In vitro gas production of a ration for dairy cows supplemented with isolated yeasts from apple by-products

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In order to assess the effect of adding yeasts isolated from apple by-products on the *in vitro* gas production of a ration for dairy cows, eight inocula were developed with the strains 2, 9, 11 and 13 of *Kluyveromyces lactis* (Kl); 3 and 8 of *Issatchenkia orientalis* (Io); 4 and 6 of *Saccharomyces cerevisae* (Sc). The *in vitro* gas production technique was applied for its assessment. The eight treatments consisted of 0.2 g of complete diet + 10 mL ruminal liquid + 20 mL artificial saliva and 1 mL of inoculum of the different strains. The inocula were evaluated in 120 glass flasks of 50 mL, with three repetitions per treatment and different sampling times: 12, 24 and 48 h for the variables ammoniac nitrogen (NH_3), lactic acid (LA) and yeast counting (YC). For the gas production (PG), intervals of 3, 6, 12, 24 and 48h were established. The data were assessed with the procedure Proc Mixed of SAS 9.0, for a random design of eight treatments in plots divided in time. The results showed effect ($P < 0.01$) of strain and fermentation time in the variables NH_3, LA and YC. The lowest values of NH_3 were observed with the strain Kl-2, and the highest ones with the Io-3, Io-8 and Sc-6, with values of 22.5, 24.8, 24.8 and 24.1 mM/mL, respectively. Effect ($P < 0.01$) was found on the YC, with best performance of the strains Kl-2, Kl-9, Kl-11, Kl-13 and Sc-6, with cell counting of 1.9, 1.3, 1.2, 1.1 and 1.0 x 10^7cel/mL, respectively at 48h. It is concluded that the *K. lactis* yeast demonstrated to be the most effctive strain for ruminal fermentation, with better performance in respect to higher cell counting and reduction of the lactic acid.

Key words: *yeasts, fermentation, gas production.*

The productive performance of ruminants is in function of the nutritional value of the diet they consume. The *in vitro* gas production techniques used in the assessment of ruminants' feeds are of great interest for the research on animal nutrition due to their low costs and because they are not invasive.

These techniques simulate the fermentative and digestive processes in the rumen, are cheaper and require only small amounts of samples. Besides, they use less time for their realization and favor better control of the experimental conditions (Fondevila and Barrios 2001 and López *et al*. 2007). The gas production technique is in the peak as it allows following easily the evolution of the ruminal fermentation and is very used to predict the nutritive value of feeds, as well as to determine the effect of substances used as additives (Colombatto *et al*. 2007 and Giraldo *et al*. 2008).

In order to improve ruminants' health and productivity (Lila *et al*. 2004), one of the alternatives used at present is the yeast feeding additives, composed mainly of *Saccharomyces cerevisiae*. The effects of using microbial additives or their derivatives modify the digestive and metabolic processes, translated into the efficiency increase in the use of feeds and increase of the productive capacity (Díaz-Reyes *et al*. 2009).

The search for alternatives of economically viable feeds for animals that improve their productivity and do not compete with human feeding; apart from being natural and safe sources for human and animals' health is one of the premises of nutritionists (Calsamiglia *et al*. 2005).

The objective of this study was to assess the effect of adding eight strains of yeasts isolated from apple by-products on the *in vitro* gas production of a ration for dairy cows.

Materials and Methods

The experiment was conducted in the animal nutrition laboratory of the Husbandry and Ecology Faculty of the Autonomous University of Chihuahua, Mexico. The strains used were *K. lactis*, strains 2, 9, 11 and 13; *I. orientalis*, strains 3 and 8; *S. cerevisae*, strains 4 and 6. They were all obtained from solid state fermentation of apple baggase for elaborating eight inocula. They were added 100 g of molasses, 1 g of yeast, obtained from the different strains; 1.2 g of urea, 0.2 g of ammonium sulfate and 0.5 g of premixture of vitamins and trace minerals. It was gauged at 1,000 mL with distilled water and oxygenators were used for each flask. The fermentation time for each inoculum was of 96 h, at average room temperature of 20 °C. Once finished the fermentation time for each inoculum, the yeasts were counted. Later, dilutions of each until adjust them to 1.8 x10^9 viable cells /mL1 were conducted.

Eight treatments were prepared with the eight inocula: 1) 0.2 g complete diet + 10 mL ruminal liquid + 20 mL artificial saliva and 1 mL inoculum strain 2; 2) 0.2 g of complete diet + 10 mL ruminal liquid

+ 20 mL artificial saliva and 1 mL inoculum strain 9; 3) 0.2 g of complete diet + 10 mL ruminal liquid + 20 mL arificial saliva and 1 mL inoculum strain 11; 4) 0.2 g complete diet + 10 mL ruminal liquid + 20 mL artificial saliva and 1 mL inoculum strain 13; 5) 0.2 g complete diet + 10 mL ruminal liquid + 20 mL artificial saliva and 1 mL inoculum strain 3; 6) 0.2 g complete diet + 10 mL ruminal liquid + 20 mL artificial saliva and 1 mL inoculum strain 8; 7) 0.2 g complete diet + 10 mL ruminal liquid + 20 mL artificial saliva and 1 mL inoculum strain 4; 8) 0.2 g of complete diet + 10 mL ruminal liquid + 20 mL artificial saliva and 1 mL inoculum strain 6.

The combinations were assessed in 120 glass flasks of 50 mL, with three repetitions per treatment (t) and different sampling hours (12, 24 and 48 h) for the variables. For yeasts counting (YC), the methodology described by Díaz (2006) was used. The ammoniac nitrogen was calculated with colorimetry, according to the procedure of Broderick and Kang (1980). The lactic acid (LA) was determined by colorimetry according to the procedure of Taylor (1996). Samplings were established at 3, 6, 12, 24 and 48 h for the *in vitro* gas production and the technique of Theodorou *et al*. (1994) was applied.

Statistical analysis. It was conducted with a mixed model, using the time and the type of strain (inoculum) as fixed effect. The repetition nested in each treatment was used as random effect. The data were assessed with the Proc Mixed procedure, of the SAS (2004) for a random design of eight treatments in plots divided in time.

Results and Discussion

Effect was found ($P < 0.01$) for the yeasts counting due to the interaction time x inoculum (table 1). This indicates differences in the amount of yeast with an increase of the different treatments in function of time. The highest concentration of yeasts proved in the gas production corresponded to the t1 with the strain Kl-2. It standed out in all sampling times. The concentration of yeasts in t5 with the strain lo-3, and in t6, with lo-8 disappeared rapidly from the ruminal environment. The yeasts of the different inocula submitted to gas production diminished gradually as fermentation time passed. This result coinicides with the report of Galindo *et al*. (2005) and Castillo *et al*. (2009) when studying the growth of *S. cerevisiae* in ruminal environment. They state that yeasts are not able to

Table 1. Influence of the type of yeast strain on the temporary evolution of cell counting $N\text{-}NH_3$ and LA

Variable	Treatments	Strain	Hours		
			12	24	48
$N\text{-}NH_3$	1	Kl-2	12.1±0.75[a]	15.3±0.33[b]	22.8±0.35[b]
(mM/mL)	2	Kl-9	12.0±1.15[a]	15.1±0.48[b]	23.2±0.33[b]
	3	Kl-11	12.6±0.76[a]	15.0±0.32[b]	23.2±0.31[b]
	4	Kl-13	12.2±0.59[a]	15.0±0.86[b]	23.2±0.21[b]
	5	Io-3	13.3±0.28[a]	17.5±1.67[a]	24.5±0.00[a]
	6	Io-8	11.7±0.72[a]	16.5±1.65[a]	24.7±0.04[a]
	7	Sc-4	13.3±0.02[a]	15.0±0.40[b]	23.8±0.10[b]
	8	Sc-6	13.2±0.14[a]	13.8±0.23[c]	24.0±0.07[a]
LA	1	Kl-2	21.7±0.31[d]	7.5±0.10e	2.4±0.01[c]
(mM/mL)	2	Kl-9	27.5±0.30[c]	10.1±0.04[d]	2.8±0.34[c]
	3	Kl-11	34.7±0.22[b]	10.4±0.18[d]	2.3±0.31[c]
	4	Kl-13	28.1±0.20[c]	15.8±0.35[c]	2.3±0.07[c]
	5	Io-3	45.7±0.39[a]	34.4±0.28[a]	17.6±0.31[a]
	6	Io-8	32.8±0.34[b]	21.9±0.51[b]	15.3±0.43[a]
	7	Sc-4	26.3±0.30[c]	17.7±0.47[c]	8.8±0.32[b]
	8	Sc-6	36.1±0.36[b]	18.2±0.18c	9.9±0.39[b]
Yeasts	1	Kl-2	$3.3x10^7$±0.01[a]	$2.5x10^7$±0.01[a]	$1.9x10^7$±0.02[a]
(cel/mL)	2	Kl-9	$2.6x110^7$±0.01[b]	$1.5x10^7$±0.02[b]	$1.3x10^7$±0.01[b]
	3	Kl-11	$2.6x10^7$±0.01[b]	$1.4x10^7$±0.03[b]	$1.2x10^7$±0.03[b]
	4	Kl-13	$2.3x10^7$±0.02[c]	$1.4x10^7$±0.03[b]	$1.1x10^7$±0.02[b]
	5	Io-3	$2.2x10^7$±0.02[c]	$8.0x10^6$±0.02[c]	$3.2x10^6$±0.26[d]
	6	Io-8	$2.4x10^7$±0.01[c]	$9.0x10^6$±0.00[c]	$1.5x10^6$±0.05[e]
	7	Sc-4	$2.8x10^7$±0.02[b]	$1.1x10^7$±0.03[b]	$9.8x10^6$±0.00[c]
	8	Sc-6	$2.6x10^7$±0.01[b]	$1.3x10^7$±0.06[b]	$1.0x10^7$±0.03[b]

[a, b, c, d, e] Different letters with superscripts show difference ($P < 0.01$) between treatments

Means (± SE) of the *in vitro* fermentative performance between treatments of eight yeasts strains

keep a productive population in ruminal environment, as it has growth inhibitor factors, like temperature. The inocula t1, t2, t3 and t4, with the yeast *K. lactis*, as well as t8, with *S. cerevisiae* showed higher yeast population in the rumen in respect to the rest of the inocula up to 48 h. This is due to the use of lactic acid as energy source that allow them keep developing. Similar results were obtained by Rodríguez (2009) and Díaz-Plascencia *et al*. (2010a).

Effect was found ($P < 0.01$) in the ammoniac nitrogen due to the interaction time x inoculum, indicating a different performance between the strains in the fermentation time (table 1). This effect increased and was more marked in the treatments t5, t7 and t8, with values of 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. The ammoniac nitrogen is the main nitrogen source for the ruminal microorganisms, being able to supply between 40 and 100 % of the nitrogen needs for the synthesis of microbial protein (Dewhurst *et al*. 2000). This effect is produced by the urea added to the substrates during the fermentation processes, which is transformed into NH_3 by the effect of ureolytic microbial strains. Several authors report so in studies related with manzarine and saccharine production, in which similar effects are shown (Rodríguez 2009 and Díaz-Plascencia *et al*. 2010b).

There was effect ($P < 0.01$) for the LA variable during the fermentation hours (table 1), specifically on the LA concentration in the treatments 1, 2, 3 and 4. There was higher loss of LA with *Kluyveromyces lactis* in respect to the other inocula, from the 12 h to 48.

In respect to the performance for this variable by the strains of the three genera, the four strains of *K. lactis* showed less LA at 48 h. The two strains of the genus *S. cerevisiae* were next, and those showing the highest concentration were the two strains of *I. orientalis*. The LA increase of some fermentations inhibits the microbial growth and induces the cell death of the yeast or microorganisms, as reported by Madrid *et al*. (1999), Ludovico *et al*. (2001) and García *et al*. (2008). It is known that the LA toxicity depends on the system pH. When the pH is low, the LA is mainly in un-dissociated form and may enter to the microbial cell through passive diffusion (Geros *et al*. 2000). It dissociates in the cytoplasm because of more neutral pH and the protons are released. The cytoplasm pH interferes with some metabolic paths (Schüller *et al*. 2004), as well as with the nutrients and ions transportation. This modifies the membrane structure of the fatty acids, the phospholipids composition and the protein synthesis (Ramos *et al*. 2006). The LA is produced by the carbohydrates catabolism and it is the best indicator of the correct fermentation of the forages under anaerobic conditions. However, the yeast *K. lactis* has a very marked on the use of the LA, when using it as energy source to survive for long periods of time.

There was effect ($P<0.01$) on the treatments 1, 2, 3 and 4 with the yeast *Kluyveromyces lactis* for gas production (figure 1). The fermentation rate and degree of carbohydrates in the rumen vary accroding to their type and structure (Ivan *et al*., 2005), and the predominant microbial population (Dehority 2003). The gas production increase obtained with these strains could be the result of the raise of propionic acid production. This is because the carbon dioxide is produced when the propionic acid is formed by any ruminal bacteria through the succinate-propionate metabolic pathway.

Tang *et al*. (2008) also found possitive effect of a yeast culture ont he gas production of Straw from

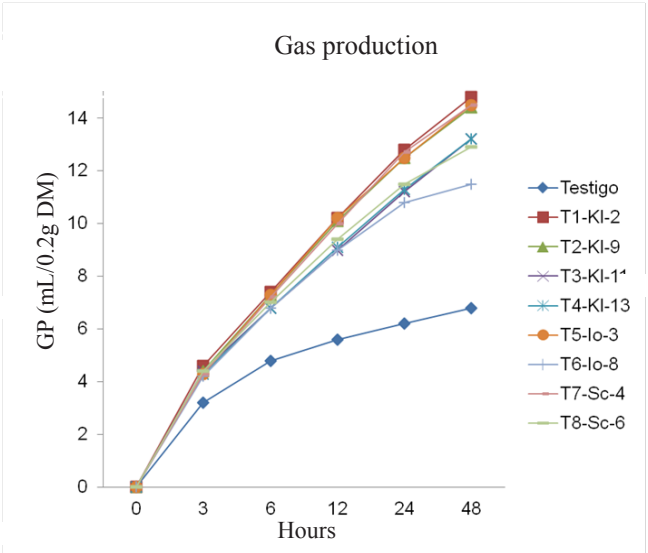

Figure 1. Comparison of the *in vitro* gas production of eight yeasts

different cereals. Other authors (Marrero 2005 and Castillo 2009) have obtained similar results when using the gas production technique to assess the yeasts performance with different substrates.

Form the yeast strains used in this experiment, the *Kluyveromyces lactis* was the most viable in gas production and the one that resisted the most the ruminal degradation. It also showed better performance in yeasts production and lactic acid reduction.

References

Broderick, G. A. & Kang, J. H. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. J. Dairy. Sci. 63:64

Calsamiglia, S., Castillejos, L. & Busquet, M. 2005. Estrategias nutricionales para modificar la fermentación ruminal en vacuno lechero. XXI Curso de Especialización FEDNA. Madrid. España. Pp. 161-185

Castillo, C. Y. 2009. Fermentación *in vitro* para obtener la levadura *Candida norvegenesis* en mezclas de alfalfa con bagazo de manzana fermentado y sus efectos sobre la actividad microbiana ruminal. PhD Thesis. Facultad de Zootecnia y Ecología. Universidad Autónoma de Chihuahua. Chihuahua. México

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

Colombatto, D., Mould, F.L., Bhat, M.K. & Owen, E. 2007. Influence of exogenous fibrolytic enzyme level and incubation pH on the *in vitro* ruminal fermentation of alfalfa stems. Anim. Feed Sci. Tech. 137:150

Dehority, B.A. 2003. Rumen Microbiology. Nottingham University Press. p. 19-43

Dewhurst, R.J., Davues, D.R. & Merry, R.J. 2000. Microbial protein supply from the rumen. Anim. Feed. Sci. Tech. 85: 21

Díaz, P.D. 2006. Producción de proteína microbial a partir de manzana de desecho adicionada con urea y pasta de soya. Master Thesis. Facultad de Zootecnia. Universidad Autónoma de Chihuahua. Chihuahua. México

Díaz Reyes, A., Galindo, J., Bocourt, R., Aldana, A.I., Moreira, O. & Saduy, L. 2009. Effect of a hydrolyzed enzymatic preparation of *Saccharomyces cerevisiae* and its different fractions on the ruminal fermentative dynamics of star grass (*Cynodon nlemfuensis*) in *in vitro* conditions. Cuban J. Agric. Sci. 43: 243

Díaz-Plascencia, D., Rodríguez. Muela, C., Mancillas-Flores, P., Angulo, C., Salvador, F. Arzola, C., Jiménez, J. A., Mena, S. & Elías, A. 2010b. Producción de proteína microbiana a partir de manzana de desecho con fermentación en estado sólido a 32 ºC. Rev. Elect. Vet.10:1-9

Díaz-Plascencia, D., Rodríguez. Muela, C. Mancillas-Flores, P., Angulo, C., Salvador, F., Ruiz, O., Rubio, H. O., Mena, S. & Elías, A. 2010a. Desarrollo de un inóculo con diferentes sustratos mediante fermentación sólida sumergida. Rev. Elect. Vet.10:1

Fondevila, M. & Barrios, A. 2001. The gas production technique and its aplication to the study of the nutritive value. Cuban J. Agric. Sci. 35:197

Galindo, J. & Marrero, Y. 2005. Manipulation of ruminal microbial fermentation. Cuban J. Agric. Sci. 39: 441

García, Y., Elías, A., Albelo, N., Herrera, F.R., Núñez, O. & Dieppa, O. 2008. Growth of acid lactic bacteria and yeasts during the liquid fermentation of broilers feces for the obtainment of probiotics. Cuban J. Agric. Sci. 42: 195

Geros, H., Cassio, F. & Leao, C. 2000. Utilization and transport of acetic acid in *Dekkera anomala* and their implications on the survival of the yeast in acidic environments. J. Food. Prot. 63: 96

Giraldo, L.A., Tejido, M.L., Ranilla, M.J. & Carro, M.D. 2008. Effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage:concentrate ratios. Anim. Feed. Sci. Tech. 141:306

Ivan, S. K., Grant, R.J., Weakley, D. & 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

Lila, Z.A., Mohammed, N., Yasui, T., Kurokawa, Y., Kanda, S. & 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

López, S., Dhanoa, M.S., Dijkstra, J., Bannink, A., Kebreab, E. & France, J. 2007. Some methodological and analytical considerations regarding application of the gas production technique. Anim. Feed Sci. Tech. 135:139

Ludovico, P., João, S.M., Silva, M.T., Leão, C. & Côrte-Real. 2001. *Saccharomyces cerevisiae* commits to programmed cell death process in response to acetic acid. Microbiol. Rev. 147: 2409

Madrid, J., Martínez-Teruel, A., Hernández, F. & Megías, M.D. 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

Marrero, Y. 2005. Las levaduras como mejoradoras de la fermentación ruminal de dietas con alto contenido de fibra. PhD Thesis. Instituto de Ciencia animal. La Habana, Cuba

Ramos, J.A., Elías, A. & 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

Rodríguez, R. 2009. Producción y evaluación de alimentos fermentados a partir de bagazo y desecho de manzana y su efecto sobre el desarrollo ruminal y parámetros sanguíneos. PhD Thesis. Facultad de Zootecnia y Ecología Universidad Autónoma de Chihuahua. Chihuahua. México

SAS. 2004. SAS/STAT® 9.1 User's Guide. SAS Institute Inc. Estados Unidos de Norteamérica.

Schüller, C., Mamnun, Y.M., Mollapour, M., krapf, G., Shuster, M., Bauer, B.E., Piper, P.W. & Kuchler, K. 2004. Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae*. MBC Online. 15:706

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 & 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

Taylor, K. A. 1996. A simple colorimetric assay for muramic acid and lactic acid. Appl. Biochem. and Biotechnol 56: 49

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

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