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ASSESSING THE IMPACT OF GRAPE POMACE ADDITION ON THE FERMENTATIVE PROFILE OF CONCENTRATE FEED

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Abstract: *Spain's agricultural sector, a significant socioeconomic and environmental activity, utilizes half of the country's land for agriculture and livestock. Among livestock, Spain ranks second in the European Union for sheep population, primarily concentrated in the Extremadura region. Ruminants, prevalent in the country, possess a digestive system capable of fermenting fibrous material into volatile fatty acids and gases, including methane (CH4), a potent greenhouse gas. The nation is also a global leader in vineyard cultivation and ranks third in wine production, generating substantial organic by-products like grape pomace, known for its polyphenolic properties.*
Given these circumstances, this study addresses the necessity of proposing environmentally sustainable management strategies. The research entails in vitro analysis of the fermentative and methanogenic profiles of a concentrate enriched with dried grape pomace at varying proportions. Grape pomace contains tannins and ruminal antimethanogenic properties. Incorporating these by-products into ruminant diets not only reduces ration costs but also yields a healthier, higher-quality end product for consumers.
Keywords: *Agriculture, Livestock, Grape pomace, Ruminants, Methane mitigation*

1. Introduction

The Spanish agricultural sector represents an important socioeconomic and environmental activity, with half of the country's surface dedicated to agriculture and livestock farming. Regarding the livestock sector, Spain is the second country in the European Union with the largest number of sheep, with most of the livestock concentrated in the Extremadura region(MAPA, 2020). Ruminants are characterized by a digestive system capable of digesting fibrous material into volatile fatty acids, fermentation gases and heat (Owens and Basalan, 2016). Fermentation gases include CH_4, a potent greenhouse gas, which accounts for about a quarter of the anthropogenic CH_4 generated (Lassey, 2008).In terms of agricultural activity, Spain is the country with the largest area of vineyard cultivation in the world, ranking third after France and Italy in wine production (OIV, 2019). The wine industry generates seasonally a high volume of by-products with high organic content. Among these byproducts, grape pomace represents between 20-25% of the weight of fresh grapes, characterized by its polyphenolic properties (Yu and Ahmedna, 2013).Considering both premises, this study arises because it is essential to propose strategies that allow an environmentally sustainable management.
In this work, an *in vitro* analysis has been carried out on the fermentative and methanogenic profile of a concentrate incorporating dried grape pomace in different proportions due to its concentration of

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tannins and ruminal antimentanogenic properties (Bhatta *et al*., 2013). The use of these by-products by incorporating them into the ruminant diet would reduce the costs of the ration, as well as the possibility of obtaining a healthy and higher quality final product for the consumer.

2. Material and Methods

2.1. Mixing system

After a previous analysis of *in vitro* fermentation of 15 grape pomace varieties (Barraso *et al*., 2021), four pomaces were selected (P1, P2, P3, P4) whose gas and CH_4 production was lower. The selected pomaces belonged to the *Tempranillo* variety.

The pomaces, previously dried and grounded, were mixed in different proportions with a base concentrate (barley). The mixing process was carried out with a mixer, after having carried out previous tests to determine the optimum mixing time. The percentage of pomace incorporated into the concentrate was: 1.25%, 2.5%, 5% and 7.5% so that we have four mixtures of each selected pomace plus the barley.

2.2. Chemical analysis

The mixtures were analyzed according to EC Regulation 152/2009 (EC, 2009), for ash/organic matter, crude fat, crude protein, crude fiber, neutral detergent fiber, and acid detergent fiber.

2.3. *Gas production Kinetics and modelling*

The in vitro fermentation process was carried out according to the technique proposed by Menke et al., 1979 using 120 ml glass syringes with Luer-type tips and measuring the displacement of the plunger produced by the fermentation gas. The Luer tip is closed with a three-way valve that ensures a hermetic seal and allows the attachment of multiple measuring devices.

Faeces are used as a source of inoculum for fermentation, collected immediately before the beginning of the study and directly of the rectum of the sheep(Axford and Chamberlain, 1987). The animals belonged to the farm of the Veterinary Faculty of Cáceres and the flock of the research farm “La Orden-Valdesequera”. This procedure consists of the following stages:

Preparation of artificial saliva from four solutions (Aghajanzadeh-Golshani et al.,2015): macromineral solution, buffer solution, reducing solution and micromineral solution.

In order toinitiate the fermentation process, 0.2 g of the study mixture together with 30 ml of faecal liquid (homogeneous mixture of feces and artificial saliva) were collected in a syringe and then incubated in a 39ºC oven.

Two sets of 12 syringes were used in each trial. Two of them act as blank (30 ml faecal fluid), two others act as control (0.2 g barley as reference fermenting substrate), and the remaining eight syringes contain the pomace mixtures (4/mix).

In order to the fermentation process was carried out under two different conditions. On the one hand, the potential production of gas and CH4 were measured after 18 h of incubation, time enough to determine the potential degradation of a concentrate (Damiran and Yu, 2010) and on the other hand, the same samples were incubated for 96 h, recording the progress of the plunger at 0, 2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 hours of incubation according to the described procedure (El-Meadaway et al., 1998; Rasouli and Amiri, 2016).The gas production curves were analysed according toMcDonald (Mcdonald, 1981) p = a + b (1 - e-c(t-tl)).

2.4. Measurement of CH4 final production

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The CH4 production was measured at the end of the fermentation test at 18 and 96 hours, using a GMI PS211 gas detector. This detector determines the percentage of CH4 in the gas produced using a closed gas circuit system connected to a 250 ml (18 h)-500 ml (96 h) Duran canister from syringe. A three-way tips system allowed the process to remain hermetic and to direct the gases in the right direction. The final concentrate was expressed as ml of CH4/g of fermented material.

2.5. Statistical analysis

The possible effect of the grape and percentage on the total gas and CH4 production were studied by a non-parametric Kruskall-Wallis test. When differences were statistically significant ($p<0.05$), differences between means were established by Dunn's test using SPSS program (V23). The modelization of the gas production kinetics was adjusted by Solver Lineal Programming in Excel, minimizing the difference of squares between the real and adjusted data as the constants of the equation (a, b, c, tl) vary.

3. Results

Chemical analysis (Table 1) indicate that the barley values are within the range of variability described for this cereal. As for the pomaces, it could be considered as fibrous byproducts, with a moderate protein content and slightly high in fat. The mixtures indicate the corresponding composition according to the percentage and type of pomace incorporated

Table 1. Chemical composition of barley, pomaces and pomace -barley mixtures expressed in g/kg dry matter

	M.O	C.Fat	C.P	C.F	N.F.D	A.F.D
Barley	97.38±8.56	2.17±0.03	9.41±0.36	3.54±0.19	20.85±0.19	5.87±0.59
P 1	92.32±6.18	6.28±0.41	11.33±0.10	25.21±1.02	56.48±0.32	47.66±1.12
1.25%	97.28±7.51	2.32±0.05	9.34±0.37	3.85±0.21	21.85±0.22	6.43±0.61
2.50%	97.03±8.45	2.29±0.05	9.48±0.35	4.01±0.23	21.72±0.17	6.94±0.68
5.00%	97.15±8.62	2.41±0.04	9.53±0.32	4.73±0.24	22.33±0.23	8.05±0.58
7.50%	96.77±7.84	5.1±0.05		5.19±0.25	23.25±0.19	9.11±0.61
	92.30±6.03	8.37±0.58		26.50±0.04	58.17±0.24	48.98±0.03
P 2	11.44±0.0					9.55±0.35
1.25%	97.43±8.35	2.18±0.04	9.28±0.38	3.83±0.19	21.33±0.23	6.16±0.43
2.50%	97.12±8.62	2.23±0.05	9.51±0.37	4.17±0.19	21.88±0.23	7.02±0.53
5.00%	97.31±8.04	2.55±0.06	9.51±0.34	4.83±0.15	22.89±0.18	8.13±0.59
7.50%	97.12±8.17	2.66±0.07	9.65±0.31	5.36±0.17	23.75±0.22	9.15±0.65
P 3	93.02±6.68	5.19±0.87	11.47±0.04	27.59±0.19	57.07±0.71	51.01±0.71
1.25%	97.23±8.45	2.21±0.03	9.48±0.33	3.72±0.16	21.19±0.25	6.41±0.62
2.50%	97.05±8.53	2.26±0.04	9.44±0.37	4.03±0.23	21.33±0.22	7.03±0.56
5.00%	97.18±8.37	2.33±0.09	9.55±0.37	4.83±0.22	22.76±0.28	8.11±0.55
7.50%						
P 4						

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1.25%	97.15±8.62	2.43±0.07	9.65±0.34	5.54±0.19	23.77±0.25	9.36±0.58
	92.47±6.53	7.28±0.74	11.50±0.00	25.14±1.27	63.50±7.78	57.00±7.07
	97.32±8.55	2.32±0.06	9.47±0.36	3.73±0.24	21.42±0.26	6.58±0.65
2.50%	97.10±8.51	2.30±0.05	9.47±0.36	4.01±0.21	22.05±0.45	7.30±0.74

(mean ± standard deviation).

5.00%	97.18±8.26	2.55±0.07	9.55±0.33	4.66±0.26	23.23±0.47	8.45±0.72
7.50%	97.10±8.41	2.61±0.08	9.62±0.31	5.19±0.29	24.25±0.75	9.71±0.94

O.M: organic matter; C.Fat: crude fat; C.P: crude protein; C.F: crude fiber; N.D.F: neutral detergent fiber; A.D.F: acid detergent fiber. P: pomace.

Regarding the effect of the pomace variety, it is observed that after a fermentation period of 18 h (Table 2), P2 and P3 show a greater inhibitory potential for gas production with respect to barley (223.71), which has the highest values, while P1 and P4 ranked an intermediate position. Regarding CH4 production, once again, P3 is the one with the highest inhibitory effect, while P1 and P4 show a production similar to barley and P2 shows a slight inhibitory effect with intermediate values.

Table 2. Gas (ml/g fermented substrate) and CH4 (ml/g fermented substrate) production after 18 h of fermentation.

		Barley	P 1	P 2	P 3	P 4	*p*
G.P 4h	*Mean*	7.23b	11.70c	0.69ab	0.00a	14.31c	***
	s.d	8.08	5.34	2.77	0.00	4.69	
G.P 6h	*Mean*	51.82bc	59.32c	46.26ab	40.10a	58.97c	***
	s.d	13.57	8.00	4.95	5.02	5.98	
G.P 8h	*Mean*	106.28bc	109.33c	97.40ab	96.41a	111.76c	***
	s.d	11.07	7.71	5.45	6.48	5.61	
G.P 10h	*Mean*	147.29b	145.71b	136.08a	139.86ab	145.05b	***
	s.d	10.83	8.59	5.37	6.55	6.99	
G.P 12h	*Mean*	171.85b	171.22b	160.35a	169.89b	163.51ab	***
	s.d	10.70	8.01	5.96	8.20	8.24	
G.P 24h	*Mean*	269.96b	266.00b	249.62a	259.36ab	252.22a	***
	s.d	12.24	12.84	7.86	18.71	14.31	
G.P 48h	*Mean*	312.01b	300.69b	285.26a	301.20b	297.65ab	***
	s.d	13,33	15,38	8,85	10,73	12,49	
G.P 72h	*Mean*	327,71c	321,62bc	297,20a	322,67bc	299,41ab	***
	s.d	14,63	15,03	9,99	11,23	41,98	
G.P 96h	*Mean*	337,76c	329,72bc	299,07a	324,10bc	316,51b	***
	s.d	21,20	18,44	8,74	9,31	11,22	
$CH_4$96h	*Mean*	57,00b	56,07b	44,66a	57,21b	48,96a	***
	s.d	7,67	3,93	2,52	2,75	3,27	

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Note: different letters in the same row indicate significant differences between means (p<0.05). P: pomace variety; %: Percentage of pomace incorporation in the pomace-barley mixture; G.P: gas production; s.d: standard deviation; p: p-value (Significance levels are: *** p ≤ 0.001, ** p ≤ 0.01, * p ≤ 0.05 and ns p > 0.05).

Table 3. Kinetics of gas production (ml/g fermented substrate) and CH4 (ml/g fermented substrate) production after 96 h of fermentation of the varieties of pomace studied.

		Barley	P1	P2	P3	P4	p
G.P	Mean	223.71b	213.45ab	204.08a	201.59a	212.42ab	***
	s.d	11.04	8.88	18.59	17.77	11.80	
CH_4	Mean	40.28b	38.71b	37.99ab	30.14a	44.32b	***
	s.d	7.85	2.80	6.62	6.78	12.78	
		Barley	**1.25%**	**2.5%**	**5%**	**7.5%**	*p*
G.P	Mean	223.71b	210.74ab	206.29a	209.12ab	205.39a	**
	s.d	11.04	15.02	21.73	5.18	15.74	
CH_4	Mean	40.28ab	42.49b	39.79ab	36.37ab	32.51a	*
	s.d	7.85	12.48	40.70	3.37	5.32	
		Barley	**P1**	**P2**	**P3**	**P4**	*p*
G.P	Mean	223.71b	213.45ab	204.08a	201.59a	212.42ab	***
	s.d	11.04	8.88	18.59	17.77	11.80	
CH_4	Mean	40.28b	38.71b	37.99ab	30.14a	44.32b	***
	s.d	7.85	2.80	6.62	6.78	12.78	
		Barley	**1.25%**	**2.5%**	**5%**	**7.5%**	*p*
G.P	Mean	223.71b	210.74ab	206.29a	209.12ab	205.39a	**
	s.d	11.04	15.02	21.73	5.18	15.74	
CH_4	Mean	40.28ab	42.49b	39.79ab	36.37ab	32.51a	*
	s.d	7.85	12.48	40.70	3.37	5.32	
		Barley	**P1**	**P2**	**P3**	**P4**	*p*
G.P	Mean	223.71b	213.45ab	204.08a	201.59a	212.42ab	***
	s.d	11.04	8.88	18.59	17.77	11.80	
CH_4	Mean	40.28b	38.71b	37.99ab	30.14a	44.32b	***
	s.d	7.85	2.80	6.62	6.78	12.78	
		Barley	**1.25%**	**2.5%**	**5%**	**7.5%**	*p*
G.P	Mean	223.71b	210.74ab	206.29a	209.12ab	205.39a	**
	s.d	11.04	15.02	21.73	5.18	15.74	
CH_4	Mean	40.28ab	42.49b	39.79ab	36.37ab	32.51a	*
	s.d	7.85	12.48	40.70	3.37	5.32	

Note:different letters in the row indicate significant differences between means (p<0.05). P: pomace variety; G.P: gas production; s.d: standard deviation; p: p-value (Significance levels are: *** p ≤ 0.001, ** p ≤ 0.01, * p ≤ 0.05 and ns p > 0.05)

Table 4. Kinetics of gas production (ml/g fermented substrate) and CH4 (ml/g fermented substrate) production after 96 h of fermentation of the different levels of pomace studied.

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Note:different letters in the row indicate significant differences between means ($p<0.05$). P: pomace variety; G.P: gas production; s.d: standard deviation; *p*: p-value *(Significance* levels are: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ and ns $p > 0.05$).

Regarding the kinetics of gas production (Table 3), differences ($p<0.05$) were observed among the different pomaces, showing that up to 8 h, P3 presented the highest inhibitory potential, repeating the pattern observed in the 18 h fermentation (Table 2), being P1 and P4 the highest gas producers. After 10 h, a change in the fermentation curve of the different substrates was observed, being barley the highest gas producer, maintaining this pattern until the end of the fermentation process, while P2 registered lower values. It should be noted that in the period from 10 to 48 h, P1 shows the same gas production as barley, as does P3 from 12 to 48 h. With respect to CH_4 production significant differences were found ($p<0.001$), P2 and P4 (44.66 and 48.96 respectively) showed the greatest inhibitory potential, while P1 and P3 (56.07 and 57.21 respectively) do not inhibit CH_4 production, showing a production similar to barley (57.00).

After 18 h of fermentation (Table 2) gas production is significantly reduced ($p<0.01$) in the mixture with 2.5% and 7.5% (206.29 and 205.39 respectively) compared to barley (223.71). In the study of the kinetics of gas production (Table 4), it was observed that after 10 h of fermentation, the 7.5% level had an inhibitory effect on gas production compared to barley. This pattern is constant throughout time, until the end of the fermentation process, with an estimated inhibition between 5.7% and 10%.

In both fermentation times, the lowest CH_4 production is observed in the 7.5% mixture while the higest values corresponded to 1.25% (Table 2) and barley (Table 4).

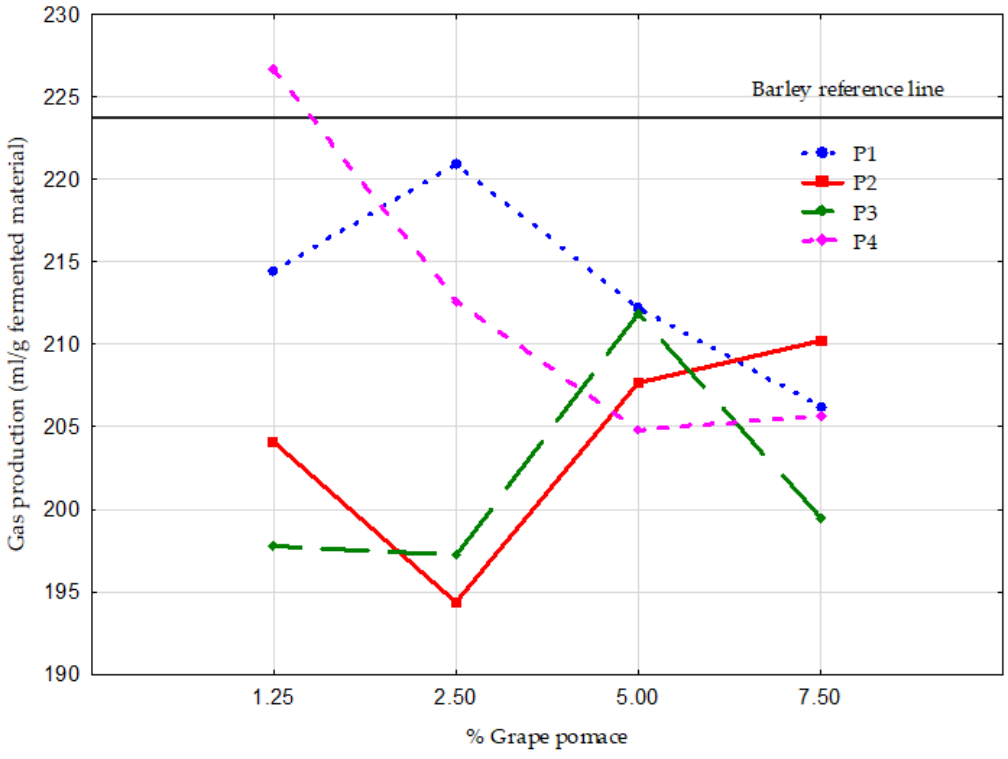

Figure 1. Gas production after 18 h of fermentation.

Figure

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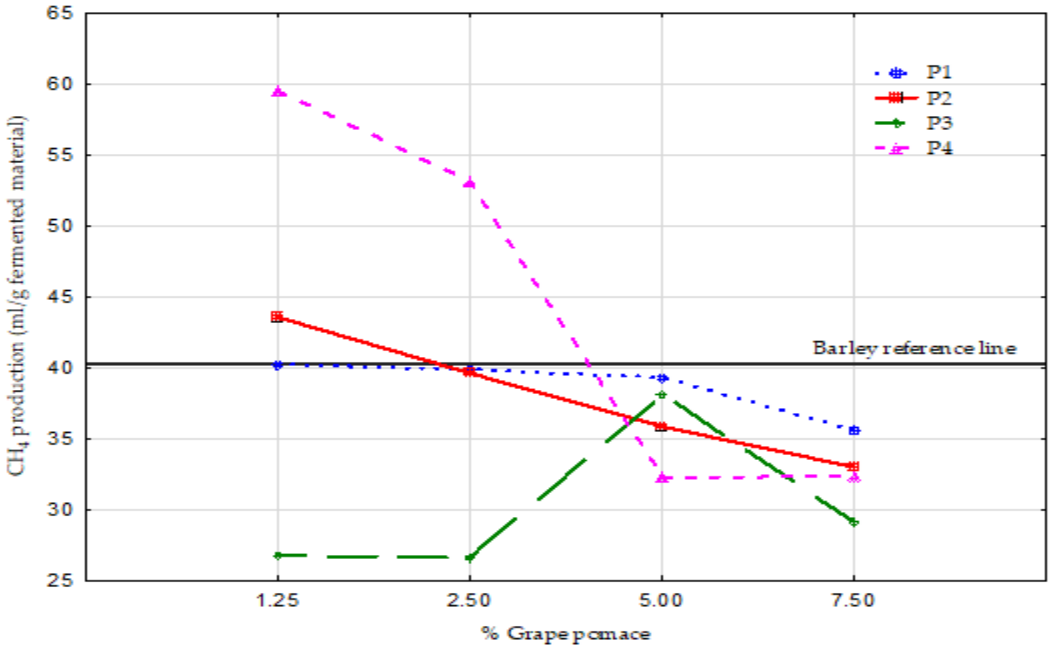

2. CH_4 production after 18 h of fermentation.

The gas production values (Figure 1), value of P1, P2, and P3 are lower than those of the control barley (reference line) when the incorporation levels are low, P4 does not show this pattern. In the case of CH_4 production (Figure 2) this effect is not observed up to 5% of pomace in the mixture. Lowest values for gas and CH_4 production are reregistered by P3.

If each pomace is analyzed separately it can be notice that:

P1 shows that the maximum gas production (Figure 1) is at 2.5% and then decreases al the concentration increases. Respect to CH_4 production (Figure 2), a dose-response effect is observed by decreasing CH_4 production as the concentration of P1 increases in the mixture.

P2, show the lowest gas production values (Figure 1) at 2.5%. CH_4 production (Figure 2) is similar to P1. Regarding P3, both gas production (Figure 1) and CH_4 production (Figure 2) show the same pattern. A potent inhibitory effect can be observed when P3 is at low concentrations (1.25%, 2.5%).

The highest values are found at concentrations of 5%. However concentration of 7.5% show an inhibitory effect similar to that observes at low percentages.

P4 has the same pattern for both, gas (Figure 1) and CH_4 production (Figure 2). A progressive decrease in the production is observed up to 5%. Higer levels do not increase the inhibitory potential.

Table 5.- Gas production modelling after 96 h of fermentation. Parameters of the equation Mcdonald, 1981

Y=a+b (1-e-c(t-tl)).

a: soluble or degradable fraction, time 0; b: insoluble but potentially degradable fraction; c: speed or rate of degradation; tl: colonization time.

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The parameter "a" is associated with the easily degradable fraction of the feed. This parameter reaches higher values for barley and lower for the 7.5% level. These results can be reflected in practice, since the highest gas production is registered by barley, while the lowest corresponds to the 7.5% inclusion level. The parameter "b" corresponds to the potentially degradable fraction of the feed, in thiscase it can be observed that the highest value is registered for barley while thelowest value coresponds to the 7.5% inclusion level.

The addition of the parameters “a+b” would express the total degradability of the material to be fermented, it can be observed a progressive decrease as the percentage of pomace in the mixture increase, in correspondence with the obtained experimental results.

The parameter "c" is an intrinsic degradability coefficient of each product. In this case, the lowest degradability would correspond to barley and the 2.5% inclusion level, while the rest present equal values.

The parameter “tl” is considered as the fermentation initiation time and allows adjusting the equation to the real model, although it does not necessarily reflect reality, since at 4 h all the pomaces have started the fermentation process.

4. Discussion

The statistical analysis of the results (Kruscal-Wallis), determined by the type of data, allows us to detect the possible effect of the main factors, but not whether there are interactions. In any case, a qualitative effect associated with the type of pomace used and a quantitative effect associated with the concentration of pomace are demonstrated.

Sheep feces were used as inoculum source for fermentation, considering the high correlation between the utilization of rumen liquid and fresh feces to compare gas and CH_4 production between different feeds and/or treatments (Dhanoa *et al*., 2004; Mauricio *et al*., 2001.; Ramin *et al*., 2015). In addition, obtaining feces is less invasive for the animal and avoids problems of scientific-ethical committees that could limit the development of research (Spanghero *et al*., 2019).

The rumen fermentation inhibitory activity of grape pomace has been previously established (Abarghuei, 2015) and is based on the interaction of tannins with rumen microbes through different processes(Goel *et al*., 2005; Mcsweeney *et al*., 2001.; Smith *et al*., 2005), such as metal chelation, which affects the functioning of

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	a	b	(a+b)	c	tl	R^2
Barley	131.79	200.54	323.33	0.075	9.60	0.99
1.25%	123.86	192.48	324.40	0.078	9.21	0.99
2.5%	125.92	194.57	320.49	0.076	9.42	0.99
5%	123.06	191.69	314.75	0.078	9.26	0.99
7.5%	116.71	185.31	302.02	0.078	9.16	0.99

metalloenzymes, or affecting the integrity of the cell wall and mem-brane. Furthermore, these compounds have the ability to form complexes with the carbohydrates and proteins of the substrate, decreasing the availability of fermentable substrate to rumen microorganisms(Jayanegara *et al.*, 2011; Patra and Saxena, 2010). This hypothesis is interpreted under *in vitro* fermentation conditions as a decrease in total gas(Abarghuei, 2015)and CH_4 production(Peter J. Moate *et al.*, 2020) and our result support it.

Regarding the type of pomace used (Table 2 and 3), it may be paradoxical that the results show different activity, taking in account that the pomaces are from the same grape variety (Tempranillo) and geographical area, but the production of tannins by the vine is a reaction to drought and cultivation conditions(Bucchetti *et al.*, 2011; Hochberg *et al.*, 2015), this could explain the observed values because the grapes come from cultivars with different levels of hydric stress, resulting in different tannin production (antimethanogenic activity).

Differences in grape processing could explain the variability in pomace activity due to the fact that content of different by-product compounds (pulp, granilla, stalk), are not always in the same proportions(Hixson *et al.*, 2016).

In line with our results, Atalay, 2020, also found differences for both gas and CH_4 production after an incubation period of 24 h. In an *in vitro* fermentation study of dif-ferent grape varieties pomaces, Sofyan *et al.*, 2017, also confirmed that the presence of tannins determines a decrease in gas production, but unlike our results, they find no differences in terms of CH_4 production. Other authors such as Moate *et al.*, 2014, demonstrated the reduction effect of grape by-products on CH_4 production, by substi-tuting 36% of alfalfa hay with grape byproducts, obtaining a decrease between 20-24% of CH_4 emissions in an in vivo study with cows.

Regarding the quantitative effect observed on gas production (Figure 1) (Table 2 and 4), a dose-response trend is observed in the range of concentrations analysed. Sallamab *et al.*, 2010, showed a clear negative correlation (R^2= 0.92) between the total polyphenols of five plants (Atriplex, Alfalfa, Leucane, Ecucalyptus, Acacia) and the total gas production. However, this dose-response relationship also depends on the substrate, which is evident in the case of a fermentable material, but not in the case of a material of low natural degradability(Hervás *et al.*, 2003). In this study, is assumed that, since barley is the fermentative base, there must not have been any limitation to the fermentative process.

Regarding CH_4 production, on the one hand, we can observe a dose-effect pattern (Table 4) (Figure 2), according to our results, Gomaa *et al.*, 2017, found differences in CH_4 production when incorporating tanniferous plants in different percentages, obtaining the best results with the highest rates.Hatew *et al.*, 2016, in an *in vitro* study, also obtained a linear reduction between CH_4 production and the level of tannin incorporated. Moate *etal.*, 2020, clearly indicate the inhibitory action of tannins on

methanogenesis determined by their chemical composition, high fat and lignin. Atalay, 2020 in an *in vitro* fermentation study, supports that the antimethanogenic effect found is due to the combination of tannins with the fat of the seeds. On the other hand, studies carried out by Gemeda and Hassen, 2015 indicate that the presence of tannins has a significant effect on the production of volatile fatty acids *in vitro*.

5. Conclusions

According to the results, it can be concluded that the incorporation of grape pomace (Tempranillo variety) to a concentrate type exerts an inhibitory effect on the *in vitro* production of gas and CH_4, obtaining a greater inhibitory potential with an inclusion of 7.5%.

6. References

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