RESEARCH PAPER | Science | Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

Design and Evaluation of a Liquidbeneficial Yeast Active Additivefor Ruminant Animal Diets

KEYWORDS: apple, yeast, lactic acid,gas production, fermentationin vitro.

D. Díaz-Plascencia
Facultad de Zootecnia y Ecología. Universidad Autónoma de Chihuahua, Chihuahua, México. Periférico Francisco R. Almada km 1. Chihuahua, México. (614) 434-0303.

C. Rodríguez-Muela
Facultad de Zootecnia y Ecología. Universidad Autónoma de Chihuahua, Chihuahua, México. Periférico Francisco R. Almada km 1. Chihuahua, México. (614) 434-0303.

P. Mancillas-Flores
Facultad de Zootecnia y Ecología. Universidad Autónoma de Chihuahua, Chihuahua, México. Periférico Francisco R. Almada km 1. Chihuahua, México. (614) 434-0303.

J. M. Mendoza-Carrillo
Departamento de Agronomía, División de Ciencias de la Vida, Campus Irapuato-Salamanca, Universidad de Guanajuato, Guanajuato, México.(462) 6241889.

P.J. Albertos-Alpuche
Departamento de Agronomía, División de Ciencias de la Vida, Campus Irapuato-Salamanca, Universidad de Guanajuato, Guanajuato, México.(462) 6241889.

R. Martínez-Yáñez
Departamento de Agronomía, División de Ciencias de la Vida, Campus Irapuato-Salamanca, Universidad de Guanajuato, Guanajuato, México.(462) 6241889.

ABSTRACT *Two experiments were performed in order to determine the in vitro fermentation behavior of yeast strains isolated from apple's byproducts and the optimum level of an additive based on this yeast added to diets for high producingHolstein cows using the In vitro gas productiontechnique. In Experiment 1, eight inocula were developed with yeast strains 2, 9, 11 and 13 of Kluyveromyceslactis (Kl); 3 and 8 Issatchenkiaorientalis (Io); 4 and 6 of Saccharomyces cerevisiae (Sc). The eight treatments consisted of 0.2 g sample of dairy cows feed + 10 mL ofruminal liquid + 20 mL artificial saliva and 1 mL inoculum of different strains. There were 3 replications per treatment and sampling times of 12, 24 and 48 h for variables ammonia nitrogen (AN), lactic acid (LAc) and yeast counts (YeC). Gas production (GP) was determined at 3, 6, 12, 24 and 48 h. In Experiment 2, four treatments were evaluated: t1) 0.2 g sample of the diet without yeast additive added; t2) 0.2 g of sample with 3x1011 CFU/kg DM; t3) 0.2 g of sample with 6x1011 CFU/kg DM and t4) 0.2 g of sample with 9x1011 CFU/kg DM. All treatments had 10 mL of rumen fluid + 20 mL of artificial saliva. According to the results obtained in the first experiment, the inoculum was prepared from 4 yeast strains of K. lactis (Kl) 2 and 11; I.orientalis (Io), strain 3 and S.cerevisae (Sc), strain 6.The evaluated variables were pH, YeC, protozoa count (PrC), AN, LAc, and for GP sampling at 3, 6 , 12, 24, 48 and 96 h. Experiment 1 results showed strain and fermentation time effect(P <0.01) in AN, LAc and YeC variables. The lowest ANvalues were observed with the Kl-2 strain and the higher with Io-3, Io-8 and Sc-6 strains, with 22.5, 24.8, 24.8 and 24.1 mM/mLvalues, respectively. In YeC differences were observed (P <0.01) with a better performance of Kl-2, Kl-9, Kl-11, Kl-13 and Sc-6 strains finding cell counts of 1.9, 1.3, 1.2, 1.1 and 1.0 x 107CFU/mL, at 48hrespectively.Experiment 2 results, show a pH increase (P <0.01) over time from 7.1 to 7.4; whereas t2, t3 and t4 with inoculum treatments showed a gradual descent from 12 to 24 h. PrC increased (P <0.01) with the highest increased production for t4 (0.01 ± 1.8x107 CFU/mL, 48 h). YeC excelled (P <0.01) in t4, with 2.0x107± 0.00 CFU/mL at 48 h. AN increased in all treatments (P <0.01) from h12 to h96. LAc decreased (P <0.01) with the additive addition in t2, t3 and t4 showing a marked decrease in LAc, from h12 to h96 with values of 7.12 ± 0.19 to 2.04 ± 0.19 mM/mL in t2, from 6.73 ± 0.19 to 1.36 ± 0.17 mM/mL in t3 and 10.73 ± 0.29 to 1.95 ± 0.16 mM/mL in t4, which shows the best LAc reduction.It is concluded that the K. lactis yeast proved to be the most effective strain for ruminal fermentation with the best performance in terms of increased CFU count and therefore remain longer in the rumen environment and better LAc reduction.Inoculum addition to diets favored GP, YeC, PrC and a marked LAcreduction in t2, t3 and t4, showing that the most appropriate inoculum level to be used in diets for high producing cows is 10mL/kg DM.*

INTRODUCTION

Diet plays an important role in animal and human health. In recent years, special attention has been dedicated to functional foods production, aiming to add beneficial microorganisms or compounds within the body through the daily diet (Dsi Criscio et al., 2010). Probiotics have shown promising results in several animal production areas. A probiotic is defined as a culture or mixed culture of live microorganisms that benefit man or animals by improving the properties of the indigenous microflora of the intestine (Champagne et al., 2005). Benefits include bacterial pathogens inhibition, reduction of serum cholesterol levels, diarrhea and intestinal cancer; improved lactose tolerance, calcium absorption, vitamins synthesis and stimulate the animal's immune system (Sánchez et al., 2009). Solid state and submerged solid fermentations are processes that allow the exploitation of unconventional carbohydrates source from the animal feed using microorganisms. Probiotics and yeast food additives, composed essentially by Saccharomyces cerevisiae, have improved the health and ruminants productivity when added to animal feed. They remain active in the digestive tract exerting important physiological effects that contribute to balancing the rumen environment, (Desnoyers et al., 2009), they produce

Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

enzymes, complex B vitamins, minerals and various amino acids (Van der Peet-Schwerin et al., 2007), and so stimulating the absorption of nutrients creating a healthy intestinal environment and improving the immune system (Desnoyers et al., 2009), resulting in beneficial effects on productivity performance. Many substrates can be fermented, between them, the apple byproducts which can be used as food (Becerra et al. 2008; Rodriguez-Grind et al., 2010). Using Kluyveromyces lactis, Issatchenkia orientalis and Saccharomyces cerevisiae yeast inocula obtained from apple bagasse, through the submerged solid fermentation has contributed to the growth of yeasts useful in nutrition and animal feed, providing efficiently beneficial active yeast, being an adequate biotech pathway for the utilization of industrial wastes rich in carbohydrates (Díaz-Plascencia et al., 2011).

The in vitro gas production (GP) is a technique that is not new but has been improved, allowing obtaining the forage's kinetics fermentation when it is incubated in jars. This technique can predict gas volume production corresponds to a certain dry matter amount degraded (Theodorou et al., 1994; Posada and Noguera, 2005; Villegas-Castañeda et al., 2010). One advantage of this procedure is that the course of the fermentation and the role of the substrate soluble components can be quantified (Pell et al., 1997). An inherent in situ and in vitro method problem that have tried to solve by GP technique is to study the early stages of fermentation as the gravimetric methods are not sensitive enough to measure small changes that occur in the substrate weight during the first hours of fermentation (Rosero, 2002). Therefore, the objective of this study was to determine the optimal level of a yeast-based additive in diets for high producing dairy cows, using the in vitro gas production technique.

MATERIALS AND METHODS

This study was conducted at the Animal Nutrition Laboratory in the Facultad de Zootecnia y Ecología, Universidad Autónoma de Chihuahua, México, latitude 28° 35' North longitude 106° 04' West, with an altitude of 1595 AMSL (Alvarez, 1992).

Experiment One: fermentative Behavior

Biological Materials and Media Culture Eight yeast strains were used, which were obtained from apple bagasse solid state fermentation, which were identified through the extraction and amplification of 18S rDNA by Polymerase chain reaction (PCR), in the animal transgenesis laboratory, Faculty of Animal Science and Ecology at the Autonomous University of Chihuahua. To identify yeast serial dilution culture was performed and 16 colonies were isolated to which DNA was extracted to amplify 18S rDNA region (752 bp). The PCR product obtained was sequenced; the analysis of the sequences was performed using the Blast program database from National Center for Biotechnology Information (NCBI). The sequences analyses showed that the 16 yeast colonies isolated corresponding to: Kluyveromyces lactis, Issatchenkia orientalis and Saccharomyces cerevisae (Villagran et al., 2009). The strains used in this work were K. lactis strains 2, 9, 11 and 13; I. orientalis strains 3 and 8; S. cerevisiae, strains 4 and 6, all obtained from solid state fermentation of strains remained viable apple bagasses. Stains were kept viable by periodic passaging into wedges and in Petri dishes. The medium used was malt extract 33.6 g/L and the incubation time was 48 hours at a temperature of 30°C. Subsequently, they were kept refrigerated 4°C.

Inocula Preparation

Eight yeasts strains mentioned above were used for the inocula preparation. Each, had the addition of 100 g of molasses, 1 g of yeast obtained from different strains, 1.2 g of urea, 0.2 g of ammonium sulfate and 0.5 g of vitamin premix and trace mineral and dilute to volume 1,000 mL with distilled water and using aerators for each flask, the fermentation time for each inoculum was 96 h at 20°C. Once the fermentation time for each inoculum was completed, we proceeded to perform yeast counts, to which each subsequent dilutions were made to align them in 1.8x109 CFU/mL.

Strains Preparation for Incubation

Using the eight strains eight treatments were prepared. Incubation was carried out in 50 mL glass flasks; each contained 0.2 g of cows offered diets composed of steam-flaked corn, cottonseed, wheat bran, animal fat, bypass fat, corn gluten meal, cottonseed meal, soybean meal, molasses, minerals trace, salt, urea, calcium bicarbonate, alfalfa, corn silage and water ad libitum. The chemical composition of the diet consumed by the fistulated cows (dry matter) was as follows: DM, 61.13; CP, 17.80; CF, 21.87; Ash, 11.13, EE, 4.45 and NFE, 44.75 according to AOAC (2000).10 mL rumen fluid, 20 mL artificial saliva and 1 mL inoculum of the respective strain. The combinations were evaluated in 120, 50 mL glass vials, with 3 replications per treatment at different sampling times (12, 24 and 48 h) for the variables count yeast (YeC), ammonia nitrogen (AN) and lactic acid (LAc) ; and 3, 6, 12, 24 and 48 h for GP. For YeC, the methodology described by Diaz (2006) was taken. AN was determined colorimetrically (Broderick and Kang, 1980) and LAc (Taylor, 1996) .The in vitro GP procedure was done according to the Menke and Steingass (1988) technique. This method is used to determine the amount of gas produced by fodder in an incubation period of 96 h. The amount of released gas is closely related to the food degradability.

Diet samples were ground with a 1mm mesh (Menke et al., 1979) and dried (105°C, 8 h). The in vitro rumen consisted of bicarbonate and phosphate buffers, a reducing agent, a nitrogen source, various minerals and resazurin. CO2 was used during preparation of the means to ensure an anaerobic environment at the time of inoculation. The solution A (micro mineral) was constituted by 13.2 g CaCl2. $2H_2O$, 10.0 g $MnCl_2.4H_2O$, 1.0 g $CoCl_2.6H_2O$, 8.0 g $FeCl_3.6H_2O$ in 100 mL with distilled water; solution B (buffer solution) with39.0 g $NaHCO_3$ ó 35.0 g $NaHCO_3$ + 4.0 g de $(NH_4)HCO_{3\ in\ 1\ liter}$ with distilled water; solution C (macro mineral) by 5.7 g Na_2HPO_4, 6.2 g KH_2PO_4, 0.6 g Mg $SO_4.7H_2O$ in 1 liter with distilled water; resazurin solution (100 mg resazurin) made of 100 mL with distilled water; and reducing solution 4 mL 1N NaOH, 625 mg $Na_2S.9H_2O$ added to 95 mL of distilled water.

To collect ruminal fluid three producing Holstein cows were used with an average weight of 600 kg, fistulated in the rumen dorsal sac and provided with a simple cannula (Bar Diamond, Inc). They were housed in individual cubicles and ate a whole grain diet consists of corn grain, cottonseed, wheat bran, animal fat, bypass fat, corn gluten meal, cottonseed meal, soybean meal, molasses, minerals trace, salt, urea, calcium bicarbonate, alfalfa, fodder silage and water ad libitum. The chemical composition of the diet consumed by the fistulated cows (dry matter) was as follows: DM, 61.13; CP, 17.80; CF, 21.87; Ash, 11.13, EE, 4.45 and NFE, 44.75 according to AOAC (2000).

Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

The ruminal fluid was collected 15 minutes before starting the test with the help of a vacuum pump. Taking the collected rumen fluid from the cows was held in the morning immediately before feeding, considering that microorganisms are less active but more consistent in their composition (Menke and Steingass, 1988; Blümmel and Orskov, 1993). Samples were transported to the laboratory in a 1000 mL sealed thermos previously tempered and subsequently filtered through muslin. The procedure was performed under a CO_2 atmosphere in order to ensure anaerobic conditions. The sample vials, ruminal fluid and artificial saliva were sealed and incubated at 39°C with constant stirring (68 rpm), protecting from light; the evaluation period lasted 48 h. The bottles internal pressure (exerted by the GP) was measured with a pressure transducer at 3, 6, 12, 24 and 48 h incubation. The pressure values were converted to volume of gas production (GP mL) according Theodorou et al. (1994) GP net/h of each sample was obtained from the GP observed difference less the average GP white. Data are expressed in milliliters of 0.2 GP per g dry matter (GP mL/0.2 g DM).

Ruminal Fermentation in vitro parameters (A, B, C) Solutions A, B, C and resazurin were added into a flask with distilled water, which was introduced in a rotary incubator (Incubator Shaker I2400) at 39°C, subsequently reducing solution was added and the whole mixture was bubbled with CO2 until the color turned from blue to pink and finally clear (transparent). The previously collected rumen fluid was filtered through a nylon stocking and mixed with artificial saliva, artificial saliva: ruminal fluid final ratio was 2:1. It must be mentioned that the parameters A, B and C were utilized to prepare the samples, but were not used in the results interpretation because only the in vitro effect of ruminal degradation was sought over this experiment time.

Experiment two: in vitro fermentation kinetics
Biological Materials and Media Culture
As result of the experiment one, a liquid yeast active additive was created. The four yeast strains used for this additive were: Kluyveromyces lactis strains 2 and 11; Issatchenkia orientalis, strain 3 and Saccharomyces cerevisae strain 6, all obtained from solid state fermentation apple bagasses, which were identified through the extraction and amplification of 18S rDNA by Polymerase chain reaction (PCR), in laboratory animal transgenesis, Facultad de Zootecnia y Ecología, Universidad Autónoma de Chihuahua (Villagran et al., 2009). To identify yeast serial dilution culture was performed and 16 colonies were isolated to which DNA was extracted to amplify 18S rDNA region (752 bp). The PCR product obtained was sequenced; the analysis of the sequences was performed using the Blast program database from National Center for Biotechnology Information (NCBI). Stains were kept viable through periodic passaging into wedges and in Petri dishes. The used medium was malt extract 33.6 g/L and the incubation time was 48 hours at a temperature of 30°C. Subsequently, they were kept refrigerated 4°C. An experimental diet meeting the NRC (2001) nutritional requirements for high producing Holstein cows (35 L/day expected production) was created. Nutrión 5 software was used for the formulation of this diet (Table 1).

Inocula Preparation
In a 1,000 mL glass flask it was added 1 g of the aforementioned strains 250 g cane molasses, 750 mL whey, 2 g of urea and 0.04 g of ammonium sulfate. To each flask an aquarium air pump was adapted to inject air and dilute to volume of 1,000 mL with distilled water. The fermentation time for the inoculum was 96 h to an average ambient temperature of 28°C. Once the fermentation time for each inoculum was completed, we proceeded to perform yeast counts, finding the amount of 1.2x109 CFU/mL to subsequently adjust the amount of yeast required for each treatment.

Rumen Fluid collection
All the animals used for ruminal fluid collection were kept and handled following the procedures mentioned in Fulwider (2014). To collect ruminal fluid three producing Holstein cows were used with an average weight of 600 kg, fistulated in the rumen dorsal sac and provided with a simple cannula (Bar Diamond, Inc). They were individually housed in cubicles and ate a whole grain diet consists of corn grain, cottonseed, wheat bran, animal fat, bypass fat, corn gluten meal, cottonseed meal, soybean meal, molasses, minerals trace, salt, urea, calcium bicarbonate, alfalfa, fodder silage and water ad libitum, similar to the one used in experiment 1. The rumen fluid was extracted through said cannula and with the help of a vacuum pump before food consumption in the morning (06 am). Samples were transported to the laboratory in a 1000 mL sealed thermos previously tempered and subsequently filtered through muslin. The procedure was performed under a CO_2 atmosphere in order to ensure anaerobic conditions. Bottles with the food sample, ruminal fluid and artificial saliva were sealed and incubated at 39°C with constant stirring (68 rpm), protecting from light; the evaluation period lasted 48 h. The bottle's internal pressure exerted by the gas production was measured with a pressure transducer at 3, 6, 12, 24 and 48 h of incubation, after taking the reading in each of the different times all pricked jars to release the gas pressure. The pressure values were converted to volume of gas production (GP mL) according Theodorou et al. (1994) GP net/h of each sample was obtained from the GP observed difference less the average GP white. Data are expressed in milliliters of 0.2 GP per g dry matter (GP mL/0.2 g DM).

Treatments
There were evaluated three different treatments (inoculum concentration) and a control treatment without inoculum (Table 2). For the preparation of each treatment a 1 kg of the aforementioned experimental diet sample was taken ground and manually mixed and homogenized with the concentration of liquid yeast additive, respectively, in a plastic tray. Then the amount of 0.2 g of the sample was taken and placed in 50 mL glass jars (Table 2).

Measured Variables
pH, yeast and protozoa count, ammoniac nitrogen and lactic acid were evaluated in 48 glass jars of 50 mL, with 3 replicates per treatment (t) and different sampling times (h) 12, 24, 48 and 96 h. pH was determined directly with a digital potentiometer within ± 0.1 units, based on the methodology described by Rodriguez et al. (2001). Yeast counts (YeC) this analysis was based on, the methodology described by Diaz (2006). 2 mL samples were deposited in 50 mL plastic bottles with cap; in each sample, two drops of formalin 10% solution were added to preserve it in the refrigerator until the count was performed. The YeC was performed by microscopy; a variable volume pipette with a range of 100 to 1000 µl with disposable tips was used to take 1 mL of liquid sample and a serial dilution was prepared using distilled water as diluent; a variable volume pipette with a range of 0.5 to 10 µl with disposable tips; 10 µl of the dilution of each sample were taken, and were placed in a hemocytometer (Neubauer chamber) for

Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

counting. Protozoa count (PrC), counted using a light microscope in a Neubauer chamber. For this protozoa were stained with gentian violet solution in 0.01% glacial acetic acid by 1% according to Painting and Kirsop method (1990). Ammonia nitrogen, 2 mL were taken of each sample were placed in 50 ml plastic jars and frozen (-5°C) for storage until the determination of $N\text{-}NH_3$ samples were thawed under refrigeration (4°C) and NH3-N concentration of the liquid samples was determined by colorimetry according to the Broderick and Kang (1980) method. For lactic acid (LAc), 2 mL were taken of each sample were placed in 50 mL plastic jars and frozen (-5°C) for storage until determining LAc. Upon determination, the samples were thawed under refrigeration (4°C) and the concentration of LAc of liquid samples was determined colorimetrically by the method of Taylor (1996). The in vitro gas production (GP) was determined in 12 in addition to the 48 glass vials of mL 50 above mentioned with 3 replicates per treatment in different sampling times, 6, 12, 24, 48 and 96 (h), according to the technique of Menke and Steingass (1988). Sample preparation involved the ground substrate in a 1mm mesh (Menke et al., 1979). Samples were dried at 105° C for 8 hours. The in vitro rumen consisted of bicarbonate and phosphate buffers, a reducing agent, a nitrogen source, various minerals and resazurin. CO2 was used during preparation of the means to ensure an anaerobic environment at the inoculation time. Artificial saliva comprising solution A (micro minerals) consists of 13.2 g CaCl2.2H2O, 10.0 g MnCl2.4H2O, 1.0 g CoCl2.6H2O, 8.0 g FeCl3.6H2O in 100 mL with distilled water; B (buffer solution), solution consisting of 39.0 g or 35.0 g NaHCO3 + or 4.0 g of (NH4) HCO3 in 1 liter with distilled water; C (macro mineral) solution consists of 5.7 g Na2HPO4, 6.2 g KH2PO4, 0.6 g Mg SO4.7H2O in 1 liter with distilled water; resazurin solution (100 mg resazurin) made of 100 mL with distilled water; and reducing solution consisting up to 4 mL 1N Na OH, 625 mg Na2S.9H2O, added to 95 mL of distilled water. Incubation was carried out in 50 mL glass flasks; within these 0.2 g of the complete ration, 10 mL of ruminal fluid and 20 mL of artificial saliva was placed and the corresponding amount of yeast was added per treatment in each glass jar.

Statistical Analysis
Data were evaluated with the procedure (Proc Mixed) of SAS (2004) for an 8 treatments randomized design (Experiment 1) and 4 treatments (Experiment 2) in split plots in time.

RESULTS
Experiment One Yeast Count (YeC) In the interaction time by inoculum an effect (P <0.01) was found, indicating that there were differences in the amount of yeast with increased in the different treatments depending on the time. The highest concentration of yeast found in the gas production (GP) was observed in t1, with the Kl-2 strain excelled in all sampling times, the t5 with strain lo-3 and t6 with I-8 strain disappeared in ruminal environment quickly as shown in Figure 1.

Ammonia nitrogen (AN)
An effect (P <0.01) was found for the interaction time by inoculum, indicating different behavior between strains over fermentation time. The AN increased, especially in t5, t7 and t8 treatments: 13.32±0.28 to 24.52±0.00 mM/mL in t5, 13.37± 0.02 to 23.85±0.10 mM/mL in t7, 13.21±0.14 to 24.05±0.07 mM/mL in t8 (Figure 2).

Lactic acid (LAc)
An effect (P <0.01) in the hours of fermentation on LAc concentration in all treatments was observed. Estimates of mean t1, t2, t3 and t4 treatments inoculum of yeast K. lactis, values had more LAc loss compared to the other inocula of the h 12 to h 48 with values of 21.75±0.31 to 2.47±0.01 mM/mL in t1, 27.54±0.30 to 2.82±0.34 mM/mL in t2, 34.77±0.22 to 2.37±0.31 mM/mL in t3, 28.14±0.20 to 2.39±0.07 mM/mL in t4. The behavior for this variable by the strains of three different genera, it is seen that at 48 hours, the four K. lactis strains had less LAc, then the two strains of the genus S. cerevisiae and the two strains of I. orientalis showed more concentration of LAc (Figure 3).

Gas Production (GP)
An effect (P <0.01) on GP was observed in all treatments. In the inocula where different yeast strains were included, it can be observed the maximum increased from the GP accumulated in the h3 and h48, with values of 4.63±0.06 to 2.00±0.05 GP mL/0.2g MS in t1, 4.36±0.08 to 2.00±0.03 GP mL/0.2g MS in t2, 4.36±0.12 to 2.03±0.03 GP mL/0.2g MS in t3 and 4.26±0.012 to 1.93±0.08 GP mL/0.2g MS in t4, all the K. lactis genus (Figure 4).

Experiment Two.
pH: This variable showed an effect (P <0.01) in treatment time interaction. Estimated pH values from h12 to h96 in t1, increased gradually over time of 7.17±0.00 to 7.44±0.00 h; whereas t2, t3 and t4 treatments showed a gradual decline from the h12 to h24, a slight increase from h48 to h96.

Yeasts counting (YeC): effect (P <0.01) was found by treatment time interaction in the amount of yeast, with a fluctuating behavior in the different treatments depending on the time. The highest yeast concentration was found in the GP was observed in t4, with a value of 5.4x107±0.00 in h12 and 1.8x107±0.01 CFU/mL-^{1} in the h48 (Figure 5).

Protozoa count (PrC): An effect (P <0.01) in treatment time interaction in the amount of protozoa, with a fluctuating increase in all treatments, highlighting t4 with a production of 1.1x106±0.02 to 2.0x167±0.00 CFU/mL-1 (Figure 6).

Ammonia nitrogen (AN): An effect (P <0.01) by treatment time interaction for all treatments from h12 to h96 was found.

Lactic acid (LAc): A significant effect (P <0.01) was observed between the inoculum concentration and time in t2, t3 and t4 treatments, where an LAc reduction, from h12 to h96 with values of 10.73±0.29 to 1.95±0.16 mM/mL was recorded in t4 being the best LAc reduction (Figure 7).

In vitro gas production (GP): An effect (P <0.01) was found for treatment time interaction in all treatments. The maximum cumulative GP increase was presented at h3 with values 3.06±0.06 GP in mL/0.2g DM in t1, 4.03±0.03 GP in mL/0.2g DM in t2, 4.70±0.05 GP mL/0.2g DM t3 and GP 4.70±0.05 mL/0.2g DM in t4, and was gradually reduced (Figure 8).

DISCUSSION
The present study provides for the first time data related to the development of a liquid yeast additive created using inherent strains from the apple bagasse which can be utilized for the ruminant animal diets.

Experiment One
Yeast Count (YeC)

Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

According to the obtained results, yeasts from different inocula under the GP gradually decreased as fermentation time went by which coincides with similar results obtained by Arambel and Rung-Syin (1987) and Marrero et al. (2014) when they studied the growth of S. cerevisiae on ruminal environment. These authors pointed that yeasts are unable to maintain a productive population within the rumen environment, as this contains inhibitory growth factors such as temperature. The inocula t1, t2, t3 and t4 with K. lactis strain and t8, S. cerevisiae strain shown to have a larger yeast population in the rumen compared to other inoculants up to h48, this probably due to the use of the lactic acid as an energy source to keep viable and further developed (Rodríguez-Muela et al., 2010; Diaz-Plascencia et al, 2011). Inocula t5, t6 and t7 provided an acceptable concentration of yeast, but immediately enter degradation phase, which confirms the point made by Williams et al. (1990). However, it is important to mention that the anaerobic fermentation process in the rumen by microorganisms makes substrates mainly in carbohydrates and microbial protein, and other fermentation final products such as volatile fatty acids, dioxide carbon (CO_2), peptides and amino acids among others (McDonald et al., 2002; Dehority, 2003).

Ammonia nitrogen (AN)

The AN is the main nitrogen source for rumen microorganisms, which can supply between 40 and 100% of this item needs for microbial protein synthesis (Dewhurst et al., 2000). This effect is given by the urea added to the substrates in fermentation processes, as it is transformed to AN affect ureolytic microbial species, as reported in the production of manzarina and saccharina mainly showing similar effects to that observed in the present study (Valiño et al., 2002; Calderón et al., 2005; Rodriguez, 2009; Diaz-Plascencia et al., 2010). If the substrate has a low energy input, microorganisms cannot incorporate them in the amino acids formation for growing or do so at a low rate. When you have a low pH AN produced is retained in the substrate (Díaz-Plascencia et al., 2011). The AN may also occur by deamination activity and thus can be used by certain microorganisms that do not hydrolyze added urea to the fermentation medium and as a result, the amount of some microorganisms in fermented substrates can be increased and may even disappear, causing the increase or lower the levels of AN; as a result, the fermentation medium is modified and causes changes in pH, the transit speed and ruminal microbial population prevailing, which in turn can alter the proteolytic activity (Cardozo et al., 2000; Cardozo et al., 2002)

Lactic acid (LAc)

The LAc increased in some fermentation inhibits microbial growth and induces cell death in yeast or microorganisms (Madrid et al., 1999; Ludwig et al., 2001). It is known that LAc toxicity is pH dependent on the system. At low pH, this LAc is found primarily in undissociated form and can enter the microbial cell by passive diffusion (Geros et al., 2000); dissociates in the cytoplasm due to more neutral pH and protons are released, lowering the cytoplasm pH, which interferes with some metabolic pathways (Schüller et al., 2004) and in the transport of nutrients and ions, changing the membrane structure, in the fatty acids, the composition of phospholipids and protein synthesis (Ramos et al., 2006). LAc is produced by the catabolism of carbohydrates and is the best indicator of the right fodder fermentation under anaerobic conditions, but the yeast K. lactis, has a very marked effect on the use of this molecule when used as an energy source to continue to survive for prolonged periods.

Gas Production (GP)

The rate and extent of fermentation of carbohydrates in the rumen varies with the type and structure thereof (Ivan et al., 2005) and according to the dominant microbial population (Dehority, 2003). The increase in GP obtained with these strains could be the result of increased production of propionic acid because the carbon dioxide is produced when the propionic acid is formed by some rumen bacteria for the metabolic pathway succinato- propionate (Wolin and Miller, 1988). Tang et al. (2008) also found a positive effect in GP when adding a yeast culture to different cereals straws. Several authors coincide in similar results when they used the GP technique to assess the behavior of yeast against different substrates (Lila et al., 2004; Castillo et al., 2009; Marrero et al., 2014).

Experiment Two.

pH: The ruminal pH strengthens the balance between the buffering capacity and the fermentation acidity considering that pH decreasing, acetate propionate ratios narrow, consequently with pH increasing reactions acetate propionate are extended (Wales et al., 2004).

Yeasts counting (YeC): Yeasts of different treatments decreased as fermentation time went by, which coincides with similar results obtained by Castillo et al. (2009), when they studied S. cerevisiae growth in the rumen environment.

Protozoa count (PrC): The increase was probably due to the fact that all treatments had pH and a supply of AN and LAc suitable for their growth and multiplication. Protozoa degradation is usually associated with a decrease in the butyrate proportion and acetate or propionate increased. These results are similar to those reported by Kumar et al. (1994), who indicate that yeast increases the total bacteria number, total viable cellulolytic, amylolytic bacteria and protozoa.

Ammonia nitrogen (AN): The found AN increase, could be given by the added urea to the substrates in fermentation processes, as it is transformed to AN affect ureolytic microbial species. A similar effect has been reported in the production of manzarina and mainly saccharina (Rodríguez-Muela et al., 2010).

Lactic acid (LAc): The increase in the concentration of LAc in t4 is mainly due to the establishment of the lactogenic bacteria. Increasing LAc in some fermentation inhibits microbial growth and induces cell death in yeast or microorganisms (García et al., 2008).

In vitro gas production (GP): The increase in GP obtained with these strains could be the result of increased production of propionic acid because the carbon dioxide is produced when the propionic acid is formed by some rumen bacteria for the metabolic pathway succinato- propionate (Tang et al., 2008). The fermentation pattern in ruminants is performed in the ruminal environment which is influenced by the interaction between diet, microbial population and the animal itself. Two important aspects in the rumen are for fermentation conditions an efficient cellulolytic activity and needs for optimal microbial protein synthesis. However, the relative importance of these processes varies according to the food characteristics.

Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

CONCLUSIONS

Of the eight yeast strains used in this study, K. lactis proved to be the most viable for the GP and the one that most resisted ruminal degradation, showing a better performance in yeast production and participation in reducing lactic acid. The yeasts S. cerevisiae and I. orientalis shown to have an important role during fermentation, being these which give the primary conditions for K. lactis, in subsequent fermentation processes. Yeasts are incorporated into diets with the purpose of improving health and especially the animal performance and improve their husbandry characteristics, so it is advisable to continue testing this yeasts inoculum obtained from apple bagasse in order to further improve it. It is concluded that the addition of the liquid yeast additive in diets favored the gas production, the yeasts count and total protozoa counting with a marked reduction of lactic acid in treatment 4 so we believe that the optimal amount to use is 30 mL/kg of offered food.

Table 1. Content and calculated analysis from the diet used in the experiment, for high producing Holstein cows (expected production 35 L cow/day). Nutritional requirements from NRC (2001).

Ingredients		%
Alfalfa		25
Steam-flaked Corn		20
Soybean meal		14
Soybean peel	13	
Wheat bran		10
Cottonseed meal		10
Molasses		4
Bypass fat (palm oil)		2.5
Vitamin and mineral trace Premix		0.5
Calcium orthophosphate		0.5
Calcium bicarbonate		0.5
Total		100
Calculated Analysis		
Net energyfor lactacting cows		1.73 MC/Kg
Dry matter		85.09 %
Total protein		17.29 %
Ether extracts		5.85 %
Neutral Detergent Fiber		32.70 %
Acid Detergent Fiber		23.10 %
Crude Fiber		17.84 %
Ash		5.93 %
Total Phosphorus		0.58 %
Calcium		0.87 %
Magnesium		0.22 %
Potassium		1.47 %

Formulation and calculated analysis using Nutrion 5 software.

Table 2. Treatment design (experiment 2)

Treatments	Sample g	Inoculum CFU/mL	Rumen fluid mL	Artificial saliva mL
t1 (0 mL)	0.2	0	10	20
t2 (10 mL/ kg)	0.2	$3x10^{11}$	10	20
t3 (20 mL/ kg)	0.2	$6x10^{11}$	10	20
t4 (30 mL/ kg)	0.2	$9x10^{11}$	10	20

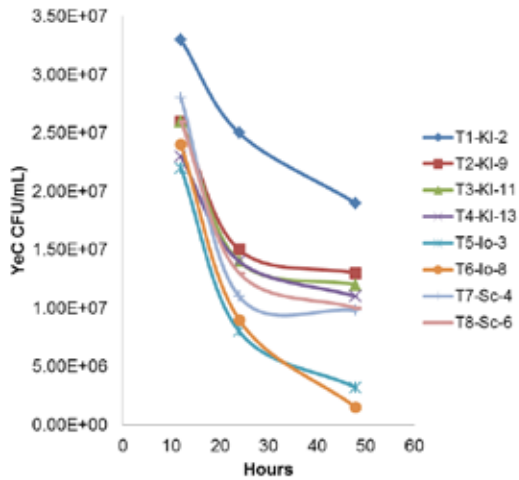

Figure 1. Yeast population degradation by time according to the used inoculum (experiment 1).

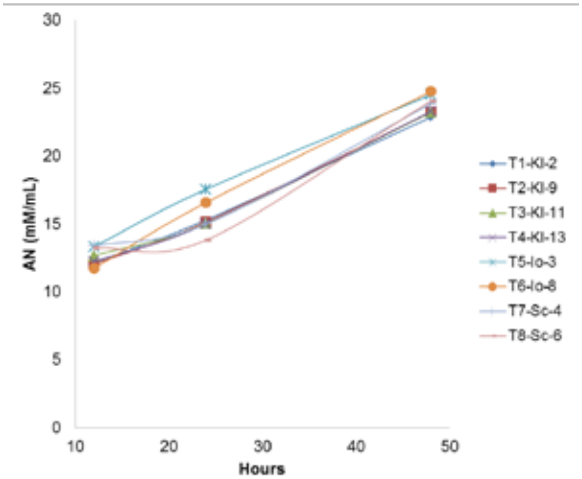

Figure 2. Ammonia Nitrogen concentration by time according to the used inoculum (experiment 1).

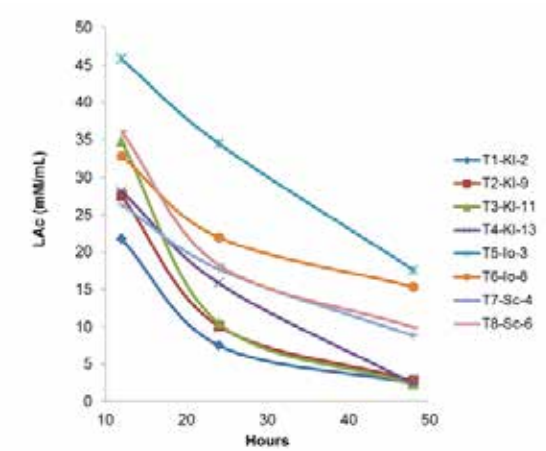

Figure 3. Lactic Acid reduction by time according to the used inoculum (experiment 1).

Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

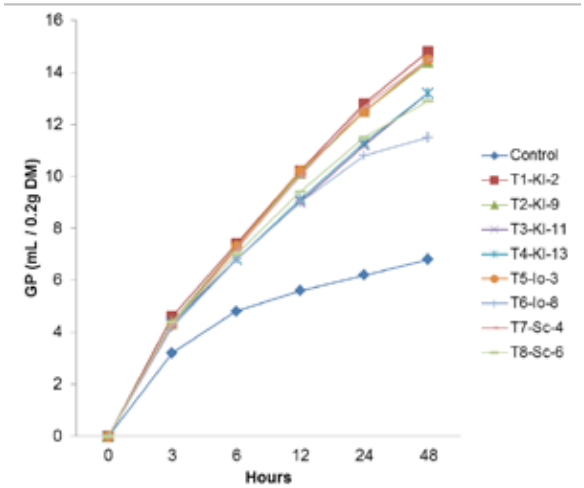

Figure 4. Gas production volume by time according to the used inoculum (experiment 1).

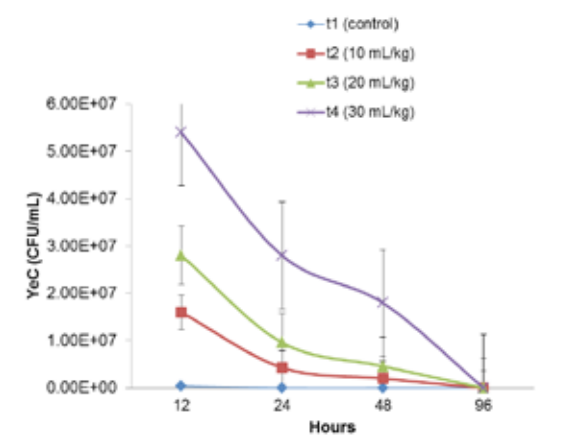

Figure 5. Yeast population degradation by time according to the inoculum concentration (experiment 2).

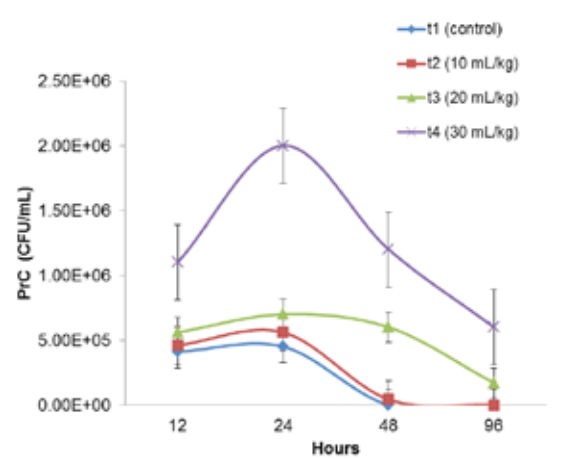

Figure 6. Protozoa population by time according to the inoculum concentration (experiment 2).

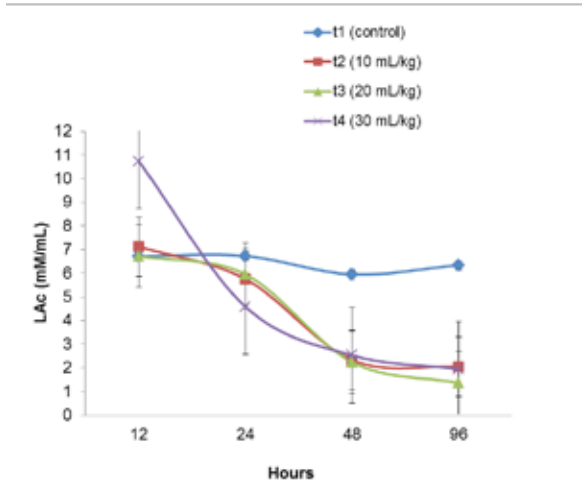

Figure 7. Lactic Acid reduction by time according to the inoculum concentration (experiment 2).

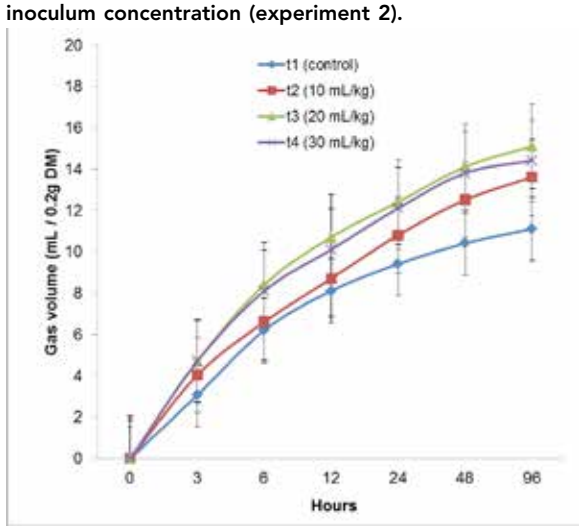

Figure 8. Gas production volume by time according to the inoculum concentration (experiment 2).

Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

REFERENCE

Álvarez, A. 1992. Compendio de temperaturas máximas extremas, mínimas extremas y precipitaciones pluviales. Boletín meteorológico. Estado de Chihuahua. Gobierno del Estado. Dirección de Desarrollo Rural. Servicio Meteorológico y Geográfico. Chihuahua, Chih. Méx. | | AOAC. 2000. OfficialMethod of analysis. 16th Ed. Ass. Off. Agric. Chem. Washington, D.C. | | Arambel, M. J. Rung-Syin, T. 1987. Evaluation of Saccharomyces cerevisiae growth in the rumen ecosystem. Memories. 19th Biennial conference on rumen function. 17-19. | | Becerra, A., C. Rodríguez, J. Jiménez, O. Ruiz, A. Elías y A. Ramírez. 2008. Urea y maíz en la fermentación aeróbica de bagazo de manzana para la producción de proteína. TECNOCIENCIA Chihuahua. 2:7-14. | | Blümmel, M. and Orskov, E.R. 1993. Comparison of in vitro gas production and nylon bag degradability of roughages in predicting food intake in cattle. Anim. Feed Sci. Techno. 40: 109-119. | | Broderick, G. A., and J. H. Kang. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J. Dairy. Sci. 63:64-75. | | Calderón A., J. O., A. Elías I. y M. Valdivie N. 2005. Dinámica de la fermentación en estado sólido de las camas de cascarilla de café en Inicio de ponedoras inoculadas con Vitafert. Rev. Elect. Vet. 5:1-7. | | Cardozo, P., Calsamiglia, S. y Ferret, A. 2000. Effects of pH on microbial fermentation and nutrient flow in a dual flow continuous culture system. J. Dairy. Sci. 83 (Suppl. 1): 265. (Abstract) | | Cardozo, P., Calsamiglia, S. y Ferret, A. 2002. Effects of pH on nutrient digestion and microbial fermentation in a dual flow continuous culture system fed a high concentrate diet. J. Dairy. Sci. 85 (Suppl. 1):182. (Abstract) | | Castillo, Y., Ruiz, O., Elías, A., Arzola, C., Rodríguez, C., Ortega, J. A., La O, O., Holguín, C. & Ricardo, Y. 2009. Kinetics of fermentation of Apple residues. J. Anim. Sci. 87:90. | | Champagne, C. P., N. J. Gardner y D. Roy. 2005. Challenges in the addition of probiotic cultures to foods. Crit. Rev. Food Sci. Nutr.45:61-84. | | Dehority, B.A. 2003. Rumen Microbiology. Nottingham University Press. 19-43. | | Desnoyers, M., S. Giger-Reverdin, G. Bertin, C. Duvaux-Ponter y D. Sauvant. 2009. Metaanalysis of the influence of Saccharomyces cerevisiae supplementation on ruminal parameters and milk production of ruminants. J. Dairy Sci. 92:1620-1632. | | Dewhurst, R.J., Davues, D.R. y Merry, R.J. 2000. Microbial protein supply from the rumen. Anim. Feed. Sci. Techno. 85: 1-21. | | Di Criscio, T., A. Fratianni, R. Mignogna, L. Cinquanta, R. Coppola, E. Sorrentino y G. Panfili. 2010. Production of functional probiotic, prebiotic and synbiotic ice creams. J. Dairy Sci. 93:4555-4564. | | Díaz P., D. 2006. Producción de Proteína Microbial a Partir de Manzana de Desecho Adicionada con Urea y Pasta de Soya. Tesis de Maestría. Facultad de Zootecnia. Universidad Autónoma de Chihuahua. Chihuahua. México. | | Díaz-Plascencia, D., Rodríguez-Muela, C., Mancillas-Flores, P., Angulo, C., Salvador, F., Ruíz, O., & Elías, A. (2011). Desarrollo de un inoculo con diferentes sustratos mediante fermentación sólida sumergida. REDVET. Revista Electrónica de Veterinaria, 12(1), 1-10. | | Díaz-Plascencia, D., Rodríguez-Muela, C., Mancillas-Flores, P., Angulo, C., Salvador, F., Arzola, C. & Elías, A. (2010). Producción de proteína microbiana a partir de manzana de desecho con fermentación en estado sólido a 32 ºC. REDVET. Revistaelectrónica de Veterinaria, 11(10), 1-9. | | Fulwider, W.K. 2014. Dairy cattle behaviour, facilities, handling, transport, automation and well-being.In: Grandin, T. (ed.). Livestock handling and transport.4thediction. CABI. | | García, Yaneisy; Elías, A.; Albelo, Nereyda; Herrera, F.R.; Núñez, Odalys y Dieppa, Oraida. 2008. Crecimiento de bacterias ácido lácticas y levaduras durante la fermentación líquida de excretas de pollos de ceba para la obtención de probióticos. Rev. Cubana Cienc. Agríc. 42: 195-197. | | Geros, H., Cassio, F. y Leao, C. 2000. Utilization and transport of acetic acid in Dekkeraanomala and their implications on the survival of the yeast in acidic environments. J. Food. Prot. 63: 96-101. | | Ivan, S. K., Grant, R.J., Weakley, D. and Beck, J. 2005. Comparison of a corn silage hybrid with high cell wall content and digestibility with a hybrid of lower cell-wall content on performance of Holstein cows. J. Dairy. Sci. 88:244-254. | | Kumar, V.K., Sareen, P.K., Singh, S. 1994 Effect of Saccharomyces cerevisiaeyest culture suplements on ruminal metabolism in buffalo calves given a high concentrate diet. Brit Soc. Animal Sci. 59:209-215. | | Lila, Z.A., Mohammed, N., Yasui, T., Kurokawa, Y., Kanda, S y Itabashi, H. 2004. Effects of a twin strain of Saccharomyces cerevisiae live cells on mixed ruminal microorganism fermentation in vitro. J. Anim. Sci. 82:1847-1854. | | Ludovico, P., João, S.M., Silva, M.T., Leão, C. y Côrte-Real. 2001. Saccharomyces cerevisiae commits to programmed cell death process in response to acetic acid. Microbiol. Rev. 147: 2409–2415. | | Madrid, J., A. Martínez-Teruel, F. Hernández y M. D. Megías. 1999. A comparative study on the determination of lactic acid in silage juice by colorimetric, high-performance liquid chromatography and enzymatic methods. J. Sci. Food. Agri. 79:1722-1726. | | Marrero, Y., Ruiz, O., Corrales, A., Jay, O., Galindo, J., Castillo, Y., & Madera, N. (2014). Producción de gas in vitro de sustratos fibrosos con la inclusión de levaduras. Revista Cubana de Ciencia Agrícola, 48(2), 119-123. | | McDonald, P., R. A. Edwards, J. F. D Greenhalgh, y C. A. Morgan. 2002. Animal Nutrition. 6th edition. Pearson Education. U.S.A. | | Menke, K. H. and Steingass, H. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Research. Development. 28: 7-55. | | Menke, K. H., Raab, L., Salewski, A., Steingass, H., Fritz, D. and Schneider, W. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. J. Agric. Sci. 93: 217-222. | | Painting, K. y Kirsop, B. 1990. A quick method for estimating the percentage of viable cells in a yeast population, using methylene blue staining. Worl J MicrobiolBiotechnol. 33: 109-116. | | Pell, A., N. Doane P H and Schofield P. 1997. In vitro digestibility and gas production. Página 109-132 emmemórias del Simpósio sobre Tópicos Especiaisem Zootecnia, Lavras. | | Posada S. L., y R. R. Noguera. 2005. Técnica in vitro de producción de gases: una herramienta para la evaluación de alimentos para rumiantes. Liv. Res. Rural. Development. 17:36-38. | | Ramos, J.A., Elías, A. y Herrera, F. 2006. Processes for production of energy protein feed for animals. Effect of four energy sources on solid state fermentation of sugarcane. Cub. J. Agri. Sci. 40: 47-53. | | | Rodríguez, Z., A. Elías, R. Bocourt y O. Núñez. 2001. Efectos de los niveles de nitrógeno ureico en la síntesis proteica durante la fermentación de mezclas de caña (Saccharumofficinarum) y boniato (Ipomea batata Lam.). Rev. Cub. Cien. Agrí. 35:29-36. | | Rodríguez-Muela, C., D. Díaz., F. Salvador., O. Ruiz., C. Arzola, A. Flores, O la O, and A. Elías. 2010. Effect of the level of urea an soybean paste on the concentration of proteins during the solid state fermentation of wasted apple (MalusDomestica) Cub. Jour. Of Agr. Sci. 44(1):23-26. | | Rosero, J., R,2002. Estudo químico, "in situ", "in vitro" e microscópico da paredes celular de cinco genótipos de sorgo colhidosemtrêsepocas de corte. Ph. D. Thesis. Belo Horizonte: Escola de Veterinária da Universidade Federal de Minas Gerais. | | Sánchez, B., C. G. De Los Reyes-Gavilán, A. Margolles y M. Gueimonde. 2009. Probiotic fermented milks: Present and future. Int. J. Dairy Technol. 62:472-483. | | SAS. 2004. SAS/STAT® 9.1 User'sGuide. SAS Institute Inc. Estados Unidos de Norteamérica. | | Schüller, C., Mamnun, Y.M., Mollapour, M., krapf, G., Shuster, M., Bauer, B.E., Piper, P.W. y Kuchler, K. 2004. Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in Saccharomyces cerevisiae. MBC Online. 15:706-720. | | Tang, S.X., Tayo, G.O., Tan, Z.L. Sun, Z.H., Shen, L.X., Zhou, C.S., Xiao, W.J., Ren, G.P., Han, X.F y Shen, S.B. 2008. Effects of yeast culture and fibrolytic enzyme supplementation on in vitro fermentation characteristics of low-quality cereal straws. J. Anim. Sci. 86: 1164-1172. | | Taylor, K. A. C. C. 1996. A simple colorimetric assay for muramic acid and lactic acid. Applied Biochemistry and Biotechnology. 56: 49-58. | | Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B. & France, J. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminants feed. Anim. Feed. Sci. Tech. 48:185. | | Valiño, E., A. Elias, V. Torres y N. Albelo. 2002. Study of the microbial contenton fresh sugar cane bagasse as substrate for animal feeding by solid state fermentation. Cub. J. Agri. Sci. 36:359-364. | | Van der Peet-Schwering, C. M. C., A. J. M. Jansman, H. Smidt y I. Yoon. 2007. Effects of yeast culture on performance, gut integrity, and blood cell composition of weanling pigs. J. Anim. Sci. 85:3099-3109. | | Villagran, D., Rodríguez-Muela, C., Burrola, E., González, E., Ortega, A. 2009. Identificación de levaduras involucradas en la fermentación en estado sólido de bagazo de manzana. Página 2 en Memorias de la XLV Reunión Nacional de Investigación Pecuaria. Saltillo, Coah, Méx. | | Villegas-Castañeda, M., Meneses-Mayo, M., Miranda-Romero, L.A., Loera-Corral, O. 2010. Producción de gas in vitro y desaparición de la materia seca del cultivo sólido con hongos ligninoliticos. Agrociencia. 44: 917-929. | | Wales WJ, ES Kolver, PL Thorne, AR Egan. 2004. Diurnal variation in ruminal pH on the digestibility on highly digestible perennial ryegrass during continuous culture fermentation. J Dairy Sci 87,1864-1871. | | Wiiliams, P. E. V., Walter, A., Macrae, J. C. 1990. Rumen probiosis: the effects of addition of yeast culture (viable yeast Saccharomyces cerevisiae plus growth medium) on duodenal protein flow in wether sheep. Proc. Nutr. Soc. 49:128 (Abstr). | | Wollin, M. J. y Miller, T. L. 1988. Microbe-microbe interactions. Rumen microbial ecosystem. P.N. Hobson, ed. Elseivers Science, Essex, U.K. p 343. |