Mode of action of the yeast *Saccharomyces cerevisiae* as a feed additive for ruminants

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Two suggested modes of action of yeast in stimulating rumen fermentation were investigated. The first, that yeast respiratory activity protects anaerobic rumen bacteria from damage by O_2, was tested using different strains of yeast that had previously been shown to have differing abilities to increase the viable count of rumen bacteria. *Saccharomyces cerevisiae* NCYC 240, NCYC 1026, and the commercial product Yea-Sacc®, added to rumen fluid *in vitro* at 1·3 mg/ml, increased the rate of O_2 disappearance by between 46 and 89%. The same three preparations also stimulated bacterial numbers in an *in vitro* fermenter (Rusitec). *S. cerevisiae* NCYC 694 and NCYC 1088, which had no influence on the viable count in Rusitec, also had no effect on O_2 uptake. Respiration-deficient (RD) mutants of *S. cerevisiae* NCYC 240 and NCYC 1026 were enriched by repeated culturing in the presence of ethidium bromide. *S. cerevisiae* NCYC 240 and NCYC 1026 stimulated the total and cellulolytic bacterial populations in Rusitec, while the corresponding RD mutants did not. Rigorous precautions to exclude air from Rusitec resulted in *S. cerevisiae* NCYC 240 no longer stimulating total bacterial numbers, although it still increased numbers of cellulolytic bacteria. The second hypothesis, that yeast provides malic and other dicarboxylic acids which stimulate the growth of some rumen bacteria, was examined by comparing the effects of yeast and malic acid on rumen fermentation in sheep. Three mature sheep were given 0·85 kg barley/d plus 0·55 kg chopped ryegrass hay/d either unsupplemented, or supplemented with 4 g *S. cerevisiae* NCYC 240/d or 100 mg L-malic acid/d either mixed with the diet or in aqueous solution infused continuously into the rumen. Yeast increased the total viable count of bacteria ($P < 0·05$) whereas malic acid did not, and no other effect of the treatments reached statistical significance. It was concluded, therefore, that the stimulation of rumen bacteria by *S. cerevisiae* is at least partly dependent on its respiratory activity, and is not mediated by malic acid.

Malic acid: Probiotics: Ruminants: *Saccharomyces cerevisiae*: Yeast

Many possible modes of action have been suggested to explain the effects that yeast culture, based on *Saccharomyces cerevisiae*, can have on rumen fermentation and ruminant production (Rose, 1987; Martin & Nisbet, 1992; Wallace & Newbold, 1992; Dawson, 1993). An increase in bacterial numbers recovered from the rumen is the most reproducible effect of dietary yeast supplementation, and it has been suggested that the increased bacterial population is central to the action of the yeast in improving ruminant productivity (Wallace & Newbold, 1992). What causes the increased bacterial count is not clear, however. Removal of O_2, which would inhibit the growth of the strictly anaerobic bacteria of the rumen, was suggested by Rose (1987), but no experimental evidence has appeared in support of this hypothesis. On the other hand, yeast has been shown to provide nutrients which stimulate the growth of certain rumen micro-organisms. Aqueous extracts prepared from *S. cerevisiae* stimulated the growth and activity of the lactic acid-utilizing rumen bacterium *Selenomonas ruminantium*, in pure culture (Nisbet & Martin, 1990, 1991). The cause of the stimulation appeared to be the high dicarboxylic acid, particularly malic acid,

content of the yeast (Nisbet & Martin, 1990, 1991). Yeast has been shown also to provide vitamins to support the growth of rumen fungi (Chaucheyras *et al.* 1995).

The objective of the present experiments was to resolve the most important mode of action of yeast in stimulating the mixed rumen population. Different strains of *S. cerevisiae* differ in their ability to stimulate bacterial numbers in the rumen (Jouany *et al.* 1991; Newbold *et al.* 1995). These strains were compared for their effects on O_2 uptake by rumen fluid. The malic acid hypothesis was tested by determining the malic acid content of *S. cerevisiae* and by comparing the effects of malic acid and yeast on rumen fermentation.

MATERIALS AND METHODS

Preparation of yeast cultures and yeast mutants

S. cerevisiae NCYC 240 and NCYC 1026 were prepared by growth on malt-extract broth in fed-batch culture as described previously (Newbold *et al.* 1995). Respiration-deficient (RD) mutants were isolated by repeated culturing in the presence of 20 μg ethidium bromide/ml as described by Rickwood *et al.* (1988). Incubations were terminated after 10–12 h. Viable cells were enumerated by plating out on a glucose (g/l; glucose 20, yeast extract 10, Bactocasitone 10, (Difco Laboratories, Detroit, Michigan, USA), agar 10) or glycerol medium (g/l; glycerol 20, yeast extract 10, Bactocasitone 10, agar 10) and incubating overnight at 30°. The yeast was harvested along with spent medium by freeze-drying. Yea-Sacc, a commercial yeast supplement (provided by Alltech Biotechnology Center, Nicholasville, KY, USA), was used as supplied.

Measurement of oxygen uptake by rumen fluid in vitro

To determine the effect of different yeast preparations on O_2 removal from rumen fluid, *in vitro* incubations were carried out with rumen fluid withdrawn from a rumen-cannulated sheep. The sheep received 1·4 kg/d of a mixed diet consisting of hay, barley, molasses, fishmeal and a mineral and vitamin mixture (500, 229·5, 100, 91 and 9·5 g/kg DM respectively) in two equal meals. Rumen fluid was removed, via the cannula, 1 h after the morning feed, strained through two layers of muslin and maintained at 39° under O_2-free CO_2 before use. Dried yeast culture (200 mg), prepared as described previously (Newbold *et al.* 1995), was incubated in rumen fluid (30 ml) mixed with anaerobic 50 mM-potassium phosphate buffer (pH 6·5) plus glucose (5 g/l; 120 ml). In control incubations, malt-extract broth (200 mg; Oxoid Ltd, Basingstoke, Hants), the main component of the medium used to grow the yeast, was added instead of yeast. Incubations were carried out at 39° under a stream of Ar. Incubations were carried out in a gas-tight plastic vessel (total volume 250 ml) and mixed using a magnetic stirrer bar. After allowing the O_2 electrode reading to stabilize (approximately 5 min), O_2 was added to the system by injecting 10 ml water bubbled with air and maintained at 39°. The decline in O_2 concentration was monitored by means of an indwelling Clark O_2 electrode (WPA, Linton, Cambs.) connected via a pico-ammeter to a chart recorder. Rates of uptake were calculated from the linear phase of the decline. The electrode was calibrated as described by Rickwood *et al.* (1988), assuming the O_2 concentration in water at 39° to be 215 μM. O_2 uptake experiments were completed within 2 h of removing the rumen fluid from the sheep; O_2 uptake by rumen fluid in the absence of yeast did not change during this time. Incubations were conducted in duplicate with rumen fluid withdrawn on two separate days (n 4). Results were analysed by a one-way ANOVA. Each incubation was considered to be an experimental unit. Following a

significant F test ($P < 0{\cdot}05$), significant differences between means due to yeast addition were determined by the Student's t test (Snedecor & Cochran, 1976).

Evaluation of yeast preparations in Rusitec

The effect of *S. cerevisiae* NCYC 240 and NCYC 1026 and their respective RD mutants on rumen fermentation was investigated using the rumen-simulating fermentor, Rusitec (Czerkawski & Breckenridge, 1977). The nominal volume in each vessel was 850 ml and the dilution rate was set at 0·88/d. Inocula for the fermentation vessels were obtained from a rumen-fistulated non-lactating Friesian cow receiving 1 kg hay, 1 kg grass nuts and 1 kg concentrate (eight parts maize, two parts oats, two parts bran, one part linseed cake, one part white fish meal, with added vitamin supplement; Eadie & Gill, 1971) twice daily. The basal diet used in Rusitec consisted of grass hay, barley, molasses, white fishmeal and a mineral and vitamin mixture (500, 299·5, 100, 91 and 9·5 g/kg DM respectively). The food for the fermentation vessels (20 g/d) was provided in nylon bags (110 × 60 mm; mean pore size 50 μm), which were gently agitated in the liquid phase. Two bags were present at any time and one bag was replaced each day to give a 48 h incubation. While the bag was being changed, the vessels were flushed with CO_2 to help maintain anaerobiosis. Artificial saliva (pH 8·4; McDougall, 1948) was pumped constantly into the vessels. Each yeast culture prepared as described previously was added to triplicate vessels at 500 mg/d. Three vessels received no addition. Yeast addition was continued for 21 d. All measurements were made during the last 4 d of the experiment. Samples were taken either during the addition of a new feed bag or from the liquid overflow at the time of feeding. Trichloroacetic acid (TCA; 250 g/l; 100 ml) was added to the overflow daily in order to stop microbial activity and preserve fermentation products. The volume of the overflow together with the concentration of fermentation products were used to calculate the daily output of fermentation products. pH was measured in samples of fermentation fluid withdrawn from around the feed bags at the time of feeding using a pH electrode connected to a Russell 660 pH meter (Russell pH, Auchtermuchty, Scotland). Fermentation products were determined on samples taken from the liquid overflow. Volatile fatty acids (VFA) were determined by GLC as described by Stewart & Duncan (1985). NH_3 was measured by the phenol–hypochlorite method of Whitehead *et al.* (1967). L-lactic acid was determined by the automated method of Goodall & Byers (1978) using porcine L-lactate dehydrogenase (*EC* 1.1.1.27).

Bacterial numbers were determined in digesta removed from bags incubated for 48 h. Solids (1 g) and 20 ml of the free liquid were homogenized, in a gas phase of O_2-free CO_2, for 60 s using an MSE top-bladed homogenizer at full speed. Dilution series were prepared under O_2-free CO_2 by the anaerobic method of Bryant (1972) using the anaerobic diluent described by Mann (1968). Total viable counts of bacteria were made in roll tubes on Hobson's medium 2, a rumen fluid-containing medium which contains lactate, glucose, maltose and cellobiose as C sources (Hobson, 1969). The medium was dispensed under O_2-free CO_2 into Hungate tubes sealed with butyl rubber stoppers (Bellco Glass, Vineland, NJ, USA). Roll tubes were incubated for 72 h at 39°. The numbers of cellulolytic bacteria capable of degrading filter paper were determined using the method of Mann (1968). Counts of ciliate protozoa were carried out microscopically in a counting chamber (Newbold *et al.* 1987).

The digestibility of the diet at 24 and 48 h was estimated from the DM remaining in the bags after incubation. The DM in incubated bags and in samples of non-incubated feed was determined by drying at 105° for 24 h. Results were analysed by a one-way ANOVA table. Each vessel was considered as an experimental unit. Following a significant F test ($P < 0{\cdot}05$),

significant differences between means due to yeast addition were determined by the Student's *t* test (Snedecor & Cochran, 1976).

S. cerevisiae NCYC 240 was evaluated further in a modified Rusitec under conditions where steps were taken in addition to those normally employed to exclude air from the fermenter. Ar was continuously flushed over the headspace to maintain a positive pressure within the vessel. The artificial saliva infused into the vessel was degassed by boiling, then allowed to cool while bubbling with Ar to displace O_2 and maintained under an atmosphere of Ar. Feed bags were stored under CO_2 for at least 24 h before use and the vessels were flushed with Ar during feeding. *S. cerevisiae* NCYC 240 was prepared as before and was tested both in normal-O_2 and low-O_2 vessels as described previously. Dissolved O_2 concentrations in the vessels were monitored using Ag–Pb electrodes, the signal from which was polarized, then amplified and displayed on a chart recorder. All treatment combinations (yeast $\times$ O_2) were evaluated in triplicate vessels. Results were compared by an ANOVA table with a factorial structure to determine the effects of O_2, yeast and yeast $\times$ O_2 interactions. All statistical analysis was carried out using the Genstat 5 computer program (Genstat 5 Committee, 1987).

Effects of Saccharomyces cerevisiae *and malic acid on rumen fermentation in sheep*

Rumen fluid was withdrawn 2 h after feeding, from the same rumen-fistulated non-lactating Friesian cow used previously, strained through two layers of muslin, autoclaved and then centrifuged at 20000 ***g*** for 15 min. *S. cerevisiae* NCYC 240 (20 mg; obtained in bulk from the National Collection of Yeast Cultures, Norwich) was added to 10 ml of the supernatant fraction and the mixture was incubated under CO_2 at 39° for 2 h. In one experiment, samples were taken into trichloroacetic acid (1 ml sample plus 0·25 ml TCA, 250 g/l), while in a second experiment incubations were stopped by placing the samples in ice. Samples were centrifuged (14000 ***g*** for 5 min) and the supernatant fraction was analysed enzymically for L-malic acid by the method of Mollering (1985). All incubations were carried out in duplicate on two separate days.

Four mature Dorset-cross sheep (average weight 70 kg) each fitted with a permanent rumen cannula were given 0·85 kg barley/d plus 0·55 kg hay/d divided into two feeds. Hay was prepared from mature mixed-grass swards. The sward consisted mainly of perennial ryegrass (*Lolium perenne*) and timothy (*Phleum pratense*). The diet was given unsupplemented, or supplemented at each meal with 2 g *S. cerevisiae* NCYC 240 (obtained in bulk from the National Collection of Yeast Cultures, Norwich) or 50 mg L-malic acid, mixed with the barley. In an attempt to simulate the possible continuous release of malic acid into rumen fluid from lysing yeast cells, L-malic acid was infused at a constant rate (100 mg/d) into the rumen in 50 ml water. The experiment was designed as a Latin square with four periods of 18 d. Rumen samples were removed on two consecutive days during the last 4 d of each period at 0, 1, 2, 4 and 6 h after the morning feeding. Rumen samples were treated and analysed for pH, VFA, L-lactate and NH_3 as described previously. Samples were withdrawn 2 h after feeding to enumerate total cultivable and cellulolytic bacteria as described previously. Lactate-utilizing bacteria were enumerated in the same samples as described by Mackie & Heath (1979). The DM loss of hay from nylon bags was determined by the method of Mehrez & Ørskov (1977). Samples were incubated in the rumen for 0, 8, 16, 24, 48, 72 and 96 h and when withdrawn from the rumen were washed in a domestic washing machine in cold water for 18 min and then dried to constant weight.

The sheep experiment was designed initially as a 4 $\times$ 4 Latin square, but one animal (receiving yeast) stopped eating after 2 d on the diet and had to be removed from the trial. Due to the removal of an animal early in period 1 the data for the three remaining sheep was

analysed as a 3 × 4 Youden square, using Genstat 5 (Genstat 5 Committee, 1987). Sampling time was considered as a subplot. However, since the effect of time after feeding did not vary between the treatments, mean values were analysed and are reported on pp. 255–257.

RESULTS

Influence of Saccharomyces cerevisiae *on oxygen uptake by rumen fluid* in vitro

O_2 concentrations before the addition of oxygenated water to strained rumen fluid were low (1 μM-O_2). They rose transiently to approximately 13 μM following the injection of water. The decline in dissolved O_2 was rapid and had reached baseline levels within approximately 90 s. *S. cerevisiae* NCYC 240, NCYC 1026, and Yea-Sacc significantly ($P < 0.05$) stimulated O_2 uptake from rumen fluid (Table 1). In contrast, *S. cerevisiae* NCYC 694 and NCYC 1088 had no effect (Table 1).

Effects of respiration-deficient mutants in Rusitec

Ethidium bromide was used to select RD mutants of *S. cerevisiae* NCYC 240 and NCYC 1026 (Rickwood *et al.* 1988). Mutant cultures were capable of growth on glucose but not glycerol and no uptake of O_2 was detected when fresh cultures (before freeze-drying) were added to phosphate buffer, consistent with the cultures being unable to use O_2 for respiration. When grown on glucose, 10·9, 10·2, 9·0 and 8·2 (SE of difference (SED) 1·45) × 10^7 cells/ml were recovered from *S. cerevisiae* strains NCYC 240, NCYC 1026, NCYC 240 RD and NCYC 1026 RD cultures respectively before drying. The freeze-dried preparations from the mutants did not stimulate O_2 uptake by rumen fluid (106, 179, 151, 110 and 108 (SED 19) nmol O_2/min per ml rumen fluid for controls with no addition, strain NCYC 240, strain NCYC 1026, mutant strain NCYC 240 RD and mutant strain NCYC 1026 RD respectively).

Yeast addition had no influence on pH, fermentation products or digestion of DM in Rusitec ($P > 0.05$; Table 2). Significant effects were restricted to changes in the bacterial population. Parent strains NCYC 240 and NCYC 1026 stimulated total and cellulolytic counts, but the corresponding RD mutants did not. Thus, selecting RD mutants seemed to eliminate the probiotic activity of the effective yeast strains.

Effects of Saccharomyces cerevisiae *NCYC 240 under decreased oxygen conditions in Rusitec*

The influence of decreasing O_2 contamination on the efficacy of *S. cerevisiae* NCYC 240 was evaluated in a Rusitec in which a number of different measures were applied to decrease the entry of air to the fermenter. The effectiveness of these measures was assessed by measuring dissolved O_2 concentrations in the vessels using Ag–Pb electrodes. Poisoning of the electrode by the dissolved sulphides in the fluid rendered the initial calibration made in water unusable. The signal appeared to stabilize after 12 h. However, attempts to recalibrate the electrodes in fermenter fluid after prolonged incubation proved unsuccessful as the inherent reducing capacity of rumen fluid made it impossible to oxygenate the system fully. A zero value could be obtained, however, and the data were interpreted by assuming that dissolved O_2 in the control vessels (no yeast, normal oxygen) was 7·5 μM (Hillman *et al.* 1985*b*). The values for the other vessels were then calculated by assuming a linear relationship between electrode output and O_2 concentration. Degassing the buffer and feed bags while maintaining the vessels under a constant stream of Ar significantly decreased the dissolved O_2 concentration. Yeast also decreased O_2 concentrations (7·5, 5·8, 5·2 and 3·2 μM-O_2 in normal-O_2, normal-O_2 plus yeast, low-O_2 and low-O_2 plus yeast vessels respectively).

Table 1. *Comparison of the effects of different preparations of* Saccharomyces cerevisiae *on the rate of removal of oxygen from rumen fluid and on bacterial numbers in Rusitec**

(Mean values for twenty-four determinations for O_2 uptake and for eighteen determinations for bacterial numbers)

Treatment†	O_2 uptake (nmol/min per ml rumen fluid)	Total viable bacterial no. recorded in Rusitec‡ ($\times 10^8$/ml)
Control	102[a]	2·7[a]
S. cerevisiae:		
NCYC 694	108[a]	2·9[a]
NCYC 1026	146[b]	3·7[b]
NCYC 240	189[c]	3·9[b]
NCYC 1088	104[a]	2·5[a]
Yea-Sacc	164[bc]	4·3[b]
SE	79	0·25

[a, b, c] Mean values in the same column with different superscript letters were significantly different ($P < 0·05$).

* Rumen-simulating fermenter developed by Czerkawski & Breckenridge (1977).

† For details, see pp. 250–252.

‡ Yeast culture preparations were added to Rusitec (500 mg/d) and bacterial numbers were determined after 21 d. For further details, see Newbold *et al.* (1995).

Table 2. *Effects of respiration-deficient (RD) mutants of* Saccharomyces cerevisiae *NCYC 240 and 1026 on fermentation in Rusitec**

(Mean values for fifteen determinations)

Treatment†...	Control	NCYC 240	NCYC 1026	NCYC 240 RD	NCYC 1026 RD	SE
pH	6·85	6·88	6·96	6·94	6·91	0·05
Volatile fatty acids (mmol/d)						
Acetate	26·0	26·7	27·4	26·9	28·4	1·45
Propionate	7·2	6·8	7·8	6·8	7·4	0·56
Butyrate	8·0	7·7	7·9	7·3	8·8	0·49
L-Lactate (mmol/d)	0·24	0·17	0·18	0·23	0·17	0·03
NH_3-N (mmol/d)	3·55	4·08	4·60	3·87	4·26	0·339
Digestion of DM (g):						
After 24 h incubation	5·2	7·2	6·1	5·7	5·6	1·09
After 48 h incubation	8·9	10·3	9·4	8·5	8·9	0·92
Total viable bacteria ($\times 10^8$/ml)	2·8[a]	5·1[c]	3·9[b]	2·7[a]	2·8[a]	0·28
Cellulolytic bacteria ($\times 10^6$/ml)	3·5[a]	37·3[b]	87·3[c]	5·3[a]	4·5[a]	8·0
Protozoa ($\times 10^3$/ml)	2·7	4·0	4·5	3·2	3·0	1·02

[a, b, c] Mean values in the same horizontal row with different superscript letters were significantly different ($P < 0·05$).

* Rumen-simulating fermenter developed by Czerkawski & Breckenridge (1977).

† For details, see pp. 250–251.

The pH at the time of feeding was significantly ($P < 0·05$) higher in low-O_2 vessels (Table 3), probably because the constant stream of Ar over these vessels removed dissolved CO_2. There were no significant ($P > 0·05$) treatment effects on the production of VFA or L-lactate (Table 3). Yeast increased the output of NH_3 significantly ($P < 0·05$; Table 3),

Table 3. *Consequences of decreasing oxygen contamination of Rusitec* on the effects of* Saccharomcyes cerevisiae *NCYC 240 on fermentation characteristics*

(Mean values for twelve determinations)

Treatment†...	Normal O_2		Low O_2			Statistical significance: $P <$		
	No yeast	Plus yeast	No yeast	Plus yeast	SED	Effect of O_2	Effect of yeast	Interaction of O_2 and yeast
pH	7·11	7·14	7·30	7·26	0·027	0·001	0·564	0·083
Volatile fatty acids (mmol/d)								
Acetate	24·9	24·2	25·6	27·1	1·71	0·060	0·697	0·803
Propionate	6·6	6·0	6·2	6·0	0·846	0·749	0·531	0·798
Butyrate	8·0	6·9	7·1	8·6	1·81	0·757	0·878	0·329
L-Lactate (mmol/d)	0·20	0·23	0·17	0·24	0·069	0·869	0·354	0·719
NH_3-N (mmol/d)	3·49	3·86	3·12	4·12	0·0408	0·975	0·035	0·240
Digestion of DM (g):								
After 24 h incubation	6·2	7·2	6·9	6·7	0·805	0·912	0·525	0·292
After 48 h incubation	8·5	8·6	8·4	8·6	0·539	0·791	0·761	0·845
Total bacteria ($\times 10^8$/ml)	2·7	3·8	4·8	5·0	1·031	0·035	0·500	0·700
Cellulolytic bacteria ($\times 10^6$/ml)	3·8	12·9	56·0	141·0	30·4	0·003	0·050	0·115
Protozoa ($\times 10^3$/ml)	2·5	1·8	3·2	4·8	1·231	0·065	0·606	0·208

SED, standard error of difference.

* Rumen-simulating fermenter developed by Czerkawski & Breckenridge (1977).

† For details, see pp. 250–252.

probably due to breakdown of yeast protein. The total and cellulolytic bacterial populations were greater ($P < 0·05$) in control vessels when *S. cerevisiae* NCYC 240 was present ($P > 0·05$; Table 3). Both populations were greater in low-O_2 vessels than in the untreated controls, and stimulation by yeast occurred only with the cellulolytic population ($P > 0·05$; Table 3). The trend towards greater DM digestion at 24 h in control vessels receiving yeast and in low-O_2 vessels (Table 3) was consistent with a stimulation of the cellulolytic flora.

Effects of Saccharomyces cerevisiae *and malic acid on rumen fermentation in sheep*

L-Malic acid was produced by *S. cerevisiae* when yeast cells were incubated in autoclaved rumen fluid (Fig. 1). The initial concentration of L-malic acid in a 2 g/l suspension of yeast was 10 mg/l, which increased to 25·5 mg/l after 2 h, equivalent to 12·8 mg L-malic acid/g yeast. Chilling the suspension followed by removal of yeast cells by centrifugation resulted in low concentrations of L-malic acid in the supernatant fraction (Fig. 1), indicating that the L-malic acid was almost wholly intracellular.

The effects of dietary yeast (4 g/d) and L-malic acid (100 mg/d) and of the continuous infusion of the same quantity of L-malic acid directly into the rumen were compared in sheep (Table 4). The amount of malic acid was chosen to be more than twice the maximum that would be supplied by 4 g yeast. Neither yeast nor malic acid had a significant effect on the mean concentrations of rumen fermentation products in sheep (Table 4), nor were any significant differences noted in the pattern of the fermentation with time after feeding (data not shown). The inclusion of malic acid in the diet caused a non-significant decrease in the concentration of L-lactic acid in the rumen (Table 4); however, this was not associated with an increase in rumen pH (Table 4). There was a trend towards a lower concentration of NH_3 in sheep supplemented with *S. cerevisiae* (Table 4).

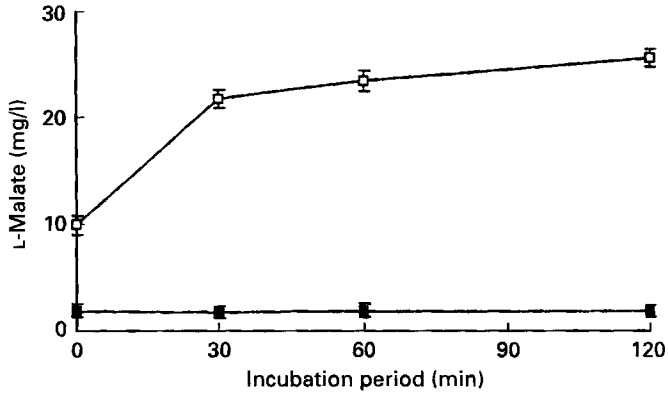

Fig. 1. Production of L-malic acid by *Saccharomyces cerevisiae* NCYC 240 incubated in rumen fluid. Yeast (20 mg) was incubated in autoclaved, centrifuged rumen fluid (10 ml) at 39° for 2 h. Malic acid was measured in the supernatant fraction when the reaction was stopped with trichloroacetic acid (final concentration 50 g/l; □) and when the reaction was stopped in ice (■). Results are the means with their standard errors represented by vertical bars for four incubations.

Table 4. *Effects of* Saccharomyces cerevisiae *NCYC 240, dietary* L-*malic acid or* L-*malic acid infused intraruminally on fermentation in the rumen of sheep*

Treatment*...	Control	NCYC 240 added to diet	Malic acid added to diet	Malic acid infused into rumen	SED (3 df)
pH	6·32	6·34	6·22	6·36	0·039
Total volatile fatty acids (mmol/l)	107	112	113	97	8·12
Acetate (mmol/mol)	661	670	659	673	8·71
Propionate (mmol/mol)	137	130	143	138	10·82
Butyrate (mmol/mol)	153	156	153	151	2·91
Branched-chain and longer-chain fatty acids (mmol/mol)	48	42	45	38	8·09
L-Lactate (mmol/l)	1·14	1·29	0·941	1·05	0·112
NH_3-N (mmol/l)	12·9	10·7	12·1	12·0	1·34
Total bacteria ($\times 10^8$/ml)	3·93[a]	5·48[b]	4·41[a]	2·72[a]	0·480
Lactic acid-utilizing bacteria ($\times 10^7$/ml)	0·96	0·85	0·92	1·19	0·283
Cellulolytic bacteria ($\times 10^7$/ml)	2·18	3·77	3·61	1·96	0·874

[a, b] Mean values in the same row with different superscript letters were significantly different ($P < 0·05$).
SED, standard error of difference.
* For details, see pp. 252–253.

Addition of yeast caused a significant increase ($P < 0·05$) of 39% in the total bacterial population, but no treatment affected the number of lactate-utilizing bacteria (Table 4). There was a trend towards an increase in the population of cellulolytic bacteria with the addition of *S. cerevisiae* and malic acid to the diet, but the effect did not reach significance (Table 4).

Table 5. *Effects of* Saccharomyces cerevisiae *NCYC 240, dietary* L*-malic acid or* L*-malic acid infused intraruminally on the degradation of hay in the rumen of sheep*

	DM loss (%) from nylon bags				
Treatment* ...	Control	NCYC 240 added to diet	Malic acid added to diet	Malic acid infused into rumen	SED (3 df)
Incubation period (h)					
0	29·5	29·5	29·5	29·5	—
8	32·8	32·5	38·3	30·4	3·87
16	38·3	42·2	42·5	37·2	1·39
24	41·8	46·9	47·4	42·6	5·55
48	51·2	53·7	54·6	52·0	7·35
72	58·1	59·9	57·3	54·9	6·58
96	62·6	64·1	60·2	58·0	4·76

SED, standard error of difference.
* For details, see pp. 252–253.

The degradation of hay incubated in nylon bags in the rumen was not affected significantly by the treatments at any of the time intervals studied (Table 5). There was, however, a trend towards a higher disappearance of hay in bags incubated in the rumen of sheep supplemented with yeast or dietary malic acid for 16 or 48 h.

DISCUSSION

Any mode of action proposed for yeast in the rumen must be consistent with certain experimental observations. These include the findings that yeast extract, which contains large amounts of vitamins, amino acids and other nutrients, does not stimulate the mixed fermentation (Newbold & Wallace, 1992) or the growth of *F. succinogenes* on cellulose *in vitro* (Dawson, 1990) in the same way as yeast; some strains of yeast are effective whereas others are not (Newbold *et al.* 1995); autoclaved *S. cerevisiae* had no effect on bacterial numbers in rumen-simulating fermenters (Dawson *et al.* 1990; El Hassan *et al.* 1993); and yeast that had been sterilized by gamma-irradiation, although it contained no cells capable of division, retained its ability to stimulate total bacterial numbers (El Hassan *et al.* 1993). Thus, either a heat-sensitive nutrient present only in some strains is destroyed by autoclaving or the yeast has a metabolic activity that is denatured by heat but not affected by irradiation and does not require cell growth for the metabolic activity to be sustained.

A beneficial effect of malic acid on rumen fermentation was found by Kung *et al.* (1982) and Martin & Streeter (1995). The quantities used were very much higher than would be supplied by yeast as a dietary additive. Malic acid present in the yeast as added to the diet was calculated to be only 5 mg/g yeast, equivalent to 20 mg/d per sheep. This amount corresponds well with that found in aqueous extracts of commercial yeast culture by Nisbet & Martin (1991) and by ourselves (unpublished results). Malic acid present in the dried yeast would be expected to survive autoclaving. However, if larger quantities were necessary to provide the stimulatory effect, a metabolically-active form of yeast, continuously producing malic acid in rumen digesta, might clearly be necessary. The 100 mg amounts of malic acid added to the diet or infused into the rumen were selected, therefore, to simulate both the malic acid present in the dried yeast plus malic acid that would be produced in the rumen by metabolically-active yeast. Since the malic acid is

intracellular and, therefore, unavailable to rumen bacteria unless yeast cells lyse, this quantity can be considered to be the maximum amount of malic acid that might be available. When the influence of malic acid was compared with that of *S. cerevisiae* NCYC 240 in sheep, the only statistically significant result was an increase in viable bacterial numbers in the rumen with the yeast. No significant changes occurred with malic acid. Thus, malic acid in yeast produces highly significant effects on growth and metabolic activities with certain pure cultures of rumen micro-organisms *in vitro* (Nisbet & Martin, 1990, 1991), but it does not appear to cause the most important effects of yeast *in vivo*.

S. cerevisiae does not grow in rumen fluid (Arambel & Tung, 1987) but retains its metabolic activity (Ingledew & Jones, 1982) and viability (El Hassan, 1994). Several of the metabolic activities of yeast could affect rumen fermentation (Rose, 1987; Wallace & Newbold, 1992). Among these is O_2 consumption. Rumen contents are essentially anaerobic, but low concentrations of dissolved O_2 can be detected during the daily feeding cycle (Scott *et al.* 1983). O_2 enters the rumen while the animal is eating, both with the feed and in saliva, such that peaks in O_2 concentration occur at approximately the time of feeding (Hillman *et al.* 1985*a*). Many rumen micro-organisms are highly sensitive to the presence of O_2 (Loesche, 1969; Stewart & Bryant, 1988). Rose (1987) speculated that the high respiratory activity of *S. cerevisiae* might allow it to scavenge O_2, thus protecting the strictly anaerobic bacteria.

Published values for O_2 uptake by *S. cerevisiae* (200–300 μmol/min per g; Barford & Hall, 1979) suggest that they have respiratory rates several orders of magnitude greater than rumen fluid. Thus, even at the low inclusions used in ruminant diets, yeast might still be expected to exert an effect on the rate of O_2 uptake in rumen fluid. It is less clear from the literature how much respiratory activity varies between strains. The incubation used here to measure O_2 consumption was similar to the system used by Ellis *et al.* (1989) except that, whereas Ellis *et al.* (1989) added O_2 by changing the composition of the gas phase, O_2 was added here via the injection of oxygenated water. Basal O_2 concentrations (approximately 1 μM) were of the same order as those described in the rumen over much of the day (Scott *et al.* 1983). The peak in O_2 concentration after the addition of water (approximately 13 μM) was four-fold higher than peaks detected in the rumen at approximately the time of feeding (Hillman *et al.* 1985*a*). Using mass-inlet mass spectrometry we found a decline in O_2 concentrations in the rumen fluid of sheep after eating, but concentrations rose again 1 h after eating stopped such that O_2 was measurable (1–3 μM) in rumen contents for much of the day (C. J. Newbold, R. J. Wallace and F. M. McIntosh, unpublished results). Rates of O_2 uptake in the control incubation were comparable with those previously reported at similar O_2 concentrations (Ellis *et al.* 1989). The respiratory activity of cultures of *S. cerevisiae* NCYC 240, NCYC 694, NCYC 1088 and NCYC 1026 before drying, measured in absence of rumen fluid, was lower than that reported previously, but did not differ between strains (155 (SE 20·8) μmol/min per g protein). Freeze-drying decreased the respiratory activity, but again the activity measured did not differ between strains (31·5 (SE 3·32) μmol/min per g DM). However, freeze-dried preparations of *S. cerevisiae* NCYC 240, NCYC 1026 and the commercial product Yea-Sacc all significantly stimulated the rate of O_2 uptake by rumen fluid, while *S. cerevisiae* NCYC 694 and NCYC 1088 failed to affect O_2 consumption. The reason for this difference between the strains, which only arose when they were incubated in rumen fluid, is unknown. The effect of *S. cerevisiae* NCYC 240, NCYC 694, NCYC 1088 and NCYC 1026 on bacterial numbers in Rusitec was described previously (Newbold *et al.* 1995); this information is included in Table 1 for comparative purposes. The ability of different yeast preparations to stimulate the viable count of bacteria in the sheep rumen appears to correspond with their ability to remove O_2 from rumen fluid. Furthermore, when RD mutants of the stimulatory strains, NCYC 240 and

NCYC 1026 were tested in Rusitec, they had lost their stimulatory activity, suggesting that O_2 consumption was required for activity. More stringent precautions to exclude air than had been used by Czerkawski & Breckenridge (1977), who developed the Rusitec fermenter, resulted in yeast stimulating only cellulolytic bacteria, which appear to be more sensitive to dissolved O_2 than the total population and, therefore, more responsive to protection by yeast.

Conclusions

Although the rumen is widely considered to be anaerobic, rumen gas contains between 5 and 10 ml O_2/l (MacArthur & Multimore, 1962), and significant concentrations of O_2 can be detected in the liquid phase, as discussed previously. Czerkawski (1969) calculated that O_2 transfer from saliva, food, and diffusion of the blood of the host animal might account for 38 litres O_2 entering the rumen of a sheep daily. In the present study, rates of O_2 uptake by rumen fluid were measured at between 60 and 100 nmol/min per ml. Assuming the rumen volume of a sheep to be 6 litres, this equates to an O_2 consuming capacity of between 11·5 and 16·1 litres/d. Thus, the indications from Table 1, where yeast added at 1·33 g/l produced a near-doubling of respiratory activity, are that dietary yeast culture will make a significant difference to the O_2-consuming activity of rumen fluid and to the residual concentrations that may persist. O_2 is toxic to anaerobic bacteria and it inhibits the growth of rumen bacteria in pure culture (Loesche, 1969; Marounek & Wallace, 1984) and the adhesion of cellulolytic rumen bacteria to cellulose (Roger *et al.* 1990). The presence of a respiring yeast, therefore, would be predicted to be beneficial to the rumen microflora. This may not be the only beneficial effect of yeast, but in view of the results presented here, O_2 consumption, as originally proposed by Rose (1987), appears to be at least partly responsible for the probiotic activity of yeast cultures.

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