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Fermentation extract effects on the morphology and metabolism of the rumen fungus *Neocallimastix frontalis* EB188

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J.S. CHANG, E.M. HARPER AND R.E. CALZA. 1999. The effects of *Aspergillus oryzae* fermentation extract, Amaferm™, on the rumen fungus *Neocallimastix frontalis* EB188 were studied. The secretion of cellulase was increased by 67% and rhyzoid development was increased 3·8-fold in the presence of extract. Strength of fungal response increased in a dose-dependent manner and demonstrated a positive correlation between cell surface area and enzyme secretion. Above certain concentrations of extract, however, the development of the fungus and enzyme secretions remained at control values or slightly diminished. Supernatant fluid appearance of the intracellular enzyme, malate dehydrogenase, paralleled the secretion of cellulase both in the presence and absence of extract. Ether solubilization of extract demonstrated that the active component(s) possessed a moderately polar value between 2·7 and 2·8. Thin layer chromatography separated extract into inert, inhibitory and intensely stimulating fractions. These results support the idea that by accelerating fungal growth and metabolism, Amaferm™ increases the rate (or extent) of fibre degradation caused by rumen fungi and that this, in turn, may contribute to enhanced animal performance.

INTRODUCTION

Animal feed additives (e.g. direct fed microbials) of varying degrees of sophistication, and based on components isolated from *Aspergillus oryzae* (AO) or *Saccharomyces cerevisiae* fermentations, have been used to enhance ruminant performance with respect to milk yield, flesh gain, feed conversion and disease resistance (Huber and Higginbotham 1986; Wiedmeier *et al.* 1987; Fondavila *et al.* 1990; Kellems *et al.* 1990; Stanley *et al.* 1993; Varel *et al.* 1993). One such product (Amaferm™, BioZyme, Inc., St Joseph, MO, USA) has been shown to be of considerable merit when fed to production animals given diets high in fibre (Huber and Higginbotham 1986; Gomez-Alarcon *et al.* 1990). However, animal response to this product can be somewhat variable (Gomez-Alarcon *et al.* 1990; Varel and Kreikemeier 1994). A complex mixture of rumen micro-organisms including several types of bacteria, protozoa and fungi, mediate fibre conversion in ruminants (Hungate 1966). Fibre conversion is inefficient in animals fed diets high in lignocellulose and low in protein (Van Soest 1994). In the absence of any of the three main types of micro-organisms, rumen function may falter, change or temporarily compensate itself through population adjustments. In the absence of anaerobic fungi, ruminants fail to convert fibre efficiently (Gordon and Phillips 1993) whereas the reintroduction of fungi restores normal rumen function. Furthermore, rumen fungi have been shown to possess fibre-degrading enzymes of the highest specific activity studied to date (Wood *et al.* 1988) and to secrete a large variety of fibre-degrading enzymes (Li and Calza 1991a). It remains possible that the beneficial effects of rumen-stimulating agents could be mediated via the endogenous populations of fungi.

In a previous study which measured the endogenous enzymes present in that preparation (Welch *et al.* 1996), it was concluded that insufficient activities are present to be responsible for the increased digestibility recorded in production ruminants. In the same study, it was demonstrated that stimulation occurred beyond that expected of nutritional factors and that furthermore, autoclaving or boiling extract did not affect its ability to stimulate fungi, yet this harsh treatment would probably have inactivated most enzymes. In this report, *Neocallimastix frontalis* EB188 is used as an *in vitro* tester system (Harper *et al.* 1996; Welch *et al.* 1996) to measure fungal morphology and secretion of various enzymes in the presence of AO fermentation extract (e.g. Amaferm™). Previous studies are extended by determining the polarity of the fungal stimulation component, outlining the differential

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impact of low concentrations of ether extract, demonstrating the presence of inhibitory components and quantifying fungal morphological changes which the extract causes. With detailed information about mode of action, it is hoped that increasingly effective products can be developed.

MATERIALS AND METHODS

Chemicals

All chemicals, unless otherwise stated, were purchased from Sigma or US Biochemicals and were of the highest grade available. *Aspergillus oryzae* fermentation extract (Amaferm^{TM}) was a gift from BioZyme, Inc. This material was supplied as a dry powder resembling wheat bran in particle size and texture.

Conditions of culture growth and sampling

Neocallimastix frontalis EB188 (ATCC no. 76100) was used throughout this work and obtained from the American Type Culture Center (Rockville, MD). The composition of growth medium, growth conditions and sampling protocols have been described elsewhere (Barichievich and Calza 1990a, b). The carbon source used was Sigma Cell 100 or rinsed, cellulose dialysis membrane (at 0·2%, w/v). The amount of Amaferm^{TM} (e.g. aqueous or ether extract) added to cultures varied from 0–100 $\mu l\ ml^{-1}$ medium but normally the dose studied was 7 $\mu l\ ml^{-1}$. This level of extract approximated the expected concentration of supplement in the rumen of mature cattle (e.g. 3 g per day per head) assuming 100% efficiency of extraction. When adding ether extract, material was added to the uppermost part of the culture vessel and the ether allowed to evaporate before mixing extract with medium.

Preparation of Amaferm^{TM} extractions

Amaferm^{TM} powder (1 or 5% by weight) was added to distilled water or carbon source-free medium (Welch *et al.* 1996) to extract soluble components. The slurry was bubbled at room temperature using oxygen-free CO_2. Time of extraction was 2 h unless described otherwise. This extract was then filtered through Whatman no. 1 filter paper and finally passed through a microfilter (0·22 μm) using a syringe. Aqueous extract was stored at − 20 °C until used. The ether extract was made by mixing an equal volume of aqueous extract with ether (usually 5 ml) and mixing. Screw-top test tubes containing liquid were placed on a rocker for various periods of time; the solubilized material (partitioned into the organic layer) was then recovered. If appropriate, additional ether was then added to continue extraction. Ether-extracted material was dried using a stream of N_2, diluted as appropriate and held at 4 °C in a dark bottle until used.

Assays

All experimental cultures were run in replicates of at least five. Assays of samples from individual cultures were run in at least triplicate. The standard deviations of all replicates were generally less that 3%. Where appropriate, indication of the deviation of sample values is provided.

Cellulase and B-glucosidase. The methods for the assay of endoglucanase and B-glucosidase in culture supernatant fluids have been published previously (Li and Calza 1991a). As culture response can be significantly influenced by the metabolism of inoculation zoospores (Welch *et al.* 1996), all values noted for comparisons were obtained from simultaneously performed experiments using a single preparation of zoospores.

Malate dehydrogenase. Malate dehydrogenase (MADH) was determined using the method described by Smith (1991). The nicotinamide adenine dinucleotide (NAD) solution was prepared by dissolving NAD to a final concentration of 3·6 mmol in the recommended diethanolamine buffer adjusted to pH 9·2. The assay was performed by adding 250 μl of fungal culture supernatant fluid to 2·0 ml of the NAD solution. The mixture was incubated for either 30 or 60 min at room temperature. Production of NADH + H^+ was determined by measuring the absorbency of u.v. light at 339 nm. This assay provides a representative measure of NADH but does not rule out the influence on NAD conversion of other intracellular NAD-reducing enzymes possibly present in culture supernatant fluid.

Protein. The Bradford (1976) method of protein determination was calibrated using bovine serum albumin as reference. All values were recorded after the background corrections for medium components or other solutions.

Evan's blue stain. The procedure used to determine cell integrity with the Evan's blue exclusion dye was as described by Kanai and Edwards (1973). Microscopic examination determined whether the reagent entered the cell cytoplasm which is a measure of cell wall leakage or damage (rupture).

Volatile fatty acids (VFA). Measurements of VFA in culture liquor were obtained using gas chromatography, after acidification, by the methods of Holdeman *et al.* (1977). All values are reported after correction for background of medium components.

TLC methods

Amaferm™ was extracted with ether as described above. Spots (2 μl) of the solubilized material were placed onto silica gel thin layer chromatography plates. The solvent system used to develop plates was 2 : 1 (v/v) benzene, ethyl acetate. The reagent used to visualize chromatographed components on the plates was vanillin-phosphoric acid reagent (Jork *et al.* 1990). Where noted, a parallel lane of sample, which remained unreacted with staining reagent, was recovered from the silica gel plate by placing the strip in 1 ml methanol. The resultant solution was vortexed, appropriately diluted and added to cultures for testing.

Scanning electron microscopy (SEM) sample preparations

Fungal cultures grown for 5 d on dialysis membrane were fixed with 4% (v/v) glutaraldehyde in 0·1 mol l^{-1} cacodylate buffer (pH 7·2) and washed with the same buffer. Osmium tetroxide (1·5%) was used for post-fixation. Stepwise concentrations of ethanol were used for dehydration and liquid CO_2 was used for critical drying. Samples were coated with gold and examined using a Hitachi S-570 (Hitachi Ltd, Tokyo, Japan). The power setting ranged from 15–25 kV. In some cases, the fungal mat was teased by stretching before fixing to assist in the presentation of sample and subsequent photography.

SEM image analysis

All electron micrographs were analysed using the NIH Image software (version 1·60). The shape of rhyzoids was assumed to be cylindrical. Experimental results are reported from an average of 30 individual cultures, i.e. they were arranged in replicas of five and each test concentration was run six times. All measurements were calibrated against both externalized and machine (internalized) sizing standards and are stated in μm or μm^2 as appropriate.

Statistical analysis

Wherever appropriate, data were analysed as a randomized block design with blocks untreated by the linear or General Linear Model Procedure (SAS 1989). Calculations were handled using (i) General Linear Model analysis, (ii) Regression Analysis for each character with dose differences and (iii) Regression Analysis with combined characters using the Forward Selection Method.

RESULTS

Secretion of culture protein and cellulase and production of VFA

Addition of aqueous extracted Amaferm™ to fungal culture increased the secretion of protein and cellulase (Table 1). Protein increased up to the 35 μl ml^{-1} dose (38% greater than controls) but at the highest dose, dropped to control levels. Cellulase increased up to 34% above the control (at 7 μg ml^{-1}) and thereafter decreased until the highest dose (100 μl ml^{-1}) where levels were not statistically different from controls. The secretion of B-glucosidase was not significantly increased at any dose except 35 μg ml^{-1} where it reached a level 31% greater than the control cultures. The specific activity of cellulase ranged between the low of 3·21 U μg^{-1} protein for controls and the high of 3·69 U μg^{-1} protein for the culture treated at 7 μl ml^{-1} and was significantly different only at the 7 μl ml^{-1} dose. Measurement of VFA suggested that cultures containing Amaferm™ possessed higher acetate to propionate ratios. Experiments measuring the uptake of Evan's blue dye in cultures grown in the presence of extract for 120 h suggested that less than 3% of the rhyzoids and/or zoospores were ruptured and therefore non-viable.

SEM of fungal morphology

Electron micrographs of fungal mass from the same cultures used in measurements of enzyme secretions show fungal morphological changes in the Amaferm™ (aqueous)-treated cultures (Fig. 1). Fungal mass was more dense and robust in the treated cultures up to 35 μl ml^{-1} but at higher concentrations, looked like control cultures. Branched buds were significantly longer in most treated cultures (data not shown). Systematic measurements provided evidence that the branch frequency of rhyzoids was tripled from the control of 3·7 to a maximum of 14·1 at 7 μl ml^{-1} but, in fact, all cultures up to 50 μl ml^{-1} showed statistically significant increases (Table 2). Branch area apparently increased in all treated cultures. The stem area was significantly increased in treated cultures up to 20 μl ml^{-1}. Calculations of the stem area divided by the number of branches (e.g. S/B) suggested that all cultures treated with up to 50 μl ml^{-1} possessed an increase in surface area. Total surface area was at a maximum in cultures treated with 20 μl ml^{-1} and most treated cultures possessed a significantly increased surface area. The total number of sporangia was not significantly increased (data not shown) in any treated culture. However, the surface area of sporangia increased, where measured, with an increase in extract concentration. A low number of long and branchless structures was observed in all cultures and may represent distinct formations found in *N. frontalis* EB 188. The R^2 values for all doses of extract against branch number is 0·279. The R^2 of

Table 1 Secretion of culture enzymes and protein and the production of VFA in the presence of Amaferm™

Dose (ul ml^{-1})	CMCase (U ml^{-1}) and % control	B-Glu-ase (mU ml^{-1}) and % control	Protein (ug ml^{-1}) and % control	VFA (A/P)
0	0·151 (100)	0·531 (100)	47·1 (100)	3·89
7	0·202 (134)^{a}	0·536 (102)	54·7 (116)^{a}	8·84^{ab}
20	0·181 (120)^{a}	ND	53·2 (113)^{a}	7·31^{a}
35	0·213 (141)^{a}	0·696 (131)^{ab}	65·2 (138)^{ab}	4·32
50	0·170 (113)^{a}	0·545 (103)	ND	6·10^{a}
100	0·164 (109)	ND	49·1 (104)	ND

ND, not determined.
Entries which are superscripted differently are significantly different at $P > 0{\cdot}05$, or greater, from the controls or other values.
A/P, ratio of the concentration of acetate *vs* propionate.

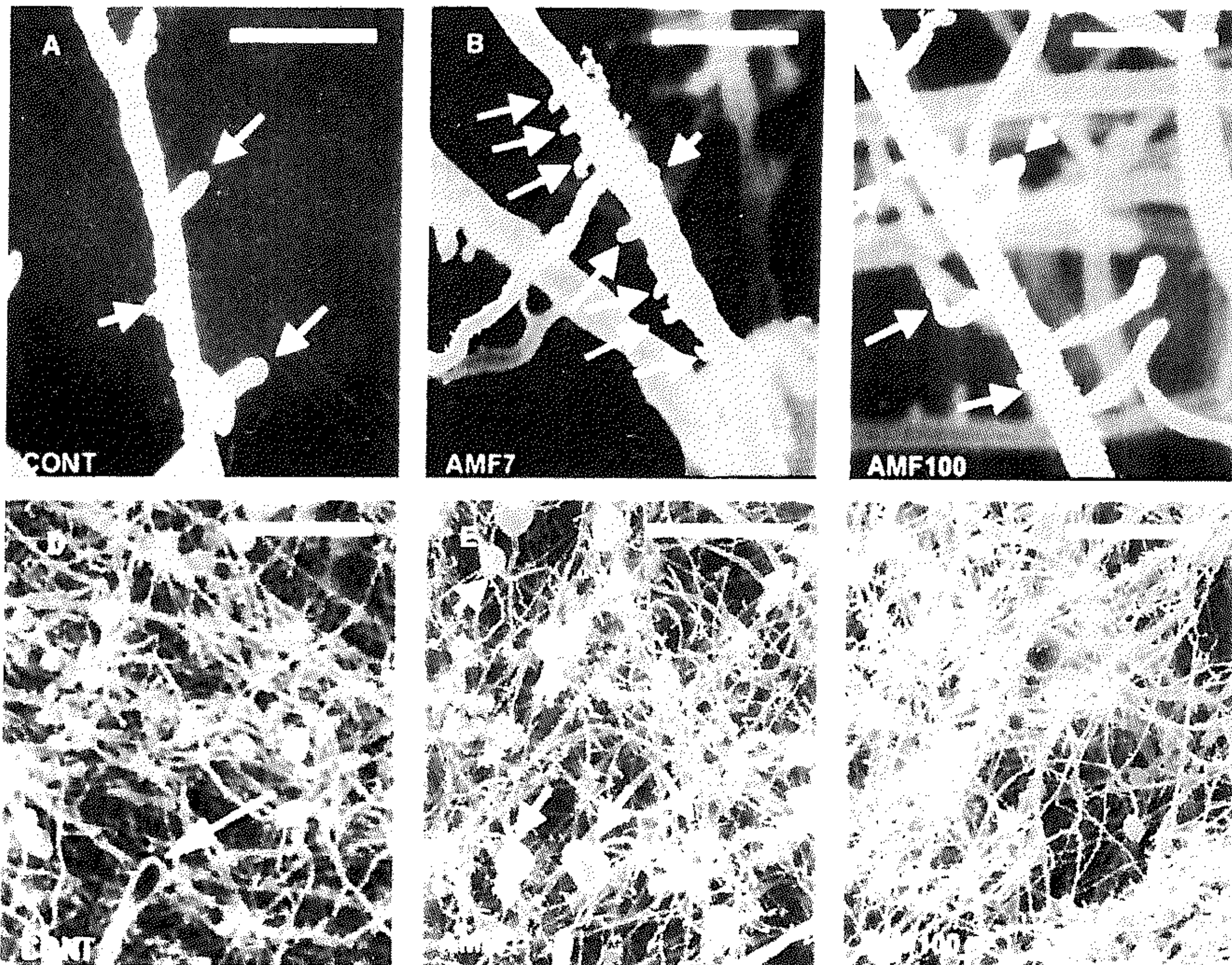

Fig. 1 SEM of fungal morphology. Electron microphotographs of various cultures (as indicated). Magnification was 5000 × for plates A and B, 6000 × for plate C and 500 × for plates D, E and F. Sizing bars represent 6 μm in plates A and B, 5 μm in plate C and 60 μm in plates D, E and F as indicated. Control cultures are indicated as Cont; Amaferm™ treated cultures at 7 μl ml^{-1} are indicated as AMF7 and Amaferm™ treated cultures at 100 μl ml^{-1} are indicated as AMF100. Small arrows indicate buds in photographs A, B and C and sporangia in photographs D, E and F. The large arrow in photograph D indicates the large unbranched structure found in cultures

Table 2 Analysis of EM of fungal morphology in the presence of Amaferm™

Dose (ul ml^{-1})	Stem (area)*	Branch (area)	Total (area)	S/B (ratio)	Sporangia (area)	No. of branches
0	73·4	17·6	91·0	4·17	68·2	3·7
7	84·9^{a}	78·8^{ab}	163·7^{a}	1·08^{a}	81·5^{a}	14·1^{ab}
20	107·6^{ab}	69·6^{a}	177·2^{a}	1·55^{a}	99·7^{a}	11·6^{a}
35	65·5	29·5^{a}	95·0	2·20^{a}	130·3^{ab}	7·0^{a}
50	73·9	35·2^{a}	109·1^{a}	2·10^{a}	ND	8·1^{a}
100	75·0^{a}	29·8^{a}	104·8	2·51	ND	4·7

ND, not determined.
Entries which are superscripted differently are significantly different at $P > 0{\cdot}05$, or greater, from the controls or other values.
*All morphological measurements are determined in microns and include complete scrutiny of an entire sample grid of replica cultures. Values are the averages of all replica.
S/B, the area of stem divided by the area of branches.

branch number and stem surface area against extract concentration is 0·564. The R^2 for dose of extract against the secretion of cellulase and branch area is 0·160. Interestingly, the R^2 value for sporangia surface area against extract concentration (e.g. measured only up to 35 μl ml^{-1}) is 0·455. The correlation of cellulase secretion against extract concentration, against stem surface area, against branch number, against sporangia surface area is 0·648. Correlation of the 7 and 20 μl ml^{-1} treatments against the secretion level of cellulase is 0·521 whereas the R^2 of the same additions against branch frequency is 0·417. Most equations were significant at ($P < 0{\cdot}01$) and all equations for the correlations were at least significant at the 95% confidence level.

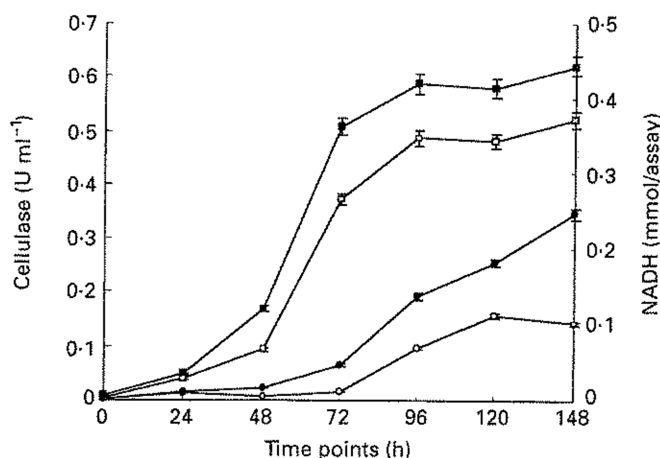

Fig. 2 Malate dehydrogenase and cellulase in the culture supernatant fluids. Culture supernatant fluid levels of cellulase and NAD converted (MADH) in the presence or absence of extract. Samples of cultures were taken every 24 h, as indicated. Deviation of replicates was less than 2% for the MADH and less than 3% for the cellulase, as indicated

Malate dehydrogenase and cellulase in the culture supernatant fluid

The concentration of MADH in culture supernatant fluid increased with the time of culture incubation and in cultures containing aqueous extracts of Amaferm™ (Fig. 2). The level of MADH present in the supernatant fluid of cultures paralleled the secretion of cellulase. The absolute difference for increase in malate dehydrogenase at time 148 h was 241% whereas the cellulase increased by only 19%. The results also show that treated cultures released more of both cellulase and MADH through their entire growth phase. The activity recorded in supernatant fluids accounted for less than 10% of the total malate dehydrogenase within the fungal mass in the culture.

Time course of ether extraction of stimulating materials

The usefulness of using ether extraction in separating the extract into differentially stimulating preparations was tested. It was necessary to extract the material for at least 15 h to obtain material which would stimulate secretion of protein (Fig. 3). By 24 h of extraction, all the cellulase-stimulating material was removed. The initial times of extraction (e.g. between 1 and 6 h) produced components which would markedly inhibit the secretion of cellulase and protein in cultures. Time points later than 24 h resulted in material which had little or no effect on the secretion of cellulase. In these experiments, the secretion of protein was less responsive than was typically recorded but nevertheless did change (e.g. stat-

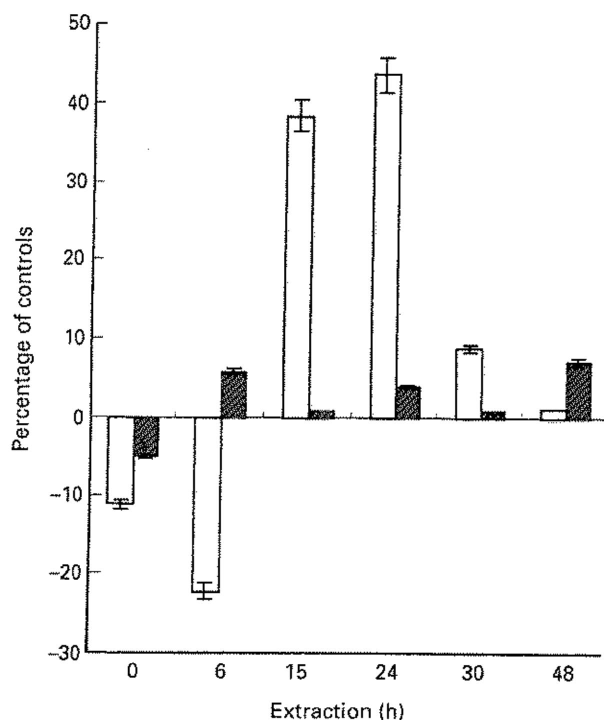

Fig. 3 Time course of ether extraction of stimulating materials. Sample represents the relative cellulase and protein secretion of culture treated at 7 μl ml^{-1} with the various extract preparations. Time of extraction is indicated. The error bars represent the deviation of 2% between triplicate samples. Cellulase ranged from 0·220 U ml^{-1} to 0·120 U ml^{-1} and protein ranged from 49 μg ml^{-1} to 42 μg ml^{-1}, with the control values being 0·150 U ml^{-1} and 45 μg ml^{-1} for cellulase and protein, respectively

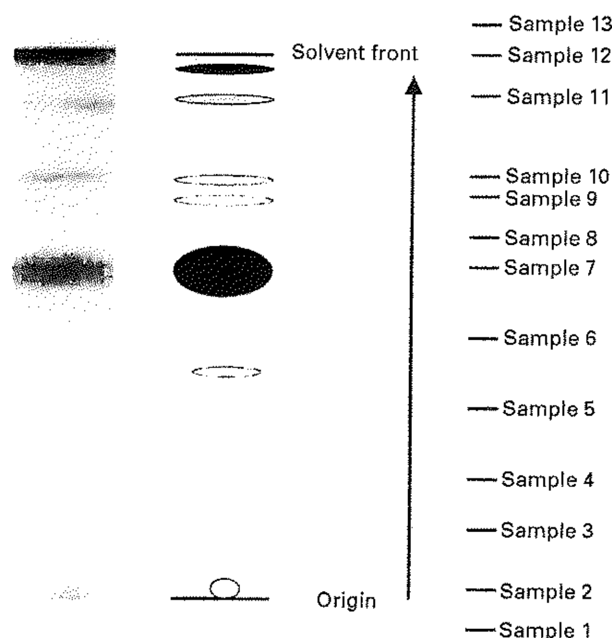

Fig. 4 Thin layer chromatography of ether extract. A photograph of the actual TLC plate and an artist's stylized tracings of the resultant spots from materials extracted from aqueous Amaferm™ using ether and chromatographed using silica gel thin layer plates. The origin is at the bottom of the drawing. The sample fraction numbers are as indicated and were used to test cultures listed below in Fig. 5

istically significant at 6 and 48 h of extraction) in cultures receiving extract.

Thin layer chromatography of ether extract sample

Ether-extracted material (24 h) yielded a complex pattern of stainable components which could be separated using thin layer chromatography (TLC) (Fig. 4). At least seven distinct and strongly staining spots were visible. Several were present in relatively large amounts (e.g. those within the samples marked 7 and 12) and a certain amount of material remained at the origin. At least three other spots were visible between the origin and the first spot but were only transiently stained and could not be quantified or traced. The polarity of the material migrating at or close to the solvent front was 2·7 or 2·8, suggesting that it will readily dissolve in benzene.

Culture production of cellulase in the presence of TLC fractions

Ether extract which had been separated using TLC was used to test effects on culture secretion of enzymes (Fig. 5).

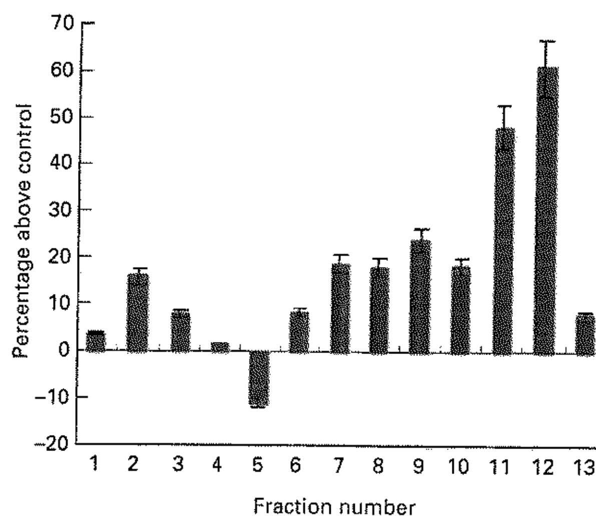

Fig. 5 Culture production of cellulase in the presence of TLC fractions. The fraction number corresponds to the section strip in Fig. 4 above. The error bars represent 5% deviation between replicate samples. Cellulase ranged from 0·242 to 0·133 U ml^{-1}, with the control value at 0·15 U ml^{-1}

Material scraped off a parallel lane on the same plate as shown above, but which had not been stained, was eluted into buffer and added to cultures in a dose approximating 7 μl ml^{-1} (assuming 100% recovery of material). It is evident that certain materials either inhibit (e.g. sample number 5), have little or no effect (e.g. samples 1 and 4) or strongly stimulate the secretion of cellulase (e.g. samples 11 and 12). The material at a polarity of 2·7 or 2·8 (e.g. at the solvent front in sample 12) had the greatest effect on culture secretion of cellulase (and protein, data not shown).

Culture response to low concentrations of ether extracts

The effect of low concentrations of ether extract on the secretion of culture cellulase was measured. Extract stimulated culture secretion of cellulase in a dose-dependent manner up to approximately 0·2 μl extract ml^{-1} culture and then again beyond 1 μl ml^{-1} (Fig. 6). At levels below 0·0001 μl ml^{-1} or between 0·2 μl ml^{-1} and 1 μl ml^{-1}, the stimulation was not detected. The 7 μl ml^{-1} treatment cultures responded much like cultures treated with aqueous extracts of Amaferm™ (see above).

DISCUSSION

This research provides the first report of physical (e.g. morphological) changes caused by a direct fed microbial within an important species of rumen micro-organism. The presence of branch buds, which were particularly long in treated cultures, suggested a more rapid growth rate. This finding was unexpected as such growth morphology alterations in fungi are generally only associated with incubation of cells with toxic compounds such as membrane depolarizers (e.g. nystatin) (Michel 1977). We cannot detail the mechanism involved in the process whereby additional (more than triple the number) branches of rhyzoids form in treated cultures. Alteration in morphology may represent a shift in the timetable of cellular events which would normally occur in the growth of the fungus. Cultures grown with xylose as the carbon source contained a greater number of branches and sporangia and therefore possessed a denser rhyzoidal development than glucose-grown cultures (Barichievich and Calza 1990b). Evidence of reduced distance between rhyzoid branches (and therefore increased overall fungal surface area) is consistent with previous findings (Harper *et al.* 1996; Welch *et al.* 1996) in which several species of rumen fungi in the presence of Amaferm™ extract produced more cellulase, protein and cell mass. Also consistent with that earlier study, the specific activity of supernatant fluid cellulase in extract-containing cultures was not consistently increased, although specific differences in the molecular weight of cellulases secreted in the presence of Amaferm™ has been recorded (Harper *et al.* 1996). Increased secretion of enzymes and protein into culture medium may be a consequence of the added surface area afforded by the increased numbers of rhyzoids. Basal (shaft) stem area is not increased, which is in sharp contrast to the large increase in branch area. Cultures treated with more than 35 μl extract ml^{-1} culture generally possessed physiological and morphological characteristics which often failed to differ significantly from controls. The correlation (R^2) values between the secretion of cellulase, morphological changes and the concentration of extract suggested that the relationship is low, even though it is positive. The same extract sample which possesses a strong stimulatory component also has inhibitory characteristics but can be separated using TLC.

The inhibitory effects of high concentrations of Amaferm™ on the secretion of cellulase from anaerobic fungi have been reported (Harper *et al.* 1996). It has been shown here that over a low and very narrow range, and over a higher range of concentrations, fungal metabolism appears to increase in activity. Culture response at the low concentrations of ether extract may mimic what occurs in cultures treated with higher concentrations of aqueous extract. As it is difficult to attribute this to a single component, and as intermediate concentrations of extract have little or no effect on the fungi, fungal response may be caused by at least two different components. When using ether extracts of Amaferm™, although cellulase secretions consistently increased, the secretion of protein was less and difficult to predict. During timed extraction experiments, inhibitory components were first removed followed by extraction of the stimulatory components at or near 24 h. This extraction

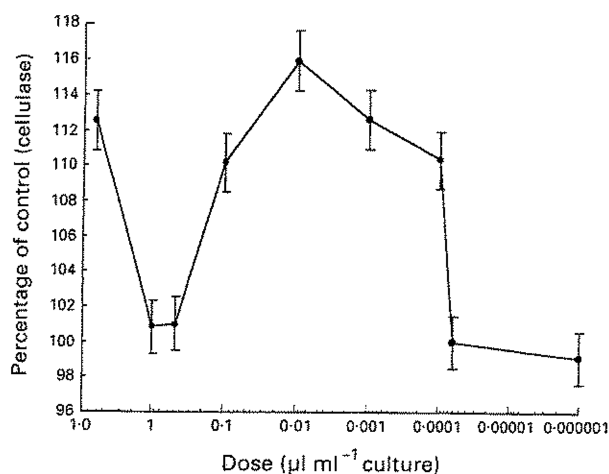

Fig. 6 Culture response to low concentrations of ether extracts. Production of cellulase is plotted against culture concentration of ether extract. Deviation of replicates was less than 3%, as indicated. Cellulase ranged from 0·168 to 0·145 U ml^{-1}, with the control value at 0·145 U ml^{-1}

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experiment contrasts with the aqueous extraction data which consistently showed active components removal within 2 h of hydration (Welch *et al.* 1996). The majority of the stimulation components migrated with the solvent front under the conditions of thin layer chromatography employed and yet other TLC fractions were inhibitory, neutral or differentially stimulating. This appears to provide evidence for the possibility of more than one stimulatory component in the preparation and that the fungi respond in a pleotrophic manner. The staining of the TLC plates suggested that several components were present and that they differed in polarity, but this experiment did not allow the identification of specific components. From the aqueous preparations of Amaferm™, fungal stimulatory components can be extracted using organic solvents. This further suggests that the components which stimulate the fungi are moderately polar in nature or possess both an aqueous and a non-aqueous functional moiety. The fungal promoting factor(s) is also of relatively high molecular weight (Harper *et al.* 1996; Welch *et al.* 1996).

Experiments measuring MADH (or possibly other enzymes which might reduce NAD) suggested that as the fungal culture mass matured, there was a leakage of what would normally be considered an intracellular enzyme. The presence of this enzyme in the culture supernatant fluid might be a result of asynchronous culture growth in which more mature mass is soon to senesce and/or perhaps lyse, thereby releasing its components. As the cultures were started using zoospores which were asynchronous (Welch *et al.* 1996), differential stages of fungal growth were expected. Staining of the cell cytosol with Evan's blue dye, which does not normally enter intact cells, showed that a very low percentage possessed a breach in the cell wall. The relative differences of cellulase secretion and MADH appearance in the culture supernatant fluid (e.g. 19% *vs* 241%, respectively) suggests that two distinct events or processes are responsible. Furthermore, evidence is provided that cellular physiology, including protein synthesis and glycosylation, were necessary for cellulase secretion (Calza 1991a; Li and Calza 1991b). Discovery of MADH in supernatant fluids of cultures not containing extract suggests that this characteristic is typical of *in vitro* growth of *N. frontalis* EB188. It is therefore unlikely that cell lysis or rupture caused leakage of both these cellular components.

Other researchers have suggested that the response of microbial populations to AO-based, direct fed microbials is unlikely to involve an increase in the numbers of anaerobic fungi (Newbold *et al.* 1991). Newbold *et al.* (1992) tested the product at levels found to be inhibitory to the fungi (Harper *et al.* 1996). The concentrations of extract tested here are within the range recommended by the manufacturer for supplementing ruminants. The study was concerned with an *in vitro* response from the fungi but the material was being tested at typical *in vivo* levels. If Amaferm™ works *in vivo* as it apparently does *in vitro*, acceleration of rumen fungal physiology might cause enhanced rumen fibre conversion.

Researchers have suggested that heat inactivation of this product was possible and would abolish any bacterial stimulation (Newbold *et al.* 1991; Martin and Nisbet 1992). Therefore, it can be concluded that both heat-labile and heat-stable stimulation components are present in the product. Although researchers have failed to find increases in populations of rumen fungi in ruminants fed Amaferm™ (Newbold *et al.* 1991), it has been shown that physiologically important changes do occur in rumen fungi *in vitro* (Harper *et al.* 1996; Welch *et al.* 1996; this work) and there has been a significant increase in fungal mass in cultures containing Amaferm™ (Welch *et al.* 1996). It has also been shown that populations of cellulolytic bacteria increase in the presence of this product (Beharka *et al.* 1991; Newbold *et al.* 1991). The ability of direct fed microbials to reduce the lag phase of rumen bacterial growth has been suggested as an indication of their effectiveness (Girard and Dawson 1994). Cell growth acceleration could no doubt place certain bacteria, and more importantly, their physiology, in a position to increase the rate of rumen fermentation of fibre. A plausible explanation has been suggested for rumen stimulation based on the accelerated transport of certain metabolic acids in the presence of fermentation extract (Martin and Nisbet 1992). No single proposal can be discounted conclusively at this time but the effectiveness of AO products differs dramatically, which could make certain modes of action unlikely. For instance, products which have been shown to be of no benefit in production animals have, nevertheless, been found to either stabilize rumen pH, reduce oxygen in the rumen, or provide various amounts of digestive enzymes. Furthermore, the dramatic increase in cellulolytic bacteria often recorded is more than the stochiometric proportions necessary to account for the modest increase in rumen cellulolysis. More than one mechanism must be at work during the action of these direct fed microbials as the heat lability experiments, inhibitory fraction analysis and pathogen exclusion experiments have conclusively shown (Huber and Higginbotham 1986; Wiedmeier 1989; Fondevila *et al.* 1990; Kellens *et al.* 1990; Beharka *et al.* 1991; Newbold *et al.* 1991; Martin and Nisbet 1992; Stanley *et al.* 1993; Varel *et al.* 1993; Harper *et al.* 1996; Welch *et al.* 1996).

CONCLUSIONS

The physiological adjustment which anaerobic fungi make in the presence of extract is potentially important during the acceleration of rumen function. It is proposed that small yet critical changes in the physiology of the fungi offer an insight into the cause and effect characteristic(s) of some AO products (e.g. Amaferm™). A cascade of events may occur and ultimately lead to increased solubilization of cellulose within

the rumen. A more active rumen fungal population and metabolism should promote a more extensive and accelerated conversion of feedstuff. Increased enzyme secretion may promote enhanced contact of fungi with plant components, resulting in increased infiltration of fungal rhyzoids. Furthermore, such events may lead to the acceleration of bacterial invasion of plant materials and afford deeper penetration as a secondary invasion regimen. Precise adjustments in the control of gene expression in anaerobic fungi might account for the physiological alterations recorded. Even if this 'extract' advantage is temporary, it remains possible that a timely and daily boost in microbial metabolism could cause production improvements in the animal. Future products must be rationally reformulated and be increasingly cost effective, possess a greater degree of predictability and be tailored to look and act naturally to satisfy consumer requirements for a safe, plentiful and sustainable food supply.

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