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Original article

Effects of amino acids on the growth of an anaerobic rumen fungus *Neocallimastix* sp N 13

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Summary — The amino acid requirements of *Neocallimastix* sp N 13 isolated from sheep rumen were studied using supplements containing either ten essential amino acids (10 EAA) or eight nonessential amino acids (8 NEAA), or both (18 AA). Although the fungus could grow in a medium containing ammonium sulphate as the sole source of nitrogen, amino acid supplements, especially 18 AA, greatly stimulated its growth. Omission of the sulphur-containing amino acids (SCAA) from the 18 AA mixture markedly reduced the growth-stimulating effect, and sulphide, but not sulphate, substituted for SCAA only partly compensated for this omission. Omission of aromatic, branched chain, basic, acidic, aliphatic and hydroxy amino acids, and proline, singly or in combination, all reduced fungal growth to some degree as compared with the 18 AA supplement. A three amino acid (leucine, methionine and histidine) supplement was a potent stimulator for the fungus, whereas another three amino acid combination (glutamic acid, methionine and serine) was ineffective. The results indicated that *Neocallimastix* sp N 13 effectively utilized amino acids for its growth, but the requirements for the different amino acids differed from those of *N patriciarum*.

rumen fungus / *Neocallimastix* / amino acids / growth / sheep

Résumé — Effets des acides aminés sur la croissance du champignon anaérobie *Neocallimastix* sp N 13 isolé du rumen du mouton. *Les besoins en acides aminés de* Neocallimastix *sp N 13 isolé du rumen de mouton ont été étudiés en utilisant des suppléments contenant soit dix acides aminés essentiels (10 EAA), soit huit acides aminés non essentiels (8 NEAA), soit les deux (18 AA). Bien que le champignon puisse croître sur un milieu contenant du sulfate d'ammonium comme seule source d'azote, les suppléments d'acides aminés, particulièrement 18AA, ont considérablement stimulé sa croissance. L'omission des acides aminés soufrés (SCAA) dans le mélange 18AA a réduit nettement l'effet stimulateur sur la croissance, alors que les sulfures, mais pas les sulfates, substitués aux SCAA compensaient seulement en partie cette omission. La suppression des acides aminés aromatiques, à chaîne ramifiée, basiques, acides, aliphatiques et des hydroxyaminoacides et de la proline, seuls ou combinés, a réduit la croissance du champignon par rapport à l'effet des 18AA. Un supplément de trois*

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acides aminés (leucine, méthionine, histidine) avait un pouvoir stimulateur, tandis qu'une autre combinaison de trois acides aminés (acide glutamique, méthionine, sérine) était sans effet. Les résultats ont montré que Neocallimastix *sp N 13 utilisait effectivement les acides aminés pour sa croissance, mais les besoins pour les différents acides aminés diffèrent de ceux de* N patriciarum.

champignon du rumen* / Neocallimastix / *acide aminé* / *croissance* / *mouton

INTRODUCTION

The free- and protein component-amino acid contents of anaerobic rumen fungi *Neocallimastix frontalis* and *Piromyces communis* are comparable to those of casein and lucern leaf protein (Kemp et al, 1985). We have also confirmed that the amino acid profiles of *Neocallimastix* sp, *Piromyces* sp and *Caecomyces* sp resemble one another and are similar to those of rumen protozoa. They are characterized by a high lysine content as compared with rumen bacterial proteins (Onoda et al, 1993). Moreover, fresh rumen fungi infused into the abomasum are extensively digested (Gulati et al, 1989). These findings suggest that the rumen fungi may substantially contribute to the amino acid supply for their host animals if notable amounts of fungi are transferred from the rumen to the lower gut.

Orpin and Greenwood (1986) demonstrated that amino acid supplements to the defined minimal medium (consisting of ammonium ion, haemin, vitamins and a reduced source of sulphur) greatly stimulate the growth of *Neocallimastix patriciarum*. The rumen fungi *Neocallimastix* sp can take up amino acids and incorporate them into proteins without requiring any modification of the amino acids (Gulati et al, 1989). When a mixture of L-(U-^{14}C) amino acids was added to cultures of rumen fungi, the radioactivity was later found located in the cellular proteins, lactate, volatile fatty acids and ethanol (Marounek and Vovk, 1992). Thus, it is highly probable that rumen fungi can synthesize their cellular proteins from amino acids which were either taken up from the surrounding environment or synthesized in the cell, or both, and that a performed amino acid supply is favourable for the rapid growth of fungi. However, the amino acid requirement of rumen fungi has not yet been fully elucidated, except that cysteine and cysteine-like compounds support the growth of *Neocallimastix* sp (Phillips and Gordon, 1991).

Maeng et al (1976) reported that substitutions of amino acid mixtures for urea stimulate rumen bacterial growth in vitro, the stimulation being greatest when a mixture of 18 amino acids (18 AA) was substituted for urea, followed by a mixture of ten essential amino acids (10 EAA) and of eight nonessential amino acids (8 NEAA). These amino acids are known to be essential or nonessential for the growth of rats and other mammals.

Argyle and Baldwin (1989) also demonstrated that rumen bacterial growth is greatly stimulated by the complete amino acid mixture consisting of 18 AA and two amides, and growth stimulation from amino acids is due to the number of amino acids provided in a given mixture rather than specific growth limiting amino acids. Fujimaki et al (1989, 1992) confirmed these previous findings and found that an amino acid mixture (leucine, methionine and histidine) is comparable to the mixture of 10 EAA in stimulating bacterial growth yields.

In the present paper, we describe the effects of different amino acid supplements, which have been found to be effective for rumen bacterial growth, on the growth of a sheep ruminal fungus, *Neocallimastix* sp N 13.

MATERIALS AND METHODS

Fungus and culture

Neocallimastix sp N 13 was isolated from the rumen of fistulated crossbred (Corriedale x Suffolk) sheep fed a diet of 400 g concentrate and 600 g lucern hay cubes daily. The isolated fungus was maintained anaerobically in Joblin's medium (1981) in which cellobiose was replaced with cellulose in the form of Toyo No 1 filter paper (equivalent to Whatman No 1) and including the antibiotics (ampicillin: 0.09 g/L, chloramphenicol: 0.09 g/L and streptomycin: 0.09 g/L). Stock cultures were subcultured every 6 days, enabling the isolate to retain its ability to use cellulose and proliferate.

Neocallimastix sp N 13 was grown on liquid Joblin's medium (1981) for 3 days and then zoospores were harvested anaerobically by filtrating the cultures through nylon meshes (10 µm). The filtrate containing the zoospores (0.3 ml) was inoculated into 5 ml of a basal medium in sealed Hungate tubes (100 x 6 mm) with or without amino acid present and after the addition of 0.2 ml of an antibiotic solution containing ampicillin (0.25%), chloramphenicol (0.25%) and streptomycin sulfphate (0.25%); it was incubated anaerobically at 39 °C for at least 5 days. Although the fungal culture was definitely axenic, the antibiotics were added to the culture for possible contamination. The basal medium consisted of a salt solution A (34.0 ml) and B (34.0 ml) (Hungate, 1969), cellobiose (0.4 g), $NaHCO_3$ (1.0 g), 0.05% haemin solution (0.8 ml), $Na_2S.9H_2O$ (0.08 g), vitamins (2.10 ml) and a volatile fatty acid solution (14.0 ml) for rumen bacteria (RGCMSA medium) (Hungate, 1969), 0.1% resazurin solution (0.2 ml) and distilled water (125.9 ml). The sole nitrogen source of the basal medium was ammonium sulphate (12 g/L) from salt solution B.

Amino acid supplements

Each of the L-amino acids was dissolved in either 0.1 M HCl or 0.1 M NaOH at a concentration of 0.1 M except for aspartic acid (0.07 M) and tyrosine (0.05 M) and mixed to give an equal molar mixture. The 10 EAA consisted of arginine (Arg), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp) and valine (Val), while the 8 NEAA contained alanine (Ala), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), glycine (Gly), proline (Pro), serine (Ser) and tyrosine (Tyr). The 18 AA was made up of both 10 EAA and 8 NEAA. The pH value of these and other amino acid mixtures was adjusted to 6.8 prior to use in the incubations.

To compare the effect of the different amino acid supplements on the rate of fungal growth, the amino acid mixtures were added to the basal medium to give an equal concentration of amino acid (5 mg/ml medium: experiment 1) or amino-nitrogen (0.69 mg/ml medium: experiments 2–4). The control tubes without amino acid supplement contained only basal medium which had ammonium sulphate (0.41 mg/ml) as a nitrogen source.

In experiment (Exp) 1, the rates of fungal growth for the three amino acid supplements (18 AA, 10 EAA and 8 NEAA) were compared with that of a control. Since the most effective supplement was 18 AA, the amino acid requirement for fungal growth was evaluated by the sequential omission of an amino acid or an amino acid group from the 18 AA medium. Special amino acid combinations (Lys + Met + His or Glu + Met + Ser) were also tested.

Analytical procedures

During the 5 day incubation, fungal protein and chitin levels were determined every day to evaluate growth rates. The culture tube was centrifuged (2 200 g for 15 min) and the resultant supernatant was saved for amino acid analysis. The precipitate was washed with distilled water four times, dried overnight at 100 °C, dissolved by standing in 1 M NaOH solution at 100 °C for 5 min and then centrifuged (2 200 g for 15 min). The aliquots were used for protein determination as described by Lowry et al (1951). The residual precipitate was washed four times with distilled water, dried overnight at 100 °C, hydrolyzed by heating at 100 °C for 4 h with 6 M HCl solution and filtered through Toyo No 1 filter paper. The filtrate was dried in vacuo and the residue was dissolved in distilled water for the determination of the amount of fungal chitin using the method of Chen and Johnson (1983) with glucosamine as the standard. The amino acids in the culture medium were analyzed by an *o*-phthal-aldehyde

method with the aid of high performance liquid chromatography (Tosoh Co, Japan).

Statistical analysis

The results were analyzed by analysis of variance and Duncan's multiple range test, whenever appropriate.

RESULTS

Exp 1: 18 AA, 10 EAA and 8 NEAA supplements

The amino acid supplements were considered to have stimulated fungal growth when it was estimated that the amount of fungal chitin was significantly different from the control after day 2 of the incubation (fig 1). The complete mixture (18 AA) was the most effective of the three supplements, and the growth rate on day 2 was two and six times higher than 10 EAA and 8 NEAA, respectively (fig 1). When the fungal growth rate was expressed in terms of protein μg/tube, the results were virtually identical with those as mentioned earlier (data not shown). The correlation coefficient between chitin and protein levels produced was 0.83 and significant ($P < 0.01$, $n = 176$) for this fungus species. Thus, the fungal growth rates in the following experiments were expressed as fungal protein increases during incubation. This allowed us to handle a large number of incubation tubes.

Exp 2: Omission of sulphur-containing amino acids (SCAA), aromatic amino acids (AAA) and branched chain amino acids (BCAA) from 18 AA

Omission of SCAA greatly suppressed fungal growth to the level of control which contained no amino acid (fig 2). Omission of AAA also suppressed the early growth of the fungus, while the suppressing effect of BCAA omission was observed only in day 2 cultures (fig 2). Either Met- or Cys-omission had less of an effect than the complete SCAA-omission. The replacement of SCAA by sodium sulphide, but not sodium sulphate, was partly effective at stimulating fungal growth (fig 3).

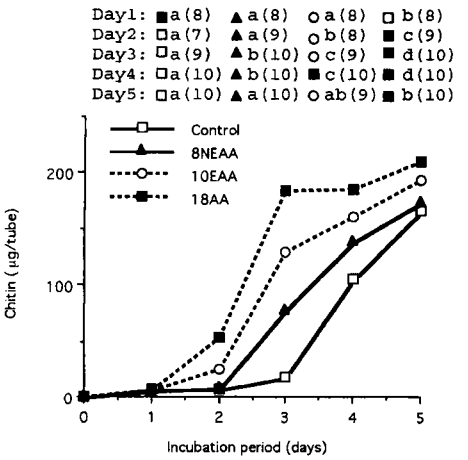
Omission of one AAA from the 18 AA supplement reduced fungal growth, and was significantly lower than 18 AA on days 2 and 3 (fig 4). Cultures lacking two AAA (Phe, Trp: Phe, Tyr: Trp, Tyr) had a greater growth reduction than those where only one AAA was omitted (data not shown).

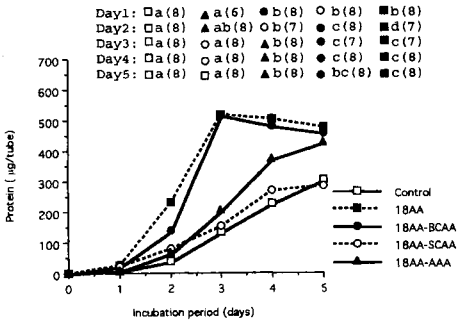
Omission of BCAA from the 18 AA supplement had no appreciable effect on fungal growth except for the day 2 cultures (fig 2).

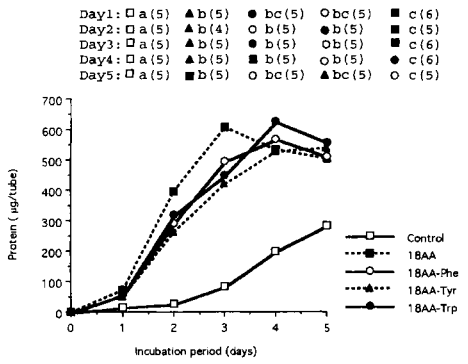
Exp 3: Omission of basic, acidic, alphatic and hydroxy amino acids and proline

Omission of the basic amino acids, singly or in combination, reduced fungal growth significantly; His and Lys had a greater effect than Arg (fig 5). Omission of Asp or Glu, or both from the 18 AA supplement reduced fungal growth during its later stages, days 3 to 5 (fig 6). When Ala or Gly, or both were removed from the 18 AA supplement, the growth curves obtained were similar to those for acidic amino acid omission (fig 7).

Omission of Thr or both Thr and Ser induced a slight but significant growth retardation in days 3 and 4 cultures, whereas in the case of Ser, no growth inhibition was observed (fig 8). Cultures lacking Pro also demonstrated a slight growth inhibition on days 3 and 4 as compared to 18 AA cultures (fig 9).







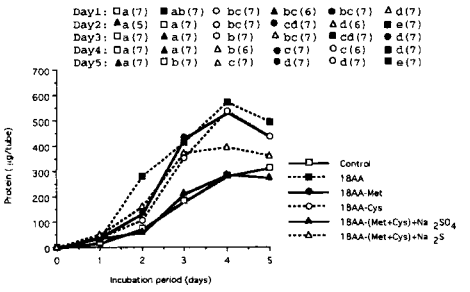
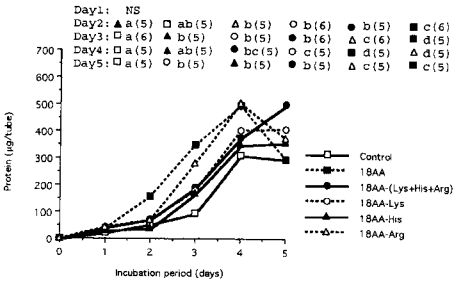
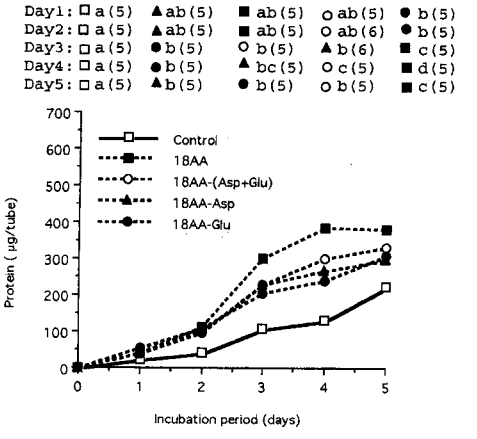
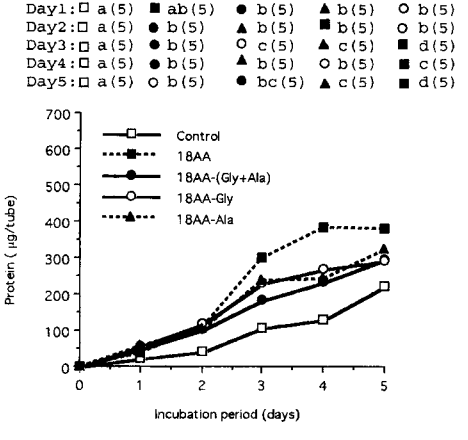


Fig 1. Effects of 18 amino acids (18 AA), ten essential amino acids (10 EAA) and eight nonessential amino acid (8 NEAA) supplements on the rate of fungal growth in terms of fungal chitin content during incubation of *Neocallimastix* sp N 13. Statistical analysis: means with different letters differ ($P < 0,01$). Numbers in parentheses indicate the number of observations. **Fig 2.** Effects of omission of branched chain (BCAA), aromatic (AAA) and sulphur-containing amino acids (SCAA) from 18 amino acids (18 AA) on the rate of fungal growth in terms of fungal protein during incubation of *Neocallimastix* sp N 13. Statistics: see figure 1. **Fig 3.** Effects of omission of Met and Cys from 18 amino acids (18 AA) and replacement by Na_2SO_4 or Na_2S on the rate of fungal growth. Statistics: see figure 1. **Fig 4.** Effects of omission of Phe, Tyr and Trp from 18 amino acids (18 AA) on the rate of fungal growth. Statistics: see figure 1.







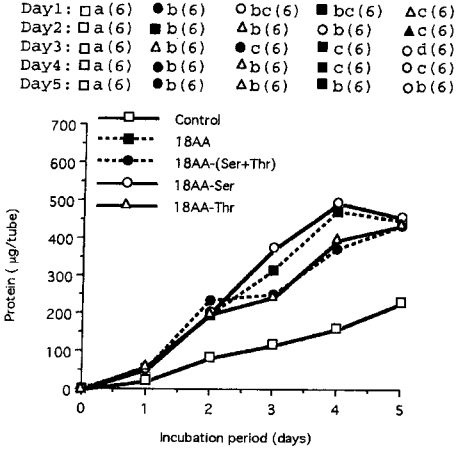


Fig 5. Effects of omission, singly or in combination, of basic amino acids from 18 amino acids (18 AA) on the rate of fungal growth. Statistics: see fig 1. NS: not significant. **Fig 6.** Effects of omission of Asp or Glu, or both from 18 amino acids (18 AA) on the rate of fungal growth. Statistics: see figure 1. **Fig 7.** Effects of omission of Ala or Gly, or both from 18 amino acids (18 AA) on the rate of fungal growth. Statistics: see figure 1.**Fig 8.** Effects of omission of Ser ou Thr, or both from 18 amino acids (18 AA) on the rate of fungal growth. Statistics: see figure 1.

Exp 4: Supplements consisting of three amino acids

An equal molar mixture of Lys, Met and His (LMH), that is as powerful as the 10 EAA supplement for rumen bacterial proliferation (Fujimaki et al, 1992), stimulated fungal growth at a level comparable to 18 AA (fig 10). The other supplement consisting of Glu, Met and Ser (GMS), each of which has proven to be a significant growth stimulator for *Neocallimastix patriciarum* (Orpin and Greenwood, 1986), was not effective at stimulating the growth of *Neocallimastix* sp N 13 (fig 11).

Amino acid concentrations in culture medium with 18 AA supplement during incubation

The concentration of Met, Cys, Arg, Ile and Leu decreased rapidly and that of the other amino acids except Ala decreased gradually during the 5 day incubation. In contrast, Ala concentration gradually increased up to the end of incubation (data not shown).

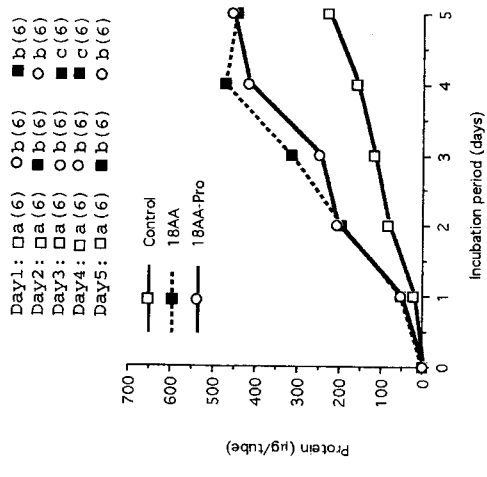
DISCUSSION

The present fungus isolate, *Neocallimastix* sp N 13 could survive and grow in a medium containing ammonium sulphate as its sole nitrogen source and sulphide. This result was similar to that for *Neocallimastix patriciarum* reported by Orpin and Greenwood (1986), suggesting that this fungus as well as most of the rumen bacteria can synthesize amino acids from ammonia and minerals. This view is supported by the finding that labelled sulphide is incorporated into Cys and Met in batch culture of *Neocallimastix* sp LM 1 from sheep rumen (Gulati et al, 1989). The amino acid synthesis in *N* sp N 13, however, seems to be a costly and time-consuming process, because amino acid supplements greatly stimulated fungal growth at an earlier stage of incubation in comparison with control without supplements (fig 1).

The effect of amino acid supplements on fungal growth was greatest with the 18 AA supplement, followed by the 10 EAA and the 8 NEAA supplement (fig 1). The EAA were more effective in stimulating fungal growth than the NEAA (figs 2 and 3). These results are in general agreement with those reported for rumen bacteria (Maeng et al, 1976; Argyle and Baldwin, 1989; Fujimaki et al, 1989, 1992), implying that rumen fungi would compete with bacteria for amino acids.

The findings that SCAA (Met, Cys) are potent stimulators for the growth of *N* sp N 13 and that sulphide, but not sulphate, can partially replace SCAA (figs 2 and 3) are in good agreement with the results from studies on *N patriciarum* (Orpin and Greenwood, 1986) and *N* sp LM 1 (Phillips and Gordon, 1991). The basal medium contained a sufficient amount of Na_2S but the addition of 17 AA containing either Cys or Met greatly stimulated growth as compared to the control culture in basal medium (fig 3), suggesting that the fungus requires these amino acids for rapid growth despite the fact that reducing conditions in the culture were maintained with reductants such as Na_2S. Such is the case with *N* sp LM 1 which grows better with sulphide plus Met or Cys than with sulphide alone (Phillips and Gordon, 1991). Therefore, the vital requirement for sulphur in rumen fungi (Akin et al, 1983) may in fact be for SCAA.

In addition to SCAA, omission of aromatic, branched chain, basic, acidic, alphatic and hydroxy amino acids or amino acid from the 18 AA supplement (figs 4–9), all decreased the rate of fungal growth to some degree, suggesting that the fungus utilizes these amino acids for rapid growth, though the precise metabolic processes



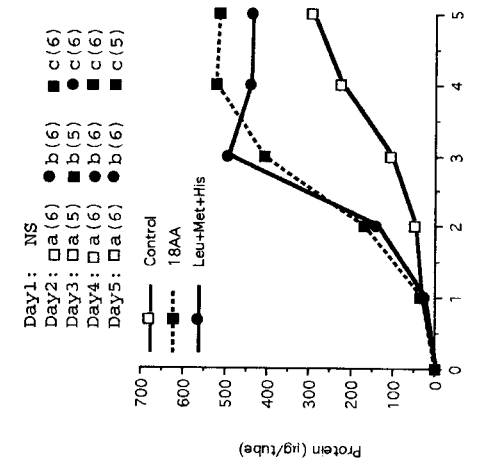


Fig 9. Effects of omission of Pro from 18 amino acids (18 AA) on the rate of fungal growth. Statistics: see figure 1. **Fig 10.** Effects of a Leu, Met and His (LMH) supplement on the rate of fungal growth. Statistics: see figure 1. NS: not significant. **Fig 11.** Effects of a Glu, Met and Ser (GMS) supplement on the rate of fungal growth. Statistics: see figure 1.

remain to be clarified. It is possible that under in vivo conditions the fungus may take up amino acids and utilize them for protein synthesis, aided by proteolysis by the fungi themselves or by bacteria. Several workers have observed that a few rumen fungi including *Neocallimastix* sp have extracellular proteolytic activity (Wallace and Joblin, 1985; Asao et al, 1993; Michel et al, 1993; Yanke et al, 1993). However, it is obscure at present to what extent fungal proteolysis contributes to amino acid release in the rumen.

It should be noted that an amino acid combination (Lys + Met + His: LMH) was just as effective for fungal growth as the 18 AA supplement (fig 10). Since the LMH combination also increases rumen bacterial yields (Fujimaki et al, 1992), this combination may be useful as a less expensive supplement for increasing rumen microbial biomass.

In contrast to *Neocallimastix patriciarum* (Orpin and Greenwood, 1986), a Glu, Met and Ser (GMS) combination supplement was ineffective at increasing the growth rate in the present fungus, suggesting that both Met and Glu are effective for the growth of *N* sp N 13 only when the media contain other amino acids (figs 2, 3 and 6), and that the nutritional requirements of *Neocallimastix* sp differ with different strains. It is possible, however, that differences in medium components may have affected the amino acid requirements of these *N* sp strains. Further study is needed to elucidate the amino acid requirements of rumen fungi, which will lead to a better understanding of rumen ecosystems.

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