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Potential of white garlic powder (*Allium sativum* L.) to modify *in vitro* ruminal fermentation

F. Sahli[1,2#], C. Darej[1] & N. Moujahed[1]

[1] Animal Resources and Food Laboratory, National Agronomic Institute of Tunisia (INAT), 43 Av. Ch. Nicolle, 1082, Tunis Belvedere, Tunisia

[2] Faculty of sciences Bizerta, University of Carthage, Bizerta, Tunisia

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Abstract

The current study aimed to evaluate the effect of increasing doses of garlic powder (GaP) on *in vitro* fermentation characteristics. Two successive 24-hour incubations were run, and gas production was measured at the end of each incubation period. Liquid samplings for each dose were reserved to determine ammonia nitrogen (NH_3-N) and true organic matter degradability (TOMD). Partitioning factor (PF) was estimated as the ratio between TOMD and the gas produced at 24 hours of incubation. Microbial biomass (MBM) was estimated on the bases of truly degraded substrate and PF. Results showed that gas production increased (P <0.001) with the addition of 32 and 64 mg GaP. An increase (P <0.0001) in NH_3-N concentration was recorded with 4 mg and 8 mg GaP compared with control, whereas adding 32 and 64 mg resulted in a NH_3-N concentration equivalent to control (averaged 39.25 mg/100 ml). The propionate (C3) increased with doses and the highest proportion was noted with the addition of 8 mg GaP (P <0.001). The TOMD was similar for all the doses except for 64 mg GaP, where a slight but significant (P <0.001) increase was noted (77.7%). GaP did not affect PF and MBM values until the dose of 64 mg. It was concluded that GaP added to a ration composed of 50% roughages and 50% concentrate did not result in drastic modifications of *in vitro* rumen fermentation parameters, except at the highest dose (64 mg), where an increase of gas production, TODM, PF and MBM were noted.

Keywords: Ammonia-nitogen, garlic powder; gas production, microbial biomass, propionate, sheep
[#] Corresponding author: sahli-fatima@hotmail.fr

Introduction

The main concern of ruminant nutritionists is to improve the efficiency of converting feed to animal products by manipulating the ruminal microbial ecosystems (Mirzaei-Aghsaghali *et al*., 2012). In this context, the use of feed additives such as antibiotics proved to be a useful tool to reduce energy and nitrogen losses from the diet (Kongmun *et al*., 2011). Additionally, the use of antibiotic growth promoters such as monensin was established as an efficient way to improve feed digestion and to prevent rumen acidosis under intensive production systems (Page, 2006; Martin-Garcia *et al*., 2011). However, their use became highly regulated because of the possible development of drug resistance in human pathogenic bacteria (CAFA, 1997). In the last few years, feed additives such as aromatic plant extracts have gained interest as alternatives to antibiotic growth promoters (Yan *et al*., 2011) and because of their ability to manipulate gastrointestinal microflora (Syadati *et al*., 2012). An *in vitro* study of natural alternatives demonstrated that certain plant derivatives, such as garlic compounds, can inhibit the rate of amino acid deamination, the number of hyper-ammonia-producing bacteria (McIntosh *et al.*, 2003), and methane production (Chiquette & Benchaar, 2005). Also, they seem to affect rumen fermentation by reducing the C2 : C3 acetate/propionate proportion in a manner similar to monensin (Busquet *et al*., 2005; Martin-Garcia *et al.*, 2011). Currently, growing interest is being given to additives such as diallyl disulfide and allyl mercaptan as natural alternatives in animal feeding (Busquet *et al*., 2005, Castillejos *et al*., 2006).

Garlic (*Allium sativum* L., Liliaceae family), has been used as a spice and as folk medicine since antiquity (Rivlin, 2001). Researchers have shown that the major sulphur-containing compounds in intact garlic are ɤ-glutamyl-S-allyl-l-cysteins and Sallyl-l-cysteine sulphoxides (alliin), which are converted into

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thiosulphinates (such as allicin, the main major bioactive component) via enzymic reactions (activation of allinase by crushing cloves or wetting powder) when raw garlic is processed (Amagase, 2006; Kamruzzaman *et al*., 2011).

Some studies carried out on ruminants have shown that garlic extracts improved the efficiency of nutrient use by decreasing energy loss as methane or ammonia nitrogen in continuous rumen culture (Cardozo *et al*., 2005; Kamel *et al*., 2008, Kamruzzaman *et al*., 2011). Additionally, Busquet *et al.* (2005), using the same *in vitro* system, showed that 300 mg/L garlic oil reduced the proportions of acetate and branched-chain volatile fatty acids (VFA), and increased the proportion of propionate and butyrate and small peptides (Busquet *et al*., 2005). These changes in the fermentation trends are consistent with those observed with methane inhibitors and could modify rumen microbial fermentation beneficially (Mirzaei-Aghsaghali & Maheri-Sis, 2011). Few studies so far have investigated the effects of garlic and its components on feed intake, digestion, ruminal fermentation, and performance in ruminants (Benchaar *et al.*, 2007; Yang *et al*., 2007). Kongmun *et al.* (2010) affirmed that GaP supplementation resulted in greater efficiency than coconut oil on ammonia nitrogen concentration by increasing it, *in vitro* true organic matter digestibility, proportion of propionate, C2: C3 ratio, and methane gas production. The authors also suggested that adding a mixture (coconut oil/garlic powder at 8:4 and 0:16 mg) could improve ruminal fluid fermentation in terms of VFA profile, and reduce methane losses and protozoal population. Wanapat *et al*.'s (2008) study showed that adding garlic powder to a mixture of concentrate containing high cassava chip enhanced ruminal propionate production, resulting in a lower C2: C3 ratio, decreased the protozoal population, and increased N retention and absorption in ruminants.

Since the differences of garlic's varieties have a direct effect on the variability of the chemical composition (Kongmum *et al.*, 2010, Anassori *et al.*, 2011) and different effects on animals, the aim of this study was to evaluate the potential of garlic powder from local varieties to modify in vitro rumen fermentation in sheep in Tunisian conditions.

Material and Methods

The white garlic *(Allium sativum)* used in this study was collected from Beja (north-west Tunisia, a humid region). Fresh garlic was ground and oven-dried at 50 °C over 48 hours, then milled to pass through a 1-mm screen. The incubated substrate was a diet (D) composed of 50% ryegrass hay and 50% commercial concentrate milled (1-mm screen) and mixed on a DM basis.

Four adult Barbarine sheep, 12 months old, and 30.0 ± 3.0 kg bodyweight, were assigned to this assay. They were slaughtered at the municipal slaughterhouse of Ariana (Tunis) and used as rumen fluid donors. The data about the sheep diets were checked from their owners. Those diets that generally consisted of oat hay supplemented with barley were retained to standardize the rumen fluid. The rumen contents of the four freshly slaughtered sheep (1 L per animal) were collected immediately after evisceration and rapidly transferred in pre-warmed (39 °C) thermos flasks to the laboratory, where the contents were mixed and filtered through four layers of surgical gauze.

The mixed diet (D) was used as a substrate to determine the effect of growing doses (0, 4, 8, 16, 32, and 64 mg per 0.5 g D) of white garlic powder on *in vitro* rumen fermentation parameters. Samples of D were incubated in 100 ml graduated glass syringes. The incubation medium (50 ml) was a mixture (1 : 1) of rumen fluid and artificial saliva prepared according to the procedure described by Menke & Steingass (1988). For each garlic dose, D was incubated in five replications (syringes) on two successive batches. The syringes were pre-warmed at 39 °C for 1 hour before filling with 50 ml of rumen inoculum's mixture in presence of D mixed with the garlic at the studied dose. Before the incubation, pH in the syringe liquid was measured (Voltcraft digital PH-100 ATC calibrated pH-metre) and the content of each syringe was saturated with CO_2. Each incubation lasted 24 hours and gas production was measured at 1, 2, 4, 6, 12, and 24 hours of incubation. At the end of incubation, two syringes from each dose were reserved for NH_3-N analysis and VFA analysis using gas chromatography (GC 2010 Plus) (18 ml of liquid + 2 ml of conservative solution) (1% $HgCl_2$ p/v and 5% H_3PO_4 v/v) and three syringes were used to determine TOMD. After 24 hours of incubation, the contents of each syringe reserved for TOMD determination were quantitatively transferred into a pre-weighed crucible. It had undergone repeated washings with 100 ml neutral detergent solution heated to reflux for 1 hour and filtered with hot water (Van Soest & Robertson (1985). The crucibles were dried in the oven overnight, and weighed, then calcined at 550 °C for about six hours and reweighed after cooling in a dryer to determine the TOMD.

Cumulative gas production data were fitted according to the model of Orskov & McDonald (1979) as:

$$y = a + b\,(1-e\,(-^{ct}))$$

where: y = gas produced at time 't'

a = the gas production from the rapid fermented fraction
b = the gas production from the insoluble fraction
c = the constant gas production rate from the insoluble fraction (b)
t = incubation time
(a + b) = the potential extent of total gas production.

TODM measured in three syringes was calculated as:

$$\text{TOMD} = \frac{(\text{OM of incubated D (mg)} - \text{residuel OM (mg)}) * 100}{\text{OM of incubated D (mg)}}$$

The partitioning factor (PF) was calculated as the ratio between TODM (mg) and the gas produced at 24 hours of incubation of substrate truly degraded (Blümmel *et al*. 1997). The microbial biomass (MBM) yield was calculated using the TODM (mg) and gas volume (24 h) and stoichiometric factor (Blümmel *et al*. 1997) as follows:

MBM (mg) = substrate truly degraded (gas volume × stoichiometric factor)

where the stoichiometric factor was equal to 2.25 (value used for roughages)

Feeds were analysed using the AOAC method for dry matter (DM) according to method 27.005, for ash according to method 27.009 and for crude protein (CP) contents according to method 990.03. Cell wall composition (NDF, ADF and ADL) in feeds was analysed as described by Van Soest *et al*. (1991). NDF was determined using sodium sulphite, but not α-amylase, in boiling neutral detergent solution. Ammonia-N was analysed according to the method of Conway (1962).

The numerical data were analysed statistically according to the general linear model (GLM) procedure of (SAS Inst. Inc., Cary, NC). with the option of MEANS multiple ranges. The model included effects of dose, incubation and interaction. Measurements from the control syringes (T), containing only buffered solution with inoculum were used as co-variables to control rumen liquid origin variation.

Results

The chemical composition of feeds is presented in Table 1. DM content of garlic was around 43.3%. Garlic is relatively high in CP (18.8% DM). The total cell wall content (NDF) of GaP was low (7.9% DM). Garlic cell wall low in lignification and the obtained value of ADL did not exceed 2% DM. Ryegrass hay contained 16% CP and 51.1% NDF. The ADF content was of 29.3% DM and ADL reached 9.3% DM. Concentrate contained CP of 15.5% DM, while the mixed diet contained 15.9% CP on a DM basis.

Table 1 Chemical composition of feeds used in the experiment (% of dry matter)

Feed	DM (%)	Ash	CP	NDF	ADF	ADL
White garlic	43.3	3.6	18.8	7.9	6.5	1.9
Ryegrass hay	95.1	14.4	16	51.1	29.3	9.3
Concentrate	89	7.6	15.5	27.3	03.9	3.6
Diet	91.9	10.9	15.9	49.9	19.1	-

DM: dry matter, CP: crude protein, NDF: neutral detergent fibre, ADF: acid detergent fibre, ADL: acid detergent lignin
Diet: ryegrass hay + concentrate

Gas production parameters are given in Table 2. Results of gas production from insoluble fractions (b) showed no differences between doses 4, 8 and 16 mg (averaged 107.4 ml) compared with 32 mg (116.1 ml) and 64 mg (133.4 ml) (P <0.001). The same trend (P <0,001) was noted for total gas production (a + b), in which the highest gas production was noted for 32 mg GaP (a + b = 115.6 ml), followed by 64 mg (133.3 ml). The lowest values were noted for the other doses, which were similar (a + b averaged 106.4 ml). The gas production constant rate (c) for the insoluble fraction (b) increased with the doses and ranged from 0.095 to 0.108 h^{-1}. Compared with control, gas production at 24 hours (GP_{24}) increased significantly (P <0.0001) for doses 16, 32 and 64 mg. The highest value was observed for the highest dose of garlic (109 ml) and was

significantly (*P* <0.001) higher than the value recorded at 16 mg and 32 mg (98.8 ml and 105.3 ml, respectively).

Table 2. Effect of increasing doses of garlic powder on cumulative gas production parameters (ml) using sheep rumen fluid

Doses (mg)	b**	(a+b)**	c**	GP_{24}***
0	107.7^{f}	106.7^{f}	0.095^{ef}	95.1^{g}
4	105.4^{f}	104.6^{f}	0.1^{def}	95.8^{dc}
8	107^{f}	105.7^{f}	0.092^{f}	96.5^{fg}
16	109.5^{f}	108.75^{f}	0.095^{bc}	98.8^{f}
32	116.1^{e}	115.6^{e}	0.102^{de}	105.3^{e}
64	133.4^{d}	133.3^{d}	0.108^{d}	109^{d}
SEM	0.60	1	0.003	0.083

b: gas production from the insoluble fraction
c: gas production rate for the insoluble fraction (b)
(a + b): potential extent of gas production
$^{d, e, f:,g}$ Values with different letters in the same column are statistically different
** *P* <0.01; *** *P* <0.001; SEM: standard error of mean

The effects of increasing doses of GaP on NH_3-N concentration are reported in Figure 1. An increase (*P* <0.0001) in NH_3-N concentration was recorded with 4 and 8 mg GaP (averaged 43 mg/100 ml) compared with control (39.4 mg/100 ml). The addition of 16 mg GaP decreased NH_3-N concentration to 40.4 mg/100 ml. The doses of 32 and 64 mg GaP resulted in an NH_3-N concentration equivalent to control (averaged 39.25 mg/100 ml).

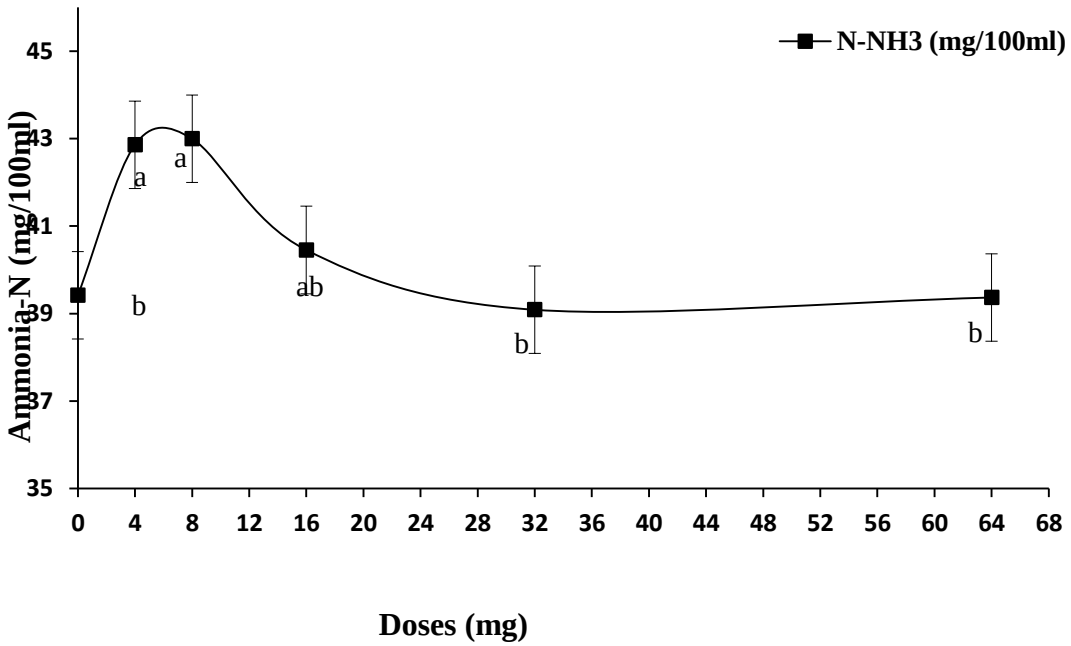

Figure 1. Effect of garlic powder on ruminal ammonia nitrogen concentration (*P* <0.001)

The results for VFAs are presented in Table 3. The lowest value of total VFA concentration was noted with control (73.6 mmolL^{-1}) (*P* <0.001). Adding GaP resulted in increased, but equivalent VFA concentrations for all the other doses (averaged 99.28 mmolL-1). No significant variations among doses were noted for C2 proportions (averaged 56.37%). Molar proportion of C3 was highest (22.75%, *P* <0.0001) when 8 mg GaP was added. The proportion of C4 exhibited no significant trend of decrease with increased doses of GaP (averaged 14.63%). The C2: C3 ratio was significantly lowest (*P* <0.001) with the 8 mg dose (2.45).

Table 3 Effect of garlic powder supplementation on volatile fatty acid production

Dose	0	4	8	16	32	64	SEM
Total VFA (m mol/l)	73.6^{b}	101.89^{a}	106.15^{a}	95.36^{a}	95.66^{a}	97.13^{a}	6.74
Acetate (C2%)	56.85	56.6	56.63	55.22	56.32	56.6	0.99
Propionate (C3%)	20.24^{c}	21.98^{b}	22.75^{a}	20.70^{c}	21.2^{bc}	21.06^{bc}	0.39
Butyrate (C4%)	15.44	14.22	14.35	14.86	14.43	14.53	0.85
C_2/C_3	2.75^{a}	2.57^{ab}	2.45^{b}	2.67^{ab}	2.66^{ab}	2.69^{ab}	0.08

VFA: volatile fatty acid
$^{a, b, c, d}$Values with different letters in the same row are statistically different
: *P* <0.01; *: *P* <0.001; SEM: standard error of the mean

Results for TOMD, PF and MBM are reported in Table 4. TOMD values did not differ among GaP doses, except for the 64 mg dose, in which the observed value (77.7%) was significantly (*P* <0.001) higher than all the others (averaged 73.1%). PF values were equivalent in doses 0, 4, 8, 16, and 32 mg GaP (averaged 3.2), while a significant (*P* <0.0001) increase was noted with 64 mg. The same trend was observed in MBM (228) and averaged 153.75 mg for 64 mg and all other doses (*P* <0.001).

Table 4. Effect of increasing doses of garlic powder on fermentation parameters

Doses (mg)	0	4	8	16	32	64	SEM	*P*-value
TOMD (%)	72.18^{b}	72.67^{b}	72.95^{b}	72.93^{b}	73.98^{b}	77.7^{a}	0.0048	***
PF (mg/ml)	3.23^{b}	3.19^{b}	3.19^{b}	3.22^{b}	3.25^{b}	3.75^{a}	0.0429	****
MBM (mg)	153.7^{b}	147.5^{b}	149.5^{b}	152.3^{b}	165.7^{b}	228^{a}	5.48	***

TOMD: true organic matter degradability; PF = partitioning factor, MBM = microbial biomass
$^{a, b:}$ Values with different letters in the same row are statistically different
: *P* <0.01; *: *P* <0.001; ****P* <0.0001 SEM: standard error of the mean.

Discussion

The chemical composition values of garlic were close to those reported by Kongmun *et al.* (2010) and Klevenhusen *et al.* (2011). Garlic is relatively high in CP (18.8% DM). This value is close to those reported by Kongmun *et al*. (2011), who found a value equal to 22.9% of DM. The total cell wall content, NDF value (7.9% DM) is higher than those found by Kongmun *et al*. (2011) and Manasri *et al.* (2012). The ADF fraction (6.5% DM) was higher than the values reported by Kongmun *et al*. (2011) and Manasri *et al*. (2012), but was closer to that reported by Patra *et al.* (2011). The differences observed in chemical composition compared with the literature may be related to genetic varieties and possibly to the initial conditioning of garlic, resulting in different proportions of remaining teguments in the analysed substrate mainly for cell wall.

The gas production results of the current study confirmed those of Anassori *et al*. (2011). These authors reported that garlic increased the gas production and thus the digestibility of the substrate. Kongmun *et al*. (2010) showed that the use of garlic powder in *in vitro* assays increased the density of the population of cellulolytic bacteria. According to Menke *et al*. (1979), there is a high correlation between *in vitro* gas production and microbial growth.

When connecting chemical composition with gas production, the current results suggest that increasing doses of garlic, mainly dose 64 mg, represented small non-lignified organic matter and protein supplies to the incubated diet. These nutrients are generally associated with the establishment of an adequate environment for microflora (Chen *et al.*, 2008) and result in higher gas production.

The results of the current study for NH_3-N presented an increasing trend until 8 mg GaP and a decreasing trend at the dose of 16 and mainly 32 mg and 64 mg. This result is somewhat concordant with that of Kongmun *et al*. (2010), who showed that in a diet containing coconut, supplemented with various doses of garlic powder, NH_3-N concentrations decreased mainly at 16 mg. However, Cardozo *et al*. (2005) affirmed that at 7.5 mg GaP, there was a decrease in the rate of NH_3-N by about 25%. The increase in NH_3-N that was noted in the current experiment at doses of 4 and 8 mg could be explained by the stimulation of proteolytic activity in the rumen.

The effect of garlic on rumen NH_3-N concentration is discussed widely in the literature. Cardozo *et al*. (2014) reported that adding garlic oil in a continuous culture reduced NH_3N, suggesting that deamination was inhibited. This inhibition was explained by Ferme *et al.* (2004) as being related to Prevotella spp., which are responsible mainly for protein degradation and amino acid deamination, suggesting a mechanism of action of garlic oil on protein metabolism. The deamination requires an optimum activity of dehydrogenases, and the reduction in deamination level could be related to reduced availability of this enzyme (Kongmun *et al*., 2010). Methane inhibitors such as garlic oil could reduce dehydrogenase activity (Hino & Russell, 1985), which might lead to decreased NH_3-N production in the rumen. Essential oils exert an anti-microbial effect on hyper-ammonia-producing bacteria and protein-degrading bacteria in the rumen (Busquet *et al*., 2005; Patra & Yu, 2014). Other studies showed that the addition of garlic powder to diet had no effect on ruminal fluid NH_3-N concentration (Wanapat *et al.*, 2008). The discrepancies in the results of these studies could be attributed to differences in the experimental conditions (for example in vivo versus in vitro, the basal diet, and the duration of the experiment), the amount and the composition of garlic, and its mode of administration in particular.

Results for VFAs were in line with those found by Wanapat e*t al.* (2008), who reported that increased supplementation of garlic powder in diets resulted in increased C3 and C4 proportions. The authors reported that the increased proportion of C3 was simultaneous with a reduction of the proportion of C2. Additionally, Kongmun *et al.* (2010) reported that supplementation with garlic powder influenced the molar proportion of propionate. On the other hand, Busquet *et al.* (2005b), using garlic oil and its compounds as additives in *in vitro* fermentation study, suggested that diallyl disulfide and allyl mercaptan compounds in garlic were responsible for most of their effects. Indeed, they were selected for evaluation in long-term continuous culture and gas production trials. Furthermore, high concentrations (300 and 3000 mg/L) of all compounds (except allicin) resulted in detrimental effects on rumen microbial fermentation by decreasing total VFA concentration, which confirms their antimicrobial activity. Numerous studies have suggested that the antimicrobial potency of allyl sulfides of garlic oil increases with each additional sulphur atom. Results by Busquet *et al.* (2005) suggested that in general additives were not detrimental to rumen microbial fermentation. The lack of detrimental effects in a long-term continuous culture study could be due to the longer adaptation time allocated to the rumen microflora, which may allow replacement of the inhibited microbial population with other resistant bacterial groups. Anassori *et al.* (2011) reported that partial or complete exclusion of protozoa from the rumen in sheep favours the production of propionate at the expense of acetate and butyrate, and reduces bacterial N recycling and rumen ammonia concentration. The increase of the C3 proportion is associated with the improvement of energy supply to ruminants through neo-glycogenesis in the liver.

GaP did not affect TODM except for the 64 mg dose. The results of the current study up to 32 mg agree with those found *in vivo* by Manasri *et al.* (2012), who reported that supplementation with garlic powder did not affect OM digestibility in cattle. Busquet *et al*. (2005) affirmed that the addition of garlic oil and its compounds did not affect true DM, OM, NDF and ADF digestibility. Moreover, Yang *et al*. (2007) observed that supplementation with garlic essential oil did not affect total digestibility of DM, OM, fibre and starch, while ruminal DM and OM digestibility values increased. In contrast, Yang *et al.* (2007) and Patra *et al.* (2011) reported that supplementation with garlic increased the digestibility values of DM and OM compared with control (Mirzaei-Aghsaghali e*t al*., 2012). The current results with the dose of 64 mg may be related to the small amounts of nutriments supplied from garlic, are in accord with the increase in gas production registered at this dose (Anassori *et al.,* 2011), and may be associated with an increase in the activity of cellulolytic bacteria (Kongmun *et al.*, 2010).

PF is an index of microbial biomass synthesis (Blümmel *et al*., 1997). According to Getachew *et al.* (1998), PF is valuable in predicting voluntary feed intake. Diets with higher microbial efficiency mean that proportionally more of the degraded organic matter is incorporated into microbial biomass (Blümmel *et al*., 1997). In the current study the PF value was constant until 64 mg at which the authors noted a significant

increase. This dose probably provided larger proportions of degradable OM, mainly protein that could be captured for the microbial synthesis in the rumen, resulting in higher microbial synthesis as showed by the MBM variation. MBM founded in this assay did not agree with those reported by Kumar *et al*. (2012), who found that MBM values decreased with increasing doses of GaP. The observed results for 64 mg may be related to the supply of digestible OM from garlic powder.

Conclusions

White garlic powder added to a ration composed of 50% roughage and 50% concentrate could induce changes in rumen fermentation as measured in vitro. The most important effects concerned VFA concentration and C3 proportion at 8 mg GaP and also the most measured parameters (GP_{24}, TODM, PF and MBM) at the highest dose of garlic (64 mg), at which an increase in their value was noted. These results may reflect a combination between a modifying effect of *in vitro* rumen fermentation trends (VFAs) and nutrient supply from GaP (fermentation parameters at high doses of white garlic). Further measurements to investigate the effects of garlic varieties on intake, in vivo diet digestibility, N balance, and microbial activity are currently being carried out in the laboratory.

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Authors' Contributions

FS was in charge of writing the manuscript and interpreting the study. NM, the doctoral student's tutor, checked the manuscript and made the corrections. FS performed tests in the laboratory and participated in the acquisition of data. CD, FS made statistical analyses. All the authors have read and approved the final paper.

Conflict of interest declaration

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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