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Enumeration of Anaerobic Chytridiomycetes as Thallus-Forming Units: Novel Method for Quantification of Fibrolytic Fungal Populations from the Digestive Tract Ecosystem

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An endpoint dilution procedure, based on the technique of most probable numbers, was developed to enumerate anaerobic chytridiomycetes as thallus-forming units. The method does not distinguish between zoospores and thalli, but does permit enumeration of fungal populations with respect to their ability to digest plant cell walls. Fibrolytic populations in batch culture, ruminal contents, and feces were compared by relating viable counts to the dry matter content of enumerated samples (i.e., thallus-forming units per gram of dry matter). Batch cultures of *Neocallimastix* sp. strain R1 grown on wheat straw were used to assess the enumeration procedure and to demonstrate the potential of the technique for quantification of anaerobic fungi in vivo. Determination of total ruminal contents from steers enabled the quantification of the entire population of fiber-degrading anaerobic fungi in the reticulorumen. The enumeration procedure revealed substantial populations of fibrolytic anaerobic fungi in fresh and air-dried feces. Populations in fresh feces were equivalent to those in ruminal contents, but declined exponentially with time in dry feces. Minimum values were obtained from dry feces 90 days after drying, and anaerobic fungi were detectable for up to 210 days thereafter.

Most of the fungal biomass in digesta contents is in the form of particle-associated thalli, and a substantial proportion of the plant fragments which enter the rumen are rapidly and extensively colonized by ruminal fungi (1, 2). These observations suggest that ruminal chytridiomycetes function in primary colonization of plant cell walls, perhaps by making plant matter more accessible to invasion by other ruminal microorganisms (19, 20). However, because of the difficulty of measuring amounts and activities of particle-associated biomass in a mixed-population ecosystem, the extent of the fungal contribution to the ruminant digestive process has yet to be determined.

The life cycle of ruminal chytridiomycetes is reported to consist of two stages, in which free-swimming zoospores alternate with particle-associated fungal thalli (6, 14). Although the population size of free-swimming zoospores in ruminal fluid has been determined (5, 15, 17), that of the particle-associated thalli remains unknown. In addition to ruminal fungi, anaerobic fungi can be isolated from the feces of a wide range of ruminant and monogastric herbivores (2, 4, 7, 11, 16). In recent studies, anaerobic fungi have been obtained from the rumens, omasa, abomasa, small intestines, large intestines, and ceca of cattle and sheep (M. K. Theodorou, C. G. Orpin, D. Davies, and A. P. J. Trinci, unpublished results). Anaerobic fungi identical in appearance to ruminal chytridiomycetes have also been cultured from cattle and sheep feces that were dried and stored in air at ambient temperature for up to 3 months (11, 21). In view of the obligate anaerobic status of these fungi and their rigid requirements with respect to survival (9, 11, 21), these results appear to be anomalous.

To obtain a more complete understanding of the role of anaerobic fungi in the digestive-tract ecosystem, a method to quantify populations from different sites relative to their ability to digest plant cell walls has been developed. The method does not distinguish between zoospores and fungal thalli and relies upon most-probable-number (MPN) tables to provide a viable-cell count. Fungal populations are enumerated as cell wall-degrading thallus-forming units (TFU), with respect to the dry-matter (DM) content of the sample. The method was originally developed and assessed by using batch cultures of *Neocallimastix* sp. strain R1 grown on wheat straw and has since been used to quantify fungal populations in all parts of the digestive tract of cattle and sheep. In this paper we present the method and report on fungal populations in batch culture and in ruminal contents and fresh and air-dried feces of cattle.

MATERIALS AND METHODS

Fungal strain and culture conditions. *Neocallimastix* sp. strain R1 is an obligate anaerobic fungus isolated from the rumens of sheep (10) and has been the subject of previous publications (6, 8, 9, 19). Since the original communication (10), cultures have been obtained from individual zoospores and maintained on straw in a defined liquid medium (6, 9).

Aseptic anaerobic techniques were used to grow cultures in a non-ruminal-fluid-containing medium (defined medium B) in 125-ml serum bottles (Phase Separations Ltd., Clwyd, United Kingdom) sealed with butyl rubber stoppers and aluminum crimp seals (Bellco Glass, Inc., Vineland, N.J.) (6, 9, 10). Cultures were also grown under similar conditions in a different medium which contained ruminal fluid (1). Prior to the inoculation of experimental cultures, *Neocallimastix* sp. strain R1 was adapted to media with and without ruminal fluid by several 48-h serial transfers. For time course studies, culture bottles contained 1 g of autoclaved wheat straw (milled to pass through a 1-mm dry mesh screen) and 95 ml of sterile medium. Each bottle was inoculated with 5 ml of culture supernatant (containing zoospores) and incubated for up to 11 days under an atmosphere of CO_2 at 39°C without agitation; some cultures were incubated for 15 days or longer. Sufficient bottles were inoculated to enable three

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replicate cultures to be harvested at each time interval. The pH was recorded immediately in harvested cultures, and 10-ml portions of supernatant from replicate cultures were removed anaerobically and combined for enumeration of anaerobic fungi as described below. The residual wheat straw and adherent fungal biomass from individual cultures were collected and washed by centrifugation (19), and resultant pellets were oven dried to constant weight.

Feeding of animals and sampling techniques. Four British Friesian steers (average initial live weight, 223 kg), fitted with large ruminal cannulae (internal diameter, 62 mm) were used. Each animal was offered 250 g of barley concentrates at 9:45 a.m. followed by 5.0 kg of chopped perennial ryegrass (*Lolium perennae*) hay (882 g of organic matter, 532 g of neutral detergent fiber, 21.9 g of N kg of DM^{-1}) at 10:00 a.m. At 4:00 p.m., additional hay was given such that the total daily allocation was 10 to 15% higher than the previous day's consumption. Water and mineral blocks were freely available. Animals were adapted to the diet for 21 days prior to the first measurement and then housed in metabolism crates for up to 21 days. Metabolism crates had the facility to record the pattern of feed consumption by measuring the weight of feed remaining in the feed bin at 5-min intervals over 24-h periods. Fecal output, measured in grams of fecal DM per day, was determined by collecting all feces over a 7-day period. Fungal populations and corresponding sample DM were determined by using 5-g (fresh weight) subsamples of the digesta contents, removed from within the raft region of the rumen 1 h prior to the morning feed and 1, 2, 3, 6, 9, and 12 h after feeding. Feces (5-g [fresh weight] subsamples) for fungal enumeration and corresponding DM determination was obtained directly from the rectum into plastic bags. Fungal populations were determined in fresh feces and in feces which were allowed to dry in air at ambient temperature and stored in air for up to 300 days. On the last day in the metabolism crates, 1 h prior to the morning feed, the total contents of the rumen of each animal were removed by hand, mixed, weighed, and subsampled (three replicates per animal) for enumeration of fungi and DM determinations. Digesta contents were returned to the rumen within 40 min, and the animals were then moved to resting pens.

Enumeration of anaerobic fungi as thallus forming units. An endpoint dilution procedure based on the MPN technique was used to enumerate populations of anaerobic fungi. The procedure involved preparing a 10-fold dilution series of the sample in an anaerobic, antibiotic-containing basal medium. Defined medium B, plus 10% (vol/vol) clarified ruminal fluid (9, 10), was used for the dilution series. The dilution medium was devoid of a carbon source, but contained freshly prepared penicillin G (Sigma Ltd., Poole, United Kingdom) and streptomycin sulfate (Sigma) at final concentrations of 240 and 75 U ml^{-1}, respectively. The medium was dispensed in 45-ml amounts in 60-ml serum bottles (Phase Separation) and prewarmed to 39°C prior to use. The dilution series was initiated either by addition of 5 ml of a pooled culture supernatant (without particles of wheat straw) to 45 ml of medium in a 60-ml serum bottle or by addition of 45 ml of medium to 5 g (fresh weight) of ruminal digesta, fresh feces or air-dried feces in a plastic bag flushed with CO_2. Ruminal and fecal samples were mixed for 1 to 3 min with a model 400 stomacher (Colworth Ltd., London, United Kingdom) to obtain a particulate suspension. The dilution series was continued down to 10^{-6} by transferring 5-ml amounts between serum bottles. Three 10-ml portions of the appropriate dilutions (10^{-2} or 10^{-3} to 10^{-6}) were then transferred to enumeration tubes (three replicate tubes per dilution [Hungate type, 16 by 125 mm; Bellco Glass]) containing ca. 100 mg of wheat straw (milled as above). To ensure representative sampling of particulate suspensions, pipettes were used inverted and in combination with an automatic pipetter (Pipetus-Standard; Camlab Ltd., Cambridge, United Kingdom) which was modified for anaerobic work. Enumeration tubes were incubated at 39°C and observed daily for the presence or absence of anaerobic fungi. The majority of tubes which contained anaerobic fungi were scored positive within 3 to 4 days of incubation, although a few required longer incubation periods. An inverted microscope was used to confirm the presence of anaerobic fungi in enumeration tubes, and tubes were scored positive if motile zoospores were present in the culture supernatant and rhizoids and zoosporangia were associated with the plant fragments. All negative tubes were investigated after 10 days of incubation to confirm the absence of anaerobic fungi. For comparative purposes, sample DM was determined and fungal populations were expressed as TFU per gram of DM. Thus, the population density of anaerobic fungi in batch cultures, ruminal contents, and fresh and air-dried feces was expressed as TFU per gram of wheat straw residue (WSR), TFU per gram of ruminal DM (RDM), and TFU per gram of fecal DM (FDM), respectively.

Microscopy. Routine observations of culture supernatants, colonized wheat straw, ruminal digesta, and fresh and air-dried feces were made by using a BH-2 light microscope with Nomarski differential interference optics or a CK-2 inverted light microscope with bright-field illumination (Olympus Optical Co., Ltd., London, United Kingdom).

Statistics. All statistical analyses and curve fittings were carried out by using a VAX 11/750 computer (Digital Equipment Corp., Marlborough, Mass.). Genstat 5 or MLP (Maximum Likelihood Program) (3) was used to calculate the standard errors, perform the analysis of variance, and fit the lines of regression. Fitted curves were subjected to parallel-curve analysis as described previously (19). Anaerobic fungi were enumerated as TFU by adapting the three-tube MPN tables cited by Thatcher and Clarke (18).

RESULTS

TFU population in batch cultures of *Neocallimastix* sp. strain R1 fermenting wheat straw. Observations with the light microscope revealed the presence of zoospores in culture supernatants and fungal thalli on wheat straw particles from 1 to 2 days after inoculation until the end of the 11-day incubation period. The majority of the fungal biomass was associated with wheat straw particles, and relatively few particle-free thalli were observed in culture supernatants. Motile zoospores were less numerous in culture supernatants toward the end of the fermentation, whereas zoospores that were nonmotile and granular in appearance (presumably dead or dying) were frequently observed.

After an initial lag, the growth of *Neocallimastix* sp. strain R1 in media with and without ruminal fluid was accompanied by a decline in medium pH (from 6.8 to 6.1), which coincided with the removal of DM from wheat straw. Growth in ruminal-fluid medium resulted in a shorter lag phase and a lower rate of DM loss compared with growth in the defined medium without ruminal fluid (Table 1). However, the maximum extent of DM loss did not differ between media and was achieved after similar incubation times of 105 and 111 h in medium with and without ruminal fluid, respectively (Table 1; Fig. 1).

Cultures of *Neocallimastix* sp. strain R1 were adapted to

TABLE 1. Digestion kinetics of wheat straw in batch culture inoculated with *Neocallimastix* sp. strain R1

Culture medium	Lag time (h)[a]	Digestion rate (h^{-1})[a]	% Digestion at 11 days
Without ruminal fluid	48a	0.37a	44.12
With ruminal fluid	34b	0.45b	43.08

[a] Values were obtained from curves fitted to the experimental data shown in Fig. 1 (dry-weight loss values) by using a biphasic polynomial. Data within columns and followed by different letters were significantly different ($P < 0.001$) by parallel-curve analysis with the F test.

media with and without ruminal fluid by several 48-h serial transfers prior to inoculation of experimental cultures. Thus, as a consequence of the shorter lag phase in medium with ruminal fluid, the inoculum for this medium contained substantially more TFU than the inoculum for the medium without ruminal fluid (Fig. 1, TFU per gram of WSR at zero time). Despite these relative differences, an exponential increase in TFU per gram of WSR occurred in both media

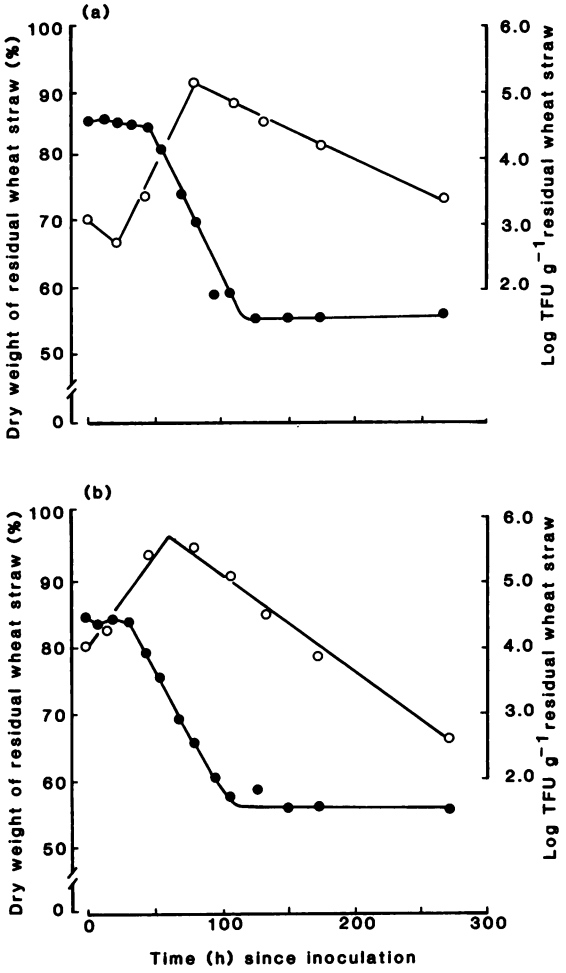

FIG. 1. Removal of DM and production of cell wall-degrading TFU during fermentation of wheat straw by *Neocallimastix* sp. strain R1 in medium without (a) and with (b) ruminal fluid. Symbols: ●, wheat straw residue; ○, TFU. For dry-weight values, the standard errors of the mean of pooled replicates across all times were 1.43 and 1.61 for medium B and medium with ruminal fluid, respectively. TFU values were fitted by using linear spline curves.

TABLE 2. Populations of anaerobic chytridiomycetes in ruminal contents of Friesian steers 1 h prior to feeding[a]

Animal	Mean TFU in ruminal contents[b]	
	Concn (10^4 TFU/g of RDM)	Amt (10^8 TFU/ rumen)
1	6.24ba	1.85ba
2	3.46cb	1.58cb
3	13.97cb	4.09cb
4	4.12ab	1.33ab
Range	2.20–28.55	0.94–8.37

[a] Digesta samples were obtained by completely removing the ruminal contents from each of four steers ca. 1 h before the morning feed.

[b] Each value represents the mean of three determinations, and coefficients of variation for animals 1, 2, 3, and 4 were 12.04, 17.47, 27.75, and 12.99%, respectively. The 95% confidence limits for the values in the table were as follows: lower limit, value in table × first letter (where a, b, and c represent 0.30, 0.40, and 0.46, respectively); upper limit, value in table × second letter (where a and b represent 4.6 and 5.3, respectively).

soon after inoculation (Fig. 1). These data were fitted to linear spline curves to determine exponential rates, breakpoints, and maximum population sizes. In cultures grown on medium without ruminal fluid, the population increased exponentially at a rate of 0.039 h^{-1} and reached a maximum size of 1.09×10^5 TFU g of WSR^{-1} after 84 h of incubation (Fig. 1a). The population increased more slowly in medium with ruminal fluid, at an exponential rate of 0.029 h^{-1}, and reached a higher maximum size (5.13×10^5 TFU g of WSR^{-1}) after 60 h of incubation (Fig. 1b). Thereafter, populations declined exponentially in both media until the end of the experimental period, 11 days after inoculation. The rate of decline of TFU per gram of WSR in media with and without ruminal fluid was 0.015 and 0.009 h^{-1}, respectively. The onset of population decline was not correlated with the lower inflection point of pH profiles and occurred without any apparent effect on the rate of DM loss of wheat straw at 50 h (medium with ruminal fluid) and 31 h (medium without ruminal fluid) prior to the maximum loss of wheat straw (Fig. 1). TFU were not detected in supernatants from cultures which were more than 15 days old; motile zoospores were absent from these cultures, and they would not produce viable cultures when used to inoculate fresh medium.

The TFU population in the reticulorumen. Results from each of the 12 enumerations (four animals with three replicate enumerations per animal) on digesta samples obtained by emptying ruminal contents 1 h prior to feeding are summarized in Table 2. Populations ranged from 2.20×10^4 to 28.6×10^4 TFU g of RDM^{-1}, with all but two of the values within a narrower range of 2.20×10^4 to 9.16×10^4 TFU g of RDM^{-1}. The mean population size across all animals (6.96×10^4 TFU g of RDM^{-1}) was smaller than the maximum populations which developed during the fermentation of wheat straw. When values were related to the total DM content of the rumen, the total population of fibrolytic anaerobic fungi in the reticulorumen was estimated to be within the range of 0.94×10^8 to 8.37×10^8 TFU, with an overall mean value of 2.09×10^8 TFU per rumen.

To determine whether the population density of fibrolytic anaerobic fungi in digesta contents was constant or varied throughout the day in response to feed intake profiles, we removed digesta samples from the raft region of three animals at intervals from 1 h before feeding to 12 h after the initial feed was offered. Intake profiles recorded for each animal during the entire feeding period showed that approximately 85% of the daily ration was consumed within the first 12 h of feeding. Each animal ate approximately 10 meals per

TABLE 3. Change in population of anaerobic chytridiomycetes in the reticulorumen of growing steers from 1 h prefeeding until 12 h after the initial feed was offered[a]

Animal	10^4 TFU/g of RDM[b] at following time (h) since initial feed was offered:						
	−1	1	2	3	6	9	12
1	22.40cd	42.20cc	20.40cd	20.70cd	25.40cd	18.0cd	3.64cc
2	3.95cc	18.80cd	3.43ab	13.50ba	8.12bb	9.44ba	8.41bb
3	3.98cc	18.46cd	20.79cd	12.86ba	3.25cc	6.46aa	7.91bb
Mean	10.1	26.5	14.9	15.7	12.3	11.3	6.7

[a] Digesta samples were obtained from three animals as grab samples from the raft region via the ruminal cannula. The ruminal contents of each animal were sampled without replication at each time interval.

[b] The 95% confidence limits were as follows: lower limit, value in table × first letter (where a, b, and c represent 0.25, 0.33, and 0.50, respectively); upper limit, value in table × second letter (where a, b, c, and d represent 3.4, 4.3, 5.3, and 7.0, respectively).

day, the largest meals being immediately after fresh feed was offered. Results for TFU enumerations (Table 3) show that the mean fungal population increased during initial feed consumption and declined slowly thereafter, although individual values varied and, as with some digesta samples obtained by the rumen-emptying procedure, large differences were evident between animals and within the same animal. Fungal populations ranged from 3.25×10^4 to 42.20×10^4 TFU g of RDM^{-1}, with the highest populations being observed in digesta samples from animal 1 (Table 3). The TFU population in individual animals increased by two- to fivefold 1 h after the first meal of the day (Table 3), and this coincided with the largest meal and highest eating rates during initial feed consumption. Further relationships between the eating behavior of individual steers and changes in population size of anaerobic fungi in digesta contents were not apparent.

TFU population in fresh and air-dried feces. The sizes of the fibrolytic population of anaerobic fungi in samples of fresh feces of animals 1, 2, and 3 were 31.9×10^4, 7.81×10^4, and 1.43×10^4 TFU g of FDM^{-1}, respectively. These values were similar to those obtained for the population density of TFU in ruminal contents and demonstrate the extent of the anaerobic fungal population in fresh feces. From fecal production data (grams of FDM per day) it was calculated that the steers excreted between 0.20×10^8 and 4.69×10^8 TFU day^{-1}. Despite repeated observations, motile zoospores were not detected in feces, although structures recognizable as zoosporangia were observed. In addition to enumeration of fungal populations in fresh feces, the MPN procedure was performed on numerous subsamples after they had been dried in air at ambient temperature and stored in air for up to 300 days. Anaerobic fungi with fibrolytic activity were detected in all dried fecal samples, although the population density declined with storage. During the initial 90 days of storage, the population declined exponentially at a rate of 0.022 TFU g of FDM^{-1} day^{-1} (Fig. 2). For 210 days thereafter, the fungal population remained approximately constant within the range of 29 to 782 TFU g of FDM^{-1}.

DISCUSSION

Enumeration of anaerobic fungi as TFU is analogous to enumeration of bacteria as CFU. In the same way that a CFU can be defined as a bacterium or collection of bacteria (rods in chains or cocci in tetrads, clusters, or chains) with the ability to produce a bacterial colony in culture, a TFU may be defined as a zoospore or collection of zoospores (inside a zoosporangium) with the ability to produce a fungal thallus in culture. Although CFU or TFU values are not absolute with respect to the total number of viable cells in a population, the enumeration has practical merit in that it represents the minimum size (effective size) of the population of viable cells. The method of enumeration, as performed in the present study, does not distinguish between stages of the fungal life cycle or between the number of thalli associated with each colonized particle. However, because populations were enumerated with respect to their ability to degrade plant cell walls, values are representative of the functional population of fibrolytic anaerobic fungi. Furthermore, by relating viable counts to sample DM content, the MPN procedure enabled comparative enumeration of anaerobic fungi from different locations such as the rumen and feces. This is unlike previous research, in which fungal enumeration was based upon direct counting of free-swimming zoospores in ruminal fluid (15) or upon the development of fungal colonies from zoospores in cellobiose-containing roll tubes (5). Although comparisons between MPN and other enumeration procedures have not yet been made, the range of values for TFU per milliliter of ruminal fluid in the present study (0.38×10^4 to 4.40×10^4 TFU ml^{-1}) is within the range quoted in the literature for the concentration of zoospores per milliliter of ruminal fluid from sheep (10^3 to 10^5 ml^{-1} [5, 15, 17]). With regard to the time required for enumeration, the majority of enumeration tubes which contained anaerobic fungi were positive within 3 to 4 days of incubation. However, an incubation period of 10 days was

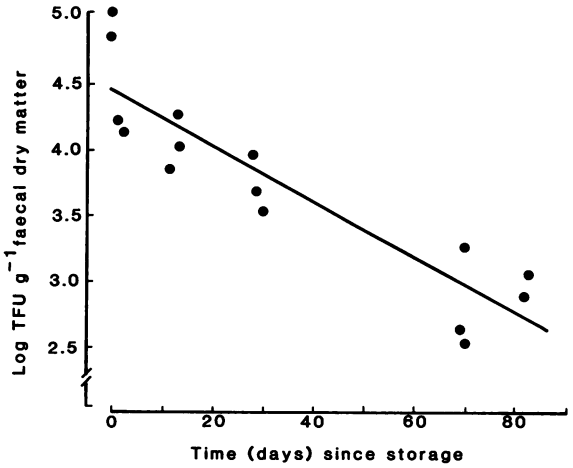

FIG. 2. Exponential decline of the TFU population of anaerobic fungi in air-dried feces after 90 days of storage in air at ambient temperature. The standard error of the mean for pooled replicates across all times was 0.44. Daily means were regressed against days, and the variance accounted for (corrected r^2) was 80.1.

adopted as a precautionary measure when looking for anaerobic fungi in feces, where dormant cysts or spores may require time to germinate. For enumeration of anaerobic fungi in batch cultures or in ruminal contents, the MPN procedure takes no longer and is technically less demanding than the roll tube method of viable counting (5).

In published accounts, when light microscopy was used to enumerate zoospores in the ruminal fluid of sheep fed a restricted diet, the zoospore population (referred to as the protozoan flagellate, *Callimastix frontalis*, in the early work of Orpin [12]) was minimal prior to feeding and increased (by up to 44-fold) and declined dramatically soon after the feed was consumed (12, 13). Orpin concluded that the population increase was in response to a water-soluble component(s) in the feed which induced zoosporogenesis and that population decline occurred as liberated zoospores attached to freshly ingested plant material (15). In the present study, TFU populations in the bovine rumen were maximal 1 h after the initial feed was consumed, but in contrast to previous work, the population increase was markedly smaller (only two- to fivefold) and populations varied considerably during feeding (Tables 2 and 3). Differences between the two studies may be due to the enumeration procedures used, but may also reflect differences in animal feeding. Whereas fungal populations were synchronized in sheep fed once daily, cattle in the present study consumed hay over a much longer period, presumably supporting the continuous production of fungal biomass.

With regard to sampling error, TFU data from batch cultures were of sufficient quality to merit curve fitting and comparative statistical analysis. With ruminal and fecal samples, however, substantial variation within and between animals was evident (Tables 2 and 3; Fig. 2). These samples were obtained from different animals which had more or less stratified rumens and were fed ad libitum; even mixing of digesta contents via ruminal emptying did not result in an improvement in the quality of enumeration in all cases. We would argue that variation encountered with in vivo samples is not solely due to the enumeration procedure, but is to be expected as a true reflection of the heterogeneity of the digestive-tract ecosystem, particularly with animals fed ad libitum. Thus, the range of values obtained may be more significant than the individual counts; fecal samples, for example, contained fungal populations within the same range of those found in ruminal digesta.

Feces contained substantial populations of anaerobic fungi which declined very slowly after drying, such that organisms identical in appearance to ruminal chytridiomycetes could still be cultured up to 10 months later. Related studies have shown that zoospores and vegetative thalli are highly sensitive to periods of exposure to low temperatures and air (9, 11, 21); thus, it is improbable that feces contained active populations of these stages of the fungal life cycle. Furthermore, motile zoospores were not detected in fecal contents, although structures recognizable as fungal zoosporangia were observed. Although their observation does not preclude the existence of other as yet unrecognized structures, such as zoospore cysts, perhaps these zoosporangia gave rise to some if not all of the fungi cultured from fresh and air-dried feces. Thus, on the basis of evidence obtained both during the course of this work and in related studies, we propose that the life cycle of anaerobic chytridiomycetes, like that of many of their aerobic counterparts, consists of three stages: the motile zoospore, the vegetative thallus, and an aerotolerant survival stage (perhaps a resistant zoosporangium). Two other reports suggest that anaerobic fungi produce survival structures (cysts or spores). Although survival was not demonstrated, Orpin (16) commented on the appearance of thick-walled zoosporangia in the cecal contents of the horse. Perhaps more significant was the report of Joblin (5) that anaerobic fungi could be maintained in sisal roll tubes for up to 7 months. A survival stage in ruminal fungi would explain their transfer through the entire digestive tract (Theodorou et al., unpublished) and the apparent anomaly of large populations of anaerobic fungi in feces. These survival structures might be disseminated via feces to herbage in the field, thus enabling the transfer of anaerobic fungi between herbivores. With respect to host nutrition, loss of fungal biomass from the digestive tract via feces represents yet another drain on the conversion efficiency of plant biomass in the ruminal ecosystem.

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