



UNIVERSITÀ DEGLI STUDI DI TORINO

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This is an author version of the contribution published on:

6

Questa è la versione dell'autore dell'opera:

7

Journal of Agricultural Science, 150, 2012, 10.1017/S0021859611000578

8

The definitive version is available at:

9

La versione definitiva è disponibile alla URL:

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

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Abstract

The effects of a commercial product containing inactivated cells of *S. cerevisiae* (Thepax) and a yeast culture with live cells of *S. cerevisiae* (Yea-sacc) on the ruminal fermentation of two different diets were investigated *in vitro*. Total mixed rations (TMR) having forage:concentrate ratios of 60:40 (medium-high forage diet) and 20:80 (low forage diet) were incubated in batch cultures of mixed ruminal microorganisms to which either inactivated (to reach concentrations of 500 and 250 mg product per l incubation medium) or live yeast (at a concentration of 150 mg product/l) were added directly as powder. To evaluate medium-term effects of the additive on ruminal microbial population, sheep used as donors of rumen fluid were allocated in three experimental groups, two of them receiving a diet with a different additive during 10 days and a control group. With both diets, yeast live cells decreased the ruminal pH compared to control, whereas inactivated yeast had no effect. Adding yeast additives to the high-fibre diet affected total volatile fatty acid (VFA) production and VFA composition, in general with a slight increase with inactivated yeast, and a significantly greater increase in response to the addition of live yeast product. 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 the live yeast supplement. Methane production was higher with Thepax inoculum, and increased even more with the Yea-sacc 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 in Yea-sacc 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 were greater with live yeast, although the asymptotic gas production was not affected. These results indicate that live yeasts affect ruminal fermentation to a slightly greater extent 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. Moreover 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.

Keywords: *Saccharomyces cerevisiae* inactivated cells; *Saccharomyces cerevisiae* live cells; Rumen fermentation; *In vitro* digestibility; Gas production technique.

Abbreviations: A, asymptotic gas production; ADFom, acid detergent fibre expressed exclusive of residual ash; c, fractional rate of gas production; G24, cumulative gas production at 24 h of incubation; IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fibre degradability; L, lag time; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; R, average fermentation rate; RSD, residual standard deviation; TMR, total mixed ration; VFA, volatile fatty acid.

1. Introduction

A yeast culture is a fermented feed additive that can contain either live or inactivated yeast cells, the culture media where the yeast cells were grown on, and the metabolic by-products produced by yeast during fermentation (Linn and 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 aiming at enhancing ruminal fermentation to reduce energy and nutrient losses and to improve production efficiency in ruminant production systems. In recent years, with increased consumer's concern about safety, quality of animal products and also environmental issues, 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 productivity, but also to decrease the risk of animal digestive transfer of potential human pathogens and to limit excretion of pollutants (Chaucheyras-Durand et al., 2008).

Some experiments have shown that supplementation of feed ration with yeast additives may improve feed intake and milk production in dairy cattle (Harris and 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 by yeast cultures (Newbold et al., 1996) that enhance 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 and Martin, 1997). *S. cerevisiae* yeasts also affect feed degradability, and the patterns of VFA production (Carro et al., 1992; Zelenak et al., 1994; Guedes et al., 2008). However, yeast responses were not always so consistent because several factors have influenced the controversial experimental results. (Arcos-García et al., 2000). Others authors, in fact, affirm 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 characteristic of different yeast strains (Newbold et al., 1995), differences between commercial additives (Mendoza 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 additives have been conducted with *S. cerevisiae* cultures that include the yeast and the medium where it was grown on, 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 the discrimination between the effects of culture products and live yeast cells on ruminal fermentation, that are processed to not include the whole culture medium and to maintain a high live cell count (Lynch and Martin, 2002). Some reports indicate that live yeast cells 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 microorganisms (Harrison et al., 1988; Martin et al., 1989; Wallace and Newbold, 1993). The main effects that have been identified are improvement of rumen environment favouring microbial establishment, stabilization of ruminal pH and interactions with lactate-metabolising bacteria, and increase of fibre degradation and interaction with plant-cell wall degrading microorganisms (Chaucheyras-Duran et al., 2008).

In addition to live cells, recently research and development have provided inactivated cells of *S. cerevisiae* with potential use as alternative additives. According to manufacturers, these products can supply more benefits and advantages compared to live cells. Even though the mechanism of action of inactivated yeast cells are partly similar to those of live cells, they ensure a uniformity of action and a faster or 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 this study was therefore to investigate and compare the effects of a commercial product containing 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. This study also aimed to test whether the potential positive effects of *S. cerevisiae* could be attributed to the yeast's viability or to its content in nutrients and essential microbial growth factors, and how the diet composition could influence the response.

2. Material and methods

2.1. Experimental design

The additives tested were a commercial product containing inactivated cells 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 yeast culture with live cells of *S. cerevisiae* (Yea-Sacc1026, with declared concentration of 1×10^9 CFU/g of strain CBS 493.94; Alltech Inc., Ireland). The experiment was carried out to evaluate two different doses of inactivated yeast (to reach concentrations of 500 and 250 mg product per l incubation medium) tested against the live yeast (at a concentration of 150 mg product/l), each of which were added directly as powder to *in vitro* batch cultures of mixed ruminal microorganisms (direct

additive treatment (7) effect). To evaluate medium-term effects of the additive on ruminal microbial population, sheep used as donors of rumen fluid were allocated in three experimental groups each receiving a diet with a different additive. There was a control group (no additive), a group of sheep receiving Thepax 100 R and a group of sheep dosed Yea-Sacc¹⁰²⁶. Additives were administered for 10 days before the starting of 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). The diets incubated *in vitro* were total mixed rations (TMR's) characterized by medium-high (60:40) and low (20:80) forage:concentrate ratios. Composition and chemical characteristics of diets are reported in Table 1.

2.2. *In vitro* experiments

Animal handling followed the recommendations of European Council Directive 86/609/EEC for protection of animals used for experimental and other scientific purposes, and experimental procedures were approved by the University of León (Spain) Institutional Animal Care and Use Committee. Twelve rumen-fistulated Assaf sheep were assigned randomly to the following treatments: four for the control group, four receiving 3.5 g Thepax 100 R/day per head and four receiving 1.5 g Yea-Sacc /day per head. Yeast products were dosed intraruminally through the cannula once daily at 08:00 h, just before feed was offered. The diet fed to the sheep consisted of alfalfa hay with free access to water. Sheep were fed *ad libitum* during the adaptation period of 10 days. Ruminal contents were collected individually for each donor sheep in thermos flasks before the morning feeding, and taken to laboratory, where ruminal contents from each sheep were strained through two layers of cheesecloth and kept at 39°C under a CO₂ atmosphere.

2.2.1. *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 and Steingass (1988). The medium was maintained at 39°C and saturated with CO₂; oxygen was reduced by the addition of a solution containing cysteine hydrochloride and Na₂S. Blanks (bottles without sample) were used to compensate for gas production in the absence of substrate. Once filled up, bottles were sealed with rubber stoppers and aluminium seals, shaken and placed in the incubator at 39°C. The head-space gas pressure released upon fermentation of feed was measured manually 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 3 inocula (control, Thepax, and Yea-sacc) x 2 sheep per inoculum x 4 additive treatments (control, Thepax1, Thepax2 and Yea-sacc) x 2 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) the asymptotic gas production; c (h⁻¹) 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 and Steingass (1988).

2.2.2. *In vitro* 24 h incubations

Samples (500 ± 10 mg) 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 3 inocula (control, Thepax, and Yea-sacc) x 2 sheep per inoculum x 4 additive treatments (control, Thepax1, Thepax2 and Yea-sacc) x 2 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 analyzed for methane (CH_4). Then fermentation was immediately stopped by swirling the bottles in ice. Then bottles were open, pH was measured (using a pH-meter), and samples of supernatant were taken for ammonia, lactate and volatile fatty acid (VFA) determinations. Finally, the contents of each serum bottle were filtered using sintered glass crucibles (pore size No. 1) under vacuum and oven-dried at 100°C for 48 h to estimate the DM disappearance 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 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 N HCl for ammonia-N ($\text{NH}_3\text{-N}$) 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 x g for 15 min at 4°C, and supernatants were collected for subsequent analysis. Ammonia-N concentration was determined as described by Weatherburn (1967), VFAs were determined by gas chromatography using crotonic acid as internal standard (Ottenstein and Bartley, 1971) and lactate was determined using a colorimetric assay as described by Taylor (1996).

2.2.3. *In vitro* digestibility

In vitro dry matter digestibility (IVDMD) was determined using the Ankom-Daisy procedure following the procedure described by Robinson et al. (1999). Rumen fluid was diluted (1:4 v/v) into the medium, prepared as described by Menke and Steingass (1988). Two incubations were completed with one jar per inocula (control, Thepax and Yea-sacc) in each incubation, each of which was added with respective treatment where necessary (control without addition, Thepax 1 and Yea-sacc) to reach the optimal concentrations of 500 mg inactivated yeast product per l incubation medium and 150 mg/l for live yeast adapted inocula. Samples of diets (250 ± 10 mg) were weighed into F57 Ankom bags with a pore size of 25 μ m, heat-sealed and then placed into an incubation jar. Nine bags per substrate were used, corresponding to 3 treatments (control, Thepax, Yea-sacc) x 3 replications. Each jar was a 5 l glass recipient with a plastic lid provided with a single-way valve which avoids the accumulation of fermentation gases, and was filled with 2 l buffered rumen fluid under anaerobiosis and placed into the DaisyII Incubator (Ankom Technology Corp., Fairport, NY, USA). Temperature (39°C) and agitation were maintained stable and uniform 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 into the fibre analyzer 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* NDF degradation (IVNDFD) was estimated from the amount of aNDFom incubated.

2.3. 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 dry matter (DM, method 934.01), ash (method 942.05), crude protein (CP, method 954.01) and ether extract (EE, method 920.39). Neutral detergent fibre (aNDFom), acid detergent fibre (ADFom) and lignin (sa) were determined with the Ankom fibre analyser (Ankom Technology Corp., 1997), following the procedure of Van Soest et al. (1991). Neutral detergent fibre was analyzed with the addition of sodium sulfite to the solution.

2.4. Statistical analysis

Data for each type of diet were subjected to ANOVA using GLM of the Statistical Package for Social Science (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, Thepax and Yea-sac), treatments (*T* effects, with levels Control, Thepax 0.5, Thepax 1 and Yea-sac) 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, Thepax 1 and Yea-sac) as the only source of variation. Multiple comparisons between treatment means were performed by the Tukey's HSD test, when a significant treatment effect ($P < 0.05$) was observed. The residual standard deviation (RSD) calculated as the square root of the residual mean square (RMS) was reported in the Tables.

3. Results

3.1. 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 in the batch of fermentation and the interaction (*I* x *T*) did not show any significant effect ($P > 0.05$). There were no differences between control and Thepax inocula in the ruminal pH, but values tended to be lower ($P = 0.008$) with the Yea-sacc inoculum. Ammonia nitrogen ($P = 0.006$), total gas production ($P < 0.001$) and *in vitro* dry matter disappearance (IVD) ($P < 0.001$) showed similar trends, where significant *I* effects were detected with feeding the live yeast supplement that showed the highest values. Methane production (mmol/g incubated matter) was higher with Thepax inoculum, and increased even more with the Yea-sacc inoculum ($P < 0.001$). When expressed on degraded substrate basis, the highest values of methane production (mmol/g degraded matter) were observed for Thepax inoculum ($P = 0.007$). Incubation in rumen fluid from sheep supplemented with live yeast (Yea-sac) 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 the sum of isobutyrate and isovalerate ($P = 0.006$) outputs, whereas the acetate:propionate ratio was increased with Thepax inoculum ($P = 0.001$). The production of propionate decreased in Thepax, but increased in Yea-sacc inoculum ($P < 0.001$). The molar proportions of individual VFAs were affected by yeast products, so that acetate decreased and valerate increased in the Yea-sacc inoculum ($P = 0.046$ and $P = 0.039$, respectively), whereas acetate increased ($P = 0.046$) and propionate decreased ($P = 0.004$) with Thepax inoculum. Table 3 shows the effects of different yeast cell products on *in vitro* fermentation when the high-concentrate TMR was incubated. There were significant differences only due to adapted inocula, whereas treatment and interaction *I* x *T* did not present differences. Similar trends were shown for lactate and methane production (either per g of incubated material or of degraded matter) and total gas production, where values tended to be higher when the high-concentrate TMR was incubated in Yea-sacc inoculum (P values of 0.055, <0.001 , 0.006 and <0.001 , respectively). The pH showed an opposite trend, decreasing with the Yea-sacc inoculum ($P = 0.033$). In general, VFA production was not affected by the effects tested, with only propionate production being decreased slightly with Thepax and slightly increased with Yea-sacc inoculum ($P = 0.003$).

3.2. *In vitro* parameters of gas production kinetics and digestibility

The effect of *Saccharomyces cerevisiae* on *in vitro* gas production kinetic parameters at 144 h of incubation for the total mixed rations 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, treatment or *I* x *T* interaction. The Yea-sacc 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 Thepax inocula, whereas *T* and *T* x *I* interaction effects were not significant ($P > 0.05$). In the high-concentrate TMR, none of the fermentation kinetics parameters were affected by inoculum, treatment and interaction effect. Data regarding the digestibility at 48 h of diets are presented in Table 5. Neither IVDMD nor IVNDFD for both TMRs 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.

4. Discussion

The use of *Saccharomyces cerevisiae* in the ruminant diets has been shown to have affect ruminal fermentation, but it is not clear whether the effects could be attributed to interactions of active yeast with ruminal microorganisms (probiotic effect) or to the supply of essential nutrients contained in the yeast culture medium that stimulate the growth of certain rumen microorganisms (prebiotic effect). On the other hand, results are somewhat inconsistent throughout the literature, partially because effects are dependent upon the ration composition. Therefore, a study was designed to test two different doses of inactivated yeast supplement against a live yeast commercial product on *in vitro* ruminal fermentation of medium-high fibre and high concentrate rations. Doses of yeast products used in the assays were calculated on the basis of the amounts administered to the animals according to the manufacturer (3.5 g/day per sheep of Thepax 100 R and 1.5 g/day per sheep of Yea-Sacc¹⁰²⁶). The theoretical concentration of the products in the rumen

was calculated assuming an average rumen volume, and the amounts added to the batch cultures were calculated to reach a similar concentration in vials and jars used in the *in vitro* assays. Live yeasts are viable microorganisms that become active in the rumen and may interact with other ruminal microorganisms (bacteria, protozoa or fungi) (Chaucheyras-Durand et al., 2008). The product will also supply the culture medium where the yeast was grown, containing some essential growth factors that may favour the ruminal microbes (Linn and Raeth-Knight, 2006). The inactivated yeast will contain culture medium, but also whole yeast cells that have the potential to stimulate fermentation (Fortina et al., 2009). Several mechanisms have been suggested for this stimulatory effect. Thus, whole yeast cells are a source of nutrients (sterols and long chain fatty acids from the cell membrane, or amino acids, nucleotides, vitamins and minerals from the cell cytoplasm), as suggested by Choudhari et al. (2008). The yeast cell wall could play an important role, as it contains glucans and mannoproteins which have been considered as valuable prebiotics for ruminal microbes.

It is well-worthy to stress that no effects of either inactivated or live yeasts were observed when the yeast was added directly 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 have received control or supplemented diets for a relatively extended period of time. 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. Our experiment was designed to test not only acute, immediate, responses to yeast additives, but also the effects on ruminal microorganisms and environment when the additive was administered regularly. The fact that the direct addition 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 yeasts products.

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. Our results could be partially consistent with those observed by Lynch and 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 and Martin, 2002). Adding inactivated yeast culture had no effect on pH values when total mixed rations with variable forage to concentrate ratios (ranging from 40:60 to 67:33) were fermented (Piva et al., 1993; Enjalbert et al., 1999; Erasmus et al., 2005) in agreement with our results. 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 our study, lactate concentration was increased when yeast was added to a high-concentrate diet in agreement with results reported by Lynch and Martin (2002) using ground corn as fermentation substrate. Therefore, these variations could be a consequence of the interaction between the *S. cerevisiae* and lactate-metabolising 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 ammonia nitrogen concentration was not affected by inactivated or live yeast supplements to a high-concentrate ration, consistently 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 Yea-sacc resulted in higher ammonia concentrations. The unexpected increases of lactate and ammonia-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 (Yea-sacc) 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 live yeast cells 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 and 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 inactivated Thepax yeast 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. In agreement with our results, Lila et al. (2004) 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 our 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 *Saccharomyces cerevisiae* addition to the diet was previously observed as 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 and Stern, 1996) or decreased (Mathieu et al., 1996) counts of cellulolytic bacteria, and no effects (Kumar et al., 1994; Yoon and Stern, 1996) in 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.

In vitro DM and NDF digestibility were not affected by yeast products with both diets considered. A similar response in digestibility of bermudagrass hay was observed by Lynch and Martin (2002) when both yeast culture and live cells were added, and by Carro et al. (2002) with addition of yeast culture on 50 forage:50 concentrate ration. With both TMR's used in our study, total gas production was 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 Yea-sacc, suggesting that propionate would derive from the succinate pathway explaining the higher total gas volume released, probably in the form of carbon dioxide amount (Wolin and Miller, 1988). As both gas production and substrate digestibility were increased with the high-forage diet when Yea-sacc 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 Yea-sacc. 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 60:40 forage:concentrate ration, 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 microorganisms could be stimulated by yeasts at initial stages of fermentation, but these effects would become negligible in the medium-term with long-lasting fermentations.

5. Conclusions

Both inactivated and live yeast products tested in the trial may stimulate ruminal fermentation, although such 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 our 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 subtle with a high concentrate diet.

Acknowledgements

This work was funded by the Regione Piemonte, Assessorato Qualità, Ambiente e Agricoltura (Project Rep. n. 13428), the Ministero dell'Università e della Ricerca, PRIN (Project n. 2007P8JMWJ_002), and Junta de Castilla y León, Spain (Project n. GR158). All the authors contributed equally to the work described in this paper. Mention of trade names is for the benefit of the reader and does not constitute endorsement by the University of Turin, Italy, and of Leon, Spain, over other products not mentioned.

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