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Influence of *Aspergillus oryzae* fermentation extract on the fermentation of a basal ration in the rumen simulation technique (Rusitec)

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SUMMARY

The effects of adding a culture extract from *Aspergillus oryzae* (AO) to the fermentation of a basal ration were investigated using the rumen simulation technique (Rusitec). The ration consisted of hay, barley, molasses, fishmeal and a minerals/vitamins mixture, at 500, 299·5, 100, 91 and 9·5 g/kg dry matter respectively. AO eliminated the transient fall in pH that occurred following the addition of substrate to Rusitec, but had no influence on the buffering capacity of the medium. Increases in the acetate:propionate ratio and in the proportion of butyrate in the fermentation products were observed in vessels receiving AO. Ammonia concentrations increased by over 30%. The number of total viable bacteria almost doubled and the cellulolytic population increased three-fold in vessels receiving AO. Protozoal numbers were reduced by almost 45% by AO. The proportion of methane in the headspace gas collected from the vessels during the day was significantly ($P < 0·01$) lower in vessels receiving AO. These results demonstrate that AO has a marked effect on the microbial population and the stoichiometry of the rumen fermentation, and that these changes can be seen in an *in vitro* system.

INTRODUCTION

Probiotics (microbial preparations and their growth media) are of increasing interest as feed additives for both ruminant and nonruminant livestock. Two main types of product based on either yeast (*Saccharomyces cerevisiae*) or fungal (*Aspergillus oryzae*) cultures, alone or in combination with other microorganisms, are available for use with ruminants. They have been shown to improve the yield of milk and milk fat by dairy cows when added to some, but not all, diets (Harris *et al.* 1983; Van Horn *et al.* 1984). These improvements were associated with changes in rumen fermentation. The stoichiometry of the fermentation moved towards increased acetate and butyrate production in the presence of probiotics, and viable counts of total and cellulolytic bacteria increased (Weidmeier *et al.* 1987).

The present study describes the use of the rumen simulation technique Rusitec (Czerkawski & Breckenridge 1977) to study the effects of a culture extract from *A. oryzae* (AO) on the rumen fermentation *in vitro*.

MATERIALS AND METHODS

Apparatus

The rumen simulation technique Rusitec was used as described by Czerkawski & Breckenridge (1977). The nominal volume in each vessel was 850 ml and the dilution rate was set at 0·88/day. Inocula for the fermentation vessels were obtained from a pooled sample from three rumen-cannulated sheep fed 1·4 kg/day of a forage/concentrate diet of hay, barley, molasses, fishmeal and a minerals and vitamins mixture (500, 299·5, 100, 91 and 9·5 g/kg dry matter, respectively).

The same diet was supplied to the fermentation vessels. The food for the fermentation vessels was provided in nylon bags (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.

Analytical methods

Fermentation products were measured in samples taken from the free liquid phase. Volatile fatty acids (VFAs) were determined by gas liquid chromatography (GLC) as described by Stewart & Duncan (1985). Ammonia was measured by the phenol-hyprochlorite method of Whitehead *et al.* (1967). L-Lactic acid was determined enzymatically (Goodall & Byers 1978). Protozoa in the liquid phase were counted as described previously (Newbold *et al.* 1987).

Headspace gas was collected as described by Czerkawski & Breckenridge (1977) and analysed by GLC on a 4 mm × 3 m glass column packed with Porapak Q mesh 60–80 (Waters Associates Inc., Milford, Mass, USA). The oven temperature was 25 °C and the carrier gas (helium) flow rate was 30 ml/min; a Katharometer detector was used. Peaks were identified by comparison with gas standards of known composition. The digestibility of the diet at 48 h was estimated from the dry matter remaining in the bags after 48 h incubation. The dry matter in incubated bags and in samples of nonincubated feed were determined by drying at 105 °C for 24 h.

Bacterial counts

Bacterial numbers were determined using digesta removed from bags incubated for 48 h. Liquid and solids from the bags were homogenized, under O_2-free CO_2, for 1 min 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 an anaerobic diluent (Mann 1968). Counts of total viable bacteria were made in roll tubes on medium 2 to which was added 2% (w/v) agar (Hobson 1969). The medium was dispensed into Hungate tubes sealed with butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ, USA). Roll tubes were incubated for 72 h at 39 °C. The numbers of cellulolytic bacteria capable of forming clearings in cellulose agar were determined using a modification of the cellulose roll tube medium of Hobson (1969) with 1·5% agar (Difco) and 1% (w/v) HCl-swollen ground filter paper (Stewart *et al.* 1981). Cellulose roll tubes were incubated for 21 days at 39 °C.

Experimental procedure

Six vessels were set up as described above and were fed at the same time every day with 18 g of the basal diet. Artificial saliva (pH 8·4) was constantly infused into the vessels (McDougall 1948). Three vessels received 250 mg *A. oryzae* fermentation extract (AO) (Amaferm; BioZyme Enterprises, Inc., St. Joseph, MO, USA) daily, while the others were controls. The duration of the experiment was 18 days with samples taken for analysis on each of the last 4 days. Samples were taken from the liquid phase during the addition of a new feed bag, or, in the case of pH determinations after feeding, by removing the top of the fermenter under a stream of CO_2 and withdrawing a portion of the liquid phase. Results are the means of duplicate samples from each vessel analysed using a standard analysis of variance table (Snedecor & Cochran 1976).

RESULTS

AO had no effect on the prefeeding pH in Rusitec (Table 1) but abolished the post-feeding dip in pH which was evident in the control vessels (Fig. 1). This was not caused by AO improving the buffering capacity of the fluid. AO added at a concentration of 0·25 mg/ml had no influence on pH changes observed in fermenter liquid or in strained rumen fluid in response to the addition of sufficient HCl to decrease the pH in controls from pH 6·5 to 6·1.

Several trends were apparent in the effects of AO on the fermentation. Total VFA concentrations were higher and there was a shift from propionate towards butyrate, valerate and branched-chained acids in vessels receiving AO (Table 1). The acetate to propionate ratio was increased by AO (Table 1). L-Lactate concentrations were low throughout, and the digestibility of dry matter at 48 h was unaffected. However, ammonia levels were significantly ($P < 0·001$) increased by AO addition (Table 1).

The number of total viable bacteria was higher in the vessels receiving AO. Cellulolytic numbers were also increased both in absolute terms ($P < 0·001$) and as a percentage of the total bacterial population (3·4 and 5·4% of the total count in the control and AO vessels respectively). Protozoal numbers were reduced by 45% in the presence of AO (Table 2).

There was a significant reduction ($P < 0·01$) in the proportion of methane in the headspace gas collected from vessels receiving AO additions (Table 1). The increase in hydrogen was not significant.

DISCUSSION

AO caused increases in the acetate:propionate ratio and in the proportion of butyrate in the fermentation products similar to those found previously *in vitro* (Arambel *et al.* 1987) and *in vivo* (Weidmeier *et al.* 1987). There were also increases in branched-chained VFA and ammonia concentrations, supporting the suggestion of Arambel *et al.* (1987) that AO increases rumen proteolysis, perhaps as a consequence of the endogenous proteolytic activity of *Aspergillus oryzae* (Boing 1983).

Stabilization of rumen pH is reputed to be one of the most important properties of probiotics for ruminants, and AO eliminated the drop in pH seen after addition of a new feed bag to the control vessels. Although the actual difference in pH between the two

Table 1. *Effect of* A. oryzae *fermentation extract (AO) on pH, dry matter (DM) digestion, volatile fatty acid (VFA), L-lactate and ammonia concentrations, and on the composition of headspace gases in Rusitec*

	AO addition (mg/day)		
	0	250	S.E.*
pH	6·87	6·87	0·041
Total VFA (mmol/l)	69·5	76·9	5·33
Acetate (mmol/mol)	441	410	19·7
Propionate (mmol/mol)	257	196	10·9
Isobutyrate (mmol/mol)	9	10	0·49
Butyrate (mmol/mol)	168	210	11·0
Isovalerate (mmol/mol)	39	46	2·0
Valerate (mmol/mol)	85	129	10·0
Acetate:propionate	1·72	2·10	0·15
L-Lactate (mmol/l)	0·41	0·51	0·06
NH_3-N (mg/l)	94	124	1·3
Digestion (g/day) of DM after 48 h *incubation* (input of DM: 18 g/day)	11·1	11·0	0·08
Headspace gas (%)			
H_2	0·071	0·074	0·020
CH_4	6·1	2·9	0·265

* D.F. = 4.

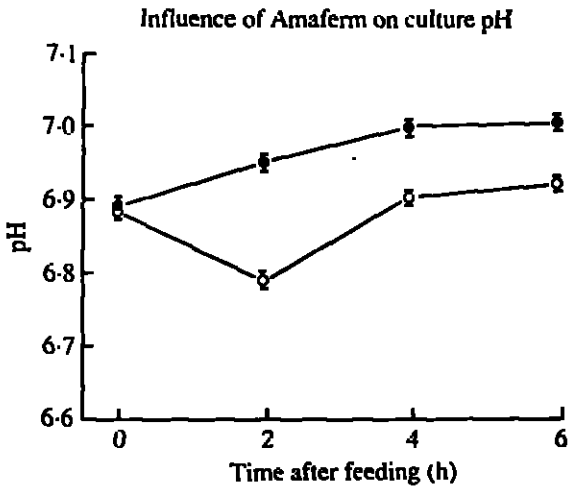

Fig. 1. Culture pH after the addition of fresh substrate to Rusitec in the absence (○) or presence (●) of *A. oryzae* fermentation extract. Results are the mean ± S.E. (I) from 3 vessels on each treatment.

Table 2. *Effect of* A. oryzae *fermentation extract (AO) on microbial numbers at time of feeding in Rusitec*

	AO addition (mg/day)		
	0	250	S.E.*
Total culturable bacteria ($\times 10^9$/ml)	15·1	27·1	0·45
Cellulolytic bacteria ($\times 10^9$/ml)	0·51	1·47	0·040
Protozoa ($\times 10^4$/ml)	3·94	2·15	0·416

* D.F. = 4.

treatments was small (0·15 units 2 h after feeding (Fig. 1)), it should be noted that Rusitec is more strongly buffered than rumen fluid. Titration of the medium used in the present experiments and of rumen fluid suggested that the drop in pH of 0·15 units in Rusitec at a pH of 6·5 would be equivalent to a 0·4 unit drop in rumen fluid withdrawn from an animal fed a similar diet. The mechanism whereby AO might stabilize rumen pH is unclear, but this property would have important implications for the cellulolytic flora, which fails as rumen pH approaches 6·0 (Stewart 1977). The bacterial cellulolytic population was stimulated by AO in the present experiments (Table 2) and *in vivo* (Weidmeier *et al.* 1987). Thus stabilization of pH might indirectly affect cellulolysis. However, the possibility that some synergism exists with the cellulase of *A. oryzae* (Boing 1983) cannot be discounted.

Despite the increased size of the cellulolytic flora, and in contrast with previous studies (Arambel *et al.* 1987; Weidmeier *et al.* 1987), AO had no influence on the extent of dry matter digestion. However, the current experimental conditions were exceptionally

favourable for fibre digestion in both the control and AO vessels, in that a long retention time was used and pH remained fairly stable. It is possible that although AO did not alter the amount of dry matter digested in 48 h it may have affected the rate of digestion. Other effects of AO in these experiments were to decrease methane production and protozoal numbers. The reduction in methane production was consistent with the increased production of reduced products such as butyrate and valerate. If this observation can be confirmed *in vivo*, it would imply that AO has a major effect on the pathways of intracellular hydrogen transfer within the rumen. Similar decreases in methane have been observed when products based on the yeast *Saccharomyces cerevisiae* were fed *in vivo* (Williams 1989). The depression in protozoal numbers could have been the reason why an increased bacterial count was observed in the presence of AO (Table 2; Weidmeier *et al.* 1987). Neither of these properties has been reported previously. Reduced predation by protozoa would presumably enable a larger bacterial flora to survive in the presence of AO. It should be noted, however, that the protozoal population in Rusitec is lower and less stable than that found *in vivo* (Hillman 1987), and the same depression in protozoal numbers may not occur *in vivo*.

This study has demonstrated that Rusitec is suitable for studying the effects of probiotics on the rumen fermentation. In addition, the results suggest that AO may have far-reaching effects on the microbial population and stoichiometry of the rumen fermentation. More studies are required to identify the mode of action by which AO exerts its effects on the rumen fermentation.

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