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Effects of *Aspergillus oryzae* on *in vitro* ruminal fermentation

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Abstract. In the last decades the nutritionists have shown a great interest in a wide variety of microorganisms that, added in small quantities to the diet, could improve the ruminal function, thereby enhancing feeds utilization. The objective of this study was to determine the effect of an *Aspergillus oryzae* culture on ruminal fermentation under *in vitro* conditions, using batch cultures of ruminal microorganisms. Forage of *Pennisetum purpureum* vc Cuba CT-169 was used as substrate for *in vitro* incubations. Treatments consisted in different doses of a culture of *Aspergillus oryzae*, and two dose-response experiments were carried out. In the first study the doses 0, 50, 100 and 200 µl of culture/50ml incubation medium were evaluated. In a second stage, five new and lower doses (0, 0.5, 5, 25 and 50 µl/50ml) were probed. In the first study, a decrease (P<0.05) of propionate, butyrate, isoacids and total volatile fatty acids was observed with the dose of 100 µl. Gas production, final pH, ammonia-N concentration, dry matter disappearance, neutral detergent fibre disappearance and true dry matter degradability were not affected (P>0.05) by any dose of *A. oryzae* culture. When lower doses were evaluated, a decrease (P<0.05) of propionate and a tendency to lower (P<0.10) butyrate and isoacids production were observed with the dose of 25 µl. This dose tended (P<0.10) to increase the acetate:propionate ratio. Doses of 0.5 and 5 µl decreased (P<0.05) final pH. The rest of indicators were not affected (P<0.05) by any treatment. The results of the study suggest that new doses of the additive must be evaluated in order to clarify their effects on ruminal fermentation, but doses to evaluate should not be higher than 100 µl of culture.

Keywords. *Aspergillus oryzae* – Rumen fermentation – *In vitro* technique – Forage diets.

*Effets d'*Aspergillus oryzae *sur la fermentation ruminale* in vitro

Résumé. *Dans les dernières décennies, les nutritionnistes ont montré un grand intérêt pour une large variété de micro-organismes qui, ajoutés dans de petites quantités au régime, peuvent améliorer la fonction ruminal, en améliorant par conséquent l'utilisation des aliments. L'objectif de cette étude a été de déterminer l'effet d'une culture d´*Aspergillus oryzae *dans la fermentation ruminal in vitro en utilisant la technique de production de gaz. Comme substrat on a utilisé le fourrage* Pennisetum purpureum *vc Cuba CT-169. Les traitements ont été différentes doses d'une culture d'*Aspergillus oryzae*. Dans le premier essai, on a évalué les doses de 0, 50, 100 et 200 µL de culture /50 ml de milleu d'incubation. Dans une seconde phase, on a essayé cinq nouvelles et plus faibles doses (0 ; 0,5 ; 5 ; 25 et 50 µl/50ml). Dans le première étude, on a observé une diminution (P<0,05) de la production de propionate, butyrate, iso acides et acides gras volatils totaux pour la dose de 100 µL, tandis que la production de gaz, le pH final, la concentration d´azote ammoniacal, la disparition de matière sèche, la disparition de NDF et la digestibilité réelle de la matière sèche n'ont pas été affectés (P<0,05) par cette dose de culture d' A. oryzae. Quand des doses plus faibles (25 µL) ont etés évaluées, on a observé une diminution (P<0,05) de propionate et une tendance à la diminution (P<0,1) dans les concentrations de butyrate et iso acides. Cette dose a puis tendu à augmenter (P<0,1) le rapport acetate:propionate. Les doses de 0,5 et 5 µl ont tendu à diminuer (P<0,05) le pH final. Le reste de paramètres n'ont pas étés affectés (P<0,05) par aucun traitement. Les résultats de l'étude suggèrent qu'on doit évaluer de nouvelles doses d' A. oryzae (non supérieurs à 100 µl de culture) dans le but de clarifier leurs effets sur la fermentation ruminale.*

Mots-clés. Aspergillus oryzae – *Fermentation ruminale* – In vitro *technique – Fourrage régimes.*

I – Introduction

In the tropical countries, animal feeding systems are based on grasses and forages of low nutritive value. The incessant search of feed additives, able to enhance the utilization of this feeds, is one of the main objectives of specialists and producers. In this sense a great number of compounds have been studied, inside which it has a special interest the microbial feed additives. Several microorganisms have been tested as feed additives, but the most commonly used in ruminant diets consist on *Saccharomyces cerevisiae* cultures and *Aspergillus oryzae* fermentation extracts. The effect of different filamentous fungi on dry matter disappearance of different substrates has been demonstrated (Campos-Montiel *et al*., 2008). Specifically, with the conidial fungus *Aspergillus oryzae* it was observed an increment of the ruminal microbial populations which degrade roughages (Wiedmeier *et al*., 1987). Consequently, increases in digestibility of feed components, feed intake and weight gains have been observed (Gómez-Alarcón *et al*., 1991; Humphry *et al*,. 2002; Kim *et al*., 2006; Di Francia *et al*., 2008). The objective of this study was to determine the effect of *Aspergillus oryzae* culture in ruminal fermentation under *in vitro* condition, using batch cultures.

II – Materials and methods

Substrate and donor animals. Forage of *Pennisetum purpureum* vc Cuba CT-169 was used as substrate for *in vitro* incubations. The samples were dried and ground at 1 mm particle size. The chemical composition of forage was the following (g per kg of dry matter): 859 g of organic matter, 69.6 g of crude protein, 693 g of neutral-detergent fibre (NDF), 356 g of acid detergent fibre (ADF) and 35.1 g of lignin. Rumen contents were obtained from four sheep ruminally cannulated. The animals were kept in indoors and were fed alfalfa hay twice daily at maintenance level. The ruminal contents were collected immediately before the morning feeding, strained through 2 layers of cheesecloth, transferred to stoppered flasks and transported to the laboratory within 30 min.

Treatments: Treatments consisted in different doses of an *Aspergillus oryzae* culture of 96 hours, obtaining from initial concentration of 10^7 spores/ml at 30ºC and 200 rpm in an orbital shaker CERTOMAT BS-1. In a first study the doses of 0, 50, 100 and 200 µL/50ml incubation medium were evaluated. In a second stage five new doses (0, 0.5, 5, 25 and 50 µl/50ml) were probed. All doses were dissolved in distillated water until completing one milliliter.

Experimental procedure: Both experiments were developed according to a completely randomized design. Samples of substrate (500 mg) were weighed into 120 ml serum bottles. One blank per treatment (bottles without substrate but with de corresponding dose of culture added) were included in each incubation run. The milliliter of the corresponding treatment was applied inside the bottles immediately before adding buffered rumen fluid. Particle-free fluid was filtered through nylon bag of 100mm pore size and mixed with the buffer solution of Goering and Van Soest (1970; no trypticase added) in a proportion 1:4 (vol/vol) at 39ºC under continuous flushing with CO_2. Fifty ml of buffered rumen fluid were added into each bottle under CO_2 flushing. Bottles were sealed with rubber stoppers and aluminum caps and incubated at 39ºC. The experiment was repeated on four different days, so that each treatment was conducted in quadruplicate. All bottles were withdrawn after 24 h. At the end of the incubation period, total gas production was measured in each bottle using a pressure transducer and a calibrated syringe. Bottles were uncapped, the pH was measured immediately with a pH meter, and the fermentation was stopped by swirling the bottles in ice. 0.8 milliliter of content was added to 0.5 mL of deproteinising solution (i.e. metaphosphoric acid (100 g/l) and crotonic acid (0.6 g/l)) for volatile fatty acids (VFA) determination and 2 mL was added to 2mL 0.5M HCl for ammonia-N analysis. Finally, contents of the bottles were transferred to previously weighed filter crucibles. Crucibles were dried at 50ºC, weighed and the residue was analyzed for a NDF and ADF to calculate true dry matter degradability (TDMD; Van Soest *et al*., 1991) and aNDF and aADF disappearance. Fibre analy-

ses were carried out according to Van Soest *et al*. (1991) using an ANKOM220 Fibre Analyzer unit (ANKOM Technology Corporation, Fairport, NY, USA). Concentrations of VFA and ammonia-N in rumen fluid were determined as described by Carro and Miller (1999).

Calculations and statistical analyses. The amounts of VFA produced were calculated by subtracting the amount present initially in the incubation medium from that determined at the end of the incubation period. The volume of gas produced (ml) was corrected for pressure (1.013×105 Pa). Data were analysed separately for each experiment using the PROC MIXED procedure of SAS (2002) for a randomized complete design. Significance was declared at P<0.05, whereas P<0.10 values were considered to be a trend.

III – Results and discussion

The effects of the first experiment (doses 50, 100 and 200 µl of culture/50 ml of incubation medium) on ruminal fermentation indicators are shown in Table 1. A decrease (P<0.05) of propionate, butyrate and isoacids production was observed with the dose of 100 µl; as a consequence, total VFA production also decreased (P<0.05).

Table 1. Effects of four doses of *A. oryzae* (0, 50, 100 and 200 µL of culture/50 mL of incubation medium) on gas production (mL), final pH, VFA production (mmol/500 mg substrate), acetate:propionate ratio (Ac/Pr; mmol/mmol), ammonia-N concentration (mg/L), dry matter disappearance (DMD; %), NDF disappearance (aNDFD; %), ADF disappearance (aADFD; %) and TDMD (%) (*n*=4)

Item	Doses (µl of culture)				Doses		SED†
	Control	50	100	200	Linear	Quadratic	
Gas	23.1^{a}	24.8^{ab}	24.4^{ab}	26.0^{b*}	0.919	0.876	1.15
pH	6.72	6.69	6.70	6.70	0.539	0.832	0.016
Total VFA	1301^{a}	1227^{a}	1092^{b}	1239^{a}	0.118	0.003	45.8
Acetate	813	789	728	781	0.177	0.022	35.4
Propionate	268^{a}	260^{a}	222^{b}	260^{a}	0.529	0.007	10.9
Butyrate	130^{a}	111^{ab}	86.8^{b}	120^{a}	0.459	0.062	11.3
Other††	90.8^{a}	67.5^{ab}	55.3^{b}	77.0^{ab}	0.337	0.196	9.65
Ac/Pr	3.32	3.60	3.73	3.23	0.717	0.855	0.232
Ammonia-N	314	303	310	314	0.674	0.245	6.58
DMD	12.0	15.0	12.3	11.9	0.320	0.682	1.89
aNDFD	12.0^{a}	19.0 ^{b*}	16.4^{ab}	12.9^{ab}	0.209	0.058	0.963
TDMD	39.1	40.4	38.8	39.6	0.662	0.222	0.774

† Standard error of the difference.

†† Calculated as the sum of isobutyrate, isovalerate and valerate acids.

^{ab}: Mean values within a row with unlike superscript letters differ (P<0.05).

* Mean values within a row with unlike superscript letters tend to differ (P<0.10).

Gas production tended (P<0.10) to be higher with the doses of 200 µl of culture. Neutral detergent fibre disappearance (aNDFD) tended (P<0.10) to increase with the doses of 50 µl. Final pH, ammonia-N concentration, DMD and TDMD not were affected by these doses of *A. oryzae* culture.

The decrease of certain parameters of ruminal fermentation after the addition of *A. oryzae* was observed by other authors. Martin and Nisbet (1990) did not find any difference in VFA concentration; however, with higher doses of the additive, they observed a decrease in the NDF and ADF digestibilities. Oellermann *et al.* (1990) did not observe differences in NDF and ADF degra-

dation and in total and individual VFA, but a decrease in proteolytic bacteria number was found. These results suggest that high levels of the additive exert an inhibitory effect on some rumen fermentation parameters.

When lower doses were evaluated (second experiment) a decrease (P<0.05) of propionate production and a trend to decrease (P<0.10) butyrate and isoacids production was observed with the dose of 25 µl (Table 2). This dose tended (P<0.10) to increase the acetate:propionate ratio. Doses of 0.5 and 5 µl decreased final pH (P<0.05). The rest of the indicators were not affected (P<0.05) by the addition of the culture of *A. oryzae*.

Table 2. Effects of five doses of *A. oryzae* (0, 0.5, 5, 25 and 50 µl of culture/50 ml of incubation medium) on gas production (ml), final pH, VFA production (mmol/500 mg substrate), acetate:propionate ratio (Ac/Pr; mmol/mmol), ammonia-N concentration (mg/l), dry matter disappearance (DMD; %), NDF disappearance (aNDFD; %), ADF disappearance (aADFD; %) and TDMD (%) (*n*=4)

Item	Doses (µl of culture)					Doses		SED†
	Control	0.5	5	25	50	Linear	Quadratic	
Gas	28.0	28.6	27.8	28.1	30.0	0.903	0.872	0,926
pH	6.94^{a}	6.91^{b}	6.91^{b}	6.93^{ab}	6.93^{ab}	0.173	0.020	0,009
Total VFA	1.710	1.81	1.69	1.65	1.74	0.371	0.332	0,071
Acetate	1.07	1.07	1.03	1.05	1.08	0.620	0.873	0,044
Propionate	0.390^{ab}	0.432^{a}	0.398^{ab}	0.370^{b}	0.396^{ab}	0.302	0.103	0,020
Butyrate	0.145^{ab}	0.171^{a}	0.153^{ab}	0.133 ^{b*}	0.149^{ab}	0.358	0.094	0,012
Other††	0.108^{ab}	0.139^{a}	0.115^{ab}	0.102 ^{b*}	0.117^{ab}	0.458	0.120	0,013
Ac/Pr	2.76^{ab}	2.53^{a}	2.59^{ab}	2.83 ^{b*}	2.75^{ab}	0.624	0.055	0,112
Ammonia-N	309	309	306	319	313	0.263	0.220	5,21
DMS	16.1	13.9	18.3	16.6	15.2	0.508	0.916	1,99
aNDFD	11.8	11.7	12.3	11.8	12.0	0.889	0.858	1,04
aADFD	12.9	13.4	14.8	13.5	13.0	0.582	0.438	1,15
TDMD	38.9	38.8	39.3	38.9	39.1	0.890	0.858	0,722

† Standard error of the difference.

†† Calculated as the sum of isobutyrate, isovalerate and valerate acids.

^{ab}: Mean values within a row with unlike superscript letters differ (P<0.05).

* Mean values within a row with unlike superscript letters tend to differ (P<0.10).

Some authors have observed an increment in fibre degradation and total VFA production with the addition of *Aspergillus oryzae* fermentation extract (Arambel *et al*., 1987; Gomez Alarcon *et al*., 1990; Beharka and Nagaraja, 1993; Varel *et al*., 1993). The effects of fungal additives on ruminal fermentation are variable, and often unpredictable. The reason for this difference is unknown, but may be explained in part by differences in culture conditions. In our study a live culture of *A. oryzae* was used, while the previously mentioned authors used a product based on the extract of fermentation of this microorganism.

The results of our study indicate that a dose-dependt response to *A. oryzae* was not observed under the ruminal conditions evaluated. Varel *et al. (*1994) found that *A. oryzae* fermentation extract increased the degradation of brome grass NDF compared with the control after fermentation for 12 hours but not after 24 or 48 hours. In our studies the parameters were measured after 24 hours of incubation; it could be postulated that the action of de fungus takes place in the first hours, and with time, some ruminal indicators like VFA production tend to be stabilized and the possible effect are not observable after 24 hours.

IV – Conclusions

The results of the study suggest that new doses of the additive must be evaluated in order to clarify its effects on ruminal fermentation, but doses to evaluate should be lower than 100 µl of culture.

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