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Effect of antimethanogenic garlic-derived compounds on amylolytic and xylanolytic activities in the rumen

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Abstract. This work assessed the effect of adding two antimethanogenic garlic-derived compounds on *in vitro* rumen amylolytic and xylanolytic activities. A 12-day trial was performed using a continuous-culture system inoculated with rumen fluid from goats. At the end of the trial, a subsequent experiment was carried out by incubating the fermenters' content in batches culture to measure methane production over 24 h. The additives and doses tested were control (without additive), propyl propane thiosulfinate (PTS, 200 µl/L), diallyl-disulfide (DDS, 80 µl/L) and bromochloromethane (BCM, 160 mg/L) as positive control. The incubated substrate consisted of 50:50 alfalfa hay:concentrate mix. After 10 days of adaptation samples of the fermenters' contents were collected at 0, 2 and 4 hours after the morning feeding and the activity of amylase and xylanase enzymes quantified. In batch cultures, the addition of PTS and BCM resulted, in a reduction ($P<0.001$) of methane production of 40 and 96% respectively, while DDS did not have an effect ($P=0.575$), compared to control. In continuous-culture a reduction ($P<0.05$) of xylanase activity in samples treated with PTS at all sampling times was observed compared to the control, while amylase activity was not affected ($P\geq0.243$) by any garlic compound. On the contrary, and despite its potent anti-methanogenic effect, BCM did not affect ($P\geq0.647$) xylanase activity but increased ($P<0.05$) amylase activity at 2 hours compared to the control. Our results suggest that the reduction of methane synthesis in the rumen by some garlic-derived compounds may be accompanied by a detrimental decrease in xylanase activity and might compromise fermentation and intake.

Keywords. Xylanolytic and amylolytic activity – Methane – Rumen – Garlic-derived compounds.

Effet des composés dérivés de l'ail sur la production de méthane et l'activité amylolytique et xylanolytique dans le rumen

Résumé. *Ce travail a pour objectif d'évaluer* in vitro *l'effet de deux composés dérivés de l'ail sur l'activité des enzymes amylolytiques et xylanolytiques dans le rumen. Un essai de 12 jours a été réalisé en utilisant des fermenteurs à flux continu inoculés avec du liquide ruminal des chèvres. À la fin du processus, une expérience a aussi été réalisée en incubant du contenu des fermenteurs dans des bouteilles afin de mesurer la production de méthane pendant 24h. Les additifs et les doses testés étaient le contrôle (sans additif), propane propyle thiosulfinate (PTS, 200 µl/l), diallyle-disulfure (DDS, 80 µl/l) et bromochlorométhane (BCM, 160 mg/l) comme témoin positif. Le substrat incubé était un mélange 50:50 de foin de luzerne et de concentré. Après 10 jours d'adaptation aux différents régimes, des échantillons du contenu des fermenteurs ont été recueillis à 0, 2 et 4 h après le repas du matin et les activités amylanase et xylanase ont été quantifiées. Dans les bouteilles le PTS et le BCM réduisaient ($P<0,001$) la production de méthane de 40-96%, tandis que le DDS n'avait pas d'effet ($P=0,575$) en comparaison avec le témoin. Dans les fermenteurs a été observée une réduction ($P<0,05$) de l'activité xylanase avec du PTS par rapport au contrôle indépendamment du moment de l'échantillonnage tandis que l'activité amylase n'a été affectée par aucun des composés provenant de l'ail. Au contraire, et malgré sa puissante activité antiméthanogénique le BCM n'a pas d'incidence sur l'activité xylanase, mais il augmentait ($P<0,05$) l'activité amylase des échantillons obtenus après 2 et 4 heures d'incubation par rapport au contrôle et à des composés dérivés de l'ail. Les résultats obtenus suggèrent que la réduction de la synthèse du méthane dans le rumen par certains composés dérivés de l'ail peut être accompagnée par une diminution préjudiciable de l'activité xylanase.*

Mots-clés. *Activités xylanolytique et amylolytique – Méthane – Rumen – Composés dérivés de l'ail.*

I – Introduction

Research in animal production is currently focusing on developing cost effective production by improving productivity and product quality but also minimizing the impact on the environment, mainly by reducing methane (CH_4) emissions and N excretion. Methane production in the rumen represents a loss of energy for the host and an important source of greenhouse gases emissions (Benchaar and Greathead, 2011). In the last decade a wide range of plant derived compounds have been tested in order to reduce methane production in the rumen and some have exhibited significant effectiveness with potential practical use (Anassori *et al.*, 2011; Benchaar and Greathead, 2011). However, the mechanisms of action involved in the antimethanogenic effect are not fully understood, especially with regards to potential negative side effects, such as the reduction of fibrolytic activity resulting from less H_2 uptake (Soliva *et al.*, 2011). Among the plant extracts studied, garlic derived metabolites such as allicin, diallyl sulphide, diallyl disulphide and allyl mercaptan have shown *in vitro* capacity for mitigating ruminal CH_4 formation (Anassori *et al.*, 2011). In our group, some of these metabolites (diallyl disulfide and propyl propane thiosulfinate) have exhibited a substantial reduction of methane emissions in short term incubations in batch cultures (Martínez *et al.,* 2011). However, less information is available from longer period of treatments. Therefore, the aim of this work was to evaluate the effects of two garlic compounds on rumen methane production and the activity of some key enzymes using a medium-term *in vitro* approach such as single-flow continuous-culture fermenters (SFCCF).

II – Materials and methods

Eight single-flow continuous-culture fermenters (SFCCF) following the model of Muetzel *et al.* (2009) were used. Eight adult Murciano-Granadina goats fitted with ruminal canula were used in total as donors of ruminal contents. For each incubation run, two groups of three fermenters were inoculated with a different pool (700 mL) each one obtained from three different animals selected randomly. This resulted in four different pools as follows: pool 1 (goats 1, 2 and 3), pool 2 (goats 4, 5 and 6), pool 3 (goats 2, 5 and 7) and pool 4 (goats 3, 6 and 8). In each incubation run two fermenters received one of the 4 experimental treatments: diallyl disulfide (DDS, 3-prop-2-enyldisulfanyl prop-1-ene purity of 80%), propyl propane thiosulfinate (PTS, purity of 75%) and bromochloromethane (BCM, halogenated aliphatic hydrocarbon, purity of 12%) that was included as antimethanogenic positive control. Donor animals were adapted for 12 days to the diet used as substrate in the fermenters, which was composed of alfalfa hay and concentrate in a 50:50 ratio. Concentrate was made of barley, fava beans, corn, sunflower meal, gluten meal and by-pass fat. Rumen contents were collected before feeding and pooled under anaerobic conditions and 700 mL were inoculated into each fermenter, with an effective volume of 1000 mL. Each fermenter received daily 16 g of fresh matter of the experimental diet ground at 1 mm, in two equal portions at 09:00 and 14:00 h. Flow through fermenters was maintained by continuous infusion of artificial saliva (Muetzel *et al.*, 2009) at a rate of 40 mL/h and CO_2 was continuously infused to keep anaerobic conditions. Fermenters were maintained in a water bath at 39ºC and the effluent from each fermenter was collected into a flask maintained in a bath at 3ºC to prevent microbial growth. The doses tested were chosen from previous experiments (Martínez *et al.* 2011): 80 µl/L/day for DDS, 200 µl/L/day for PTS and 160 mg/L/day for BCM. After 10 days of incubation 1 mL of the fermenters content was collected at 0, 2 and 4 h after the morning feeding and frozen at -80ºC for determination of amylase and xylanase activities.

A 24 hours batch culture trial, based on Theodorou *et al.* (1994) protocol, was used by incubating fermenters content collected on day 12 to measure methane production. The content of each fermenter was filtered through two layers of cheesecloth while bubbling with CO_2. Basal diet (0.5 g) was incubated in 120 ml serum bottles with 60 ml of the fermenter content. Three replicates of each treatment previously described and a blank were used. Bottles were sealed

with rubber stoppers and aluminum caps and incubated at 39ºC in a water bath. Headspace pressure and volumes of gas and were measured with a Wide Range Pressure Meter (Sper Scientific LTD, Scottsdale, AZ) and a glass calibrated syringe, respectively, at 2, 4, 6, 8, 12, 24 h after inoculation. At 24 h a sample of the gas in each bottle was collected in a graduated syringe and transferred to a 5 ml vacuum tube and then kept at room temperature before methane content was measured by gas chromatography. An aliquot of the bottles' content was collected to measure total volatile fatty acids (VFA) concentration.

To measure the enzymatic activities in fermenters content, cells were lysed using a Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK, USA) to release intracellular enzymes. Cell material was removed by centrifugation (10,000×g, 10 min, 4ºC) and the supernatant was used for analyses. Xylanase (EC 3.2.1.8.) and amylase (EC 3.2.1.1.) activities were determined as described by Giraldo *et al.* (2008) using oat beachwood xylan and soluble starch, respectively, as substrates. Enzymatic activities were expressed as mol of glucose or xylose released from the corresponding substrates per mL of sample in 1 min at 39ºC and pH 6.5. Data were analyzed comparing the additive effect as one-way ANOVA using SPSS (IBM SPSS Statistics v.19, IBM Corp., Somers, NY). Effects were considered significant at P≤0.05. When significant differences were detected, differences among means were studied using the LSD comparison test.

Table 1. Chemical composition (g/kg DM) of diets ingredients (n=2)

Item	Alfalfa hay	Concentrate
DM, g/kg fresh matter	907	915
OM	875	884
CP	203	168
NDF	513	245
ADF	330	118
ADL	99.2	36.3
Ether extract	8.1	15.3
GE, MJ/kg DM	18.4	19.5

III – Results and discussion

Total gas produced over 24 hours in batch culture and total VFA concentration were only significantly decreased by PTS and DDS treatments, respectively, as compared with the control (Table 2). Methane produced in batch cultures after 24 h of incubation was reduced (P< 0.001) with PTS (40%, P=0.008) and BCM (90%) as compared to the control, while DDS had no effect (P=0.575). Soliva *et al.* (2011) showed 90% inhibition of methane production with garlic oil (300 mg/L) in Rusitec fermenters and Busquet *et al.* (2005a) observed a decreased methane emission of about 70% after 17 h of incubation in batch cultures with a dose similar to the one used in the present study (300 mg/L) of garlic oil and DDS. However, Kamel *et al.* (2008) did not observe effect of DDS on methane emission after 24 h of incubation in batch cultures, although doses were lower than those used in other experiments. In agreement with the results observed with DDS in our experiment, Klevenhusen *et al.* (2011) reported no effect on methane production of DDS in sheep suggesting an adaptation of the rumen microbiota. The antimicrobial effect of garlic derived compounds has been suggested to be due to the inhibition of HMG-CoA reductase (Busquet *et al.*, 2005a; Busquet *et al.*, 2005b). The strong methane inhibition effect of BCM (up to 90%) is in agreement with observations made by Goel *et al.* (2009) *in vitro*. Tomkins *et al.* (2009) observed *in vivo* a reduction in methane emissions close to

90% after 14 days of treatment in beef and Abecia *et al.* (2012) reported a reduction of methane production of around 30% in lactating dairy goats after 60 d of BCM treatment.

Table 2. Effect of additives on total gas production, VFA concentration and methane produced (CH_4, mL) between 12 and 24 h of incubation in batch cultures inoculated with single-flow continuous-culture fermenters content collected after 12 days of incubation

	Treatments†				SEM	*P*-value
	Control	DDS	PTS	BCM		
Total gas, ml	59.1	59.0	43.8	59.6	3.11	<0.001
Total VFA, mmol/l	77.4[a]	65.7[b]	78.1[a]	78.9[a]	11.14	<0.001
Methane production	2.02[a]	1.87[a]	1.21[b]	0.08[c]	0.22	<0.001

†Treatments= PTS (propyl propane thiosulfinate), DDS (diallyl disulfide) and BCM (Bromochloromethane). SEM: standard error of the mean. [a-c] Within a row doses means without a common superscript letter differ, P < 0.05.

In SFCCF a reduction (P≤0.049) of xylanase activity by PTS at all sampling times was observed compared to the control (Table 3), while the other treatments did not have any effect (P≥0.157). The effect of PTS might be due to its antimicrobial activity (Ruiz *et al.*, 2010), that could specifically affect fibrolytic microorganisms, such as protozoa since it has been reported that about 38% of cellulase activity is associated to protozoa (Agarwal *et al.*, 1991). In this sense, we have recently observed that the addition of PTS in batch cultures reduced the abundance of protozoa (Martinez *et al.,* 2011). This is in agreement with Patra *et al.* (2006), who observed decreased xylanolytic activity with plant extracts in *in vitro* experiments using buffalo rumen fluid as inoculum. Also, the decreased xylanase activity with PTS agrees with the detrimental effect on total gas production. The amylolytic activity was only modified (P≤0.031) by BCM, increasing after 2 and 4 hour of incubation compared to the control and other treatments, respectively. Other authors (Hristov *et al.*, 2003) reported decreased amylolytic activity by several bioactive agents using bovine rumen fluid, in contrast to our results. These differences could be due to the different activity and chemical structure of compounds used in both studies.

Table 3. Effect of additives on xylanase and amylase activities in single-flow continuous-culture fermenters content sampled on day 10 after inoculation

	Hour	Treatments†				SEM	*P*-value
		Control	DDS	PTS	BCM		
Xylanase	0	6.91[a]	6.08[ab]	5.22[b]	7.10[a]	0.25	0.026
	2	5.53[a]	5.32[ab]	4.67[b]	5.33[ab]	0.13	0.123
	4	5.77[a]	5.69[ab]	5.11[b]	5.78[a]	0.12	0.133
Amylase	0	1.10	1.06	1.08	1.23	0.04	0.586
	2	0.59[b]	0.41[b]	0.57[b]	1.03[a]	0.08	0.014
	4	0.55[ab]	0.45[b]	0.46[b]	0.97[a]	0.09	0.085

†Treatments= PTS (propyl propane thiosulfinate), DDS (diallyl disulfide) and BCM (Bromochloromethane). SEM: standard error of the mean. [a-b] Within a row doses means without a common superscript letter differ, P<0.05. Amylase activity is expressed as nanomoles of glucose released from soluble starch by 1 mL of ruminal fluid in 1 min at 39ºC and pH=6.5. Xylanase activity is expressed as nanomoles of xylose liberated from oat beachwood xylan by 1 mL of ruminal fluid in 1 min at 39ºC and pH=6.5.

Janssen (2010) suggested that cellulolytic microbes produce more acetate and H_2, while amylolytic microbes produce less H_2 and more propionate, so more CH_4 is formed from forage

based diets. That could explain the different mechanisms of action of PTS and BCM, as while PTS could affect cellulolytic microorganisms, and reduce H_2 formation, BCM could affect competitors of amylolytic microorganisms, thus increases amylolytic activity and produces less H_2. This is important as often the anti-methanogenic effect of some compounds is confounded with a decrease in OM (mainly fibre) digestibility, which in turn will reduce animal intake and therefore productivity (Martin *et al.*, 2010).

IV – Conclusions

The results obtained in this work show that some garlic-derived compounds, such as PTS, have antimethanogenic effect over 12 days of *in vitro* incubation. Such reduction may be accompanied by a decrease in xylanase activity and gas production *in vitro*, which would need to be further investigated *in vivo* as could imply a detrimental effect on overall rumen fermentation and ultimately animal intake.

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References

Abecia L., Toral P., Martín-García A., Martínez G., Tomkins N., Molina-Alcaide E., Newbold C. and Yáñez-Ruiz D., 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. In: *Journal of Dairy Science.,* 95, p. 2027-2036.

Agarwal N., Kewalramani N., Kamra D., Agarwal D. and Nath K., 1991. Hydrolytic enzymes of buffalo rumen: comparison of cell free rumen fluid, bacterial and protozoal fractions. In: *Buffalo J.,* 2, p. 203-207.

Anassori E., Dalir-Naghadeh B., Pirmohammadi R., Taghizadeh A., Asri-Rezaei S., Maham M., Farahmand-Azar S. and Farhoomand P., 2011. Garlic: A potential alternative for monensin as a rumen modifier. In: *Livestock Science,* 142, p. 276-287.

Benchaar C. and Greathead H., 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. In: *Animal Feed Science and Technology.*

Busquet M., Calsamiglia S., Ferret A., Carro M. and Kamel C., 2005a. Effect of garlic oil and four of its compounds on rumen microbial fermentation. In: *Journal of Dairy Science,* 88, p. 4393-4404.

Busquet M., Calsamiglia S., Ferret A., Cardozo P. and Kamel C., 2005b. Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. In: *Journal of Dairy Science,* 88, p.2508-2516.

Giraldo L., Tejido M., Ranilla M. and Carro M., 2008. Effects of exogenous fibrolytic enzymes on in vitro ruminal fermentation of substrates with different forage: concentrate ratios. In: *Animal Feed Science and Technology,* 141, p. 306-325.

Goel G., Makkar H.P.S. and Becker K., 2009. Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. In: *British Journal of Nutrition,* 101, p.1484-1492.

Hristov A., Ivan M., Neill L. and McAllister T., 2003. Evaluation of several potential bioactive agents for reducing protozoal activity in vitro. In: *Animal Feed Science and Technology,* 105, p.163-184.

Janssen P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. In: *Animal Feed Science and Technology,* 160, p. 1-22.

Kamel C., Greathead H., Tejido M., Ranilla M. and Carro M., 2008. Effects of allicin and diallyl disulfide on in vitro rumen fermentation of a mixed diet. In: *Animal Feed Science and Technology,* 145, p. 351-363.

Klevenhusen F., Zeitz J.O., Duval S., Kreuzer M. and Soliva C.R., 2011. Garlic oil and its principal component diallyl disulfide fail to mitigate methane, but improve digestibility in sheep. In: *Animal Feed Science and Technology,* 166-167, p. 356-363.

Martin C., Morgavi D. and Doreau M., 2010. Methane mitigation in ruminants: from microbe to the farm scale. In: *Animal,* 4, p. 351-365.

Martinez G., Abecia L., Martin-Garcia A.I., Ramos E., Ranilla M., Molina-Alcaide E. and Yañez-Ruiz D.R., 2011. In vitro evaluation of some plant extracts as a methane-inhibiting agents with different degradability diets and rumen inoculum from goats. In: *Poster presentations. Advances in Animal Biosciences,* 2, p. 488.

Muetzel S., Lawrence P., Hoffmann E.M. and Becker K., 2009. Evaluation of a stratified continuous rumen incubation system. In: *Animal Feed Science and Technology,* 151, p. 32-43.

Patra A., Kamra D. and Agarwal N., 2006. Effect of plant extracts on in vitro methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. In: *Animal Feed Science and Technology,* 128, p. 276-291.

Ruiz R., García M., Lara A. and Rubio L., 2010. Garlic derivatives (PTS and PTS-O) differently affect the ecology of swine faecal microbiota in vitro. In: *Veterinary microbiology,* 144, p. 110-117.

Soliva C.R., Amelchanka S.L., Duval S.M. and Kreuzer M., 2011. Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). In: *British Journal of Nutrition,* 106, p. 114-122.

Theodorou M.K., Williams B.A., Dhanoa M.S., McAllan A.B. and France J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. In: *Animal Feed Science and Technology,* 48, p. 185-197.

Tomkins N., Colegate S., Hunter R. 2009. A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. In: *Animal Production Science,* 49, p. 1053-1058.