Effects of inactivated and live cells of *Saccharomyces cerevisiae* on *in vitro* ruminal fermentation of diets with different forage: concentrate ratio

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SUMMARY

The effects of yeast *Saccharomyces cerevisiae*, either inactivated (by osmotic pressure, designated IY) or provided as a culture containing live yeast cells (YC), on ruminal fermentation of two different diets were investigated *in vitro*. Total mixed rations (TMR) having forage:concentrate ratios of 0·6:0·4 (medium–high forage diet) and 0·2:0·8 (low-forage diet) were incubated in batch cultures of mixed ruminal micro-organisms to which either IY (to reach concentrations of 500 and 250 mg product/l incubation medium) or YC (at a concentration of 150 mg product/l) were added directly as powder. To evaluate the effects of the additive on ruminal microbial population, sheep used as donors of rumen fluid were allocated to three experimental groups: Control (no additive), IY and YC, that received a diet with the corresponding additive for 10 days. With both diets, YC decreased ruminal pH compared to control, whereas IY had no effect. Adding yeast products to the high-fibre diet affected total volatile fatty acid (VFA) production and VFA composition, in general with a slight increase in IY and a significantly greater increase in response to the addition of YC. Ammonia nitrogen ($P = 0.006$), total gas production ($P < 0.001$) and *in vitro* dry matter disappearance (IVD) ($P < 0.001$) showed the highest values with YC. Methane production was higher than the control when the IY inoculum was used, and increased even more with the YC inoculum ($P < 0.001$). With the high-concentrate TMR, no effects on total VFA concentration were observed when yeast additives were used. Similar trends were shown for lactate and methane production and total gas production, where values tended to be higher when using the YC inoculum (P values of 0·055, <0·001, 0·006 and <0·001, respectively). After 144 h of incubation, differences were observed only with the high-fibre diet in the cumulative gas production at 24 h of incubation and in the average fermentation rate, which was greater with YC, although the asymptotic gas production was not affected. These results indicate that live yeasts affect ruminal fermentation slightly more than inactivated yeasts, although both products require a regular administration and some adaptation of the ruminal microbial population for the stimulatory effects to become apparent. The effects of yeasts on ruminal fermentation are diet-dependent, being more noticeable with a high-fibre substrate, and subtle with a high-concentrate diet.

INTRODUCTION

A yeast culture is a fermented feed additive that can contain either live or inactivated yeast cells, the culture medium on which the yeast was grown and the metabolic by-products produced by yeast during fermentation (Linn & Raeth-Knight 2006). The most common yeast additive used in ruminant diets is obtained from cultures of *Saccharomyces cerevisiae*. This additive has been used for many years to enhance ruminal fermentation, reducing energy and nutrient losses and thus improving production efficiency in ruminant production systems. In recent years, with increased consumer concern about safety, quality of animal products and environmental impact, antibiotics and synthetic chemical products have been banned as feed additives by the European Union (Anadón 2006). Alternative additives such as yeasts are used not only to increase

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productivity but also to decrease the risk of transfer of antibiotic resistance or potential human pathogens and to limit excretion of pollutants (Chaucheyras-Durand *et al.* 2008).

Some experiments have shown that yeast additives may improve feed intake and milk production in dairy cattle (Harris & Webb 1990; Williams *et al.* 1991; Piva *et al.* 1993; Kung *et al.* 1997; Dann *et al.* 2000; Nocek *et al.* 2003). These responses are usually related to stimulation of cellulolytic bacteria (Newbold *et al.* 1996) enhancing potential fibre digestion in the rumen, and to their potential to prevent a fall in rumen pH by decreasing lactic acid production and/or increasing utilization of lactic acid by some bacteria (Chaucheyras-Durand *et al.* 1996; Callaway & Martin 1997). Yeasts also affect feed degradability, and the patterns of volatile fatty acid (VFA) production (Carro *et al.* 1992; Zeleňák *et al.* 1994; Guedes *et al.* 2008). However, yeast responses are not always consistent (Arcos-García *et al.* 2000). Some authors, in fact, have observed that milk yield, milk composition, body weight gain and feed intake were not affected by the addition of *S. cerevisiae* to the diet (Chiquette 1995; Kamalamma *et al.* 1996), with no noticeable effects of this yeast on bacterial counts or bacterial colonization of roughage in the rumen (Chiquette 1995). Some of the possible causes for the inconsistency could be associated with the characteristics of different yeast strains, the amount of added yeast (Mendoza *et al.* 1995; Newbold *et al.* 1995), type of animals and diet composition (Carro *et al.* 1992; Wallace 1994; Zeleňák *et al.* 1994).

Most of the above-mentioned *in vivo* and *in vitro* research with yeast products has been conducted with *S. cerevisiae* cultures that include the yeast and the medium on which it was grown, along with components such as vitamins and other fermentation products, potentially able to stabilize the rumen environment. Over the last few years, attention has turned towards discriminating between the effects of culture products and live yeast cells (YC) on ruminal fermentation, which are processed to remove the culture medium and to maintain a high live-cell count (Lynch & Martin 2002). Some reports indicate that YC do not grow in the rumen but show some degree of viability (Dawson *et al.* 1990; Hession *et al.* 1992) and influence the course of rumen fermentation through interactions with ruminal micro-organisms (Harrison *et al.* 1988; Martin *et al.* 1989; Wallace & Newbold 1993). The main effects that have been identified are improvement of the rumen environment favouring microbial establishment, stabilization of ruminal pH and interactions with lactate-metabolizing bacteria and increase of fibre degradation and interaction with plant-cell-wall degrading micro-organisms (Chaucheyras-Durand *et al.* 2008).

In addition to live cells, recent research and development have provided inactivated cells of *S. cerevisiae* with potential use as alternative yeast derivatives. According to the manufacturers, these products can supply more benefits and advantages compared to live cells. Even though the mechanism of action of inactivated yeast extracts (IY) could be similar in part to those of live cells, they ensure a uniformity of action and a faster or even immediate availability of the substances contained within the cells (vitamins or other growth factors) to autochthonous microbiota. However, experimental data regarding the use of this product are scarce (Piva *et al.* 1993; Mimosi *et al.* 2008; Fortina *et al.* 2009).

The objective of the current study was, therefore, to investigate and compare the effects of inactivated cells of *S. cerevisiae* and a yeast culture with live cells of *S. cerevisiae* on *in vitro* ruminal fermentation of diets with different forage:concentrate ratio.

MATERIALS AND METHODS

Experimental design

The additives tested were an IY of *S. cerevisiae* (Thepax 100 R, with declared composition of 5×10^9 inactivated cells/g of strain GSH351; Dox-Al Italia SpA, Sulbiate, Italy) and an extensively used additive with YC of *S. cerevisiae* (Yea-Sacc^{1026}, a yeast culture with declared concentration of 10^9 CFU/g of strain CBS 493·94; Alltech Inc., Ireland). The procedure of inactivation of Thepax 100 R was based on changes in osmotic pressure, so the cells were dehydrated and rehydrated again, leaving the external membrane of the micro-organism unaltered (European Patent EP0904701A2).

The experiment was carried out to evaluate two different doses of inactivated yeast (to reach concentrations of 250 (IY250) or 500 (IY500) mg product per litre of incubation medium) tested against the live yeast (at a concentration of 150 mg product/l, additive treatment YC), each of which were added directly as powder to *in vitro* batch cultures of mixed ruminal micro-organisms (direct additive treatment (*T*) effect). To evaluate the effects of the additive on ruminal microbial population, 12 rumen-fistulated Assaf sheep

were assigned randomly to the following treatments (four sheep per group): control group (no additive), IY group (receiving 3·5 g Thepax 100 R/animal/day) and YC group (receiving 1·5 g Yea-Sacc[1026]/animal/day). Additives were dosed intraruminally through the cannula once daily at 08.00 h for 10 days before starting the *in vitro* assay to test the effects of the additives on the fermentative activity of the rumen fluid used as inoculum in the *in vitro* trials (adapted rumen fluid or inoculum (*I*) effect). Sheep were fed *ad libitum* a diet consisting of alfalfa hay with free access to fresh water during the adaptation period of 10 days. Animal handling followed the recommendations of European Council Directive 86/609/EEC for protection of animals used for experimental and other scientific purposes (CEC 1986), and experimental procedures were approved by the University of León (Spain) Institutional Animal Care and Use Committee.

Two total mixed ratios (TMR) of the following forage: concentrate ratios (DM basis): 0·6:0·4 (high-forage TMR) and 0·2:0·8 (high-concentrate TMR) were used to be incubated *in vitro*. Composition and chemical characteristics of diets are reported in Table 1.

In vitro experiments

Ruminal contents were collected individually from each donor sheep in thermos flasks before the morning feeding and taken to the laboratory, where ruminal contents from each sheep were strained through two layers of cheesecloth and kept at 39 °C under a CO_2 atmosphere.

In vitro gas production

In vitro gas production measurements were conducted using a pressure transducer as described by Theodorou *et al.* (1994). Samples of the diet to be incubated (500 ± 10 mg) were weighed out in 120 ml serum bottles to which 50 ml of diluted rumen fluid were dispensed. Rumen fluid was previously diluted (1:4, v/v) with a culture medium containing macro- and micro-mineral solutions, a bicarbonate buffer solution and resazurin, prepared as described by Menke & Steingass (1988). The medium was maintained at 39 °C and saturated with CO_2; oxygen was reduced by the addition of a solution containing cysteine hydrochloride and sodium sulphide. Blanks (bottles without samples) were used to compensate for gas production in the absence of substrate. Once filled, bottles were sealed with rubber stoppers and aluminium seals, shaken and placed in the incubator at 39 °C. The

Table 1. *Composition and nutrient content (g/kg DM) of experimental TMR*

	High-forage TMR	High-concentrate TMR
Ingredient		
Maize silage	423	250
Maize meal	221	200
Soybean meal	179	200
Ryegrass hay	141	163
Minerals and vitamins*	17	90
Buffer salt†	12	65
Sodium bicarbonate	7	15
Calcium carbonate		8·2
Sodium chloride		4
Wheat bran		1·8
Dicalcium phosphate		1·7
Calcium soap (blend)		1·3
Nutrient		
DM, as fed	481	892
Ash	91	101
CP	146	187
EE	35	26
NDF	416	254
ADF	242	156
Lignin	27	21

* Containing (per g): Beta carotene, 0·12 mg; vitamin A, 4000 IU; vitamin D_3, 500 IU; vitamin E, 1 mg; vitamin B_1, 0·014 mg; vitamin B_2, 0·015 mg; vitamin B_6, 0·003 mg; vitamin B_{12}, 0·01 mg; vitamin C, 7 mg; biotin, 0·0015 mg; choline, 2·5 mg; Zn, 20 mg; Mn, 12 mg; Fe, 5 mg; Cu, 2·5 mg; I, 0·5 mg; Co, 0·2 mg; Se, 0·04 mg.
† Containing (per g) : Ca, 200 mg; Na, 280 mg; Mg, 70 mg.

head-space gas pressure released upon fermentation of feed was measured using a pressure transducer at 3, 6, 9, 12, 16, 21, 26, 31, 36, 48, 60, 72, 96, 120 and 144 h after inoculation time. Incubations were performed in two batches, carried out in two consecutive weeks with different sources of inocula. Within each batch, 48 vials were incubated per substrate corresponding to three inocula (control, IY and YC) × two sheep per inoculum × four additive treatments (control, IY250, IY500 and YC) × two serum bottles (duplicates). Gas volume was estimated from pressure measurements using the equation proposed by López *et al.* (2007). In order to estimate the fermentation kinetics parameters, gas production data were fitted to the exponential model proposed by France *et al.* (2000):

$$G = A[1 - e^{-c(t-L)}]$$

where *G* (ml/g DM) is the cumulative gas production at time *t; A* (ml/g DM) is the asymptotic gas

production; e is Napier's constant; c (/h) is the fractional rate of fermentation and L (h) is the lag time.

The average fermentation rate (R, ml gas/h) was defined as the average gas-production rate between the start of the incubation and the time at which the cumulative gas production was half of its asymptotic value, and was calculated as

$$R = Ac/[2(\ln 2 + cL)]$$

Volume of gas (ml/g DM) produced after 24 h of incubation (G24) was used as an index of digestibility and energy feed value, as suggested by Menke & Steingass (1988).

In vitro 24 h incubations

Samples (500 ± 10 mg) of TMR (high forage and high concentrate) were incubated in serum bottles with diluted rumen fluid in a culture medium as described in the previous paragraph. Incubations were performed in two batches carried out in two consecutive weeks with different sources of inocula. Within each batch, 48 vials were incubated per substrate, corresponding to three inocula (control, IY and YC) × two sheep per inoculum × four additive treatments (control, IY250, IY500 and YC) × two serum bottles (duplicates). At 24 h of incubation, gas pressure and volume were recorded using a pressure transducer and a graduated syringe, and a gas sample (10 ml) was taken from each bottle and kept in vacuum tubes (Venoject®, Terumo Europe N.V., Belgium) until being analysed for methane (CH_4). Fermentation was stopped immediately by swirling the bottles in ice; the bottles were opened, pH measured (using a pH-meter) and samples of supernatant were taken for determination of ammonia, lactate and VFA. Finally, the contents of each serum bottle were filtered using sintered glass crucibles (pore size No. 1) under a vacuum and oven-dried at 100 °C for 48 h to estimate the disappearance of dry matter (IVD) at 24 h of incubation. Methane was determined by gas chromatography. The volume of methane (M, ml) produced at the end of incubation was calculated from the volume of gas and the gas composition data, as proposed by López *et al.* (2007):

$$M = (G + Vh)C$$

where G is the volume (ml) of total gas produced at the end of incubation (24 h), Vh is the volume (ml) of the headspace in the serum bottle and C is the proportion of methane in the analysed sample. Samples of diluted rumen fluid, collected at 0 h (before incubation started)

and 24 h of incubation, were processed. A 2 ml aliquot was acidified with 2 ml of 0·5 normal hydrochloric acid (HCl) for ammonia-N (NH_3-N) and lactic acid determination and a 0·8 ml sample of the supernatant was added to 0·5 ml of a deproteinizing solution (5 g metaphosphoric acid and 1 g crotonic acid in 250 ml of 0·5 N HCl) for VFA determination. Both samples were centrifuged at 14 500 ***g*** for 15 min at 4 °C, and supernatants were collected for subsequent analysis. NH_3-N concentration was determined as described by Weatherburn (1967), VFA contents were determined by gas chromatography using crotonic acid as internal standard (Ottenstein & Bartley 1971) and lactate was determined using a colorimetric assay as described by Taylor (1996).

In vitro digestibility

In vitro dry matter digestibility (IVDMD) of TMR samples was determined using the Ankom–Daisy procedure described by Robinson *et al.* (1999). Rumen fluid, obtained as described above, was diluted (1:4, v/v) into the medium as reported by Menke & Steingass (1988). Two incubations were completed with one jar per inocula (control, IY and YC) in each incubation, each of which was added with respective treatment where necessary (control without addition, IY and YC) to reach the optimal concentrations of 500 mg inactivated yeast product per litre incubation medium and 150 mg/l for live yeast adapted inocula. Samples of diets (250 ± 10 mg) were weighed in F57 Ankom bags with a pore size of 25 μm, heat-sealed and then placed into an incubation jar. Nine bags per substrate were used, corresponding to three treatments (control, IY, YC) × three replications. Each jar was a 5 litre glass recipient with a plastic lid provided with a one-way valve that avoids the accumulation of fermentation gases, filled with 2 litres of buffered rumen fluid under anaerobiosis and placed into the DaisyII Incubator (Ankom Technology Corp., Fairport, NY, USA). Temperature (39 °C) and constant levels of agitation were maintained in the controlled chamber with continuous rotation. After 48 h of incubation the jars were emptied and the bags were gently rinsed and dried in an oven at 60 °C. Bags were then washed in the fibre analyser with a neutral detergent solution at 100 °C for 1 h and rinsed with distilled water, so as to remove bacterial cell walls and other endogenous products. *In vitro* neutral detergent fibre digestibility (IVNDFD) was estimated from the amount of neutral detergent fibre (NDF) incubated.

Chemical analysis

Samples of feed were oven dried at 60 °C for 48 h, then ground in a Buhler mill to pass through a 1 mm screen and assayed in duplicate according to the AOAC (2000) methods for DM (method 934·01), ash (method 942·05), crude protein (CP, method 954·01) and ether extract (EE, method 920·39). NDF, acid detergent fibre (ADF) and lignin were determined with the Ankom fibre analyser (Ankom Technology Corp. 1997), following the procedure of Van Soest *et al.* (1991). NDF was analysed using a heat-stable amylase and with the addition of sodium sulphite, and expressed exclusive of residual ash.

Statistical analysis

Data for each type of diet were subjected to ANOVA using the general linear model (GLM) of SPSS (v 17.0, SPSS Inc., Chicago, Illinois, USA). Separate analyses were performed for each TMR used in the incubations. The statistical model used for gas production kinetics and *in vitro* 24 h fermentation data included the fixed effects of inocula (I effects, with levels Control, IY and YC), treatments (T effects, with levels Control, IY250, IY500 and YC) and their interaction, and random effects of donor sheep within each inoculum. *In vitro* digestibility data were analysed by one-way ANOVA with the fixed effect of additive treatment (T effects, with levels Control, IY and YC) as the only source of variation. The standard error of difference and the number of observations (or replicates) for each experimental treatment within each source of variation are reported in the Tables.

RESULTS

Fermentation parameters at 24 h of incubation

The results of the influence of different yeast cell supplements on *in vitro* fermentation parameters at 24 h of incubation for high-forage TMR are given in Table 2. Different adapted inocula (I) tested in the experiment affected some parameters, whereas treatments (T) added to the batch of fermentation and the interaction ($I \times T$) showed no significant effect ($P > 0·05$). Ruminal pH was similar in control and IY inocula, but values were lower ($P = 0·008$) with the YC inoculum. With the inoculum from sheep feeding the YC supplement, higher NH_3-N ($P = 0·006$), total gas production ($P < 0·001$) and *in vitro* dry matter disappearance (IVD, $P < 0·001$) were observed.

Methane production (mmol/g DM incubated) was higher with IY inoculum, and increased even more with the YC inoculum ($P < 0·001$). When expressed on degraded substrate basis, the highest values of methane production (mmol/g DM digested) were observed for IY inoculum ($P = 0·007$). Incubation in rumen fluid from sheep supplemented with YC led to increased total VFA production ($P < 0·001$), and similar trends were shown for acetate ($P < 0·001$), butyrate ($P = 0·020$), valerate ($P = 0·002$) and iso-acids ($P = 0·006$) outputs, whereas the acetate:propionate ratio was increased with IY inoculum ($P = 0·001$). The production of propionate decreased in IY, but increased in YC inoculum ($P < 0·001$). The molar proportions of individual VFAs were affected by yeast products, so that acetate decreased and valerate increased in the YC inoculum ($P = 0·046$ and 0·039, respectively), whereas acetate increased ($P = 0·046$) and propionate decreased ($P = 0·004$) with IY inoculum. Table 3 shows the effects of different yeast cell products on *in vitro* fermentation when the high-concentrate TMR was incubated. Significant differences were seen for adapted inocula only, whereas treatment (T) and interaction $I \times T$ showed no significant differences. Similar trends were shown for lactate and methane production (either per g of DM incubated or DM digested) and total gas production, where values were higher when the high-concentrate TMR was incubated with YC inoculum (P values of 0·055, <0·001, 0·006 and <0·001, respectively). The pH showed an opposite trend, decreasing with the YC inoculum ($P = 0·033$). In general, VFA production was not affected by the effects tested, with only propionate production being decreased slightly with IY and slightly increased with YC inoculum ($P = 0·003$).

In vitro parameters of gas production kinetics and digestibility

The effect of *S. cerevisiae* on *in vitro* gas production kinetic parameters at 144 h of incubation for the TMR, are presented in Table 4. The A, c and L parameters of high-forage diet showed no statistically significant differences due to inocula used in the experiment, to the treatment or to the $I \times T$ interaction. The YC inoculum gave rise to a significantly ($P = 0·010$) higher cumulative gas production at 24 h of incubation ($P = 0·010$) and average fermentation rate ($P = 0·014$) than control and IY inocula, whereas T and $T \times I$ interaction effects were not significant ($P > 0·05$). In the high-concentrate TMR, none of the fermentation

Table 2. *Influence of different* S. cerevisiae *products (inactivated yeast extract, IY; live yeast cells, YC; IY at 250 and 500 mg product per litre of incubation medium, IY250 and IY500, respectively) on* in vitro *fermentation of high-forage TMR (24 h, DM basis)*

	Inoculum $n=8$					Treatment $n=6$						
	Control	IY	YC	S.E.D.	P value I	Control	IY250	IY500	YC	S.E.D.	P value T	P value I×T
pH	6·65	6·66	6·61	0·016	0·008	6·64	6·64	6·64	6·64	0·018	NS	NS
IVD (g/g)	0·61	0·62	0·73	0·016	<0·001	0·65	0·66	0·65	0·65	0·018	NS	NS
Total gas (ml/g)	143	153	182	4·5	<0·001	155	157	163	161	5·1	NS	NS
CH$_4$ (mM/g DM incubated)	1·4	1·7	1·8	0·04	<0·001	1·6	1·6	1·7	1·6	0·04	NS	NS
CH$_4$ (mM/g DM digested)	2·4	2·7	2·5	0·08	0·007	2·5	2·5	2·6	2·5	0·09	NS	NS
CH$_4$ (mM/M gas)	233	241	227	9·6	NS	232	244	230	231	11·0	NS	NS
NH$_3$-N (mg/l)	276	261	339	20·2	0·006	280	296	304	288	23·3	NS	NS
L-lactate (mg/l)	10·8	11·2	11·4	0·91	NS	10·0	11·2	11·2	12·1	1·05	NS	NS
VFA (mM/g)												
Acetate	2·80	2·94	3·39	0·104	<0·001	2·96	3·13	3·06	3·02	0·120	NS	NS
Propionate	1·12	0·96	1·26	0·016	<0·001	1·08	1·14	1·12	1·12	0·018	NS	NS
Butyrate	0·50	0·58	0·69	0·055	0·020	0·57	0·61	0·60	0·59	0·063	NS	NS
Valerate	0·06	0·07	0·09	0·006	0·002	0·07	0·07	0·07	0·08	0·007	NS	NS
	0·09	0·11	0·17	0·022	0·006	0·12	0·13	0·13	0·12	0·026	NS	NS
Isobutyrate + isovalerate												
Total VFA	4·6	4·7	5·6	0·16	<0·001	4·9	5·1	5·0	4·9	0·18	NS	NS
Acetate:propionate (M:M)	2·5	3·1	2·7	0·11	0·001	2·7	2·8	2·8	2·7	0·12	NS	NS
VFA (mM:mM)												
Acetate	0·61	0·63	0·60	0·016	0·046	0·61	0·62	0·62	0·60	0·018	NS	NS
Propionate	0·24	0·21	0·23	0·009	0·004	0·23	0·22	0·22	0·23	0·010	NS	NS
Butyrate	0·11	0·13	0·12	0·008	NS	0·12	0·12	0·12	0·12	0·009	NS	NS
Valerate	0·01	0·01	0·02	0·001	0·039	0·01	0·01	0·02	0·02	0·001	NS	NS
	0·02	0·02	0·03	0·004	NS	0·03	0·02	0·03	0·03	0·004	NS	NS
Isobutyrate + isovalerate												

* *I*, inoculum effect; *T*, treatment effect; *I × T*, inoculum × treatment interaction; VFA, volatile fatty acid; IVD, *in vitro* dry matter disappearance; S.E.D., standard error of difference.

Table 3. *Influence of different* S. cerevisiae *products (inactivated yeast extract, IY; live yeast cells, YC; IY at 250 and 500 mg product per litre of incubation medium, IY250 and IY500, respectively) on* in vitro *fermentation of high-concentrate TMR (24 h, DM basis)*

	Inoculum n=8					Treatment n=6						
	Control	IY	YC	s.e.d.	P value I	Control	IY250	IY500	YC	s.e.d.	P value T	P value I×T
pH	6·7	6·7	6·7	0·01	0·033	6·7	6·7	6·7	6·7	0·01	NS	NS
IVD (g/g)	0·78	0·78	0·79	0·007	NS	0·78	0·78	0·78	0·79	0·008	NS	NS
Total gas (ml/g)	152	159	169	3·1	<0·001	160	157	161	162	3·6	NS	NS
CH_4 (mM/g DM incubated)	1·6	1·7	1·8	0·03	<0·001	1·7	1·7	1·7	1·7	0·03	NS	NS
CH_4 (mM/g DM digested)	2·1	2·2	2·3	0·05	0·006	2·1	2·2	2·2	2·2	0·06	NS	NS
CH_4 (mM/M gas)	230	233	235	5·6	NS	231	237	232	230	6·5	NS	NS
NH_3-N (mg/l)	406	380	431	24·3	NS	391	412	423	397	28·0	NS	NS
L-lactate (mg/l)	8·4	9·3	11·0	0·97	0·055	8·8	9·9	9·9	9·7	1·12	NS	NS
VFA (mmol/g)												
Acetate	3·14	3·18	3·38	0·098	NS	3·31	3·23	3·30	3·24	0·113	NS	NS
Propionate	1·02	0·94	1·08	0·032	0·003	1·01	1·01	1·03	1·02	0·036	NS	NS
Butyrate	0·71	0·72	0·72	0·048	NS	0·71	0·72	0·71	0·73	0·055	NS	NS
Valerate	0·11	0·10	0·10	0·007	NS	0·10	0·10	0·11	0·10	0·007	NS	NS
	0·21	0·21	0·22	0·022	NS	0·21	0·22	0·21	0·20	0·026	NS	NS
Isobutyrate+isovalerate												
Total VFA	5·2	5·2	5·5	0·02	NS	5·3	5·3	5·4	5·3	0·02	NS	NS
Acetate:propionate (mol:mol)	3·1	3·4	3·1	0·13	NS	3·3	3·2	3·2	3·2	0·15	NS	NS
VFA (mM:mM)												
Acetate	0·60	0·62	0·61	0·008	NS	0·62	0·61	0·61	0·61	0·009	NS	NS
Propionate	0·20	0·18	0·20	0·007	NS	0·19	0·19	0·19	0·20	0·008	NS	NS
Butyrate	0·14	0·14	0·13	0·007	NS	0·13	0·14	0·13	0·14	0·008	NS	NS
Valerate	0·02	0·02	0·02	0·001	NS	0·02	0·02	0·02	0·02	0·001	NS	NS
	0·04	0·04	0·04	0·003	NS	0·04	0·04	0·04	0·04	0·003	NS	NS
Isobutyrate+isovalerate												

* *I*, inoculum effect; *T*, treatment effect; *I×T*, inoculum×treatment interaction; VFA, volatile fatty acid; IVD, *in vitro* dry matter disappearance; s.e.d., standard error of difference.

Table 4. *Influence of different S. cerevisiae products (inactivated yeast extract, IY; live yeast cells, YC; IY at 250 and 500 mg product per litre of incubation medium, IY250 and IY500, respectively) on* in vitro *parameters of fermentation of diets (144 h, DM basis)*

	Inoculum n = 8					Treatment n = 6						
	Control	IY	YC	S.E.D.	P value I	Control	IY250	IY500	YC	S.E.D.	P value T	P value I×T
High-forage TMR												
A (ml/g)	334	340	342	7·8	NS	336	340	343	336	8·9	NS	NS
C (/h)	0·05	0·04	0·05	0·002	NS	0·05	0·05	0·05	0·05	0·002	NS	NS
L (/h)	2·84	3·10	2·73	0·619	NS	2·88	2·68	2·82	3·18	0·715	NS	NS
G24 (ml/g)	205	203	220	5·0	0·010	209	209	214	207	5·8	NS	NS
R (ml/h)	9·12	8·95	10·08	0·345	0·014	9·37	9·35	9·61	9·21	0·398	NS	NS
High-concentrate TMR												
A (ml/g)	307	306	304	6·6	NS	304	303	309	308	7·6	NS	NS
C (/h)	0·05	0·05	0·05	0·002	NS	0·05	0·05	0·05	0·05	0·002	NS	NS
L (/h)	1·10	0·79	1·02	0·356	NS	0·93	1·04	0·98	0·93	0·410	NS	NS
G24 (ml/g)	199	199	205	6·3	NS	204	196	200	206	7·3	NS	NS
R (ml/h)	9·28	9·53	10·31	0·424	NS	9·73	9·30	9·76	9·93	0·490	NS	NS

* *I*, inoculum effect; *T*, treatment effect; *I×T*, inoculum×treatment interaction; *A*, asymptotic gas production; *c*, fractional rate of gas production; *L*, lag time; G24, cumulative gas production at 24 h of incubation; *R*, average fermentation rate; S.E.D., standard error of difference.

Table 5. *Influence of different* S. cerevisiae *products (inactivated yeast extract, IY; live yeast cells, YC) on* in vitro *digestibility of diets (48 h, DM basis)*

	Treatment n = 3				
	Control	IY	YC	S.E.D.	P value
High-forage TMR					
IVDMD (g/g)*	0·82	0·81	0·81	0·012	NS
IVNDFD (g/g)	0·56	0·55	0·53	0·026	NS
High-concentrate TMR					
IVDMD (g/g)	0·87	0·87	0·86	0·005	NS
IVNDFD (g/g)	0·47	0·49	0·45	0·026	NS

* IVDMD, in vitro dry matter digestibility; IVNDFD, in vitro neutral detergent fibre degradability; S.E.D., standard error of difference.

kinetics parameters were affected by inoculum, treatment or their interaction. Data regarding the digestibility at 48 h of diets are presented in Table 5. Neither IVDMD nor IVNDFD for TMR were affected by the different yeast cells products used in the *in vitro* trial, showing values of IVDMD between 0·805 and 0·818 for high-forage TMR and between 0·860 and 0·871 g/g for high-concentrate diet, and values of IVNDFD between 0·530 and 0·561 for high-forage TMR and between 0·447 and 0·490 g/g for high-concentrate TMR.

DISCUSSION

The current study was designed to test two different doses of inactivated yeast supplement against a live yeast product on *in vitro* ruminal fermentation of medium–high fibre and high concentrate ratios. Doses of yeast products used in the assays were calculated on the basis of the amounts administered to the animals according to the manufacturers (3·5 g/day per sheep of Thepax 100 R and 1·5 g/day per sheep of Yea-Sacc[1026]). It must be stressed that no effects of either inactivated or live yeasts were observed due to the direct addition of these products to batch cultures, with none of the diets used as fermentation substrate. Any significant differences observed in the present study were among the inocula used in the fermentation, obtained from animals that received control or supplemented diets for 10 days. This is an important observation and could explain the disparity of responses to live yeasts and yeast culture found in several *in vitro* studies reported in the literature. The current

experiment was designed to test not only acute, immediate, responses to yeast additives but also the effects on ruminal fermentation when the additive was administered regularly. The fact that the direct addition of a single dose of yeast product to batch cultures (with 24-h or 144-h incubations) had no effect on *in vitro* ruminal fermentation, whereas some noticeable differences were observed among inocula from animals receiving no yeast or one of the yeast products tested, indicates that yeasts induce changes in the rumen microbial population, requiring some long-standing adaptation to and interaction with yeasts for changes in ruminal fermentation pattern to become apparent. In contrast, yeast would not induce prompt changes in the ruminal microbial population, and thus short-term shifts in fermentation pattern cannot be expected in response to yeast products. Thus, Koul *et al.* (1998) did not observe changes due to the addition of a single dose of autoclaved yeasts to the rumen of buffalo calves. However, the current results are consistent with those reported by Oeztuerk *et al.* (2005) and Oeztuerk (2009), who reported long-term effects (decline in pH and increase in ammonia concentration) of autoclaved and live yeast added to Rusitec fermenters, although the effects were more pronounced when live yeast culture was used. The adaptive effect could be mediated by a prebiotic effect of yeast cells or some heat labile components (Oeztuerk *et al.* 2005), which may derive from the microbial degradation of the yeast cells. The latter could partly explain the differences observed between live and inactivated yeasts.

With both diets, yeast live cells decreased the ruminal pH compared to control, whereas inactivated yeast had no effect. In all cases the pH values remained above 6·5, the physiological range of a healthy rumen. Inconsistent effects of *S. cerevisiae* on ruminal pH have been reported in numerous *in vivo* and *in vitro* studies. The current results are consistent with some of those observed by Lynch & Martin (2002), where live cells decreased ruminal pH when alfalfa hay was incubated, with final values above 6·0. However, live yeast did not affect ruminal pH when a more concentrate substrate was fermented (Lynch & Martin 2002). The current results are consistent with previous findings that adding inactivated yeast culture had no effect on pH values when TMR with variable forage to concentrate ratios (ranging from 0·4:0·6 to 0·67:0·33) were fermented (Piva *et al.* 1993; Enjalbert *et al.* 1999; Erasmus *et al.* 2005). In contrast, other authors have reported a slight rise in ruminal pH in response to the addition of a live yeast supplement to the diet (Nocek

et al. 2002; Bach *et al.* 2007; Thrune *et al.* 2009). Guedes *et al.* (2008) observed that when maize silage was fermented, the effect of the yeast culture on pH was consistent with the changes observed in ruminal lactate concentration. Increasing activity of lactate-utilizing bacteria and/or decreasing of activity of lactate-producing bacteria will cause a decrease in lactate concentration giving rise to higher pH values in the rumen. Decreased lactate concentration has been reported in response to the addition of live yeast when forage or concentrates were fermented (Lila *et al.* 2004). In the current study, lactate concentration was increased when live yeast (YC) was added to a high-concentrate diet, which agrees with results reported by Lynch & Martin (2002) using ground maize as fermentation substrate. Therefore, these variations could be a consequence of the interaction between the *S. cerevisiae* and lactate-metabolizing bacteria, such as *Streptococcus bovis, Megasphaera elsdenii* or *Selenomonas ruminantium*, when competing for the utilization of sugars, regarding yeast live cells, or through the supply of growth factors (such as amino acids, peptides, vitamins and organic acids) in the case of yeast culture. Ruminal NH_3-N concentration was not affected by inactivated or live yeast supplements to a high-concentrate ratio, which is consistent with other studies (Lila *et al.* 2004; Erasmus *et al.* 2005; Guedes *et al.* 2008) using different substrates in their experiments. However, with the high-fibre diet the addition of YC resulted in higher ammonia concentrations. The unexpected increases of lactate and NH_3-N production observed upon the addition of yeast live cells could be due to the level of yeast inclusion used in the experiment, as suggested by Newbold *et al.* (1995), who concluded that greater outputs of fermentation end-products detected in the Rusitec fermenters supplemented with yeast additives could represent a response to increased substrate supply rather than a shift in the fermentation pattern.

Supplementation of the high-fibre diet with yeast additives had effects on total VFA production and VFA composition, in general with a slight increase when inactivated yeast culture was added, and a significantly greater increase in response to the addition of live yeast (YC) product. This result is consistent with the slight decline in the rumen pH discussed above, and with data obtained when yeast culture was added to maize silage (Guedes *et al.* 2008) or to a high forage diet (Enjalbert *et al.* 1999) or when YC were added to a hay plus concentrate diet (Lila *et al.* 2004). No effects on total VFA concentration were observed when yeast

additives were used with the high-concentrate TMR, in agreement with other published data (Piva *et al.* 1993; Doreau & Juoany 1998). The increase in the molar proportion of acetate observed when inactivated yeast culture was added to a high-forage TMR is also in agreement with results reported by Mutsvangwa *et al.* (1992) testing a yeast culture. Increased acetate to propionate ratios in response to IY supplementation occurred because acetate increased at the expense of propionate but, in general, acetate to propionate ratio measured in the batch cultures would be within the range of a good fibre digestion. The current results are in agreement with Lila *et al.* (2004), who also observed an increase in propionate molar proportion when live yeast was added to starchy substrates. The change in VFA concentration and/or molar proportion observed in the current experiment can be explained by modification of bacterial population in response to yeast supplementation. Acetate formation is mainly due to structural carbohydrate fermentation by cellulolytic bacteria, whereas the fermentation of non-structural carbohydrate by amylolytic bacteria leads to a relatively greater production of propionate. Microbial changes that occur within the rumen in response to *S. cerevisiae* addition to the diet may be increased (Newbold *et al.* 1996) or decreased (Mathieu *et al.* 1996) numbers of total viable bacteria, increased (Wiedmeier *et al.* 1987; El Hassan *et al.* 1996; Newbold *et al.* 1996), unchanged (Dawson *et al.* 1990; Erasmus *et al.* 1992; Yoon & Stern 1996) or decreased (Mathieu *et al.* 1996) counts of cellulolytic bacteria, and no effects (Kumar *et al.* 1994; Yoon & Stern 1996) on amylolytic bacteria. These reported trends towards an increased ratio of cellulolytic to amylolytic bacteria could therefore lead to a change in VFA production and an increased acetate:propionate ratio.

Neither diet considered in the current study had any effect on *in vitro* DM and NDF digestibility. A similar response in digestibility of bermudagrass hay was observed by Lynch & Martin (2002) when both yeast culture and live cells were added, and by Carro *et al.* (1992) with the addition of yeast culture on 50 forage:50 concentrate ratio. With both TMRs used in the current study, total gas production increased when live yeast was added, in agreement with Lila *et al.* (2004), whereas the inactivated product had no effect on fermentation gas. Both fermentation gas and propionate production were increased when substrates were fermented in rumen fluid obtained from animals supplemented with YC, suggesting that propionate

would derive from the succinate pathway explaining the higher total gas volume released, probably in the form of carbon dioxide (Wolin & Miller 1988). As both gas production and substrate digestibility were increased with the high-forage diet when YC inoculum was used, fermentation efficiency (mg DM degraded/ml gas production) was not affected. Methane production was increased in response to the addition of inactivated yeast and, to a greater extent, in response to YC. This increase is consistent with the higher acetate production, suggesting that fermentation may have been shifted to an acetogenic pathway.

The results obtained from 144 h incubations showed differences, only with the 0·6:0·4 forage:concentrate ratio, in the cumulative gas production at 24 h of incubation and in the average fermentation rate, which were greater with live yeast, although the asymptotic gas production was not affected. Dawson (1990) and Williams *et al.* (1991) suggested that ruminal micro-organisms could be stimulated by yeasts at initial stages of fermentation, but these effects would become negligible in the medium-term with long-lasting fermentations.

CONCLUSIONS

Both inactivated and live yeast products tested in the trial may stimulate ruminal fermentation, although such an effect requires a regular administration of the product and some adaptation of the ruminal microbial population for the stimulatory effects to become apparent. Based on the current results, live yeasts would affect ruminal fermentation to a slightly greater extent than inactivated yeasts, but none of them showed immediate acute effects. On the other hand, effects of yeast on ruminal fermentation were diet-dependent, being more noticeable with a high-fibre substrate, and less evident with a high concentrate diet.

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