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SHORT COMMUNICATION

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Effect of pre-activated *Saccharomyces cerevisiae* or malate salts on fermentation of ground barley grain under in vitro conditions simulating intensive ruminant feeding

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Abstract

Aim of study: To determine the dose response of *Saccharomyces cerevisiae* on rumen fermentation of concentrates, and to compare it with the effect of malate salts.

Material and methods: S. cerevisiae (0.7, 1.4 and 2.1 mg/g) and malic acid salts (4, 8 or 12 mg/g) were added to barley and compared with barley alone (CTL), in three 24 h *in vitro* incubation series, using rumen inocula from beef cattle receiving a high concentrate diet. Yeasts were pre-activated by aerobic incubation for 24 h at 30ºC. Incubation pH was recorded at 8 and 24 h and volatile fatty acids (VFA) and lactate at 8 h were analysed.

Main results: Gas produced with *S. cerevisiae* was higher than malate ($p<0.001$). Yeast addition linearly ($p<0.01$) and quadratically ($p<0.05$ at 4 h and from 10 to 18 h) increased gas production, but no dose response to malate levels was observed. Dry matter disappearance at 24 h was not affected by *S. cerevisiae* but increased linearly with malate. Microbial mass linearly increased with the level of yeast ($p<0.01$) and malate ($p=0.09$). Adding yeasts did not affect 8 h total VFA concentration compared with CTL, but linear valerate ($p<0.01$) and butyrate ($p=0.092$) increases, and a decrease of acetate ($p=0.064$) were detected. Malate salts linearly increased ($p<0.05$) total VFA concentration but did not affect VFA proportions.

Research highlights: Addition of active yeasts linearly increased barley fermentation and microbial synthesis, whereas the effect of malate salts was of minor magnitude.

Additional key words: activated yeasts; malic acid; gas production; beef cattle; high concentrate diets

Abbreviations used: DM (dry matter); DMd (dry matter disappearance); OM (organic matter); VFA (volatile fatty acids).

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Introduction

Ruminant production under intensive feeding systems is a common practice in countries where both availability and price make using high proportions of forage sources difficult. Despite it increases animal performance, it promotes a risk of acute (below 5.0) or subacute (5.0 to 5.6) rumen acidosis (Owens *et al.*, 1998; Krause & Oetzel, 2006), especially during the adaptation to diet of animals reared under extensive conditions. Using of certain additives that either reduce the rate and extent of rumen fermentation or modify the fermentation pattern by reducing

lactate production has been proposed for minimising this problem (González *et al.*, 2012).

Live yeasts have been used as probiotics for promoting microbial establishment, stabilise rumen pH and reduce lactic acid, and increase fibre degradation (Chaucheyras-Durand *et al.*, 2008). A meta-analysis on the effect of live yeasts (*Saccharomyces cerevisiae*) on rumen environmental conditions showed increasing pH and volatile fatty acids (VFA) concentration, and lower lactic acid concentration (Desnoyers *et al.*, 2009). In addition to a higher rumen pH and VFA concentration, Pinloche *et al.* (2015) reported a selective effect of yeasts on certain fibrolytic and lactate utilising bacteria. Although the magnitude of observed effects has been higher with mixed or concentrate diets (Erasmus *et al.*, 1992; Desnoyers *et al.*, 2009), response has been studied mostly on fibre digestion (Chaucheyras-Durand *et al.*, 2008), and there is not much information about their role on starch digestion. However, results are variable, and some experiments have recorded minor or no responses. A wide range of differences between nominal and actual concentration of active yeasts has been observed (Sullivan & Bradford, 2011), due to the fact that, despite they are often called "active live yeasts" (Chaucheyras-Durand *et al.*, 2008; Thrune *et al.*, 2009; Malekkahi *et al.*, 2016), these probiotics are added directly onto the feed in dry form. It has to be considered that the optimum growth conditions for yeasts (30 to 35ºC, pH 4.4 to 5.6, presence of oxygen in the medium; Walsh & Martin, 1977) are far from those occurring in the rumen, and thus direct addition of dry yeasts does not ensure an active metabolic stage of these organisms when entering in the fermentation site. A previous activation phase by culturing yeasts under their optimal growth conditions should therefore improve their potential activity into the rumen (Amanzougarene *et al.*, 2020).

Callaway & Martin (1997) partly associated the positive effect of yeast in the rumen to the release of malic acid that might stimulate lactate use, but Newbold *et al.* (1996) discarded this possibility. Organic acids such as malic acid, either in its acid form or as malate salts, can maintain a higher pH and increase production of propionate and reducing lactic acid (Callaway & Martin, 1996; Carro *et al.*, 1999; Montaño *et al.*, 1999). It is assumed that malate salts selectively promote growth of *Selenomonas ruminantium*, which uses lactic acid to produce succinate and propionate (Nisbet & Martin, 1993). It has been observed that the magnitude of the response to malate salts depends on diet characteristics (Carro & Ranilla, 2003; Gómez *et al.*, 2005).

This study compares the response to different doses of active living *S. cerevisiae* yeast and malate salts on *in vitro* fermentation of barley meal, in terms of gas production and VFA pattern, and their potential interest for preventing acidosis by maintaining incubation pH.

Material and methods

Substrate and additives

Barley grain was chosen as a reference incubation substrate for ingredients commonly included in high concentrate diets. A mixture of calcium and disodium malate salts (Rumalate, provided by NOREL Animal Nutrition, Madrid, Spain; 0.763 malate proportion) was included in doses of 4 (LM), 8 (MM) or 12 (HM) mg/g substrate. *S. cerevisiae* live yeast (MUCL39885, E1710; 9×10^9 cfu/g) was provided by AMBIOTEC Balance (Toledo, Spain). Yeast was pre-activated by aerobic culture (at 30ºC for 24 h) in a nutritional mixture, as described in Amanzougarene *et al.* (2020). Then, 0.35 (LY), 0.7 (MY) and 1.05 (HY) mL were added to each incubated bottle (resulting 0.7, 1.4 and 2.1 mL/g substrate) as experimental treatments.

Incubation procedures

A 500 mg amount of barley grain substrate, ground in a hammer mill to 1 mm maximum particle size, was introduced in nylon bags (4 × 4 cm; 45 μm pore size) and incubated into glass bottles (116 mL total volume) with 80 mL incubation solution, at 39ºC and under a CO_2 stream. Incubation conditions were arranged according to Theodorou *et al.* (1994), except that microminerals and resazurin were not included (Mould *et al.*, 2005) and concentration of bicarbonate ion in the buffer solution was adjusted to 0.029 M to get an incubation pH around 6.20 (Amanzougarene & Fondevila, 2018). Three incubation series (24 h) were performed, with triplicated bottles for barley alone (CTL) or supplemented with three levels of activated yeast (LY, MY and HY) or malate salt (LM, MM and HM). Three additional bottles with rumen fluid but without substrate or additives were also included as blanks. As inoculum, the incubation solution included a 0.10 proportion of rumen liquid filtered through gauze. Three beef calves (8 months of age, around 300 kg weight), provided with a 2 cm i.d. cannula fitted in the dorsal sac of the rumen were used as donors of inoculum, using a different donor animal on each incubation series. Calves were housed in the facilities of the ´Servicio de Apoyo a la Experimentación Animal´ of the ´Universidad de Zaragoza´, and were daily fed *ad libitum* with a concentrate mainly composed by 0.59 kg/kg barley, 0.15 kg/kg maize, 0.17 kg/kg soybean meal and 0.06 kg/kg maize gluten feed, together with chopped barley straw at a 0.09 proportion of total ration. Management and extraction procedures of rumen inoculum from donor animals were approved by the Ethics Committee for Animal Experimentation. Care and management of animals agreed with the Spanish Policy for Animal Protection RD 53/2013 (BOE, 2013), which complies with EU Directive 2010/63 (OJEU, 2010) on the protection of animals used for experimental and other scientific purposes.

After 8 h of incubation, one of the bottles from each treatment was opened, its pH recorded (CRISON micro-pH 2001, Barcelona, Spain) and samples of the incubation medium were taken for VFA analysis (2 mL, collected over 0.5 mL of a deproteinizing mixture of 0.5M PO4H3 with 2 mg/mL 4-methyl valeric acid) and lactate (2 mL) and immediately frozen and stored at -20ºC until analysis. Along the experimental period, pressure into the remaining two bottles was recorded at 2, 4, 6, 8, 10, 12, 18 and 24 h, by means of an HD 2124.02 manometer fitted with a TP804 pressure gauge (Delta Ohm, Caselle di Selvazzano, Italy). Readings were converted into volume by a pre-established linear regression equation between the pressure recorded in the same bottles under the same conditions and known air volumes (n= 103; R^2= 0.996), and expressed per unit of incubated organic matter (OM). The average of the remaining two bottles for each treatment on each incubation series was considered as the experimental unit. At the end of the incubation (24 h) pH was measured and the incubation medium sampled for estimation of microbial mass (10 mL sample). Besides, bags of substrate removed, squeezed and dried (60ºC, 48 h) to estimate dry matter disappearance (DMd).

Chemical and statistical analysis

Dry matter (DM) in barley substrate and incubation residues and OM content in the substrate were analysed following the AOAC (2005) procedures (methods ref. 934.01 and 942.05). The VFA were determined by gas chromatography on an Agilent 6890, apparatus equipped with a capillary column (HP-FFAP Polyethylene glycol TPA, 30 m × 530 µm id). The lactate concentration was determined by the colorimetric method proposed by Barker & Summerson (1941). Microbial mass in the liquid medium was approached by centrifuging at 10000 × g, 20 min, based on Hsu & Fahey (1990).

Results were analysed by ANOVA using the Statistix 10 software package (Analytical Software, 2010), considering the incubation series as block. The Dunnet t test was used to compare treatment means with the control. Besides, polynomial (lineal and quadratic) contrasts were planned to estimate the trend in the response of each additive, and orthogonal contrasts were established to compare the two additives between them. Differences were considered significant when $p<0.05$, and a trend for significance was considered when $0.05 \leq p<0.10$.

Results and discussion

Gas production from barley, alone (CTL) or supplemented with the three levels of each *S. cerevisiae* and malate salts are shown in Figure 1. At all times of control, inclusion of yeasts increased gas production from barley respect to CTL ($p<0.05$), whereas no differences were recorded between CTL and any level of malate. Among levels of yeasts, the pattern of response was lineal ($p<0.01$) from 2 to 24 h incubation, but also followed a quadratic trend ($p<0.05$ at 4 h and from 10 to 18 h, and $p<0.10$ at 2, 6, 8 and 24 h) because no further response was observed from HY compared with MY. In contrast, no significant pattern was observed in the response to the inclusion of increasing levels of malate, except for a linear trend ($p<0.01$) at 2 h incubation. When the two additives were contrasted, the volume of gas produced from barley when supplemented with the different levels of *S. cerevisiae* was higher ($p<0.001$) than that from malate.

A linear negative effect of adding pre-activated yeasts was detected on incubation pH at 8 and 24 h ($p<0.01$) compared with CTL, although maximum differences were always below 0.07 and 0.16 pH units, respectively, whereas no effect was detected with malate (Table 1). Comparing between additives, the pH at 24 h was lower with *S. cerevisiae* than with malate ($p<0.001$). Inclusion of *S. cerevisiae* did not affect DMd after 24 h incubation, but malate salts promoted a linear increase in DMd ($p=0.03$). Microbial mass in the liquid fraction linearly increased with the level of yeast ($p<0.01$), whereas a trend in the same sense was observed with malate ($p=0.09$).

When both additives were compared in terms of gas production (Fig. 1), throughout incubation (from 2 to 24 h) volumes from barley were higher when it was supplemented with *S. cerevisiae* than with malate salts ($p<0.001$), differences at 24 h reaching on average 23 mL gas/g OM. No differences were detected in pH at 8 h (Table 1), but at 24 h this parameter resulted higher with malate salts (5.79 *vs.* 5.89, $p<0.001$). There were no differences between additives in DMd or in microbial mass ($p>0.10$).

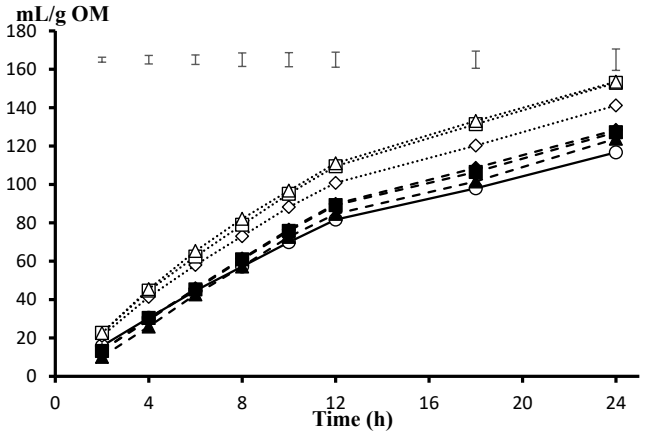

Figure 1. Gas production pattern from barley, as the only substrate (◯) or supplemented with live *Saccharomyces* yeast (mL/g: 0.7,◇; 1.4, □; or 2.1, △; dotted lines) or malate salts (mg/g: 4,◆; 8, ■; or 12, ▲; scattered lines). Upper bars show standard error of means.

Table 1. Average values and pattern of response (*p*-value of linear, L, or quadratic, Q, trend) of incubation pH at 8 and 24 h of incubation, and dry matter disappearance (DMd) and microbial mass (mg/mL) in the liquid fraction after 24 h, when barley was incubated as the only substrate (CTL) or supplemented with low, medium or high levels of *S. cerevisiae* (LY, MY and HY) or malate salts (LM, MM and HM).

	CTL	*S. cerevisiae*			Malate salt			SEM[1]	Pattern[2]	
		LY	MY	HY	LM	MM	HM		Yeast	Malate
pH 8 h	6.18	6.15	6.12	6.11	6.13	6.15	6.14	0.016	L (**)	
pH 24 h	5.91	5.83	5.80	5.75	5.90	5.90	5.88	0.028	L (**)	
DMd	0.424	0.477	0.469	0.467	0.454	0.444	0.501	0.0207		L (*)
Microbial mass	1.99	2.10	2.23	2.33	2.10	2.22	2.16	0.076	L (**)	L (T)

[1] SEM: standard error of the means. [2] Pattern: linear (L) or quadratic (Q); **: $p<0.01$; *: $p<0.05$; T: $p<0.10$

Lactate concentration from barley (Table 2) was not affected by the inclusion of additives at any dose ($p>0.10$), partly because of the large magnitude of the error term (coefficient of variation 0.93). Similarly, no differences among doses of either *S. cerevisiae* or malate, nor between additives, were recorded on the concentration of this metabolite ($p>0.10$). Inclusion of *S. cerevisiae* did not affect total VFA concentration after 8 h incubation compared with non-supplemented barley ($p>0.10$). However, a linear increase ($p=0.022$) in total VFA concentration with increasing levels of malate salts was observed. With *S. cerevisiae*, a linear increase in molar proportion of valerate ($p=0.003$) was observed, and HY differed from the control ($p<0.05$). Besides, a linear trend of both an increase of butyrate ($p=0.088$) and a decrease of acetate ($p=0.064$) were detected. In contrast, no response to malate inclusion was recorded on molar proportions of the different VFAs. On average, higher concentration of VFA were recorded with malate treatments compared with yeasts ($p<0.05$), showing lower ($p<0.01$) molar proportions of butyrate and valerate and a trend ($p=0.058$) for a higher acetate proportion.

In order to simulate environmental conditions induced by intensive feeding, the concentration of bicarbonate in the incubation solution was reduced to adjust pH to 6.2 (Kohn & Dunlap, 1998), instead of the conventional buffering to pH 6.7-6.9 in *in vitro* closed batch systems (Mould *et al.*, 2005). Under such conditions, however, exhaustion of buffer capacity because of bicarbonate consumption after 10-12 h allows for an environment in which the acidification properties might drop pH below adequate levels for microbial fermentation (Amanzougarene & Fondevila, 2018). Therefore, the low-buffered environment allows for studying acidification properties of treatments on results from 10 to 24 h.

Use of yeasts as probiotics for enhancing rumen activity has been applied in the last three decades (Newbold, 1996). A nutritive role for rumen microbiota has been assumed even for dead added yeasts (Chaucheyras-Durand *et al.*, 2008). However, provided that a probiotic exerts its positive effects in the rumen through its activity on environment and microbiota, it is logical to assume that the more active, the higher magnitude of response, as it has been admitted as a general rule for probiotics (Fuller, 1989). Thus, if a dry live product needs activation before starting its effect, this should be achieved at a higher extent under optimal growth conditions. Yeasts are aerobic-microaerophillic organisms that grow at 30 to

Table 2. Lactate (mM) and total volatile fatty acid (VFA) concentration (mM), together with molar proportions of the main VFA of barley incubated for 8 h as the only substrate (CTL) or supplemented with low, medium or high levels of *S. cerevisiae* (LY, MY and HY) or malate salts (LM, MM and HM).

	CTL	*S. cerevisiae*			Malate salt			SEM[1]	Pattern[2]	
		LY	MY	HY	LM	MM	HM		Yeast	Malate
Lactate	0.175	0.324	0.357	0.366	0.591	0.375	0.136	0.178		
Total VFA	31.2	33.0	33.7	32.2	35.2	34.9	35.7	1.12		L(*)
Acetate	0.512	0.499	0.502	0.488	0.510	0.510	0.507	0.0075	L(T)	
Propionate	0.278	0.281	0.281	0.289	0.279	0.279	0.282	0.0048		
Butyrate	0.166	0.172	0.170	0.173	0.164	0.164	0.165	0.0026	L(T)	
Valerate	0.023	0.026	0.027	0.028d	0.024	0.025	0.024	0.0009	L(**)	
BCFA	0.0210	0.022	0.021	0.021	0.022	0.022	0.022	0.0005		

[1] SEM: standard error of the means. [2] Pattern: linear (L) or quadratic (Q); **: $p<0.01$; *: $p<0.05$; T: $p<0$. Within the same row, letter *d* indicates treatment differences with the control, recorded by the Dunnet t test ($p<0.05$)

35ºC and pH range of 4.4 to 5.6 (Walsh & Martin, 1977), whereas rumen conditions are strictly anaerobic and temperature and pH are around 39ºC and 6.2 to 6.8 (Hungate, 1969). Under this reasoning, it is logical to think that a pre-activation of yeasts under their optimal growth conditions should potentiate their response in the rumen. Results from our laboratory with the same yeast strain (Amanzougarene *et al.*, 2020) indicate that concentration of *S. cerevisiae* incubated under rumen environmental conditions (CO_2 atmosphere, 39ºC, pH 6.2) is maintained at a higher level if previously activated for 24 h at 35ºC in aerobiosis, and concentration at 24 h is well over the concentration of 10^5 CFU/mL considered as the minimum effective concentration (Durand-Chaucheyras *et al.*, 1998; Jouany & Morgavi, 2007).

In this work, *S. cerevisiae* increased gas production from barley in a quadratic trend, indicating that the response is not dose-dependent from 1.4 mL/g. This behaviour was partly supported by the concentration of total VFA since, although it increased in the same trend, differences did not reached significance. A low magnitude effect of yeasts on total VFA concentration has also been reported in the meta-analysis by Desnoyers *et al.* (2009). The positive effect of yeasts on rumen bacterial fermentation has been attributed to the capacity to scavenge oxygen from the medium, thus optimising activity conditions of strict anaerobe bacteria (Newbold, 1996). The increased fermentation when *S. cerevisiae* was added contributed to a lower pH after 24 h incubation, in opposition with the mentioned buffering effect of yeasts on rumen pH *in vivo* (Chaucheyras-Durand *et al.*, 2008). This may be because of an enhanced absorption capacity that is not observed *in vitro*; however, the magnitude of such reduction was below 0.16 pH units, in agreement with results observed by Desnoyers *et al.* (2009). In any case, such response is not related with lactate, which concentration remained unaffected. Besides, incubation pH at 6.0 did not markedly affect potential of starch fermentation by rumen microbial community.

In other way, a qualitative effect on fermentation was observed, linearly increasing butyrate (p=0.092) and valerate (p<0.01) at the expense of acetate (p=0.064). Modifications of VFA molar profiles with yeasts addition in the same way have been previously addressed (Carro *et al.*, 1992; Newbold, 1996; Thrune *et al.*, 2009). Besides, the estimation of microbial mass in the liquid fraction linearly increased with yeasts (p<0.01). Despite the lack of specificity of the method of estimation, based on weight of soluble mass in the liquid fraction, this parameter should indicate an increase in microbial mass synthesis, as it has also been indicated by other authors (Newbold, 1996), thus suggesting a potentially increased microbial nitrogen flow to the duodenum. Chaucheyras-Durand *et al.* (2008) suggested that fermentable energy may be partly shifted from fermentation to microbial synthesis, which should justify the minor response in VFA concentration.

Compared to that observed with *S. cerevisiae*, malate salts had a minor effect on *in vitro* gas production from barley, reaching a 0.06 proportional increase to that observed with the control. Carro & Ranilla (2003) reported a non-significant increase of 0.03 gas volume from barley supplemented with increasing levels of malate and, in fact, Tejido *et al.* (2005) attributed the slight increase observed in gas production in their case to the malate fermentation itself. However, other fermentation parameters behaved differently in our work, and total VFA concentration linearly increased (p<0.05) with levels of malate, and a trend for a quadratic increase (p=0.055) in DMd was also observed, both indicating that malate salts might enhance barley utilisation. In contrast to that observed by Carro & Ranilla (2003) and Tejido *et al.* (2005), addition of malate salts did not alter incubation pH, not lactic acid concentration or molar VFA pattern. Theoretically, addition of malate salts should enhance utilisation of lactate by *Selenomonas ruminantium* to produce propionate (Nisbet & Martin, 1993), but the lack of any effect in this sense suggests that malate supplementation does not affect starch utilisation from barley grain.

As conclusions, results indicate that the addition of pre-activated yeasts up to 1.4 mL yeast inoculum per gram of substrate increases fermentation of barley grain as a model of cereal rumen utilisation, at the time it may increase microbial synthesis. This effect was also qualitative, since butyrate and valerate proportions increased at the expense of acetate. In any case, the lack of a major effect on medium acidification suggests this additive may be useful for enhancing rumen starch utilisation without increasing the risk of acidosis. The effect of malate salts was of minor magnitude, in both quantitative and qualitative aspects, thus discarding the association of the effect of yeasts with malic acid releasing, and reduces the potential interest of malate as additive for ruminants under intensive feeding conditions.

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