a protein ingredient in diets of dairy cows in early lactation because some variables improved and showed no adverse effects in any of the variables evaluated.
Keywords: apple residue; solid-state fermentation; milk quality; lactation; microbial contamination; fermentation; production; protein; feeding; animals

Resumen
El bagazo de manzana fermentado en estado sólido (AP-SSF), enriquecido con nitrógeno no proteico, ha sido estudiado como un ingrediente alternativo para la alimentación del ganado lechero. El presente estudio tuvo como objetivos, evaluar el efecto de la inclusión de AP-SSF en la alimentación de vacas lecheras en lactancia temprana, sobre la producción y calidad de la leche, contaminación microbiana y parámetros de fermentación ruminal, así como la concentración de amoniaco y el número de protozoarios. Se utilizaron 20 vacas Holstein de 660 kg en promedio, de entre 2 y 4 lactancias y con un promedio de 89 días en leche y 30 lts de producción. Se formaron dos grupos con 10 vacas, aleatoriamente distribuidas, cada uno en un cuadrado latino de 2 x 2, con tiempos comunes. Las dietas experimentales se formularon con ingredientes tradicionales y a uno de ellos se le

agregó AP-SSF. Las raciones se incorporaron gradualmente dando un período de 10 días para la adaptación, seguido de otros 20 días de muestreo. Se encontró diferencia estadística ($P < 0.05$) en el porcentaje de grasa en la leche para el tratamiento con AP-SSF. Para *Staphylococcus sp.*, *Streptococcus sp.* y Coliformes totales se observaron diferencias en su disminución CFU/mL ($P < 0.05$) a favor del tratamiento AP-SSF. En relación con los AGVs, se observó diferencia estadística ($P < 0.05$) a favor para el tratamiento AP-SSF con Acético, Propiónico y Butírico. Se concluye que es posible incorporar AP-SSF como ingrediente proteico en dietas de vacas lecheras en lactancia temprana, debido a que algunas variables mejoraron y no mostraron efectos adversos en ninguna de las variables evaluadas.

Palabras clave: residuo de manzana; fermentación en estado sólido; calidad láctea; lactancia; contaminación microbiana; fermentación; producción; proteína; alimentación; animales

1. Introduction

There is immense environmental pressure to reduce the pollution arising from agro-industrial activities. Recently, worldwide by-products production was estimated at 3.5 billion tons per year (Robinson and Tigan, 2003; Graminhha *et al*., 2008). Within those, apple pomace is an important source. Lately, it has been an increasing concern to improve apple pomace (AP) by enriching it with non-protein nitrogen under solid-state fermentation (SSF). The main aim of the referred process is to increase crude protein content, which could decrease direct competition for cereals between human and animal nutrition, generating an ingredient (AP-SSF) that can be used for feeding ruminants. Published results showed that crude protein increased from 4 to 24% (Hernández *et al*., 2007). One animal target for the mentioned ingredient (AP-SSF) is dairy cattle (Weinberg Z.G., 2000). The milk industry has been recognized as one of the most dynamic areas of the agriculture field due to an increasing milk demand around the world and human overpopulation (Goff and Griffiths, 2006). Moreover, there is an increasing concern about the nutritional and microbial quality of milk by consumers, which has been pushing producers to improve milk components like fat, protein, and lactose, and decrease foodborne milk pathogens like total coliforms, *Streptococcus spp.*, *Staphylococcus spp.*, and *Salmonella spp*, which are directly related to organoleptic properties of milk and its products (Hettinga, 1989). The simplest way to improve milk quality is by nutritional strategies, with immediate effects and lower costs, since feedstuffs can modify ruminal parameters such as pH, volatile fatty acids, and ammonia affecting milk yield, quality, and composition. This project evaluated milk yield and quality, microbial contamination, and ruminal fermentation parameters. Also, ammonia concentration and protozoa number on dairy cows in early lactation supplemented with AP-SSF.

2. Method

Experimental design

The Institutional Animal Care and Use Committee at the Autonomous University of Zacatecas approved the experimental procedures (UAZ-2013-36268). Twenty Holstein lactating dairy cows were selected (2 to 4 lactations) with an average age between 4 and 6 years old, an average weight of 660 kg, and a body condition score of 3. They were randomized and assigned to two treatment groups (10 animals in each group) on a 2 X 2 Latin square design (2 treatment diets and two periods of time). The apple bagasse is spread out along a concrete slab with a height of 15 to 20 cm to incorporate the NNP, the next day after mixing the ingredients, it is turned twice a day until the sixth or eighth day, the temperature and height of the bagasse bed were taken daily, depending on the humidity it retains, the material is allowed to lose the rest of the humidity until it reaches only 12 or 13 %, which takes a period of 4 to 6 days. The control diet was based on corn silage, protein concentrate, corn grain, alfalfa hay, corn hay, oats hay, and mineral/vitamin supplement, with a nutrient profile of CP, CF, NDF, ADF, Ca, and P; treatment diet was the same than control group only it rested 5% of the concentrate and added 5% of AP-SSF. The ingredients and nutritional contributions of the diets are shown in table 1. Each group was fed an average of 23 kg. Diet was offering 50 percent in the morning and 50 percent in

the afternoon. Apple pomace-SSF was elaborated by adding ammonium sulfate (0.5%), urea (1.5%), and commercial mineral supplement (0.5%) to apple pomace and fermented in solid state by 14d before being used as feed supplement (Becerra, 2006). The adaptation period to diet was 10 d long, after that, the first trial period of 20 d began with calculated milk yield. 60 mL milk samples per treatment were taken to determine foodborne pathogens (standard plate count).

Table 1. *Ingredients and nutritional contribution of diets with and without apple pomace-SSF.*

	Control	Apple pomace-SSF
Ingredient		
Corn silage %	43	43
Concentrate %	24	19
Corn grain %	14	14
Alfalfa hay %	10	10
Corn hay%	4	4
Oat hay %	4	4
Mineral and Vitamins %	1	1
Apple pomace-SSF %	0	5
Nutrients		
CP %	18.2	18.0
CF %	15.0	15.0
NDF %	34.7	34.5
ADF %	19.8	19.9
Ca %	1.2	1.2
P %	0.5	0.5

Note: CP = Crude Protein; CF = Crude Fiber; NDF = Neutral Detergent Fiber; ADF = Acid Detergent Fiber; Ca = Calcium; P = Phosphorus.

Microbial quality of milk

A sample of 60 mL per cow, taken in the afternoon, was placed in sterile containers and immediately refrigerated at 4ºC, identified with the number of the cow and treatment. In the laboratory, 1 mL of milk was taken and diluted in a phosphate buffer solution in a ratio of 10 to 1, then 1 mL was taken to seed it with a specific agar plate (IDF., 1991; IDF., 2004). The culture medium for *Salmonella spp.,* was Muller Kaufmann Broth Base with brilliant green, for *Staphylococcus spp.,* blood agar, for *Streptococcus spp*., Granada medium, and for Total Coliforms bile violet. For each of the bacteria, for incubation at 38°C by 72h and subsequent microbiological counting, including *Salmonella spp*., *Staphylococcus spp*., *Streptococcus spp*., and Total Coliforms. After incubation time, CFU/mL were counted by Miles and Misra technique (Miles and Misra, 1938), using a Colony Counter. Also, 10 mL of the total milk sample kept in refrigeration was deposited in the sample container of the JuliC2 ® analyzer, the sensor was introduced into the milk, taking 1 minute and 30 seconds for the analysis of the components of each sample, fat, protein and lactose, determinated by ultrasonography, approved by the AOAC (2000).

Ruminal fermentation parameters and protozoa

The ruminal fluid was obtained from each animal by esophageal gavage, suction and vacuum were performed for extraction, the first outgoing content was removed, and the sample was filtered with a double layer of gauze, approximately 100 mL was collected in sterilized containers, pH was determined immediately, and kept the sample in refrigeration. For determining of VFA, a sample of 5 mL of ruminal fluid was taken for esterification of these with meta-phosphoric acid for later analysis. Determination of acetic, propionic and butyric acid, were obtained by gas chromatography (Galyean, 2006). Ammonia was measured by the phenol-hypochlorite technique (Broderick & Kang, 1980), and protozoa by Total Protozoa Numbers in Rumen Contents (Dehority,

2006). At the end of the first trial period, diets and groups of animals were exchanged, meaning that control group of animals were now supplemented with AP-SSF and vice versa with the same adaptation and sample time procedure.

Statistical Analysis

Statistical analysis for variables, milk yield, milk components, foodborne pathogens and ruminal parameters were calculated using an ANOVA with main effects of treatment and period time. The Tukey power test evidenced the differences between means ($P<0.05$). All statistical analysis was completed using the SAS software (SAS, 2006).

3. Results and discussion

The aim of this project was to evaluate the nutritional and microbiological quality of milk and ruminal fermentation parameters in dairy cattle after feeding apple pomace with non-protein nitrogen. Milk yield was calculated daily for the experimental phase (40 d), with non-statistical differences ($P < 0.05$) between treatments. Nevertheless, both treatments produced more than 30 kg of milk per day (Table 1), showing that AP-SSF treatment accomplished protein nutritional requirements for dairy cattle. It has been reported that deficiency in dietary protein decreases milk production (Rodríguez-Muela, 2009). However, AP-SSF protein is considered microbial protein due to its processes, which include yeast metabolism (Hernández *et al.*, 2007). The amino acids profile of microbial protein is also recognized as similar to those required by dairy cattle (Stockdale, 2006). Moreover, AP-SSF could give sufficient nitrogen for rumen microbial growth, improving both fiber degradability and volatile fatty acid production, which will accomplish with Net Energy for lactation (NEl), maintaining milk yield, which has been previously published (Cressman *et al*., 1980; Forster *et al*., 1983; McGuffey *et al*., 1990; Zimmerman *et al*., 1991; Reyes *et al.,* 1993; O´Mara *et al*., 1998; Kalscheur *et al*., 1999; Beigh *et al,* 2015).

Table 2. *Effects on Milk Components and Dairy Yield of diets with and without apple pomace-SSF.*

Item	Apple pomace-SSF	Control
Milk Components and Dairy Production		
Production/kg/cow/day	30.43 ± 0.23[a]	30.16 ± 0.24[a]
Protein %	3.0 ± 0.01[a]	3.5 ± 0.01[b]
Fat %	4.3 ± 0.05[a]	4.0 ± 0.05[b]
Lactose %	4.4 ± 0.01[a]	4.4 ± 0.01[a]

Note: [a,b] = lines with different literals indicate significant differences with $p < 0.05$, ANOVA with Tukey power test. ± =SD.

Within the nutritional quality of milk, milk protein differs between treatments ($P<0.05$). Control groups contain more amount that AP-SSF treatment (3.5 vs 3.0 %), however, both quantities are within international protein milk parameters (3-3.2%) (Alais, 2003). Diverse factors could affect moderate changes in fat:protein proportion, being negatively correlated, when fat milk content increases, protein decreases (Bauman *et al*., 2006), which probably could explain the present results obtained. Gutierrez (2007) obtained 3.2% of protein content after feeding dairy cattle with AP-SSF at 5% of the total diet, which was in a similar range to Villegas de Gante (Villegas de Gante, 2004). Moreover, acetate is important to cows because it is the primary substrate for synthesizing fatty acids, resulting in increased milk fat (Urrutia and Harvatine, 2017).

Fat milk is greatly influenced by dairy cattle nutrition (Chilliard, 1993). We observed statistical differences in fat milk between the treatment and control group, being AP-SSF greater than control (4.3 *vs* 4.0 %) values falling within international parameters (Alais, 2003). Rumen microbial population and good quality fiber are responsible for VFA production; even more acetate and, in minor proportion butyrate, are directly

responsible for fat milk synthesis (McDonald, 2002). In this study, the increment in fat milk content obtained is partially explained by fermentation parameters. VFA measures were statistically different between treatments, with AP-SSF being higher than control group. Acetic, propionic, and butyric acids were higher in the treatment group, 56.94, 38.22 y 3.71% than in the control group. Acetate and butyrate have been identified as building units for fatty acids, which attached to the glycerol form triacylglycerols, being this molecule the base for fat milk synthesis (Bauman *et al*., 2006).

Lactose results did not show statistical differences (P>0.05), averaging 4.4 %, being slightly lower than normal ranges (4.5%) (26). Jenkins y Mc-Guire (Jenkins and McGuire, 2006) mentioned that changes in milk lactose concentration are abnormal through feeding strategies, but the biological basis of lactose synthesis is still under study by molecular techniques. Lactose regulates osmotic pressure, limiting its quantity modification and maintaining milk yield equilibrium (Park *et al*., 2006).

Foodborne pathogens were quantified on milk in this project. Total coliforms (CFU/mL) were 25% lower (P < 0.05) at the AP-SSF treatment (Table 3). The reported amount of polyphenols (antioxidant) at the AP-SSF accounted by 8.4±2.1 mg/g (DM basis) (Rodríguez-Muela, 2009). Lauzon *et al*., (2005) decreased the counting of *Escherichia coli* by adding antioxidants *in vitro*, which partially explains the results observed in this trial. *Staphylococcus* sp. and *Streptococcus* sp. (CFU/mL) decreased (P < 0.05) at the AP-SSF treatment by 25 and 29%, respectively. The latest pathogens are considered the most important microorganisms responsible for mastitis (Koskinen *et al*., 2006). Many studies show that antioxidants decrease the duration, incidence, and severity of subclinical mastitis (Goff and Griffiths, 2006; Erskine, 1993; Smith *et al*., 1993; Smith *et al*., 1985; Smith *et al*., 1984; Chew, 1993; Smulski *et al*., 2020). Gallegos Acevedo (2007) fed dairy cattle with the addition of AP-SSF, and reported an increment in monocytes. Also, Tizard (1995) mentioned that antioxidants increase the bactericidal capacity of neutrophils. *Salmonella* account (CFU/mL) did not show a statistical difference (P>0.05).

Table 3. *Effects on components of milk microbiology, in cows fed apple pomace.*

Item	Apple pomace SSF	Control
Milk Microbiology		
Total coliforms CFU/mL	107 ± 5^{a}	141 ± 6^{b}
Salmonella sp CFU/mL	136 ± 5^{a}	161 ± 4^{a}
Streptococcus sp CFU/mL	68 ± 3^{a}	95 ± 4^{b}
Staphylococcus sp CFU/mL	52 ± 3^{a}	66 ± 3^{b}

Note: ^{a,b} = lines with different literals indicate significant differences with p < 0.05, ANOVA with Tukey power test. ± =SD. CFU = Colony Forming Units.

Statistical difference (P < 0.05) was observed in VFA production, being acetic, propionic, and butyric higher with the AP-SSF treatment. Hernández (2007) accounted for $51.3X10^{6}$ yeasts from AP-SSF samples. Those microorganisms are related to improving the number of cellulolytic bacteria, enhancing fiber digestibility, and VFA yield (Lascano, 2008). We also hypothesized that the high quality of protein (18%) contained in this ingredient (primarily by microbial source) produced habitually by AP-SSF yeasts during the fermentation step improves the fermentation process and VFA yield. Moreover, more robust microbial populations improved the fermentation of fiber and non-fiber carbohydrates, increasing VFA yield (Hoover and Miller, 1991).

It was concluded that the 18% protein content of the AP-SSF was enough to cover the protein requirements of the dairy cow. It has been reported that low ammonia content in the rumen liquid is related to feed protein deficiencies (Ramírez-Lozano, 2003). Ammonia was not affected by the treatment (Table 4) and was maintained within normal ranges (85-300 mg/L). Protozoa content was not affected by the treatment (P>0.05); either way, the concentrate diets have been reported to increase populations of those microorganisms related to high soluble sugar content (Makkar and McSwnney, 2005).

Ruminal pH is a cardinal item for the appropriate function of the rumen; there was no treatment effect of the pH (P>0.05) in the present research, maintaining levels within 6.4 to 7 (Dehority, 2003; Valdéz *et al*., 1997).

Future research should evaluate increasing the amount of AP-SSF in the diets of dairy cattle. Alternatives for the use of agro-industrial by-products must be studied and validated.

Table 4. *Results of the components of ruminal fermentation parameters in cows fed apple pomace.*

Item	Apple pomace SSF	Control
Ruminal Parameters		
Acetic acid mmol/dL	61.05±5.2[a]	38.9±5.4[b]
Propionic acid mmol/dL	17.25±2.2[a]	12.48±2.1[b]
Butyric acid mmol/dL	7.82±0.1[a]	7.54±0.1[b]
Ruminal pH	6.52 ± 0.07[a]	6.6 ± 0.08[a]
Protozoa/mL	7.7 X 10^6 ± 0.6[a]	7.1 X 10^6 ± 0.7[a]
Ammonia mg/L	87.04 ± 1.4[a]	82.89 ± 1.2[a]

Note: [a,b] = lines with different literals indicate significant differences with $p < 0.05$, ANOVA with Tukey power test. ± = SD.

4. Conclusions

Incorporating fermented apple pomace in a solid-state in the diet maintains the yield of 30 L per cow per day, increases milk fat by 8%, reduces the normal count range of *Staphylococcus* sp., *Streptococcus* sp. and total coliforms in milk, increases volatile fatty acids and maintains ruminal conditions for fermentation and microbial growth in early lactating dairy cows. For all of the above it is possible to incorporate more AP-SSF as a protein ingredient in diets of dairy cows in early lactation.

Authors information

Alberto Muro Reyes [1] - https://orcid.org/0000-0001-5894-2736
Francisco Javier Gutiérrez Piña [1] - orcid.org/0000-0001-5743-254X
Carlos Rodríguez Muela [2]
Carlos Abel Amaya Guerra [3] - orcid.org/0000-0002-9010-684X
Héctor Gutiérrez Bañuelos [1] - orcid.org/0000-0002-9204-4291
Luis Humberto Díaz García [1] - orcid.org/0000-0003-0620-5587
Laura Alicia Polín Raygoza [1] - orcid.org/0000-0002-6354-975X
Alejandro Espinoza Canales [1] - orcid.org/0000-0002-5031-4791

Contribución de los autores en el desarrollo del trabajo

The authors declare that they contributed equally to the realization of this research.

Conflicto de interés

The authors declare that there is no conflict of interest.

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