Isolation and fermentative activity of rumen anaerobic fungi in dairy cows

Isolamento e atividade fermentativa de fungos anaeróbios do rúmen de vacas leiteiras

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ABSTRACT

The possibility of biotechnological application of anaerobic fungi and their biomass-degrading enzymes has been a growing area of research and development over the past decade. In this context, the occurrence and isolation of rumen fungi were evaluated in three Holstein-Gir dairy cows grazing *Urochloa* spp., in addition to the fermentation capacity of theses isolates in media containing different carbohydrates. Motile zoospores were found in all of the rumen samples. Twelve fungal strains were recovered and were capable of degrading different carbohydrates and lignocellulosic components, suggesting that these strains are able to produce various degradative enzymes when grown on glucose, xylose, cellobiose, fructose, and cellulose. Results pointed out the first insight into the isolation of rumen anaerobic fungi in dairy cattle in Brazil and suggested that further studies are needed to show the potential of some species for commercial application, especially in tropical ruminants.

KEYWORDS: *Chytridiomycetes*, rumen microbiology, dairy cattle, tropical forage.

RESUMO

A possibilidade de aplicação biotecnológica dos fungos anaeróbios, e suas enzimas de degradação de biomassa, tem sido uma área cada vez maior de pesquisa e desenvolvimento na última década. Neste contexto, a ocorrência e o isolamento de fungos ruminais foram avaliados em três vacas Holandês x Gir sob pastejo de *Urochloa* spp., além da capacidade de fermentação dos isolados em meios de cultura contendo diferentes fontes de carboidratos. Zoósporos móveis foram encontrados em todos os conteúdos ruminais analisadas. Doze isolados foram recuperados e todos foram capazes de degradar diferentes carboidratos e componentes lignocelulósicos, sugerindo que estes isolados são capazes de produzir várias enzimas degradativas. Estes resultados revelaram as primeiras descobertas sobre o isolamento de fungos anaeróbios ruminais em gado leiteiro no Brasil e sugere que estudos adicionais são necessários para evidenciar o potencial de algumas espécies para a aplicação comercial, principalmente em ruminantes tropicais.

PALAVRAS-CHAVE: *Chytridiomycetes*, microbiologia ruminal, gado leiteiro, forrageira tropical.

The rumen is a complex microbial ecosystem (bacteria, fungi, and protozoa) where lignocellulosic plant biomass is efficiently degraded and reduced to microbial end-products, providing nutrition for the host animal (CHENG et al. 2009, ARCURI et al. 2011). Despite their potential in industrial bioprocessing, studies have indicated an important role for anaerobic fungi in fiber digestion due to the presence of highly active celluloses and a wide range of fiber-degrading enzymes (LEE et al. 2004). In this point, production of diverse enzymes has recently raised interest due not only their distinctive physiology but also their efficient animal nutrition and biotechnological potential (YUE et al. 2013).

Anaerobic fungi are reported in lower numbers (10^3-10^5 mL^{-1}) (ORPIN & MOUNTFORT 1994), however the application of culture-dependent approaches to assessing the diversity of anaerobic fungi provided valuable insights on the prevalence and association of specific genera with certain animals (JIN et al. 2011). More than three decades have elapsed since rumen fungi were first recognized by Orpin (ORPIN 1975), and few advances have been made in understanding their ecology, physiology, and enzymology in ruminants in Brazil. Thus, this study reports the growth and fermentation capacity in different carbohydrates of rumen fungi isolated from dairy cattle.

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Ruminal contents were collected into pre-warmed thermos flasks from three rumen cannulated, non-lactating, Holstein-Gir dairy cows, grazing brachiaria grass (*Urochloa* spp.) at the Experimental Field José Henrique Bruschi (Embrapa Dairy Cattle, Coronel Pacheco, MG, Brazil). Twenty rumen fluid samples (100 mL) were collected at different times between January and March (approximately 2 weeks apart), and a total of 4 samples were collected for each animal. The digestive pH was determined by using a mobile pH meter. Motile zoospores of anaerobic fungi were observed microscopically in all fresh rumen fluid samples according to ORPIN & MOUNTFORT (1994).

Three aliquots (0.5 mL) of rumen fluid were inoculated into Hungate tubes (Bellco Glass, Inc., USA), containing 8 mL of GSM – "Growth Study Medium" (ODENYO et al. 1991) supplemented with antibiotics (penicillin, 12.1 mg mL^{-1} [Sigma Chemical Co., USA]; Streptomycin sulfate, 2 mg mL^{-1} [Vetec Chemicals Ltd., Brazil], and chloramphenicol, 0.300 mg mL^{-1} [Sigma Chemical Co.]). The antibiotics were added as described by TRIPATHI et al. (2007). The Hungate tubes were sealed with butyl rubber septa and aluminum crimp-seals (Bellco Glass Inc., USA) and incubated (39 ºC). All laboratory handling of rumen fluid was carried out under continuous flushing with CO_2.

For isolation of fungal colonies, roll-tubes technique was used (HUNGATE 1966). After 5 days of incubation, cultures of anaerobic fungi were examined (uniformity of morphological characteristics such as monocentric or polycentric, sporangium shape, and zoospore flagellation). The fungal strains were sub cultured every 3-4 days.

Fermentation tests for each isolate were carried out in GSM, substituting glucose with alternative carbon sources, and the growth was determined visually after 24, 48, and 72 h of incubation in triplicate. Cellobiose, fructose, arabinose, galactose, xylose, sucrose (3 g), carboxymethyl cellulose (CMC 0.3%), or filter papers were used as the sole carbon source in the culture medium. Strips of filter paper (Whatman n.1, weight: 0.02 g) were pretreated with 85% phosphoric acid and 1% sodium bicarbonate (ODENYO et al. 1991), and then added to tubes containing GSM.

The average pH of samples was 6.6, and motile zoospores and fungal structures of anaerobic fungi were found in all of the rumen fluid samples examined microscopically. Some of the zoospores observed possessed 1-4 flagella and their shape was ovoid. The observation of zoospores confirms the presence of these microorganisms in the rumen of the animals used in this investigation. Analysis of the population composition of anaerobic fungi in the initial rumen inoculum showed the range of morphological diversity of the zoospores, sporangia, and rhizoidal systems, which suggests the presence of multiple species. In all 12 rumen fluid samples (100%) monocentric fungi were detected, and the shape of the sporangia were showed to be diverse, as spherical (Figure 1a) or ovoid (Figure 1b). Polycentric sporangia were observed in all animals, but in only 9 samples (75%). Similar results were observed by ABRÃO et al. (2010) in rumen contents of cattle in Brazil. In the present study, dairy cattle were fed a high fiber diet and this may promote the concomitant developing of fungi monocentric and polycentric.

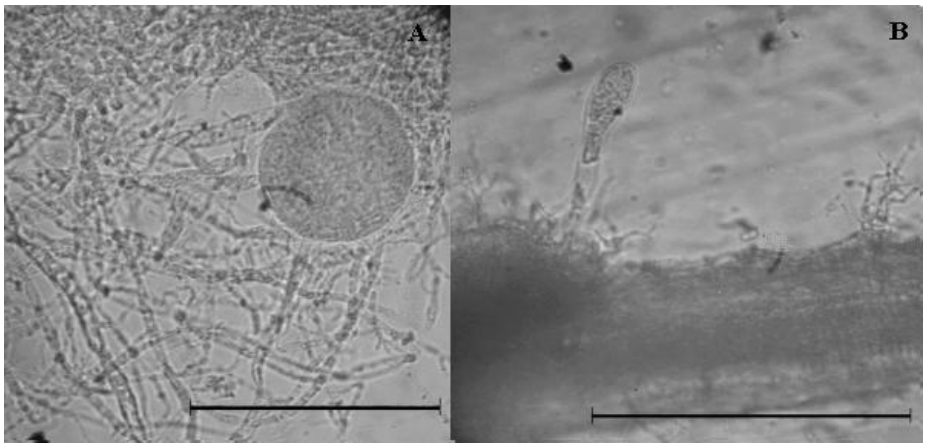

Figure 1 - Morphology of rumen anaerobic fungi observed from dairy cattle (A) Spherical sporangium with extensively branched rhizoid, Scale bar: 0.2 mm. (B) Ovoid sporangia on fibrous particles, Scale bar: 0.2 mm.

Despite difficulties in growing these microorganisms, twelve fungal strains were isolated among all samples. However, only morphological data were not sufficient for the identification of isolates. According to FLIEGEROVÁ et al. (2006) and LWIN et al. (2011), the classification of anaerobic fungi by physiological data and growth in culture are not conclusive. All strains isolated were able to utilize the tested disaccharides, cellobiose, and sucrose. Among the five monosaccharides tested, only fructose and xylose supported growth. Arabinose and galactose did not support growth. The isolates also grew on filter paper but not on carboxymethyl cellulose (CMC). The colonization on filter paper was demonstrated qualitatively after 48 h of incubation, as well as results found by DENMAN et al. (2008). It was observed that the strains grew more rapidly and with more biomass on the xylose and cellobiose substrates, which may suggest that anaerobic rumen fungi possess high cellulolytic and xylanolytic enzyme activities. The results strongly suggest that polycentric and monocentric fungi found in the rumen possess common characteristics for carbohydrate utilization and fermentation end-products. However, other isolates need to be studied for comparison between monocentric and polycentric fungi.

Altogether, these results revealed first insights into isolation of anaerobic fungi in dairy cattle in Brazil. All isolates were capable of degrading different soluble carbohydrate and lignocellulosic components to a great extent, suggesting that these isolates may have potential in cellulolytic and hemi-cellulolytic enzymes productions. Taken together, these properties make anaerobic fungi attractive in the long term for the scientific community (FLIEGEROVÁ et al. 2006). Studies on affinities and capacities for soluble sugar utilization by anaerobic fungi provide useful information on the likely contribution of these organisms to overall soluble carbohydrate fermentation in the rumen.

Efforts to study more anaerobic fungal genera using the application of next-generation sequencing techniques will improve our understanding of their identification and classification. Attempts to isolate robust strains of anaerobic fungi will result in extensive biotechnological application of anaerobic fungi and/or their enzymes in the future; making it more viable to work with these fungi on a commercial scale and/or in continuous culture (GRUNINGER et al. 2014). Moreover, further studies of these microorganisms are needed to show the higher potential of some species for commercial application than others, mainly in tropical ruminants.

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