Isolation of Anaerobic Phycomycete Fungi from Some Herbivores and Their Chemical Composition

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Abstract Anaerobic phycomycete fungi were found in gut and faecal samples from sheep, cattle, goat, sika deer, Japanese serows, the musk ox, horses and ponies. The population size of the fungi estimated by the roll tube method was of the order of 10^3 /ml for the gut samples and 10^2 /g for the faecal samples. A *Neocallimastix* sp. and a *Piromyces* sp. were isolated from sheep rumen samples and a *Caecomyces* sp. from horse faecal samples. The chemical composition of these isolates was examined: there were no distinct differences in amino acid and fatty acid composition among the three isolates, and the amino acid composition was similar to that of rumen protozoa. The fatty acid composition of the isolates was rich in long-chain fatty acids, especially in oleic acid, which amounted to 45% of total fatty acids (w/w) in the lyophilized samples of the *Piromyces* sp. The isolates all contained chitin ranging from 3.4% (*Piromyces* sp.) to 4.3% (*Caecomyces* sp.).

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A wide distribution of anaerobic phycomycete fungi in domestic and wild herbivores has so far been reported.¹¹⁾ However, we have only scanty information on fungi in herbivores which are reared or live freely in this country. It is well established that anaerobic fungi play a significant role in fiber digestion in the rumen of domestic animals¹¹⁾.

On the other hand, their nutritional contributions as protein and lipid sources to the host animal are not clear. The present paper describes the isolation and enumeration of anaerobic phycomycete fungi from the gut and faeces of various animals, together with the chemical composition of these isolated fungi.

Materials and Methods

The following animals were employed for the

fungal sampling: cattle (Holstein), sheep (a Corriedale $\times$ Suffolk cross), goats (Saanen), sika deer (*Cervus nippon*), Japanese serows (*Capri-cornis crispus*), musk ox (*Ovibos moschatus*), horses (Thoroughbred) and ponies. Cattle, sheep and horses were reared with appropriate roughage (hay, straw or haycube) and concentrate on the university farm. The other animals were maintained in a city park (goat and ponies) and in a mountain park in Mie prefecture with hay and wheat bran.

In sheep, fungal inoculum was prepared from ruminal and caecal contents and fresh faeces, while in cows from rumen contents and fresh faeces. In the other remaining animals, fresh faeces were used for the inoculum. Rumen and caecal contents were strained through two layers of gauze and aliquots were then sus-

pended in a diluent solution with vigorous stirring. Inocula thus prepared were inoculated into roll tubes with JOBLIN's medium⁵⁾ containing streptomycin (2 mg/ml) and penicillin (2×10^4 iu/ml), and the tubes were incubated at 37°C for 5 days. These procedures were carried out under anaerobic conditions. Faecal samples were treated in a manner similar to the gut samples described above and their inocula were incubated in roll tubes to enumerate colony counts, from which zoospores in terms of number/g or ml of samples were calculated. Three species of phycomycete fungi were isolated through these separation experiments. Based on the morphology of zoospores, these were a *Neocallimastix* (*N.*) sp., a *Piromyces* (*P.*) sp. and a *Caecomyces* (*C.*) sp., though each species name was unknown.

Each of the isolates was cultivated and submitted to analyses of chemical composition as follows. Five days after incubation, colonies developed in roll tubes were picked under anaerobic conditions, inoculated into a liquid medium described by JOBLIN⁵⁾ and incubated at 37°C for 5 days. Fungi thus cultivated were collected with centrifugation (1500 rpm, 5 min), washed 3 times with 0.9% saline and lyophilized. Amino acids of fungal freeze dried samples were analyzed by an

o-phthalaldehyde method with the aid of HPLC (Tosoh Co.)¹³⁾. Medium- and long-chain fatty acids were methylated with HCl-methanol (5:95, v/v) and the resultant methyl esters were determined by gas chromatography¹⁵⁾. Concentrations of chitin in fungal samples were determined as described by CHEN and JOHNSON⁴⁾. Amino acid and fatty acid composition of *Entodinium* protozoa and mixed rumen bacteria from the sheep rumen were also analyzed in a similar way to compare them with fungi.

Results

Anaerobic phycomycete fungi ranging from 0.2×10^2 to 3.4×10^3 were found in all tested gut and faecal samples (Table 1). Rumen samples from sheep and cows contained more numerous zoospores than the faecal samples. The number of zoospores detected in faecal samples were mostly of the order of 10^2 but those from sika deer and horses were less than 10^2 .

The amino acid and fatty acid composition of fungal isolates is shown in Tables 2 and 3. A *N.* sp. and a *P.* sp. were isolated from sheep rumen samples, while a *C.* sp. was isolated from horse faecal samples. Amino acid analysis of the fungal isolates failed to estimate

Table 1. Enumeration of anaerobic phycomycete fungal colonies in roll tubes inoculated gut and faecal samples from various herbivores

Animal	No. of animals	Sample	No. of colonies
Sheep (<i>Ovis aries</i>)	9	rumen	3.4×10^3 /ml
		caecum	0.8×10^3 /g
		faeces	0.3×10^3 /g
Cattle (<i>Bos taurus</i>)	4	rumen	3.4×10^3 /ml
		faeces	0.8×10^3 /g
Goat (<i>Capra hircus</i>)	1	"	0.6×10^3 /g
Sika deer (<i>Cervus nippon</i>)	3	"	0.2×10^2 /g
Japanese serow (<i>Capricornis crispus</i>)	2	"	1.0×10^2 /g
Musk ox (<i>Ovibos moschatus</i>)	1	"	2.6×10^2 /g
Horse (<i>Equus caballus</i>)	3	"	0.3×10^2 /g
Pony (<i>Equus caballus</i>)	2	"	1.7×10^2 /g

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Table 2. Amino acid composition of a lyophilized sample of three fungal isolates (*Neocallimastix*(*N.*) sp., *Piromyces*(*P.*) sp. and *Caecomyces*(*C.*) sp.) in comparison with sheep rumen ciliates and mixed rumen bacteria

Amino acid	Fungus			Rumen	Mixed rumen
	<i>N.</i> sp.	<i>P.</i> sp.	<i>C.</i> sp.	ciliates	bacteria
	(Molar %)				
Aspartic acid	13.0	13.2	11.7	13.2	8.6
Threonine	6.9	6.8	5.7	5.8	6.0
Serine	7.5	7.3	6.3	5.7	5.9
Glutamic acid	13.0	12.6	13.1	13.7	8.8
Proline	5.3	5.3	4.4	4.6	4.8
Glycine	8.3	8.6	9.0	8.0	8.7
Alanine	8.7	8.5	11.5	6.9	11.0
1/2 Cystine	0.6	0.3	0.5	0.1	0.6
Valine	8.0	7.6	7.1	5.5	8.6
Methionine	1.9	1.6	1.4	0.4	2.1
Isoleucine	7.8	8.4	6.7	7.1	5.3
Leucine	ND	ND	ND	8.4	8.4
Tyrosine	ND	ND	ND	2.2	5.7
Phenylalanine	5.6	5.1	6.4	4.6	5.3
Histidine	2.9	3.1	3.2	2.0	1.8
Lysine	6.7	7.5	9.1	8.3	4.5
Arginine	3.7	4.0	3.9	3.5	4.0
	(mM/mg)				
Total	0.49	0.30	0.38	0.78	1.84

ND: not determined

leucine and tyrosine concentrations because of the overlapping peaks of these amino acids with some glucosamines. Accordingly, the amino acid molar percent was calculated with the exception of leucine and tyrosine. The amino acid composition of the three fungal isolates was similar, with all having abundant aspartic and glutamic acids. The fungal amino acid composition was also similar to protozoal amino acid composition but the amino acid content of fungi was about one-half that of protozoa (Table 2).

There were no distinct differences in fatty acid composition among the three fungal isolates. The prominent feature of fungal fatty acid composition was a large amount of oleic acid, which was approximately 2 times as large as that of protozoa. In contrast to protozoa and bacteria, fungal samples contained a

relatively high degree of medium-chain fatty acids. (Table 3). The chitin content of fungal isolates was as follows; 4.3% in the *C.* sp., 4.0% in the *N.* sp., and 3.4% in the *P.* sp.. Chitin, however, was not detected in either *Entodinium* protozoa or mixed rumen bacteria.

Discussion

This study confirmed the presence of anaerobic phycomycete fungi in foregut-fermenters (sheep, cattle, goats, sika deer, Japanese serows and the musk ox) as well as hindgut-fermenters (horses and ponies). In addition to these animals, a distribution of these fungi has been found in impalas, kangaroos, elephants¹¹, reindeer and camels¹¹. However, this is the first ever demonstration that the sika deer and Japanese serow are both herbivores maintaining strictly anaerobic fungi in the gut.

Table 3. Fatty acid composition of lipids in a lyophilized sample of isolates (*Neocallimastix* (*N.*) sp., *Piromyces* (*P.*) sp. and *Caecomyces* (*C.*) sp.) in comparison with sheep rumen ciliates and mixed rumen bacteria

Fatty acid	Fungus			Rumen ciliates	Mixed rumen bacteria
	<i>N.</i> sp.	<i>P.</i> sp.	<i>C.</i> sp.		
	(% in weight)				
12:0	6.8	7.7	9.9	0.6	1.6
13:0	—	—	—	0.8	1.1
14:0	13.6	10.1	8.4	2.0	6.0
15:00	—	—	—	—	8.5
15:0	—	—	—	1.3	4.2
16:0	25.5	23.3	33.4	54.1	31.4
18:0	12.3	13.8	15.3	7.6	39.4
18:1	41.8	45.1	33.2	21.1	5.3
18:2	—	—	—	12.6	—
19:0	—	—	—	—	2.5
	(mg/g)				
Total	8.5	11.0	7.8	12.4	31.4

—: not detected

The zoospore population density of sheep and cattle rumen was in the range of 10^3 /ml, in accordance with previous reports^{5,11,14}. Compared to the rumen, faecal samples showed a greater decrease in zoospore population density, suggesting that rumen fungi resist outflow from the rumen and/or that most of the flowed zoospores are digested in the lower gut of the host animal.

The amino acid composition of the present fungal isolates was similar to that of rumen protozoa, indicating that fungal proteins are very excellent nutrients for host animals if notable amounts are transferred from the rumen to the intestine and hydrolyzed there. KEMP *et al.*⁷ determined the free- and protein-amino acids of *N. frontalis* and *P. communis*, and found that the amino acid profiles of the two fungi were similar and comparable to casein and lucerne protein. In comparing our results with those of KEMP *et al.*⁷, there was reasonable consistency in amino acid profiles except for the molar percent of alanine. The minor differences in amino acid composition between fungi belonging to the same genus may

be due to differences in species, growth stages and the cultivation of fungi employed.

The present fungal isolates were characterized by their high oleic acid content as compared to those of rumen protozoa and bacteria (Table 3). This is consistent with the data provided by BODY and BAUCHOP³ and KEMP *et al.*⁶ which analyzed the lipids of *N. frontalis* and *P. communis* in detail. In their study⁶, ¹⁴C-oleic acid as a tracer was incorporated into fungal lipids without being appreciably metabolized to other fatty acids, and nearly one-half of labelled glucose and acetate was used for oleic acid synthesis. These findings, together with the high oleic acid content in fungal lipids, suggest that rumen fungi, including the *P.* sp. as well as the *N.* sp. and *C.* sp., require this fatty acid to a greater degree. However, the functional role of oleic acid in these fungi remains to be clarified.

In the present culture condition, it is highly probable that both methanogens² and mycoplasmas⁸ are associated with fungal samples, in that these associated organisms may contaminate the data on amino acid and fatty

acid composition in Tables 2 and 3. However, the magnitude of this contamination is obscure at present, and a chemical analysis of bacteria- and mycoplasma-free fungi remains to be determined.

The present study confirmed the presence of chitin in the fungal isolates, in good agreement with previous reports^{7,10}). The proportion of chitin content in our isolates varied, according to species, from 3.4% to 4.3%, whereas chitin content ranging from 8-22%⁸) to 8-12%⁷) was reported for *N. frontalis*, *P. communis* and *C. communis*. These different chitin content values are attributable to differences in analytical methods, fungal species and growth stage. Although the chitin content of our isolates was low, polyoxin (an inhibitor of chitin synthesis) completely suppressed the growth of isolates (CANN *et al.*, unpublished), which is consistent with the case of nikkomycin on an *N. sp.*⁹). This indicates that chitin plays an essential role in the growth of anaerobic phycomycete fungi, as previously reported¹¹).

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草食動物から嫌気性藻菌類の分離とそれらの化学組成

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ヒツジ，ウシ，ヤギ，日本シカ，日本カモシカ，ジャコウウシ，ウマ，ボニーの消化管内容物または新鮮糞から嫌気性藻菌類を Joblin の培地を用いたロールチューブ法で分離した。得られた藻菌類のコロニー数は，試料 1 ml 当り消化管内容物（ルーメンおよび盲腸）で 10^3 ，糞試料 1 g 当りで 10^2 のオーダーであった。ヒツジのルーメン試料から *Neocallimastix* 属，*Piromyces* 属，ウマの糞試料から *Caecomyces* 属の藻菌を単離し，それらの化学組成を調べた。アミノ酸組成は 3 種の藻菌とも類似しており，*Entodinium* 属のルーメンプロトゾアのそれとよく似た組成であった。脂肪酸組成も藻菌種間に大きな差異はないものの長鎖脂肪酸，とりわけオレイン酸含量が多く，凍結乾燥菌体の総脂肪酸の 33～45%（w/w）も占めていた。また，3 菌種ともチキンが検出でき，3.4～4.3% 含まれていることが分った。

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