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FIBROLYTIC ACTIVITY AND EFFECTS OF AUTOCHTHONOUS FUNGAL STRAINS FROM DIGESTIVE TRACT IN DIET FOR WEANED LAMBS

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ABSTRACT

The aim of this study was to evaluate the cellulase and xylanase activity of fungi isolated from the digestive tract of sheep and the effects of supplementation with two selected fungi on intake, digestibility, microbial protein (MP) synthesis and nitrogen balance in lambs. The first experiment evaluated the carboxymethylcellulase (CMCase) and xylanase activities from two filamentous fungi and two yeasts, which were grown in a culture medium containing Urochloa decumbens hay. The second experiment was performed with twenty-one male Santa Inês x Dorper crossbred lambs, with initial body weight of 18.80 ± 0.55 kg, distributed in a completely randomized design lasting 78 days. The diets consisted of 30% U. decumbens hay and 70% concentrate, supplemented or not with Trichoderma longibrachiatum fungus (TL B13M2; 30 ml) or Rhodotorula mucilaginosa yeast (RM O166; 30 ml) isolates. In the first experiment, the CMCase and xylanase from Aspergillus terreus and T. longibrachiatum were more active and efficient ($P < 0.05$) than from yeasts. In the second experiment, the nutrient intake, digestibility, MP synthesis and body nitrogen retention were not influenced ($P > 0.05$) by fungal supplementation. Microbial efficiency was

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lower (P < 0.05) with the use of RM O166. The inclusion of TL B13M2 showed increased activity of CMCase and xylanase.

Keywords: Carboxymethilcellulase. Microbial Additive. Microbial Efficiency. Probiotics. Xylanase.

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INTRODUCTION

Faced with concerns about food safety and the risks of antibiotic resistance due to the release of these chemical residues into the environment and persistence in animal products, there are a plethora of natural alternatives to growth promoting antibiotics which produce similar effects on animal performance (Eicher et al., 2006; Collier et al., 2010; Martínez-Vaz et al., 2014; Yamamoto et al., 2014; McCann et al., 2017; Mani et al., 2021). Among them is the direct addition of microorganisms, also known as direct-fed microbials, explored as safer alternatives to the antibiotics to improve balance in the gastrointestinal microbiota and enhance ruminants' health and productivity (Mani et al., 2021). At present, *Saccharomyces cerevisiae* is the main probiotic in the market, and most of the probiotics are not from the rumen (Han et al., 2021). In the rumen the ingested microorganisms show benefits and modifications to the ecosystem improving fermentation characteristics (Díaz et al., 2014; Elghandour et al., 2015; Uyeno et al., 2015; McCann et al., 2017; Han et al., 2021; Abrão et al., 2022). However, these strategies may vary considering environment, management, age, feed characteristics and production purpose (Elghandour et al., 2015, Liu et al., 2021).

Fungi supplementation can elicit a "fibrolytic effect" in the rumen by colonizing and physically disrupting plant fractions to increase the access of fiber surfaces for bacteria and their fiber-degrading enzymes (Dagar et al., 2011; McCann et al., 2017; Liang et al., 2020). The development of research contemplating the dietary use of rumen autochthonous filamentous fungus and yeast strains, whose probiotic effect needs to be tested, may provide an alternative to meet the challenge of producing quality meat and in a sustainable way (Han et al., 2021; Abrão et al., 2022; Wang et al., 2022). In this sense, the strains isolated from digestive tract of sheep, such as *Trichoderma longibrachiatum*, *Aspergillus terreus*, *Rhodotorula mucilaginosa* and *Pichia kudriavzevii* present themselves as an alternative, standing out in previous tests within the research group, when the in vitro degradability of dry matter and fiber in neutral detergent was evaluated.

In tropical regions, the dry season is a limiting factor for the supply of forage with nutritional quality to livestock. Tropical grasses in the dry season reduce the supply of soluble nutrients in the cell content and have a greater proportion of lignified fiber carbohydrates in the cell wall.

The addition of fungi and their enzymes to ruminant diets can stimulate dry matter intake, promote the use of high fiber diets, and improve the performance of these animals

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(Mohamed et al., 2013; Abdelrahman et al., 2016; López-Aguirre et al., 2016; Vallejo et al., 2016; Liang et al., 2020).

It is also known that, in addition to the strict anaerobic fungi population, facultative anaerobic fungi have been detected in the rumen environment (Abrão et al., 2014; 2017). These ruminal fungi can assume fundamental importance in the degradation of tropical forages, producing enzymes with activity to degrade cellulose (Almeida et al., 2014; Abrão et al., 2017). We previous study carried out by Freitas et al., (2012), pointed to the occurrence of the genera *Aspergillus* and *Trichoderma*, isolated from the digestive tract of lambs raised in *Megathyrsus maximum*, which could be used for the development of microbial additives or probiotic. The inclusion of *T. longibrachiatum* and *R. muscilaginosa* strains from digestive tract of sheep showed increase in the intake and body weight gain of weaned lambs fed low quality hay (Magaço et al., 2020; Martins Júnior et al., 2022).

The aim of this study was to evaluate the cellulolytic and xylanolytic activity of facultative anaerobic fungi isolated from the digestive tract of sheep and to analyze the effects of supplementation with two selected fungi on intake, total apparent digestibility, microbial protein synthesis and nitrogen balance in lambs.

MATERIAL AND METHODS

All the animal care and handling procedures were approved by the Animal Use Ethics Commission under Protocol No. 128/2013, Federal University of Minas Gerais (UFMG), Montes Claros Campus, Minas Gerais, Brazil.

EXPERIMENT 1

Local, fungal isolates evaluated and molecular identification

The experiment 1 was carried out at the Animal Physiology Laboratory of the State University of Southwest Bahia, Itapetinga, Bahia, Brazil (15° 15’ 19.3” S latitude and 40° 16’ 21.2” W longitude). Four fungi/yeast, two enzymes and four fermentation times were evaluated. The fungus *Trichoderma* sp. (TL B13M2) was isolated from feces, *Aspergillus* sp. (AT O45M1), *Pichia* sp. (PK O151) and *Rhodotorula* sp. (RM O166) were isolated from the rumen of healthy crossbred Santa Inês × Dorper sheep. These animals were fed on pastures of *Megathyrsus maximum* or *Cynodon dactylon* hay and isolated during the dry period of the year.

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Fungi and yeast were grown on Sabouraud agar (Acumedia, Lansing, USA) for seven and two days, respectively, and DNA was extracted using glucanase (Glucanex, Ferment Ltd., CH) in one phenol extraction followed by two phenol/chloroform extractions according to Neuhauser et al. (2009). For fungi, the internal transcribed spacer (ITS) region of rDNA was amplified from the extracted DNA by polymerase chain reaction (PCR) using primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) according to White et al. (1990). Furthermore, fragments of the gene encoding the β-tubulin (β-tub) protein were amplified using Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGTAGTGACCCTTGGC) (Glass and Donaldson, 1995), and fragments of the elongation factor (EF-1α) gene were amplified using primers EF-728M (CATYGAGAAGTTCGAGAAGG) and EF2 (GGARGTACCAGTSATCATGTT) (Samson et al. 2014).

The PCR reactions, with a final volume of 50 µL, contained 5 µL of 10 X buffer, 5 µL of 25 mM MgCl, 1 µL of 10 mM dNTPs, 2 µL of each primer at 10 µM, 2.5 µL of 20% DMSO, 2 µL of 150 ng DNA, 0.5 µL of Taq DNA polymerase (Sinapse, BR), and 30 µL of ultrapure H_2O. Amplifications were performed with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing of the ITS primers at 58 °C for 1 min and 20 s, of the β-tub primer at 55 °C for 1 min, and of the EF-1αa primer at 57 °C for 1 min, extension at 72 °C for 1 min, and 30 s followed by a final extension at 72 °C for 5 min. For purification of the PCR products, 11.25 µL of EDTA (125 mmol/L) and 135 µL of absolute ethanol were added to the tubes containing the reaction. The mixture was incubated for 15 min at 28 °C and then centrifuged at 20215 × g for 25 min to precipitate the amplicons. The supernatant was discarded and 120 µL of 70% (v/v) ethanol was added to the microcentrifuge tubes. These tubes were homogenized by inversion, centrifuged at 20215 × g for 10 min, and the supernatant discarded again. After complete evaporation of the residual ethanol, the product was resuspended in 10 µL of nuclease-free water. Amplified products were quantified with a NanoDrop (1000ND, ThermoFisher Scientific, USA), and the concentration was adjusted to 100 ng/µL for use in sequencing reactions.

The sequencing reaction was performed in 96-well plates using a final volume of 10 µL. 20 ng of the purified amplification product was added to the reaction along with 1.6 µL of reaction buffer, 0.8 µL of BigDye® Terminator v3.1 Ready Reaction Mix (AppliedBiosystems®), 1 µL of the same amplification primers at a concentration of 5 µmol/L, and water. Amplification was performed with denaturation at 96 ºC for 1 min,

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followed by 35 cycles of denaturation at 96 ºC for 15 s, primer annealing at 50 ºC for 15 s and extension at 60 ºC for 4 min. For precipitation of the sequencing reaction, a volume of 2.5 µL of EDTA (125 mmol/L) and 30 µL of ethanol (95%) were added to each well. After 15 min, centrifugation was performed at 2500 × g for 45 min, the supernatant was discarded and 30 µL of ethanol (70% v/v) was added to each well. A new centrifugation was performed at 2500 × g for 15 min, the supernatant was discarded and the plate remained at room temperature until the ethanol was completely dry (White et al., 1990). The purification product was suspended in Hi-DiTM formamide and sequenced on an ABI Prism 3100 sequencer (Applied Biosystems, Foster City, USA). The sequences obtained were trimmed for quality using Bioedit software version 7.2.5 and Asparargim (http://asparagin.cenargen.embrapa.br/phph/) to exclude low-quality regions (Phred Score < 20). To assemble the contigs, the nucleotide sequences obtained were edited and compared with sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/), using the Blast N program (Altschul et al., 1997). To be considered belonging to a given species, the isolate had to present 97% similarity to another already deposited in GenBank (Stackebrandt et al., 1994).

For yeast, the variable domain sequences D1–D2 of the 28S rRNA gene were amplified by polymerase chain reaction (PCR) using primers NL1 (5′-GCATATCAAAAGGAAGAGTAAGCC-3′) and NL4 (5′-GGTAAGCTTCCGCTGTCCGG-3′). The DNA concentration was adjusted to 100 ng/µL using a NanoDrop (1000ND, ThermoFisher Scientific, USA) for use in sequencing reactions. ADYEnamic (Amersham Biosciences, USA) was used for sequencing on a Mega-BACE 1000 automated sequencing system. rDNA sequences were analyzed using BLASTn (v.2.215) from BLAST2.0 on the National Center for Biotechnology Information (NCBI) website. Conspecific strains differed by no more than three within the 500–600 nucleotides of the D1/D2 domains, and isolates with 99% sequence similarity to the deposited sequences were considered to be the same species (Kurtzman et al., 2011).

Forage utilized

Urochloa decumbens hay (UDH) was purchased during the dry season (March to October). Duplicate analyses were performed to determine dry matter (DM; method 934.01), ash (method 942.05), crude protein (CP; method 954.01), ether extract (EE; method 920.39), neutral detergent fiber (NDF; method 973.18), acid detergent fiber (ADF;

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method 973.18), lignin (method 973.18), calcium (Ca; method 984.27), phosphorus (P; 984.27), potassium (K; method 984.27), magnesium (Mg; method 984.27), sulfur (S; method 990.28), sugars (ethanol-soluble carbohydrates; 985.29), neutral detergent insoluble protein (NDIP; method 973.18), acid detergent insoluble protein (ADIP; method 973.18), cellulose (Cel; method 991.46) and hemicellulose (Hem; method 2002-04) according to the Association of Official Analytical Chemists (AOAC). (Table 1).

The non-fibrous carbohydrate (NFC) contents were calculated according to the model proposed by Hall (2003):

NFC = (100 – %NDF – %CP – %EE – %ash).

Table 1. Chemical composition of *U. decumbens* hay sampled during the dry season

Component	***U. decumbens* (g/kg)**
Dry matter (%)	95.38
Crude protein	30.63
NDIP	215.68
ADIP	205.92
NDF	822.56
ADF	530.44
Lignin	75.01
Total sugars	30.18
Ether extract	10.22
Ash	58.88
Ca	1.45
P	1.04
K	5.77
Mg	2.02
S	0.64
Cellulose	455.36
Hemicellulose	292.21
NFC	84.30

DM = Dry matter; NDIP = Neutral detergent insoluble protein; ADIP = Acid detergent insoluble protein; NDF = Neutral detergent fibre; ADF = acid detergent fibre; Ca = Calcium; P = Phosphorus; K = Potassium; Mg = Magnesium; S = Sulphur; NFC = non-fibrous carbohydrates.

Fermentation

The selected fungi *Trichoderma* sp. (TL B13M2), *Aspergillus* sp. (AT O45M1), *Pichia* sp. (PK O151) and *Rhodotorula* sp. (RM O166) were grown on agar-potato-dextrose (Acumedia, Lansing, MI) with chloramphenicol (150 mg/L) during seven days at 37ºC. The sporulated cultures of mycelial fungi and yeast cells were suspended in 0.01% tween 80 solution, where the number of spores or cells in suspension was counted using a Neubauer chamber with the aid of a binocular microscope. After this period, each isolate was reinoculated in medium C.

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Fermentation took place in Erlenmeyers with 50 mL of liquid medium (medium C) containing 0.5% ammonium sulphate, 0.1% monobasic potassium phosphate, 0.05% magnesium sulphate heptahydrate and 1% carbon source (UDH ground in a Wiley mill and standardized at 1 mm) added to the inoculum containing approximately 10^7 colony-forming unit (CFU)/mL.

Submerged fermentations were carried out at 39 °C in a CO_2 incubator (Forma Direct Heat 311, Thermo Fisher Scientific, USA), at 24, 48, 72 and 96 h. The pH of the medium was measured before and after incubation with the aid of a digital potentiometer (Q440BC, Quimis, BR). After the fermentation process, the enzymatic extract was collected and centrifuged at 11000 × g (Himac CF 16RX II, Hitachi, JP) for 30 minutes at 4 ºC, and the supernatant was used as a source of microbial enzymes.

It was determined the fungal biomass based on dry weight, which was obtained after the filtering process in the broth (enzymatic extract) and washing in running water, followed by drying in a circulating oven (TE-394/3, Tecnal, BR) for four days at 55 ºC until they have a constant weight. For the dry weight of the yeasts, after obtaining the enzymatic extract with centrifugation at 11000 × g, the liquid phase was discarded, and the yeasts were transferred to crucibles and followed by drying in a circulation oven (TE-394/3, Tecnal, BR) for four days at 55 ºC until they have a constant weight.

Determination of enzymatic activities

Carboxymethylcellulase (CMCase) was determined according to the methodology adapted from Ghose (1987) modified by Siqueira et al. (2020). A solution of carboxymethylcellulose 1% (m/v) (Vetec, BR) was used as a substrate in the dosage of CMCase. For the reaction, 0.5 mL of 1% carboxymethylcellulose substrate solution (m/v), 0.5 mL of enzymatic extract and 1 mL of sodium phosphate buffer (0.1 mol/L; pH 6,8). The reaction was incubated at 39 ºC for 60 min and the quantification of reducing sugars was estimated according to the methodology from Miller (1959) modified by Gonçalves et al. (2010).

To measure xylanase activity, a mixture was prepared containing 1 mL of phosphate buffer (0.1 mol/L; pH 6.8), 0.5 mL of supernatant and 0.5 mL of xylan (D-Xylana, Vetec, BR) (0.25%). The reaction was incubated for 15 min, at 39 ºC and the quantification of reducing sugars was estimated according to the methodology adapted from Miller (1959) modified by Gonçalves et al. (2010).

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All samples were tested in triplicate and 1 mL of mixtures were removed for determination of Total Reducing Sugars (TRS) and placed in test tubes together with 1 mL of DNS. The tubes were shaken and heated in a water bath at 96 ºC for five min. Subsequently, the tubes were cooled in an ice bath for five minutes, then adding 16 mL of double potassium and sodium tartrate. The blanks of each reading were carried out with the enzymatic extracts evaluated without the addition of the respective carbohydrates to be degraded. The absorbance reading was performed on a spectrophotometer (UV-M51, Bel Engineering, IT) at 540 nm, the glucose concentrations were expressed in g/L. A unit of activity or productivity of cellulase and xylanase was defined as the amount of enzyme needed to produce a micromol (1 µmol) of glucose or xylose milliliter per minute (U/mL), under the conditions described (Miller, 1959; Ghose, 1987). The productivity calculations were performed using the formula:

$U = Abs_{means} \times (1/\varepsilon) \times (1/t) \times (1/V_i) \times 10^3 \times V_f$

Where:

U = activity unit;

Abs = absorbance;

ε = glucose or xylose absorptivity: a x W (a = coefficient obtained from the glucose and xylose standard line, W = glucose or xylose molecular weight);

t = reaction time;

10^3 = transformation from mg to µg;

V_i = size of enzyme extract;

V_f = final reaction size.

EXPERIMENT 2

Local, animals and experimental diets

The experiment 2 was in Montes Claros city of North of Minas Gerais, Brazil (16° 44' 13" S latitude and 43° 51' 53" W longitude), humid tropical climate with a dry summer. The experimental period was 12 days for adaptation to diets and pens and 63 days for collection of data.

Twenty-one male Santa Inês x Dorper sheep, four months old, with initial body weight of 18.80 ± 2.34 kg, were evaluated. They were distributed in a completely randomized design, with three diets and seven replications. The experimental diets consisted of a standard diet, supplemented with the fungus/yeast strain or without probiotic

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additive (control). At the moment of feeding, a group of animals was supplemented with 30 mL of the culture medium containing 10^9 CFU/mL of the strain *R. mucilaginosa* (yeast; RM O166) or 30 mL of the culture medium containing 10^7 CFU/mL of the strain *T. longibrachiatum* (fungus; TL B13M2). These were mixed with 100 g of the concentrate, which allowed total intake of the supplement. The lambs of the control group received the same the culture medium without the fungal strains. Water was supplied ad libitum to animals in individual water trough. The microbial additives were isolated from the digestive tract (feces) and rumen (fluid) of sheep raised on pastures of *Megathyrsus maximum* or *Cynodon dactylon* hay as reported by Freitas et al. (2012) and Martins Júnior et al. (2022).

After identification and weighing, the lambs were treated with 1% ivermectin and vaccinated against clostridiosis. The animals were housed in individual pens measuring 1.20 m in width, 2 m in length and 1.30 m in height, equipped with buckets for water, and feed bunks.

To formulate the diets, the foods were analyzed by the methods described in experiment 1, according to the Association of Official Analytical Chemists (AOAC) and balanced for a gain of 200 g/day according National Research Council (NRC, 2007). The UDH was used as roughage (30% DM in the diet) and the concentrate (70% DM in the diet) consisted of ground corn and soybean meal, as detailed in Table 2.

Table 2. Chemical composition of the ingredients and the experimental diet containing *U. decumbens* hay

Ingredients	DM%	OM%	EE%	NFC%	CP%	NDF%	TDN%[1]
Corn	89.0	98.8	7.9	63.2	9.0	16.5	83.2
Soybean meal	88.8	93.6	1.8	30.0	46.0	15.5	80.7
Urea + AS	100	-	-	-	277.0	-	-
Minerals	100	-	-	-	-	-	-
U. decumbens hay	95.4	94.1	1.0	8.4	3.1	82.3	30.9
Inclusion %, dry matter							
Corn	59.1	58.4	4.7	37.3	5.3	9.8	49.2
Soybean meal	8.3	7.8	0.1	2.5	3.8	1.3	6.7
Urea + AS	0.5	-	-	-	1.4	-	-
Minerals	2.0	-	-	-	-	-	-
U. decumbens hay	30.0	28.2	9.3	2.5	3.1	24.7	0.9
Total diet	100	94.4	14.1	42.4	11.4	35.8	65.2

AS = Ammonium sulphate; DM = Dry matter; EE = Ether extract; TDN = Total digestible nutrients; CP = Crude protein; NDF = Neutral detergent fiber; [1]Estimated according to NRC equations (2007).

The animals received *ad libitum* feeding, being divided into two daily meals (07:00 h and 15:00 h), in order to allow orts of 15% of the supplied. At the time of feeding, one group of animals was supplemented with 30 mL of culture medium containing TL B13M2, another group supplemented with 30 mL of culture medium containing with RM O166. The control

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group received the same sterile culture medium without fungi. These inoculums were mixed to 100 g of the concentrate, which allowed total consumption of microbial or the control solution.

The animals were weighed after 12 h solid fast on the 1st day and on the 63rd day of the experimental period to determine the average body weight (BW) and express the daily intake of energy (g/kg BW and in g/kg $BW^{0.75}$).

Intake and digestibility

The feed supplied and the orts were weighed daily to assess the DM intake. The daily intake of total DM was obtained by the difference between the total amount of roughage and concentrate supplied and the orts every day throughout the collection period. animal. In collection period, samples of the feed supplied (hay and concentrate) and orts were placed in labeled plastic bags and stored in a freezer at -20 ºC. After thawing, samples were pre-dried in a forced-air oven at 65 ºC. Next, these were ground in a Wiley knife mill with a 1 mm sieve. For feed, it was made a composite sample per period and for orts, a composite sample was formed per animal, and labeled for subsequent laboratory analyses.

For digestibility analyses, feces were collected with the aid of collecting bags attached to the animals for six days, three days of adaptation and another three days for the collection of feces. The total feces excreted per day was weighed and 10% aliquots from each day were sampled and mixed to form a composite sample for each animal and stored at -20 ºC for further analysis. These samples were pre-drying in a forced-air ventilation oven at 55 ºC for 72 h and ground through a 1 mm screen (Wiley mill; A. H. Thomas, Philadelphia, PA).

Chemical analysis

The average concentrations of the nutritional components of concentrate ingredients and experimental diet are shown in Table 2 and of UDH, Table 1.

Samples of feces were pre-dried in a forced-air ventilation oven at 55 ºC for 72 h and ground through a 1 mm screen (Wiley mill; A. H. Thomas, Philadelphia, PA). The contents of dry matter (DM, method INCT–CA G–003/1), mineral matter (MM, method INCT–CA M–001/1), crude protein (CP, method INCT–CA N–001/1) and ether extract (EE, method INCT–CA G-004/1) contents were determined according to Detmann et al. (2012). For the

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sequential analyses of neutral (NDF) and acid (ADF) detergent fiber, samples were treated with thermostable alpha-amylase, without sodium sulphate, and corrected for residual ash (Mertens, 2002). The NDF correction for nitrogen compounds and estimate of the neutral (NDIN) and acid (ADIN) detergent insoluble nitrogen compounds were carried out according to Licitra et al. (1996). Lignin (method INCT–CA F–005/1) was obtained based on the methodology described by Detmann et al. (2012), with the ADF residue treated with 72% sulfuric acid. Non-fiber carbohydrates (NFC) content was calculated according to Hall (2003) with modifications, utilizing NDF corrected for ash and protein.

The levels of non-fiber carbohydrates (NFC) were calculated according to the model proposed by Hall (2003), being: NFC = 100 - ((%CP - %CPU + %U) + %NDF + %EE + %ash), where, %CPU = crude protein content from urea and %U = urea content.

The total digestible nutrients (TDN) were calculated according to Weiss (1999), using the following equation: TDN = DCP + DNDF + DNFC + 2.25 × DEE. Where: DCP = digestible CP; DNDF = digestible NDF; DNFC = digestible NFC; and DEE = digestible EE.

Nitrogen balance, microbial protein synthesis and urea excretion

On the 20th day of each experimental period, approximately 4 h after providing the morning feed, collections of spot urine samples were performed by spontaneous urination of the animals. The samples were filtered through four layers of gauze and 10 mL aliquot was taken and diluted in 40 mL of sulfuric acid (0.018 mol/L) and stored at -20 ºC. Subsequently, they were used to quantify concentrations of urea, total nitrogen, creatinine, allantoin, uric acid, and xanthine-hypoxanthine.

For the analysis of creatinine, uric acid and urea concentrations in urine samples, commercial kits (Bioclin®) were used with the respective codes: K016, K139 and K047. The conversion of urea values to urea nitrogen was carried out by multiplying the values obtained by the factor 0.4667.

The urinary concentrations of allantoin and xanthine-hypoxanthine were obtained by colorimetric and enzymatic method, respectively, according to methodologies proposed by Chen and Gomes (1992), and the total nitrogen content obtained by the method of Kjeldahl (AOAC 2010).

The daily excretion of creatinine was 20.37 mg/kg BW, obtained in the test with lambs from the same genetic group (Santos et al., 2021). Thus, the urine volume was estimated for each animal in the different experimental diets, dividing the daily excretion of

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creatinine (mg/kg CP) by the concentration of creatinine (mg/L) in the spot urine sample, multiplying the result by the respective body weight of the animal (kg). The daily excretion of urinary metabolites was obtained by multiplying the estimated urine volume by the concentration of each metabolite determined in the spot urine. The nitrogen balance (retained N, g/day) was calculated with the formula: retained N = ingested N (g) - N in feces (g) - N in urine (g).

The excretion of total purine derivatives (PD) was obtained by adding the amounts of allantoin, uric acid and xanthine-hypoxanthine, excreted in the urine. The amount of absorbed microbial purines (mmol/day) was estimated from the excretion of total purine derivatives (mmol/day), using the equation proposed by Chen and Gomes (1992), for sheep: $PD\ (mmol/day) = 0.84 \times AP + (0.150 \times BW^{0.75} \times e^{-0.25 \times AP})$, where: PD = total purine derivatives (mmol/day) and AP = absorbed purines (mmol day^{-1}).

The ruminal microbial protein synthesis (g MP/day) was calculated as a function of the absorbed purines (X, mmol/day), using the equation described by Chen and Gomes (1992): $MP = 70 \times AP \times (0.83 \times 0.116 \times 1000)^{-1}$, where: 70 is the N content of purines (mg N/mmol); 0.83 the digestibility of the absorbed microbial purines and 0.116 is the ratio of purine N to total N in the bacteria.

STATISTICAL ANALYSIS

The experiment 1 was conducted with a completely randomized factorial design (4 x 2 x 4), with the following fixed factors: four fungi, two enzymes and four fermentation times with three replications, in which each treatment consisted of a fungal strain. The data were subjected to analysis of variance and the non-significant interactions were removed from the statistical model, adopting a significance level of $P < 0.05$. The Mixed Procedure was used, with the analyzing period as a random factor, in the SAS Software, version 9.1 (SAS Institute, Cary, NC, USA). Regression analysis was performed using the PROC GLM of the SAS for the variables as a function of incubation time that showed a significant effect for the linear, quadratic or cubic components of the polynomial contrasts.

The means for the qualitative factors were compared using the Tukey test and polynomial contrast was used for the time, adopting a level of 5% probability for the Type I error.

The statistical analysis for experiment 2 was performed using the PROC GLM of SAS software, version 9.1 (SAS Institute, Cary, NC, USA). With the following model:

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Yij = μ + Tri + error

Where: μ = general constant; Tri = effect related to treatment or diet i; ei = random error. After data collection, these were grouped and subjected to analysis of variance, in case of significant differences, the Tukey test was applied at 5% significance level.

RESULTS

CARBOXYMETHYLCELLULASE AND XYLANASE ACTIVITIES

The analyses of enzymes produced by *A. terreus*, *T. longibrachiatum*, *P. kudriavzevii* and *R. mucilaginosa* showed interaction between fungus type and incubation times (P < 0.001) for specific and reducing sugars production. With the results split for the incubation times, an increase in the linear effect (P < 0.001) of these variables was detected for *A. terreus* and quadratic (P < 0.001) for *T. longibrachiatum* (Table 3).

The activity of CMCases and xylanases from four evaluated fungi varied significantly (P < 0.001) for reducing sugar production (μmol/mL), showing interspecific differences. An interaction enzymes × fungus type (P = 0.045) and time × fungi (P = 0.001) were also observed. *A. terreus* showed greater CMCase activity than other fungal strains (Table 3).

In the evaluation of enzyme activity, interaction of enzymes × fungal isolate was detected (P < 0.001), when considering the enzyme activity (μmol/mL/min) of cellulase and xylanase. The CMCase produced by *A. terreus* was the most active and the xylanase activity was similar between *T. longibrachiatum* and *A. terreus* isolates. The *A. terreus* isolate showed maximum enzyme activity (0.479 μmol/mL/min) at 59.7 h incubation while *T. longibrachiatum* presented an increase in the enzyme activity by 6.7 μmol/mL/min for each 1 h of incubation (Table 3).

Table 3. Specific enzyme activity (μmol/mg), production of reducing sugars (μmol/mL) and enzyme activity rate (μmol/mL/min) for carboxymethylcellulase and xylanase produced by mycelial fungi and yeasts from the digestive tract of sheep up to 96 h of fermentation in vitro with *U. decumbens* hay as substrate

	Fungi								SEM[1]	P-value		
	A. terreus		*T. longibrachiatum*		*P. kudriavzevii*		*R. mucilaginosa*			Fung	Enzym	Time
	CMCase	Xylanase	CMCase	Xylanase	CMCase	Xylanase	CMCase	Xylanase				
	Specific enzyme activity (μmol/mg)											
Fermentation time (h												
24	0.00	2.11	0.00	0.00	0.00	0.21	0.34	3.83	0.50	< 0.00	0.155	0.004
48	17.20	12.03	0.73	5.39	1.80	0.28	0.53	0.52	2.27			
72	6.06	9.76	2.46	12.19	0.81	0.82	0.70	0.07	1.66			
96	7.82	3.01	1.54	13.20	0.36	0.51	0.00	0.31	1.68			

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Interaction	Means								SEM	P-value		
E × F	7.77a	6.73a	1.18b	7.69a	0.74b	0.46b	0.39b	1.19b	1.22	0.050		
	Contrast											
T vs F	< 0.001[2]		< 0.001[3]		n.s.		n.s.		0.00	< 0.001		
	Production of reducing sugars (µmol/mL)								SEM	P-value		
Incubation time (h)										Fung	Enzym	Time
24	0.00	109.98	0.00	0.00	0.00	18.35	25.35	274.80	34.24			
48	774.01	586.75	35.40	264.81	128.16	20.65	40.83	39.30	102.5	< 0.00	0.137	0.008
72	350.39	563.44	139.20	553.00	49.77	47.96	39.16	4.00	83.48			
96	570.76	217.44	82.83	714.64	18.01	29.53	0.00	18.50	99.21			
Interaction	Means								SEM	P-value		
E × F	423.79	369.40	64.36b	383.11	48.99b	29.12b	26.34b	84.15b	63.05	0.045		
	Contrast											
T vs F	< 0.001[4]		< 0.001[5]		n.s.		n.s.		0.00	< 0.001		
	Enzyme activity (µmol/mL/min)								SEM	P-value		
Incubation time (h)										Fung	Enzym	Time
24	0.00	0.14	0.00	0.00	0.00	0.01	0.01	0.26	0.03			
48	0.29	0.80	0.01	0.36	0.03	0.02	0.01	0.03	0.10	< 0.00	< 0.00	0.114
72	0.10	0.65	0.04	0.81	0.01	0.05	0.01	0.00	0.12			
96	0.13	0.20	0.03	0.88	0.01	0.03	0.00	0.02	0.11			
Interaction	Means								SEM	P-value		
E × F	0.127a	0.449a	0.020b	0.513a	0.012b	0.030b	0.007b	0.079b	0.07	< 0.001		
	Contrast											
T vs F	0.025[6]		0.002[7]		n.s.		n.s.		0.01	0.069		

Means followed by different lowercase letters on the lines differ by the Tukey test at the 5% probability level; [1]Standard error of the mean; [2]Y = - 2.15 + 0.110X; [3]Y = - 14.41 + 0.862X – 0.007X²; [4]Y = 0.149 + 0.083X – 0.00063X²; [5]Y = - 124.3 + 5.80X; [6]Y = - 0.589 + 0.036X – 0.0030X²; [7]Y = - 0.133 + 0.007X.

INTAKE AND DIGESTIBILITY

There was no difference for intake (P = 0.664) and total apparent digestibility (P = 0.376) of DM and nutrients by lambs fed diets supplemented or not with isolates of *T. longibrachiatum* and *R. mucilaginosa* (Table 4).

Table 4. Intake and apparent digestibility of nutrients in weaned lambs fed a diet supplemented or not with *T. longibrachiatum* (fungus) *and R. mucilaginosa* (yeast) from the digestive tract of sheep

Item	Diet			SEM[1]	P-value
	Control	Fungus	Yeast		
	Intake (g/day)				
Dry matter	921.34	972.69	978.34	27.17	0.664
Organic matter	883.02	931.73	936.64	25.97	0.671
Crude protein	80.64	87.13	86.10	2.42	0.525
Ether Extract	27.44	29.45	29.74	0.87	0.509
NDF[2]	398.37	407.68	426.74	12.58	0.666
NFC[3]	391.12	416.22	409.04	11.30	0.670
TDN[4]	760.45	800.53	805.95	19.02	0.704
ME					

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MJ/day	11.09	10.38	11.42	0.07	0.424
kJ/kg BW	0.46	0.38	0.46	0.005	0.243
kJ/kg BW$^{0.75}$	1.05	0.88	1.00	0.009	0.258
	Apparent digestibility (g/kg)				
Dry matter	724.80	684.22	731.74	18.90	0.376
Organic matter	732.47	693.66	756.43	18.57	0.399
Crude protein	808.20	829.54	809.58	28.61	0.431
Ether extract	929.33	930.32	946.37	8.10	0.650
NDF[2]	597.44	531.12	621.12	28.15	0.425
NFC[3]	921.58	918.51	931.52	8.93	0.749
TDN[4]	676.13	641.42	693.76	16.30	0.439

[1]Standard error of the mean; [2]Neutral detergent fibre; [3]Non-fibre carbohydrates; [4]Total digestible nutrients.

MICROBIAL PROTEIN SYNTHESIS AND NITROGEN BALANCE

No differences were detected (P > 0.05) among the diets for nitrogen intake, fecal nitrogen excretion, urinary nitrogen, digested nitrogen and for retained nitrogen (Table 5).

Microbial protein synthesis was not influenced (P > 0.05) with the use of probiotic fungi, and there was lower efficiency of MP synthesis (P < 0.05) with the inclusion of the *R. mucilaginosa* strain in the diet (Table 5).

Table 5 Nitrogen balance, synthesis and microbial efficiency in lambs in weaned lambs fed a diet supplemented or not with *T. longibrachiatum* (fungus) and *R. mucilaginosa* (yeast) from the digestive tract of sheep

Item	**Diet**			**SEM[1]**	**P-value**
	Control	**Fungus**	**Yeast**		
Nitrogen (g day^{-1})					
Ingested	12.71	13.92	13.33	0.42	0.528
Fecal	2.34	2.33	2.42	0.04	0.510
Urine	2.88	2.27	1.91	0.31	0.454
Urine (% ingested N)	22.74	16.57	14.37	2.38	0.350
Digested nitrogen					
g day^{-1}	10.37	11.57	10.88	0.42	0.553
% ingested N	81.47	82.93	80.95	0.70	0.516
Retained nitrogen					
g day^{-1}	7.49	9.29	8.97	0.52	0.339
% ingested N	58.72	66.36	76.24	3.42	0.106
% digested N	72.05	79.95	82.12	2.92	0.355
Nitrogen microbial (g day^{-1})	10.56	8.95	10.31	0.56	0.289
Microbial efficiency (g MP kg^{-1} TDN)	100.98a	84.15ab	78.24b	3.50	0.012

Means followed by different lowercase letters on the lines differ by the Tukey test at 5% probability; [1]Standard error of the mean.

DISCUSSION

It was possible to observe great variability between the averages of CMCase and xylanase production (µmol/mL/mg) determined by the release of reducing sugars by the specific action of the enzymes. The isolate of *T. longibrachiatum* showed only 16% of CMCase activity when compared to *A. terreus*, while that of xylanase was similar. The

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fermentation time with the highest activity of fibrolytic enzymes differed between the filamentous fungi. *A. terreus* isolate showed increasing activity with the incubation time and to *T. longibrachiatum* strain with approximately 62 h. This variation could be justified by the genetic differences between the species and/or strains of these genera analyzed, since the environmental factors, such as initial pH, cultivation time, incubation temperature, inoculum concentration, and mainly substrate were the same. Therefore, the mechanisms of control of enzymatic synthesis can vary considerably among different microorganisms due to their non-specific nature.

Fungi of the genus *Trichoderma* are producers of cellulases and hemicellulases. Additional studies are needed to explain why UDH may be able to cause a reduction in cellulase production by *Trichoderma* under in vitro cultivation. However, these fungi showed low level of activity of the enzyme β-glycosidase, and this deficiency restricts the conversion of cellobiose into glucose, providing inhibition of cellulases activity by the accumulation of cellobiose (Tiwari et al., 2013).

The lower specific activity (μmol/mL/mg) of the CMCases and xylanases of *P. kudriavzevii* and *R. mucilaginosa* yeast, in comparison with filamentous fungi, is justified by the different performance characteristics on the substrate. Mycelial fungi have advantages over unicellular microorganisms because their hyphae allow physical action to penetrate the substrate and to form multi-enzyme cellulosome, which is composed of a large number of glycosylhydrolases (β-glucosidase, exoglucanase, endoglucanase and xylanase) for efficient lignocellulose degradation (Liang et al., 2020). That is, mycelial fungi make their action more efficient, allowing them to enter the substrate, increasing accessibility to nutrients available in the cellular content (Aloulou-Abdelkefi et al., 2017). The production of CMCases and xylanases by microorganisms extends to the use mainly of bacteria and filamentous fungi, with few reports on the use of yeasts (Gusmão et al., 2018; Intasit et al., 2021), which are cited for the production of other enzymes, as invertase (Kulshrestha et al., 2013).

The *A. terreus* isolate showed higher CMCase and xylanase activity, with greater production of reducing sugars within in the same incubation period, estimated in 60 h of fungal growth. Differently, *T. longibrachiatum* isolate produced greater amounts of reducing sugars with the main action of xylanase. The rate of formation of reducing sugars was 5.80 μmol/mL for each unit of hour of incubation. This linear increase may be related to the concomitant increase in the activity of CMCase, whose exposure of hemicellulose of the cell

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wall may induce the production of xylanase. The inducing effect of xylan on xylanase synthesis is cited by several authors for bacteria of the various species of *Streptomyces* (Sanjivkumar et al., 2017; Nascimento et al., 2020), and for fungi such as *Thermomyces* and *Trichoderma* (Oliveira et al., 2014; Morgan et al., 2017). Yeasts showed the lowest averages of CMCase and xylanase activity, which did not differ throughout the incubation period.

The potential of fungi of the same genus studied in this research has been evaluated for the degradation of plant residues. Sari et al. (2017) isolated fungi of the *Penicillium*, *Aspergillus*, *Paecilomyces* and *Thielaviopsis* genera found in *Salacca zalacca* leaf litter and observed that fungi isolated from plant residues are good producers of cellulolytic enzymes, which corroborates the results found in this research, as the fungus of the genus *Aspergillus* was able to convert the substrate into a product more quickly and efficiently.

In a study carried out by Jun et al. (2013), who evaluated the CMCase and xylanases activity of the fungus *T. reesei* grown in different carbon sources, found activity values of 6.58 and 4.91 µmol/mL respectively. In contrast to these results, the present study showed greater values for the production of CMCase and xylanases produced by *T. longibrachiatum*.

The productivity (µmol/mL/min) of CMCases and xylanases differed between filamentous fungi. It may be due to the fact that most filamentous fungi have a broad spectrum of metabolic ability, mainly, efficient cellulose degradation (Arntzen et al., 2020). *A. terreus* showed greater activity with 60 h of incubation and *T. longibrachiatum* showed linear increase until 96 h of incubation.

The linear increase of productivity with the incubation time of fibrolytic enzymes produced by *T. longibrachiatum*, may be important in the diet of ruminants consuming low quality fibers and high fiber retention time in the rumen. The action of *T. longibrachiatum* enzymes may remain in the fiber for a longer time, increasing the extent of fiber degradation in the rumen. These results demonstrated the potential of using this fungus as a microbial additive in the diet of ruminants fed on tropical forages and corroborates with other research that evaluated the inclusion of mycelial fungi enzymes to improve the digestibility of forages (Nurudeen et al., 2015; Liang et al., 2020).

The diets supplemented with the fungi did not change the DM intake and the total apparent digestibility. Although the hay was of low quality (high content of ADF and lignin in the fiber), possibly it was included in a proportion in the diet, which did not influence the DM

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intake and the passage rate. The low proportion between the hay and concentrate used could be the main factor in maintaining intake, regardless of the probiotic.

In other study, Qureshi et al. (2020) also did not observe an increase in DM intake when adding the probiotic to the creep feeding diet of suckling lambs. Likewise, Yuangklang et al., (2017) also did not identify any influence on DM intake in research with goats fed rice straw and supplemented with fibrolytic enzymes produced by *Aspergillus* spp. and *Trichoderma* spp.

The effects of microbial additives on intake are variable because of the diversity of composition of the microbial products, diets and category of animals studied. However, several factors may influence animal performance when supplementing the diet with fungi and yeasts, among them, the factors that cause stress, type of forage available, feeding strategy and proportion of roughage and concentrate present in the diet stand out (Magaço et al., 2020; Martins Júnior et al., 2022).

Extracellular cellulolytic enzymes, when act into the rumen environment during fermentation, can improve the microbial activity of the rumen. Fiber degradation products, such as free sugars, can be used as energy sources during microbial growth and as fermentation substrates (Liang et al., 2020).

However, the extension of fiber degradation depends on the number of cellulolytic enzymes with activities linked to fiber composition and synchronized with retention time in the rumen. It was observed that the *T. longibrachiatum* strain presented lower CMCase activity compared to xylanase in the culture medium with UDH. On the other hand, the UDH presented higher cellulose content than hemicellulose.

Regarding the extent of fiber degradation, it can be reduced if the rate of passage through the rumen exceeds its rate of degradation (Siqueira et al., 2017). Nevertheless, in the current study, changes in the intake and digestibility have not been significant with the use of probiotics, possibly because the high proportion of concentrate and lower content of NDF from the roughage in the diets promoted an increase in the passage rate (Stensig and Robinson, 1997), exceeding the time required to extent fiber degradation in the rumen.

Dietary starch inclusion and faster passage rate at high DM intake can decrease degradability in the rumen (Firkins, 2021). Also, source of starch or soluble fiber that is highly degradable still could induce acidosis in sheep (Lettat et al., 2010). The rumen needs to process forage to maintain enough rumen fill to prevent subacute rumen acidosis. However, too much rumen filling limits DM intake. To improve any sort of rumen efficiency

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for NDF digestibility is necessary dietary or other managerial changes. However, there was a numerical increase in DM intake with the use of both microbial additives, but the numerical values of NDF digestibility and TDN were higher for supplementation with the yeast than filamentous fungus. It can be explained by the fact that *R. mucilaginosa* exhibits CMCase action within 24 h of incubation.

The depression of TDN by increasing intake level is mainly assumed when digestible NDF is discounted. This effect has been lessened considerably since then in large part by dilution of starch with by-product fiber (more digestible) (Firkins, 2021). However, in the current study such slight increases observed with *R. mucilaginosa* could result from the fibrolytic enzymes acting earlier during his growth, in addition to possible beneficial contributions on nutrient digestibility, volatile fatty acids (VFA) production, rumen microbial community structure, and rumen pH (Liang et al., 2020). The regulation of yeast on rumen microorganisms is reflected in increasing fiber-degrading and lactate-utilizing bacteria, which improves further rumen fermentation efficiency and subsequent production capacity (Martins Júnior et al., 2022; Wang et al., 2022).

Additionally, the supplementation of fungi can enhance bacterial growth, microbial protein production, and digestibility of dry matter, resulting in the production of multiple types of fibrolytic enzymes to break down the structure of lignocelluloses and enhance the nutrient digestibility (Liang et al., 2020).

However, supplementation with *T. longibrachiatum* showed lower numerical value for NDF digestibility, possibly due to faster passage rate in diet with 24.7% of NDF from forage (Azevêdo et al., 2024), possibly because the CMCase activity of *T. longibrachiatum* only appears after 24 h of incubation.

The urinary and retained nitrogen did not differ among diets with the use of *T. longibrachiatum* and *R. mucilaginosa*. The loss of nitrogen in the urine turns out not to be viable in sustainable systems.

Also, Bueno et al. (2013), evaluating the addition of *S. cerevisiae* in diets for lambs with proportions of roughage and concentrate of 20:80 and 40:60, did not reported significant differences in the amount of retained nitrogen. Although the diets did not influence the amount of dietary retained nitrogen, there were changes in rumen microbial efficiency where the diet supplemented with *T. longibrachiatum* was higher than the diet with *R. mucilaginosa*, which was lower than the control diet.

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The increase in degradation and, consequently, greater fermentation does not necessarily result in greater synthesis and/or microbial efficiency in the rumen. Rumen bacteria have an inverse relationship between growth and fermentation rates (substrate degradation) (Schären et al., 2018). Increasing growth efficiency routes a greater proportion of anabolic carbon from degraded feed into cells rather than VFA (Russell and Cook, 1995). Catabolic processes are largely from fermentation of sugars and amino acids to VFA (or organic acid intermediates, especially lactate and succinate) to produce adenosine triphosphate (ATP) while disposing of reducing equivalents. In addition, Russell et al. (1992) stated that increasing starch would increase the proportion of ATP being used for maintenance and so decreased efficiency of microbial protein synthesis (EMPS). The ATP produced during fermentation can be less efficiently converted into cellular matter and is therefore more likely to be wasted (Firkins, 2021). Results of microbial efficiency are not coherent with results of digestible OM intake calculated from values of Table 4. There have been many published reports of an increased EMPS resulting from decreased ruminal OM degradability without a decrease in microbial protein flow from the rumen. Faster passage rate can decrease degradability in the rumen, but undegraded NDF passing to the duodenum might be an important vehicle to export microbial N flow because about 75% of the bacteria adhere to particles (Sauvant and Nozière, 2016). In contrast, negative associative effects (i.e., increasing dietary starch inclusion decreases NDF digestibility at high dry matter intake (DMI) depend on dietary variables and also differ among animals at least in part because of their unique microbial community structures (Firkins, 2021).

However, the use of yeast *R. mucilaginosa* reduced microbial efficiency and the calculated digestible OM intake was 8.6% higher than the other diets. Two aspects that can explain these results, first would be the pH stability provided by yeast, which can help increase resilience against subacute rumen acidosis (Ishaq et al., 2017; McCann et al., 2017). Huws et al. (2018) noted that efficient animals had less rumen bacterial diversity. In a negative way, lower bacterial diversity can be associated with rumen acidosis. Second would be the blurring anabolism and catabolism that sometimes leads to lost opportunity to gain improvement in EMPS. For example, increasing starch in the diet typically increases microbial protein flow to the duodenum while also increasing rumen OM or carbohydrate degradability (Firkins, 2021). Consistently, the results demonstrated the principle that while microbial protein flow increases, o EMPS decreases because the gain in EMPS was less than it could have been (Hespell, 1979; Russell, 2007).

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According to Firkins (2021), TDN should not be used for derivation because increasing DMI decreases NDF and starch degradability by increasing passage rate (i.e., greater passage of potentially degradable NDF and starch); in contrast, increasing ruminal degradability of NDF by feeding higher quality forages or co-products allows for greater DMI on an equal NDF basis. The UDH that is more degradable when associated with probiotics may be less resistant to particle size reduction and may reduce satiation and intake limitation, thus improving weight gain, as observed by Magaço et al. (2020) and Martins Júnior et al. (2022).

Thus, the diet with the fungus *T. longibrachiatum*, even favoring fiber degradation, did not differ from the diet without microbial additive, possibly the amount of fungus used was not enough to provoke this response. In addition, the concentrate supplied in a greater proportion was effective in maintaining the efficiency of microbial protein synthesis in the rumen. The growth rate of fungi is slower when compared to other microorganisms in the rumen, which use soluble carbohydrates.

The efficiency of nitrogen metabolism in ruminants depends on the complex interaction of various nutrients and energy. Nitrogen is only used efficiently for microbial synthesis when the diet provides a balance of energy and protein utilization. Bacteria, fungi and protozoa are essential to extent fiber digestion which maintains the supply of substrate for microbial growth in the rumen (Castillo-González et al., 2014; Hackmann and Firkins, 2015). However, stimulating their growth reduces the need to use excess protein because the proportion of fiber-bound nitrogen and rumen recycled nitrogen can be enhanced (Jin et al., 2018).

CONCLUSION

The production of CMCase and xylanase by *T. longibrachiatum*, *A. terreus*, *R. mucilaginosa* and their applications in the hydrolysis of lignocellulosic material are promising for the elaboration of a probiotic or direct-fed microbials. *T. longibrachiatum* shows increased CMCase and xylanase activity during 96 h incubation time, which may be an important feature acting to extent degradation of low-quality fiber in the rumen. On the other hand, *R. mucilaginosa* shows activity of CMCase and xylanase at 24 h of incubation, it may be used to supplement diets with a high proportion of concentrate and low-quality fiber from the forage. Further research should evaluate how these additives seem to feeding behavior based on their specific role on microbial community structure and EMPS.

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In addition, trials using a diet with a greater amount of inoculum and proportion of forage with a high content of lignified cell wall are recommended.

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