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Effects of garlic compounds on the *in vitro* ruminal fermentation promoted by diets based on alfalfa hay

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Abstract. The antimicrobial properties of garlic are influenced by the type of compound and dose. This study aims to state the effect of two garlic organosulfur-compounds: propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO) on the *in vitro* dry matter and organic matter disappearance (DMD and OMD, respectively) after 48 h of incubation and, on the total gas production after 24 and 48 h of incubation. Samples (500 mg) of alfalfa hay (AH) and a mixture (1:1) of alfalfa hay and concentrate (AHC) were incubated in syringes with 50 ml of buffered rumen inoculum from goats and PTS or PTSO were added at 0, 10, 25, 50, 300, 750, 1,000 and 2,000 mg/l. At 50 mg/l both compounds reduced ($P < 0.001$) DMD and OMD for AH. With AHC the PTS at 25 mg/l decreased ($P < 0.001$) DMD and OMD while 300 mg/l of PTSO were necessary to achieve that effect. For diet AH 50 mg/l of PTS and 300 mg/l of PTSO reduced ($P < 0.001$) the gas produced over 24 h while over 48 h only 25 mg/l of PTS and 50 mg/l of PTSO were necessary to decrease ($P < 0.001$) gas production. For diet AHC the addition of PTS had no effect ($P > 0.05$) on total gas produced over 24 h but it increased ($P < 0.001$) with 10, 50, 300, 750 or 1,000 mg/ml after 48 h of incubation. However, it was necessary to add 300 mg/l and 750 mg/l of PTSO to decrease ($P < 0.001$) gas produced at 24 h and 48 h of AHC diet incubation, respectively. Observed interactions between concentration, incubation time and diet ($P > 0.001$) both for PTS and PTSO suggest the need of carrying out longer term experiments using concentrations varying between 25 and 300 mg/l.

Keywords. Alfalfa hay – Garlic compounds – Gas production – Ruminal fermentation.

Effet des composants de l'ail sur la fermentation ruminale* in vitro *des régimes á base de foin de luzerne

Résumé. *Les propriétés antimicrobiennes de l'ail sont influencées par le type de composant et la dose. L'objectif de cette étude est de montrer l'effet de deux composants organe-sulfuriques de l'ail : propyl propane thiosulfinate (PTS) et propyl propane thiosulfonate (PTSO) sur la perte de matière sèche et organique* in vitro *(DMD and OMD, respectivement) après 48 h d'incubation et sur la production de gaz totale après une incubation de 24 h et 48 h. Les échantillons (500 mg), du foin de luzerne (AH) et d'un mélange (1:1) du foin de luzerne et concentré (AHC) ont été incubés dans des seringues contenant 50 ml du salive artificiel avec du jus de rumen de chèvre et PTS ou PTSO été ajoutés à 0, 10, 25, 50, 300, 750, 1000 et 2000 mg/l. A 50 mg/l les deux composants ont réduit ($P < 0,001$) DMD et OMD du AH. Sur AHC, le PTS à 25 mg/l a diminué ($P < 0,001$) DMD et OMD alors que 300 mg/l de PTSO ont été néccesaires pour aboutir au même résultat. Pour le régime AHC, l'ajout de PTS n'affecte pas ($P > 0,05$) la production de gaz totale après 24 h, mais une augmentation significative ($P < 0,001$) a été observé avec 10, 50, 300, 750 1000 ou 2000 mg/ml, après 48 h d'incubation. Cependant, il faut ajouter 300 mg/l et 750 mg/l de PTSO pour baisser ($P < 0,001$) la production de gaz du AHC à 24 h et à 48 h d'incubation. Les interactions observées entre la dose des composants, le temps d'incubation et le régime alimentaire ($P < 0,001$) pour PTS et PTSO suggérent le besoin de mener des expérimentations de longue durée utilisant des concentrations variables d'entre 25 et 300 mg/l.*

Mots-clés. *Foin de luzerne – Composants de l'ail – Production de gaz – Fermentation ruminale.*

I – Introduction

The use of antibiotic growth promoters as monensin has proven to be a useful means to improve feed efficiency and to prevent rumen acidosis under intensive production systems (Page, 2006). However, concerned by the possibility of the development of multidrug-resistant bacteria as a threat for human health, the European Union recently banned the non-therapeutic use of antibiotics in animal feeding. As a consequence, the interest on natural alternatives has increased and different *in vitro* studies have demonstrated that some plant derived essential oils, like garlic compounds, can inhibit the rate of amino acids deamination, the number of hyper-ammonia-producing bacteria (McIntosh *et al.*, 2003) or methane production (Chiquette and Benchaar, 2005), as well as they can alter rumen fermentation by reducing the acetate/propionate proportion in a manner similar to monensin (Busquet *et al.*, 2005). Propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO) are organosulfurate compounds obtained by decomposition of initial compounds present in garlic bulbs as alliin and allicin by DMC Research Center. Busquet *et al.* (2005) observed in a multi-concentration additive *in vitro* screening an important dose effect of five different garlic compounds on the fermentation characteristics. The aim of this work was to evaluate the effect of addition of different doses of PTS or PTSO on the in vitro ruminal fermentation promoted by diets based on alfalfa hay using batch cultures of mixed rumen micro-organisms.

II – Materials and methods

Experimental diets were alfalfa hay (AH) and a 1:1 mixture of alfalfa hay plus concentrate (AHC). The chemical composition is shown in Table 1. One millimeter ground aliquots of 500 mg of those diets were weighed in triplicate into 45x55 mm Ankom bags which had 25 mm of pore size, and PTS or PTSO was added at a concentration of 0, 10, 25, 50, 300, 750, 1000 or 2000 mg/l. Each bag was sealed and placed into a 100 ml glass tube inoculated with 50 ml of a 1:4 filtrated rumen liquor:McDougall (1948) buffer mix and provided with a hermetic rubber cap that was connected to a gas-tight 60 ml syringe. Total gas produced was recorded after 24 and 48 h of incubation at 39ºC. Two identical incubation runs were carried out in two consecutive weeks and a blank was incubated in each run as well with the purpose of the correction of total gas production due to the inoculum contribution. The fermentation was stopped introducing tubes in ice-bath and bags were washed with cold tap water and kept frozen at -20ºC until they were dried at 60ºC during 48 h to determine residual dry matter (DM) and organic matter (OM). DM, OM, ether extract (EE) and crude protein (CP) were analyzed according to the AOAC (2005). The NDF and ADF were analyzed according to van Soest *et al.* (1991) using an ANKOM Model 220 Fiber Analyzer (Macedon, NY, USA) with amylase for NDF analysis and NDF and ADF contents referred to ash-free weight. The ADL was determined by solubilisation of cellulose with 72% sulphuric acid. Rumen liquor was obtained from three adult dry non-pregnant rumen-fistulated Granadina goats (46.9 ± 2.15 kg body weight) that were fed alfalfa hay supplied to meet their energy maintenance requirements (Prieto *et al.*, 1990). The experimental model was the following: $Y = m + D_i + T_j + Q_k + D{\times}T_{ij} + D{\times}Q_{ik} + T{\times}Q_{jk} + D{\times}T{\times}Q_{ijk} + e$, where m is the overall mean, D the type of diet, T the garlic compound type, Q the garlic compound dose and e the model error. With the aim to observe interactions among main effects data were analyzed using the GLM Univariate procedure of SPSS 15.0® (Chicago, IL, USA) where diet, additive and dose were considered as fixed-effects factors and differences between runs were considered as random effect. Additionally, in each type of diet and additive, concentration data were tested by one-way ANOVA (data reported in Table 2 and 3). The effects were considered significant at $P < 0.05$ and differences among means were tested using the Tukey comparison test.

Table 1. Chemical composition of the experimental diets (g/kg dry matter)

	Alfalfa hay	Alfalfa hay + concentrate (1:1)
Organic matter	905	902
Crude protein	165	166
Neutral detergent fibre	514	414
Acid detergent fibre	382	278
Acid detergent lignin	93	48

III – Results and discussion

The effect of the addition of different concentrations of PTS and PTSO on the DM (DMD) and OM (OMD) disappearance of the experimental diets after 48 h of incubation is shown in Table 2. The values of AHC degradation were higher (P < 0.001) than those of AH due to his concentrate content. A dose of 50 mg/l of PTS or PTSO was necessary to reduce (P < 0.001) both the DMD and OMD with the AH diet, while with AHC diet 25 mg/l of PTS was enough to reduce both the DMD and OMD but 300 mg/l of PTSO were necessary to obtain the same effect. In general, the DMD and OMD of both AH and AHC diets response to the addition of increasing amounts of PTS or PTSO was similar. Nevertheless with high concentrations (from 300 mg/l of PTS or PTSO) the inhibition of the DM and OM degradation was higher (P < 0.001) with PTSO (degradation reduction from 33 to 52%) than with PTS (25 to 35%). A threshold value could be set at 300 mg/l as the addition of PTS and PTSO at concentrations above it did not modify the degradation of AH and AHC diets. In accordance with our results Busquet *et al.* (2005) found that, incubating a diet similar to AHC in batch culture for 24 h, doses between 30 and 300 mg/l of garlic compounds decreased volatile fatty acids concentration, indicating an inhibition of the microbial activity. Nevertheless these authors observed with a long term trial a lack of persistence of such detrimental effect, which could be due to the adaptation of the microbial ecosystem. No interaction between diet and garlic compound (P > 0.05) was observed, which would allow us to use only one diet in further studies.

Table 2. Effect of the addition of different doses of propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO) on the dry matter (DMD) and organic matter (OMD) disappearance (%) of the experimental diets after 48 h of incubation

Dose of garlic compounds (mg/l)	Alfalfa hay				Alfalfa hay + concentrate			
	DMD		OMD		DMD		OMD	
	PTS	PTSO	PTS	PTSO	PTS	PTSO	PTS	PTSO
0	54.5[a]	54.5[a]	55.6[a]	55.6[a]	66.8[a]	66.8[a]	66.8[a]	66.8[a]
10	54.2[a]	55.5[a]	54.5[a]	48.4[ab]	63.9[ab]	63.4[a]	63.9[ab]	63.8[a]
25	52.3[a]	54.5[a]	42.9[bc]	53.2[ab]	59.4[b]	62.5[a]	59.7[b]	60.6[a]
50	46.6[b]	45.6[b]	46.7[b]	45.9[b]	59.8[b]	61.1[a]	59.8[b]	63.5[a]
300	40.6[c]	36.2[c]	41.0[c]	35.5[c]	48.3[c]	45.2[b]	47.8[c]	44.7[b]
750	39.6[c]	30.4[d]	39.2[c]	29.9[c]	43.5[c]	38.1[c]	45.5[c]	37.4[c]
1000	39.1[c]	34.5[cd]	38.8[c]	33.7[c]	45.6[c]	37.2[c]	45.1[c]	36.0[c]
2000	39.6[c]	30.3[d]	40.0[c]	29.0[c]	45.3[c]	36.7[c]	38.7[d]	32.3[c]
SEM	0.80	0.99	0.91	1.53	1.04	0.99	0.97	1.25

In a same column, values with different superscripts letter differ (P < 0.001); SEM: standard error of the mean.

The effect of the addition of increasing amounts of PTS and PTSO on the total gas production at 24 and 48 h after incubation of the experimental diets is shown in Table 3. Diet- and time-depending effects of both compounds were observed. In syringes incubating AH diet, 50 mg/l of PTS or 300 mg/l of PTSO reduced significantly ($P < 0.001$) the gas produced after 24 h but after 48 h only 25 mg/l of PTS or 50 mg/l of PTSO were required to obtain the same effect. With AHC diet, PTS addition had no effect ($P > 0.05$) on the gas production after 24 h but it was increased ($P < 0.001$) with 10, 50, 300, 750 or 1,000 mg/ml after 48 h of incubation. Concerning PTSO 300 and 750 mg/l respectively after 24 and 48 h were necessary as minimum concentration to reduce the gas production. Independent of the diet, at concentrations over 300 mg/l PTSO had much higher ($P < 0.001$) gas production inhibition power than PTS. Gas production and degradation values are directly related, but the effect was more pronounced when gas production was observed, probably due to the absence of a blank correction related to the losses of feed particles from the bags during the washing process. Higher ($P < 0.001$) gas volumes were recorded when AHC was incubated in comparison with AH diet. The addition of 300 mg/l of PTS or PTSO had a higher impact on total gas production compared to what Busquet *et al.* (2005) observed using garlic oil, diallyl disulfide or allyl mercaptan, which might be due to differences in the mechanisms of action. A highly significant interaction ($P < 0.001$) between diet and garlic compound was observed, that could be explained by more erratic results when AHC was incubated.

Table 3. Effect of the addition of different doses of propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO) on the total gas production (ml) after 24 and 48 h of the experimental diets incubation

Dose of garlic compounds (mg/l)	Alfalfa hay				Alfalfa hay + concentrate			
	24 h		48 h		24 h		48 h	
	PTS	PTSO	PTS	PTSO	PTS	PTSO	PTS	PTSO
0	46.3^{a}	46.3^{ab}	74.8^{a}	74.8^{ab}	51.5^{ab}	51.5^{ab}	71.0^{d}	71.0^{b}
10	45.5^{a}	53.0^{a}	69.5^{ab}	80.0^{a}	52.0^{ab}	38.8^{b}	80.3^{bc}	67.3^{b}
25	40.0^{a}	39.5^{ab}	65.0^{b}	65.0^{bc}	43.5^{ab}	42.0^{b}	71.0^{d}	65.5^{b}
50	30.5^{b}	38.3^{b}	55.8^{c}	59.3^{c}	50.0^{ab}	58.5^{a}	75.5^{bc}	89.5^{a}
300	21.0^{cd}	12.0^{c}	55.0^{c}	42.0^{d}	53.0^{a}	10.0^{c}	97.5^{a}	63.5^{b}
750	18.3^{d}	1.00^{c}	62.3^{bc}	5.50^{e}	44.0^{ab}	0.00^{c}	81.0^{bc}	2.00^{c}
1000	26.0^{bc}	1.50^{c}	66.5^{b}	6.00^{e}	43.0^{ab}	0.00^{c}	85.0^{b}	1.50^{c}
2000	28.0^{b}	0.75^{c}	69.5^{ab}	2.75^{e}	39.5^{b}	0.00^{c}	73.5^{d}	0.25^{c}
SEM	1.19	1.42	2.57	1.85	2.31	1.02	2.35	2.45

In a same column, values with different superscripts letter differ ($P < 0.001$); SEM: standard error of the mean.

IV – Conclusions

Propyl propane thiosulfinate and especially propyl propane thiosulfonate seem to have a pronounced antibacterial activity.

Observed compound dose × incubation time × diet interactions both for PTS and PTSO suggest the need of carrying out longer-term experiments in a concentration from 25 to 300 mg/l. Diet AHC could be the most adequate diet for this experiment. Thus, for a practical antimicrobial use and a better understanding of the mechanisms of action of those additives a similar study must be performed together with a deeper fermentation characterization, including gas composition and short chain fatty acid production.

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