COLONIZATION OF ALKALI-TREATED FIBROUS ROUGHAGES BY ANAEROBIC RUMEN FUNGI

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Summary

This study reports light and electron microscope examination of rumen fungal colonization of alkali-treated roughage feeds incubated in decron bags in the rumen of cannulated sheep for varying time intervals.

Six roughages, pre-treated with ammonium hydroxide or sodium hydroxide at 4% (w/w) level were examined together with untreated control samples.

Alkali pre-treatment was associated with an earlier and more pronounced fungal colonization than all control roughages. Sodium hydroxide pre-treatment was significantly more effective than ammonium hydroxide in improving the susceptibility of roughages to rumen fungal colonization and studies by SEM showed that the pre-treatment permitted greater penetration of feeds by fungi. Sodium hydroxide pre-treatment also significantly increased dry matter disappearance from feed held in dacron bags in the rumen with all feeds except lucerne stem. It is not known to what extent fungal activity contributed to increased breakdown of the feeds.

(Key Words: Fungi, Roughage, Alkali, Sheep, Colonization, Rumen)

Introduction

Since anaerobic rumen fungi were isolated from sheep (Orpin, 1975) several species have been identified. Although anaerobic fungal function in ruminant digestion is not fully understood (Orpin 1976a, 1977), their close association with the slow degrading fragments of feed indicates a possible role in fibre digestion (Bauchop, 1979).

In vitro studies using inoculated wheat straw indicated that fungal digestion contributes to the overall digestibility (Gorden and Ashes, 1984), however, digestive benefits appeared to be marginally less than those obtained with grass hay (Orpin, 1981a). Further, Elliott et al. (1985) did not detect any fungal colonization upon sugarcane stem and barley straw but found extensive colonization on pangola grass when incubated in sheep rumen. Such differences, at least partially reflected the characteristics of feedstuffs and partially were due to fungal ability *per se*. Certainly the agro-by product type low quality roughages seemed to be more resistant to fungal colonization.

Improved fibre digestibility of low quality roughages, including those having massive deposits of silica, such as rice hull, has been obtained after pre-treatment with alkalis (McManus and Choung, 1976; Jackson, 1977; McManus, 1978), but nothing is known of the effects of such pre-treatment upon rumen fungal activity. The present study examines the effect of exposure time in dacron bags incubated in the rumen on fungal colonization with

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a range of roughages either not treated or pre-treated with ammonium hydroxide and sodium hydroxide.

Materials and Methods

Alkali pre-treatment of substrates

Six roughages, including lucerne stem (*Medicago sativa*), sugarcane bagasse (*Saccharum officinarum*), wheat straw (*Tritium aestivum*), oat straw (*Avena sativa*), oat hull and sorghum hay (Sudax: *Sorghum bicolor sudenenese*) were obtained. The lucerne stems (LS), wheat straw (WS), oat straw (OS), and sorghum hay (SH) were fine chopped, while sugarcane bagasse (SB) was coarsely milled and oat hull (OH) was used whole. Subsamples each of 500g were taken for control and pre-treatment with 4% (w/w) ammonium hydroxide (NH_4OH), and 4%(w/w) sodium hydroxide (NaOH). Subsamples received either 50ml water (c) or 50ml of either 40%(w/v) NH_4OH (t_1) or NaOH (t_2) to provide final concentration of 4% for both alkalis in feeds then were sealed in plastic bags. Pre-treated substrates were allowed to react with alkali for 48hr at room temperature, and then were oven dried at 80°C. Half of each substrate was ground through a 1.5 mm screen and stored in air-tight containers for determination of dry matter disappearance by the dacron bag technique in the rumen. The remainder was further prepared to examine fungal colonization in rumen incubation.

Incubation of roughage substrate samples in rumen

Four Merino wethers (live weight c. 45kg) fitted with permanent rumen cannulae were employed for incubation of dacron bags. Sheep were fed a lucerne chaff diet once daily at the maintenance feeding level.

Duplicate incubation bags, each containing 1 g of test feed, were prepared for six different incubation time intervals. The incubation bags were formed by 10x10 cm dacron cloth of pore size 37 μm. Feed samples enclosed in incubation bags were in range of 2 to 8 mm in length. Incubation bags were numbered and allocated randomly on weighted holding strings as well as between sheep so that each string represented a test time interval. Those were incubated in the rumen for 2, 8, 12, 24, 48, 60hr respectively. After the designated times, the bags were retrieved from sheep and the samples were gently washed with tap water to remove contamination, then fixed in buffered (pH:7) formaldehyde solution for examination with the microscope.

Determination of dry matter disappearance

Ground roughage samples were sieved to exclude fine dust using a sieve aperture of 75 μm. The rumen incubation bags were prepared as described above. Feed (0.5g dry matter) was placed in each bag with 8 replicates for each treatment. Sample bags were incubated for 48hr in coincide to examination of fungal colonization using the same sheep. At the termination of incubation, bags were removed with forceps and immediately stored at -20°C. Prior to analysis, the bags were thawed, thoroughly rinsed in tap water (c.10 min.) and dried at 80°C for 24hr. Dry matter disappearance rates were determined by differences between initial and post incubation weights.

Light microscopy examination of fungal colonization

At every time interval of incubation of each substrate, 30 pieces (or chips) were randomly chosen equally from the duplicate bags and placed into a sintered glass crucible. The specimens were stained for 60 seconds with lactophenol cotton blue (Akin et al., 1983), subsequently rinsed several times using distilled water and sucked to dry. Then specimens were mounted onto glass microscope slides for sporangium counting.

The sporangia were counted in one field randomly on surface of one specimen under a 10x objective lens with light microscopy (LM), 30 fields were counted for each time interval to estimate sporangium numbers of a substrate. Then the sporangium number of each field was converted to per mm^2 area of substrate surface. Where no fungi were observed that field was denoted as zero. The converted data of fungal sporangium count per mm^2 at various times within substrate between treatments were analysed for F-test after normalization by square root transformation of data ($\sqrt{n+0.5}$) and the means were compared by least significant difference (LSD).

Light micrographs were produced on selective materials.

Scanning electron microscopy examination

Ten pieces (or chips) of fixed roughage speci-

TABLE 1. ANAEROBIC FUNGAL SPORANGIA COUNT/mm^2 ON VARIOUS ROUGHAGES INCUBATED FOR DIFFERENT TIMES FOLLOWING PRE-TREATMENT WITH WATER (c), 4% NH_4OH (t_1) OR NaOH (t_2) (MEANS ± S.E)

Feeds	Incubation times in rumen (hour)												Overall means
	2hr		8hr		12hr		24hr		48hr		60hr		
SBc	0.11^{aA}	0.09	0.69^{aA}	0.05	0.47^{aA}	0.02	2.42^{bA}	0.26	0.98^{abA}	0.08	1.09^{abA}	0.02	0.87^{A}
SBt_1	0.64^{aA}	0.04	0.70^{aAB}	0.08	3.06^{bcA}	0.36	2.30^{bcA}	0.24	0.73^{aA}	0.05	1.06^{abA}	0.09	1.33^{A}
SBt_2	0.97^{aA}	0.11	2.53^{bB}	0.17	2.74^{cB}	0.48	8.57^{cB}	0.79	1.37^{abA}	0.08	1.22^{abA}	0.11	2.53^{B}
WSc	1.09^{aA}	0.11	1.76^{aA}	0.38	2.03^{aA}	0.29	3.05^{aA}	0.32	1.43^{aA}	0.09	1.38^{aA}	0.20	1.75^{A}
WSt_1	1.24^{aAB}	0.15	1.68^{aA}	0.13	6.23^{bB}	0.81	2.79^{aA}	0.32	2.30^{aAB}	0.24	1.19^{aA}	0.11	2.38^{A}
WSt_2	3.59^{abB}	0.28	5.10^{bB}	0.40	9.97^{cB}	1.19	10.15^{cB}	1.12	4.69^{bB}	0.28	1.76^{aA}	0.21	5.48^{B}
LSc	0.65^{aA}	0.07	1.21^{abA}	0.12	1.96^{abA}	0.18	1.56^{abA}	0.18	3.50^{bA}	0.28	2.27^{abA}	0.26	1.77^{A}
LSt_1	2.30^{abA}	0.30	4.43^{bB}	0.58	3.68^{abA}	0.43	8.73^{cB}	0.89	3.09^{abA}	0.19	1.38^{aA}	0.14	3.67^{B}
LSt_2	4.46^{abB}	0.52	3.80^{abB}	0.29	4.48^{bA}	0.49	9.22^{cB}	0.82	1.81^{aA}	0.13	2.98^{abA}	0.15	3.95^{B}
SHc	1.14^{aA}	0.12	2.49^{aA}	0.38	3.54^{aA}	0.04	2.85^{aA}	0.53	1.58^{aA}	0.15	2.27^{aA}	0.26	1.73^{A}
SHt_1	1.52^{aA}	0.23	2.00^{aA}	0.26	1.21^{aA}	0.12	5.37^{bA}	0.52	6.30^{bB}	0.64	6.37^{bB}	0.92	3.49^{B}
SHt_2	3.84^{abA}	0.35	12.45^{dB}	1.58	8.12^{cdB}	0.79	5.66^{abcA}	0.75	7.85^{bcB}	0.51	2.89^{aAB}	0.41	5.21^{C}
OSc	2.00^{aA}	0.19	4.22^{abA}	0.50	5.84^{bA}	0.67	3.40^{abA}	0.17	4.50^{bA}	0.40	1.08^{aA}	0.09	3.33^{A}
OSt_1	6.67^{bB}	0.77	6.04^{bA}	0.77	8.94^{bA}	0.54	8.28^{bB}	0.39	1.73^{aA}	0.10	0.94^{aA}	0.09	4.92^{A}
OSt_2	12.37^{bB}	1.03	12.79^{bB}	0.95	15.62^{cB}	1.50	21.21^{cC}	2.04	4.38^{aA}	0.44	2.13^{aA}	0.17	10.33^{B}
OHc	0.62^{aA}	0.10	1.80^{abA}	0.21	4.85^{bA}	0.87	9.05^{bcA}	0.56	13.28^{cA}	0.13	9.91^{bcA}	2.16	5.69^{A}
OHt_1	1.05^{aA}	0.06	3.06^{abA}	0.49	9.03^{bAB}	1.21	40.84^{dB}	1.59	34.38^{dB}	1.25	19.63^{cA}	4.06	14.28^{B}
OHt_2	9.09^{aB}	0.88	10.54^{aB}	1.23	18.06^{abB}	1.40	38.47^{cB}	1.97	21.74^{bAB}	0.52	8.74^{aA}	1.15	16.49^{B}

Within substrate, different lowercase letters denote significant difference at $P < .05$ between columns; different uppercase letters denote significant difference at $P < .05$ between rows (SB: sugarcane bagasse; WS: wheat straw; LS: lucerne stem; SH: sorghum hay; OS: oat straw; OH: oat hull).

mens were selected from each time interval of treatment, and gently rinsed 2 to 3 times with normal saline (0.9% NaCL). Specimens were blotted dry with absorbent tissue and air dried under vacuum in a fume cup board for 24hr. Dry specimens were adhered with conducting cement onto scanning electron microscope (SEM) stubs and vacuum coated with gold (approximately 500Å). The specimens were examined in the SEM (JSM-U3) Texas Instrument). Features of fungal colonization on different materials were observed and the morphological size was measured on a random sample of 100 fungi at 24, 48 and 60hr incubation intervals. Electron micrographs were produced using Agfaortho-25 films at 25 KV accelerating voltage.

Results and Discussion

Fungal sporangium density on roughage

Alkali pre-treatment of all these low quality roughages improved fungal colonization (table 1). Improvement appeared to be more dramatic at the early time intervals of 2, 8 and 12hr. The highest sporangia count per unit area was found after incubation for 12 and 24hr and then gradually declined.

The overall means of sporangia count per mm^2 indicated that NaOH pre-treatment significantly improved the susceptibility of these roughages for rumen fungal colonization. While NH_4OH pre-treatment was less effective, fungal counts were competitive on LS, SH and OH to that of NaOH pre-treated substrates. Of these roughages, OH, OS, SH and WS were better colonized by rumen fungi than LS and SB, regardless of pre-treatments.

The incubation times appeared important to allow the substantial colonization by rumen fungi; however, such differences were gradual and even decreased at longer time intervals of 48 and 60hr. A wide variation was observed between substrates across times, however, as incubation time increased the uncolonized specimens were obviously reduced, though the density of sporangia did not significantly differ.

Dry matter disappearances of roughages

Pre-treatment of roughages with NH_4OH slightly increased dry matter disappearance (DMD) but the change was significant only with WS ($p < .05$) (table 2). By contrast NaOH treatment substantially and significantly increased DMD with all forages except lucerne stem, the significance with different substrates varying between $p < .05$ to $p < .001$. Increased DMD was positively associated with increased sporangia count per area upon alkali pre-treated roughages, though that may not have been solely due to fungal digestion.

TABLE 2. DACRON BAG DRY MATTER DISAPPEARANCES (%) OF ROUGHAGES PRE-TREATED WITH WATER (control) OR NH_4OH (Treatment$_1$) AND NaOH (Treatment$_2$) AT 4% LEVEL (MEANS ± S.E)

Feeds	Control		Treatment$_1$		Treatment$_2$	
Sugarcane bagasse	20.9^{a}	1.6	23.5^{a}	0.8	42.1^{b**}	2.0
Oat hull	37.1^{a}	2.5	41.0^{a}	2.7	55.5^{b**}	2.0
Wheat straw	37.4^{a}	0.8	46.2^{b*}	3.7	51.7^{b**}	3.2
Lucerne stem	52.6^{a}	1.5	50.1^{a}	2.9	50.2^{a}	1.0
Sorghum hay	54.7^{a}	1.4	58.4^{a}	2.3	65.8^{b*}	3.5
Oat straw	57.8^{a}	2.0	60.9^{ab}	2.0	66.0^{b*}	3.2

Lowercase letters differ between columns differ significantly; *: $P < .05$; **: $P < .001$.

Characters of fungal invasion and development on roughages

Most roughages, especially hull types were characterised by cell wall surface silicification as revealed under SEM (figure 1). NaOH pre-treatment, particularly of OH, produced substantial erosion and weakening of the overlying silica plaque, as well as cracking patterns and fissures (figure 2). Germinating fungal spore appeared to anchor upon silica and gain access to its underlying fibrous bundles by using stomata as portals of invasion as well as using alkali eroded or fractured surfaces and other physical damage (figure 3). Massive sporangia arising from vegetative hyphae appeared to lift and displace silica deposits and so expose the underlying plant fibre to further fungal and presumably bacterial attack (figure 4). By 24hr incubation most colonies were found to be fully matured with heavy colonized or "over growth" fields frequently observed under both LM and SEM (figure 5). In areas of extensive fungal growth plant tissues, where exposed, showed evidence of pitting and digestive degradation but it could not be ascertained if this was due

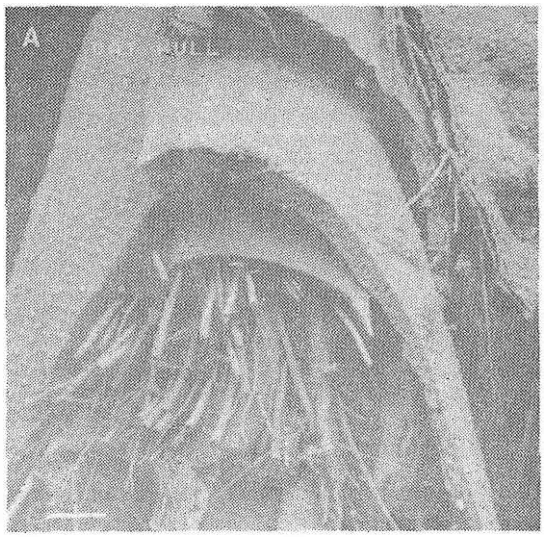

Figure 1. Electron micrograph of raw untreated oat hull before being placed in rumen. All structures have heavy deposits of silica (bar=200μm).

Figure 2. Electron micrograph of alkali pretreated oat hull after 2hr in rumen. Note erosion and cracking of silica plaque with displacement to reveal underlying fibre and silica phytoliths (bar=30μm).

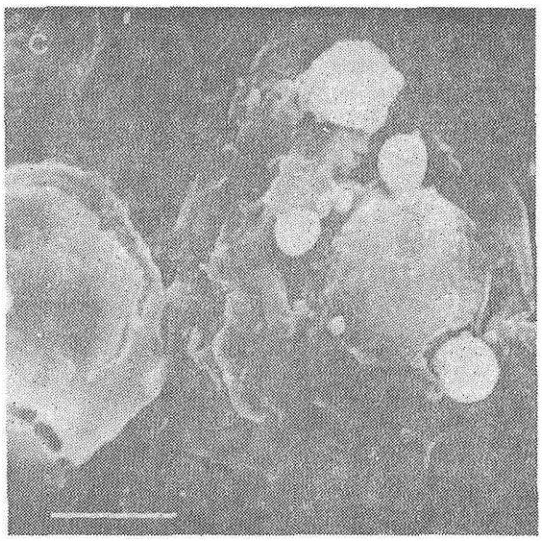

Figure 3. Electron micrograph of oat hull after 2hr in rumen, showing fungal spores beginning to germinate near silicified guard cells of stomata. These eroded and cracked structures are portals of entry of hyphe to underlying fibres (bar=10μm).

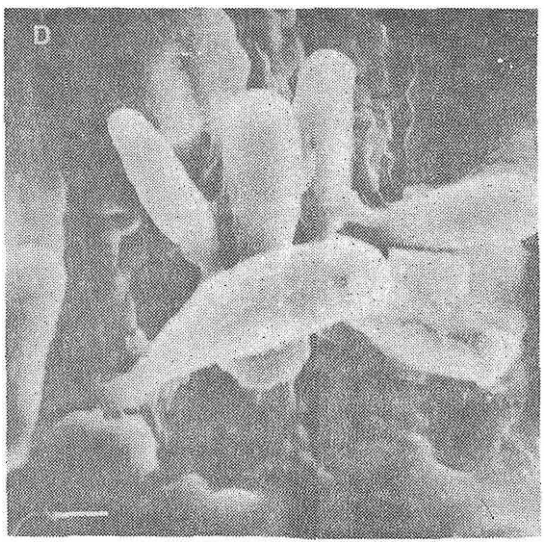

Figure 4. Electron micrograph of alkali pretreated oat hull after 24hr in rumen showing sporangia emerging through and lifting alkali-weakened silica of stomata and general surface silica plaque (bar=10μm).

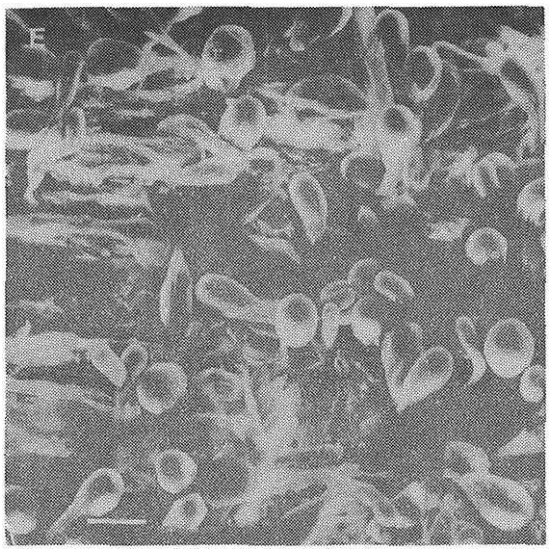

Figure 5. Electron micrograph of alkali pre-treated oat hull after 24hr in rumen showed a heavy colonized by rumen fungi and totally displaced the silicified maks (bar=30μm).

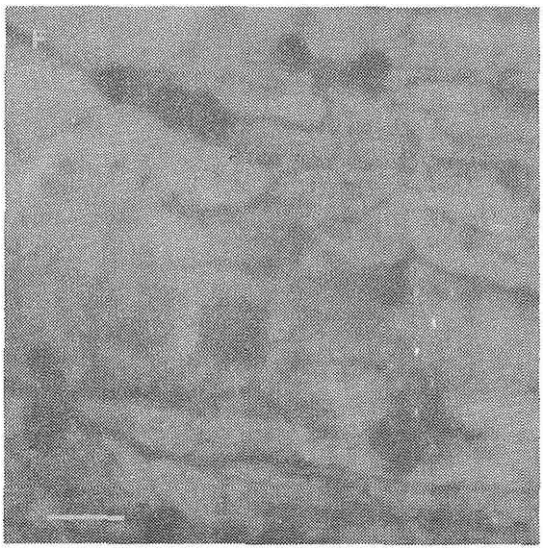

Figure 6. Light micrograph of alkali pre-treated oat straw after 8hr in rumen developed an extensive hyphal system within plant tissues (bar=30μm).

to fungal action or combined fungal and bacterial action.

The fungal sporangia were observed under lower magnification by LM (10x objective lens) at an early stage of germination following 2hr incubation and considerably developed hyphae and spores were visible on straw type roughages at 8hr incubation (figure 6), however, on LS, SB and OH such hyphal development could not be seen under LM.

Morphotypes of fungal sporangia greatly varied between oval, cylindrical and long thin tubule forms which were similar to those previously described (Bauchop, 1979). Those types were often found mixed within the same specimen regardless of material of origin; however, the cylindrical types was dominant upon plant stalks and hulls while oval types was as equally present as cylindrical types on leaves. Such morphological differences might be reflected by density of fungal colonies, development stages and nutrients of substrates so that those features may only be indication of mixed fungal species at their different developing stages.

The morphological sizes of fungal sporangia were extremely varied from germinating to maturing stages at incubation times of 24, 48 and 60hr, indicating that fungal colonies were continually established which accounted for the secondary or tertiary colonies. The fungal dimension measured by SEM on selected specimens from those substrates at 24, 48 and 60hr wer 89.5 μm (S.D: 89.5) x 39.4 μm (S.D:19.0), 84.7 μm (S.D:59.4) x 38.3 μm (S.D:22.6) and 59.3 μm (S.D:55.7) x 26.2 μm (17.4) respectively. These cyclic changes were closely consistent with the duration of fungal life cycles described by Orpin (1981b).

Role of anaerobic fungi in roughage digestion

The anaerobic rumen fungi were able to colonize a wide range of fibrous feeds including those which were alkali pre-treated; however, the degree of fungal colonization varied with nature of roughages. Highly lignified substrates, such as sugarcane bagasse were poorly colonized eventhough subjected to alkali pre-treatments indicating limits to the ability of fungi to degrade fibrous materials. This observation was supported by earlier findings that cellulose and hemicellulose were major susceptible components of feedstuffs to rumen fungi (Bauchop, 1979; Orpin and Lecther, 1981a; Akin et al., 1983).

Alkali pre-treatment of low quality roughages has been demonstrated to promote a positive effect on pioneering fungal colonization, especially

on silicified surface tissues. In treated feeds there was both earlier onset of and a greater density of fungal sporangia development along with increased DMD values, with the exception of lucerne stem. NaOH pre-treatment appeared superior to NH_4OH in terms of fungal sporangia count per unit area and DMD values. These may be attributed to the increased removal and weakening of masking substances and solubilization of lignin and hemicellulosic components. The estimation of sporangia numbers in relation to feed dry matter mass in this experiment showed that rumen fungi can be considerable sources of biomass with sporangia at the vegetative stage on feed particles in a range of 10^3 to 10^4/g in addition to the free zoospore of 10^3 to 10^4/ml in rumen fluid (Orpin, 1977).

The significance of anaerobic fungal colonization upon roughage feeds in ruminant was not only its role as a fibre degrader but also a physical destructor of plant tissues by the deep penetration of multiple branched hyphae and subsequent formation of hyphal net work within plant tissues which would provide a niche for other microbial digestion.

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