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The effect of fungal probiotics added to a high-grain diet on the gastrointestinal tract of sheep

O efeito de probióticos fúngicos adicionados a uma dieta rica em grãos no trato gastrointestinal de ovinos

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Abstract
This study aimed to evaluate the microbiological and physicochemical characteristics of the ruminal fluid and histological characteristics of the gastrointestinal tract (GIT) of sheep on a high-grain diet containing the probiotic fungi *Aspergillus terreus* and *Rhizomucor* spp. The study included four treatment groups (without probiotic, with *Rhizomucor* spp., with *Aspergillus terreus*, and with a mixture of both fungi), and two types of corn (ground/whole), in a Completely Randomized Design (CRD) arranged in 4 x 2 factorial design. Santa Inês x Dorper lambs were housed in eight pens with five lambs each for 75 days. Rumen fluid was collected to study the rumen microbiological profile, macroscopic characteristics, ammonia nitrogen concentration, and microbiological activity. In addition, GIT samples were taken for histological analysis. Fluid analyses showed that the animals presented a low acidosis index. The samples presented a predominantly aromatic odor and blackish-brown color, indicating a neutral pH and high microbial activity. The rumen pH differed ($P < 0.05$) according to the level of processed corn consumed, being higher for ground grain corn (GGC). There was no difference for any of the microbiological communities analyzed ($P > 0.05$) (Lac+ and Lac- bacteria, fungi, yeasts, and protozoa). Six genera of facultative anaerobic fungi were identified in 15 observations. *Cladosporium* spp. was the most prevalent genus (46.66%), followed by *Aspergillus* spp. (26,66%). The width of the base of rumen papillae showed significant correlation being greater for GCG ($P < 0.05$) with *Rhizomucor* and for the control ($P < 0.05$). The rumen fluid of sheep on a high-grain diet with added *Aspergillus terreus* and *Rhizomucor* spp. showed no microbiological and physicochemical changes.
Keywords: rumen bacteria; rumen microbiota; protozoa; small ruminants; rumen.

Resumo
Objetivou-se avaliar as características microbiológicas e físico-químicas do fluido ruminal e histológicas do trato gástrico intestinal (TGI) de ovinos sob dieta de alto grão com probiótico fungos *Aspergillus terreus* e/ou *Rhizomucor* spp. Analisou-se quatro probióticos (sem inóculos, com *Rhizomucor* spp., com *Aspergillus terreus* e com mistura dos dois fungos) e dois processamentos de milho (moído/inteiro), em fatorial 4x2 em em Delineamento Inteiramente Casualizados (DIC). Borregos Santa Inês/Dorper foram alojados em oito baias com cinco borregos em cada, durante 75 dias. Coletou-se fluido ruminal para o estudo do perfil microbiológico do rúmen, da característica macroscópica, da concentração de nitrogênio amoniacal e da atividade microbiológica, além dos fragmentos do TGI para análises histológicas. Pelas análises dos fluidos, os animais apresentaram baixo índice de acidose. O odor aromático e a cor castanho-enegrecido predominaram, o que caracteriza ambiente com pH neutro. As amostras do fluido apresentaram alta atividade microbiana. O pH ruminal diferenciou-se ($P<0,05$) quanto ao tipo de processamento, sendo maior para milho grão moído (MGM). Não houve diferença para nenhuma das comunidades microbiológicas analisadas ($P>0,05$) (bactérias Lac+ e Lac-, fungos, leveduras e protozoários). Seis gêneros de fungos anaeróbicos facultativos foram identificados num total de 15 observações. O *Cladosporium* spp. foi o gênero mais prevalente (46,66%), seguido do *Aspergillus* spp. (26,66%). A largura da base das papilas ruminais apresentou interação significativa, sendo maior para MGM ($P<0,05$) com *Rhizomucor* e o controle ($P<0,05$). O fluido ruminal de ovinos sob dieta de alto concentrado de grão com adição dos fungos *Aspergillus terreus* e *Rhizomucor* spp. não tiveram afetadas as características microbiológicas e físico-químicas.
Palavra-chave: bactéria ruminal; microbiota ruminal; pequenos ruminantes; protozoários; rúmen.

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Introduction

Feeding animals high levels of non-structural carbohydrates, such as the high-grain concentrate (HGC) diet, cause major changes in the rumen environment[1,2]. This diet type can cause serious metabolic problems in ruminant animals when poorly managed[2,3]. Using HGC demands a good knowledge of rumen and microbiota changes caused by the diet[4,5]. Thus, it is essential to study rumen modulation in

animals on a high energy low fiber diet to understand these changes and implement nutritional management actions to avoid or reduce metabolic disorders.

The rumen of animals fed an HGC diet present low pH (< 5.5)[6], reduced microbial diversity, decreased protozoan populations, changed bacterial populations[3,7,8], and a reduced or eliminated anaerobic fungal population[5,9] If ruminal pH continues to decrease, *Lactobacillus* spp. can replace *Streptococcus bovis*, causing excessive lactate accumulation and leading to ruminal acidosis[8,10,11]. Thus, the relevance of using nutritional additives in ruminant feed to help maintain pH at adequate levels (> 5.5) is evident, especially in animals confined for a longer period and fed with a high-energy concentrate.

Different ruminal microbiota modulators (e.g., ionophores, antibiotics, propolis, essential oils, and symbiotic substances) have been studied to improve microbial activity in the rumen[12] when animals are exposed to diets with high levels of starch or low-digestible fiber[13]. Hill et al.[14] and Markowik and ´Slizewska[15] stated that ionophores and antibiotics are the most widely used additives. However, these additives are not advised due to the risk of residual compounds in products and by-products of animal origin. As an alternative, many studies have analyzed natural additives, such as probiotics. Unlike antibiotics, probiotics are microorganisms that can benefit the host without leaving residues[14].

Studies on the ruminal microbiota of animals on high-grain diets will help predict the main species involved in whole or ground grain degradation to modulate this microbiota more efficiently, improving animal production[13,16,17].

Limited studies evaluated the prevalence of fungi in the GIT of ruminants fed concentrate only. Recently, Abrão et al.[5] demonstrated that high-grain diets eliminate the ruminal anaerobic fungal populations, increasing the concentration of *Streptococcus* spp. and decreasing microbial diversity in the rumen, probably due to the consequent significant pH reduction (< 5.5).

Other studies showed that ruminal anaerobic fungi produce various enzymes that usually degrade more substrate than rumen bacteria[18]. In addition, they have a mechanical action, with hyphae that penetrate the food, facilitating the action of other microorganisms such as bacteria[13,19].

Therefore, this study was conducted to analyze whether *Aspergillus terreus* and *Rhizomucor* spp. fungi, naturally present in the rumen of sheep, present probiotic action, consequently influencing microbiological and macroscopic parameters of the ruminal environment of animals on high-grain diets; and whether they influence the ruminal environment's balance.

Material and methods

In vivo assay with potential fungal probiotics

The in vivo assay was conducted at the sheep farming department of Instituto Federal Goiano, Campus Ceres, located on GO 154 Highway, km 3, Rural Zone of Ceres, GO, Brazil (geographical coordinates: latitude 15°21'00" S, longitude 49°36'05" W, altitude: 542 m above sea level). Confined feeding started on September 15 and ended on November 29, 2017. It totaled 75 days, with 15 days for adaptation and 60 days for data collection divided into four experimental periods of 15 days each.

The project was approved by the Animal Ethics Committee (AEC) of Instituto Federal Goiano on September 20, 2016, under protocol number 9356170616.

The study included 48 crossbred ½ Santa Inês x ½ Dorper lambs with a mean age of seven months and an initial live weight of approximately 35 kg (± 5.00 kg). The animals were randomly distributed between eight 5 x 5 m stalls with rustic concrete flooring. Each stall contained six animals (three males and three females). The experimental treatments consisted of two types of corn (whole and ground) and four types of probiotic treatments (without fungal probiotic (TE), with *Rhizomucor* spp. (RZ), with *Aspergillus terreus* (AT), and a mixture of both fungi (MX). A Completely Randomized Design (CRD) was used in a 4 x 2 factorial arrangement with the following treatments: Whole grain corn without probiotic(WGC-TE), ground grain corn without probiotic (GGC), whole grain corn with *Rhizomucor* spp. (WGC-RZ, ground grain corn with *Rhizomucor* spp.(GGC-RZ), whole grain corn with *Aspergillus terreus* (WGC-AT), ground grain corn with *Aspergillus terreus* (GGC-AT), whole grain corn with a mixture of the two fungi (WGC-MX), and ground grain corn with a mixture of the two fungi (GGC-MX).

At the end of the 75-day confinement, the heaviest animal per stall was selected for the digestibility test. The five remaining animals in each stall were slaughtered to collect ruminal fluid and GIT segments.

All treatments were composed of 85 % corn (whole or ground grain) and 15 % commercial base mix diet. The animals in each stall received the treatments described in Table 1.

Table 1. Description of treatments

Treatments	Grain corn	Ground corn	Base mix	Probiotics
		(%)		spore/animal/day[a]
WGC-TE	85	-	15	No
GGC-TE	-	85	15	No
WGC-RZ	85	-	15	*Rhizomucor spp.*
GGC-RZ	-	85	15	*Rhizomucor spp.*
WGC-AT	85	-	15	*Aspergillus terreus*
GGC-AT	-	85	15	*Aspergillus terreus*
WGC-MX	85	-	15	*Rhizomucor spp.* + *Aspergillus terreus*
GGC-MX	-	85	15	*Rhizomucor spp.* + *Aspergillus terreus*

[a]Concentration of 9.33 x 10^{11} spore per animal per day. WGC: whole grain corn; GGC: ground grain corn; TE: control; RZ: *Rhizomucor spp.*; AT: *Aspergillus terreus*; MX: mixture of the two fungi.

Analysis of the composition of the base mix pellets and total diet

Engordin® is a base mix for high-grain diets. The base mix pellets were supplied whole in the whole grain treatments, and ground separately and then mixed with the ground corn for the ground grain treatments. According to manufacturer's information, it is composed of phosphorus (6,000 mg kg^{-1-1}), calcium (minimum 34 g kg^{-1}), ethereal extract (10 g kg^{-1}), neutral detergent fiber (minimum 220 g kg^{-1-1}), mineral matter (minimum 200 g kg^{-1}), crude protein (minimum 380 g kg^{-1}), non-protein nitrogen (minimum 116 g kg^{-1}), cobalt (5 mg kg^{-1}), copper (175 mg kg^{-1}), chromium (1.4 mg kg^{-1}), sulfur (4,500 mg kg^{-1}), iodine (5 mg kg^{-1}), manganese (180 mg kg^{-1}), magnesium (3,000 mg kg^{-1-1}), sodium (minimum 9,700 mg kg^{-1}), potassium (15 g kg^{-1}), zinc (minimum 420 mg kg^{-1}), virginiamycin (150 mg kg^{-1}), Monensin sodium (150 mg kg^{-1}), vitamins A (21.000 IU kg^{-1}), D (3 600 IU kg^{-1}), and E (135 IU kg^{-1}), and other essential minerals with a moisture content of 120 g kg^{-1} (90% DM).

Table 2 shows the chemical composition of the corn used in the experimental diets and the composition of the experimental whole grain (WGC) and ground grain (GGC) diets based on the natural matter supplied to the confined animals.

Collection and preparation of rumen fluid samples

Rumen fluid was collected from five animals per treatment, totaling 40 samples. At the end of the 75-day confinement period, the animals were weighed (mean of 49.5 kg) and transported for slaughtering at the Agribusiness department of IF Goiano, Ceres campus. The animals were stunned with electrodes, and the jugular vein and carotid artery were cut. Pre-slaughter management included a 12-hour fasting period and respecting the hygienic-sanitary animal welfare norms.

Table 2. Chemical composition of the corn and experimental diets (g/kg) in natural matter

Components (g/kg)	Corn	Whole grain corn diet	Ground grain corn diet
Dry matter	925.98	890.95	889.20
Mineral matter	10.88	36.50	39.45
Organic matter	989.12	963.50	960.55
Neutral detergent fiber	323.18	250.24	183.63
Acid detergent fiber	242.73	31.57	38.10
Ether extract	11.00	27.00	23.50
Non-fibrous carbohydrate[4]	572.59	562.94	620.23
Crude protein	82.35	123.33	124.09

[4]Non-fibrous carbohydrates (NFC) calculated based on NDF (NFC = 100 - MM - CP - EE - NDF).

Collection and preparation of rumen fluid samples

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The ruminal fluid of the slaughtered animals of each treatment was collected in order to elucidate the possible modulatory effect of probiotics on the autochthonous rumen microbiota,

Immediately after evisceration, the ventral region of the rumen (the region with the highest concentration of microorganisms) was located, exposed, sanitized with 75% alcohol, and a cut of approximately 5 cm in length was made using a sterile scalpel. The ruminal content from each sample was filtered through sterile gauze into sterile 120 mL Falcon tubes and kept in a cooled isothermal box at -8 °C for no more than one hour.

Microbiological evaluation and rumen metabolism parameters

Immediately after collection, a glass tube containing 5 mL of the rumen fluid[20] was macroscopically analyzed for the color, odor, and viscosity parameters. Ruminal microbial activity was evaluated by the methylene blue redox potential (MBRP) test at a concentration of 0.03% (redox potential). Ruminal liquid pH was estimated using a digital pH meter[21]. A 20 mL sample of the liquid was filtered and kept frozen at 0 °C for subsequent ammonia concentration (NH_3) determination according to

the INCT-CA method N006/1 described by Detmann et al.[22].

Gram staining for bacterial and yeast groups

The micro-morphological and staining characteristics of the predominant bacterial and yeast groups in the collected fluid were observed in dry smears fixed and stained on microscopy slides using Gram staining[23].

Enteric zoonotic bacteria

Serial decimal dilutions of rumen fluid were prepared in sterile saline solution to investigate the influence of probiotics on the ruminal enterobacterial population. After each dilution, the tubes were homogenized for three minutes, 100 µL aliquots of 10^{-2} and 10^{-4} dilutions were plated onto sterile plates and embedded with MacConkey agar medium. The inoculation were spread with a sterile Drigalski spatula, and the plates were incubated in a BOD oven at 39 ºC and monitored for bacterial colony growth for up to 21 days[23].

The bacterial genera most prevalent in the rumen fluid samples were identified through re-isolation and cultured MacConkey agar plates in an incubator at 37 °C for 24 hours. After exponential growth, each isolate was inoculated into tubes containing Rugai and Araújo medium modified by Pessoa and Silva[24]. The tubes were incubated in a BOD oven at 39 °C, and the results were interpreted after 24 hours using an illustrative table for the presumptive identification of Enterobacteriaceae, according to Pessoa and Silva[24].

Facultative anaerobic fungi

Serial dilutions of rumen fluid were prepared in tubes containing sterile saline solution. After each dilution, the tubes were homogenized for three minutes, and 100 µL aliquots of 10^{-2} and 10^{-4} dilutions were plated onto sterile plates containing Sabouraud agar medium. The inoculation were spread with a sterile Drigalski spatula, and the plates were incubated in a BOD oven at 37 °C and monitored for fungal colony growth for up to seven days[25].

The isolated mycelial fungal colonies were identified using microculture technique. The micromorphological characteristics analyzed by optical microscopy were associated to those described for fungi of biotechnological and veterinary relevance[25,26].

Ruminal protozoa

After filtering the ruminal fluid, a 1 mL aliquot was diluted in 9 mL of 10% formaldehyde solution to preserve the micromorphological structures of the protozoa. When necessary, saline solution was used for serial dilutions. Small, medium, and large protozoa were quantified by inoculating 1 mL of the solution in a Sedgwick-Rafter cell counting chamber and visualized under an optical microscope using a magnification power of 103.

To identify the genera of these microorganisms, a drop of the 10^{-1} dilution described above was used with a drop of Lugol's solution on a microscopy slide. A coverslip was placed on the sample for subsequent visualization of the protozoon microstructure using an optical microscope[27,28].

Histological analyses of the gastrointestinal system

Rumen, small intestine, and large intestine sections were washed under running water and pre-fixed in a 10% formalin solution for six hours. A fragment of each, approximately 1 cm^2 in length, was extracted from the ventral part of the rumen and one meter from the beginning of the small and large intestines. The tissues were then preserved in 70% alcohol and mounted, at most, ten days after collection. The histological slides were mounted by embedding tissue fragments in paraffin, cutting 5-mm thick slices in a microtome, and Hematoxylin and Eosin staining[29].

The histological slides were used for measuring villus height and crypt depth of the small intestine, crypt depth of the large intestine, and width of the base of the papillae and the space between papillae of the rumen. The slides were visualized using an optical microscope connected to a computer, and the images were analyzed using the Image J software[30].

Statistical analyses

After exploratory analysis, the parametric data were subjected to analysis of variance in the easynova statistical package of the R environment[31] and the means were compared using the Tukey test at 5% probability. Non-parametric tests were used for microbiological analyses in the easynova statistical package of the R[31] environment, with the chi-square for detection rates, the Wilcoxon test for two-group comparison analysis, and the Friedman or Kruskal-Wallis test for data quantification[31]. Sensory characteristics of rumen fluid (color, odor, viscosity, and MBRP) were subjected to descriptive analyses.

Results and discussion

Characteristics of rumen fluid

Macroscopic analyses of the ruminal fluid showed a predominance of blackish-brown (BB) coloration among the samples collected (14 out of 40 samples = 35%), including nine samples of the GGC treatment and five samples of the WGC treatment. Brown (BN) was the second most common color (11 out of 40 samples = 27%), five in GGC and six in WGC (Table 3). These colorations characterize a rumen environment with a more neutral pH. Abrão et al.[5] and Vieira et al.[32] corroborate Dirksen[20] when stating that a clearer rumen fluid suggests a more acidic environment. Rumen fluid samples with lower levels of BB coloration were from animals treated with

Aspergillus terreus (AT) and WGC control (0% and 10%, respectively) and *Rhizomucor* spp. (RZ) and *Aspergillus terreus* with GGC treatments, at 10% each. A darker coloration is characteristic of rumen fluid from animals on a diet with roughages[5,32]. Thus, fluid coloration evaluation showed that the animals presented a low acidosis rate. In this sense, the diets were appropriate to fulfill the physiological requirements of the animals, not causing metabolic problems such as acidosis.

An aromatic odor (AR) was predominant in 21 of the 40 samples (52%), with 11 (27%) samples in WGC and ten (25%) GGC (Table 3). This odor was more prevalent (19.04%) in the GGC treatment associated with the mixture containing both fungi (MX). The samples with weaker odors were those described as acid penetrating (AP), with one sample in the WGC-AT group, followed by odorless to acidic with one sample in the WGG-TE and another in the GGC-RZ group. The aromatic odor was described by Vieira et al.[23] in 100% of samples from animals feeding on pasture, and slightly acidic odor for samples from animals on HGC. Animals presenting AR odor tend to have the most appropriate pH for developing microbiota. Thus, these diets are more appropriate to the physiological requirements of the animals, presenting fewer metabolic disorders.

Aqueous viscosity (AQ) was observed in 16 out of 40 (40%) samples, nine from animals in the WGC and seven in the GGC group. As for the fungal probiotic factor, the highest AQ was observed in the control treatment and the mixture of both fungi with the whole grain diet (three each) (Table 3). The second-most prevalent viscosity was thick (TC), present in 13 samples (32%), seven in WGC, and six in GGC. Vieira et al.[23] reported that grazing animals presented the characteristics described by Dirksen[20], who described rumen fluid as thick with intense gas bubble production, indicating intense microbial activity[20].

In this study, even though most samples were AQ, they presented high levels of bubble formation, and this activity was confirmed by the MBRP where it was observed in less than three minutes in all the samples (Table 3). Abram et al.[5] reported MBRP of less than one minute in animals receiving a high-concentrate diet; however, Dirksen[20] reported that an MBRP time greater than 15 minutes indicates low microbial activity potential in animals on poor energy and protein diets or in ruminants with prolonged inappetence.

Table 3. Predominant physicochemical analysis of the rumen fluid of confined sheep fed a high-grain diet associated with fungal probiotics

Physicochemical factor	Whole				Ground				TOTAL		Total
	RZ	AT	TE	MX	RZ	AT	TE	MX	WGC	GGC	
Coloration											
Blackish-brown - BB	2	0	1	2	1	1	4	3	5	9	14
Brown - BN	1	2	1	2	1	2	0	2	6	5	11
Greenish brown - GB	0	1	0	0	1	1	1	0	1	3	4
Grayish milky - GM	0	2	3	1	0	0	0	0	6	0	6
Milky brown - MB	2	0	0	0	2	1	0	0	2	3	5
Odor											
Ammoniacal - AI	1	1	0	0	0	0	0	0	2	0	2
Acid - AC	0	0	0	0	2	0	0	0	0	2	2
Aromatic - AR	3	3	2	3	1	2	3	4	11	10	21
Penetrating acid - PA	0	1	0	0	0	0	0	0	1	0	1
Insipid to acid - IA	0	0	1	0	1	0	0	0	1	1	2
Odorless - OL	1	0	2	2	1	3	2	1	5	7	12
Viscosity											
Aqueous - AQ	1	2	3	3	2	2	1	2	9	7	16
Aqueous to foamy - AF	2	1	0	1	1	0	1	0	4	2	6
Thick - TC	2	2	2	1	0	2	2	2	7	6	13
Thick to aqueous - AT	0	0	0	0	2	1	1	1	0	5	5
MBRP (min)	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3	< 3

WGC: whole grain corn; GGC: ground grain corn; RZ: *Rhizomucor* spp.; AT: *Aspergillus terreus;* TE: control; MX: mixture of the two fungi*;* and MBRP: methylene blue redox potential/activity potential.

N-NH_3 concentration and ruminal pH

No differences (P > 0.05) were observed in the evaluation of ammoniacal nitrogen concentration in the ruminal fluid of animals subjected to the different treatments (Table 4). Savari et al.[33] also found no significant difference in RDP:RUDP (rumen degraded protein: rumen undegraded protein) ratio in an experiment with dairy cows fed high-concentrate diets with ground or flaked corn.

The level of ruminal ammoniacal nitrogen should be above 8 mg/dL to increase dry matter intake. However, to increase microbial protein availability in the intestine,

this level should be around 15 mg/dL to maximize production[34]. The results obtained in this experiment ranged from 8.14–9.66 mg/dL above the minimum recommended level.

Table 4. Ammoniacal nitrogen (N-NH_3) concentration and pH of rumen fluid from confined sheep fed a high-grain diet associated with fungal probiotics

Variables	Processing		Probiotics				P-value			CV
	Whole	Ground	RZ	AT	MX	TE	Proc	Prob	Proc:Prob	
N-NH_3, mg/dℓ	8.99	9.34	9.66	9.30	9.54	81.473	0.786	0.808	0.554	33.32
pH	5.51[b]	6.15[a]	5.77	5.73	5.99	5.838	0.001	0.579	0.748	7.60

Means followed by different low case letters in the rows present significant differences at 5% significance by the Tukey test. WGC: whole grain corn; GGC: ground grain corn; RZ: *Rhizomucor* spp.; AT: *Aspergillus terreus;* TE: control; MX: mixture of the two fungi - RZ+AT. Proc: processing; Prob: probiotic; Proc:Prob: processing:probiotic

In the present study, the lack of ammoniacal nitrogen fluctuation in the rumen over time after feeding may be related to the fact that the analysis was performed at a single time point when the animals were slaughtered after being conditioned by prolonged fasting.

Rumen pH was lower in animals on a whole grain diet ($P < 0.01$). A diet rich in NDF may increase microbial growth by increasing the passage rate[34]. Ground grain diets increase NDF availability compared with a whole grain diet. In fasting animals, the passage rate of whole grain is lower than that of ground grain. Therefore, even with food restriction, whole grain fermentation occurs in the rumen, producing fermentation fatty acids and, consequently, reducing the pH[3].

Gram staining for bacterial and yeast groups

Gram analyses showed a similar pattern for the different groups of bacteria between treatments (Table 5). Regardless of the treatment, all groups showed similar variations in cocci and their clusters, spirillum, vibrio, bacilli, and yeasts. Similar characteristics were also observed in Gram-negative and Gram-positive bacteria, with the Gram-positive group ($P < 0.05$) having the highest concentration in all treatments.

The population profile of microorganisms that constitute the rumen microbiota was analyzed (Tables 5 and 6). No significant differences were observed in the bacterial populations in the samples regarding the fungal probiotic factor (Table 5). However, a higher percentage of Gram-positive bacteria was observed in all treatments (Table 5). Despite the significant difference in pH values between food processing types (Table 4), no difference was observed for any of the microbiological communities (Lac+ and Lac- bacteria, fungi, yeasts, and protozoa) analyzed in the treatments (Table 6).

Table 5. Direct testing for detecting bacterial groups and yeast identified by Gram staining of the rumen of confined sheep fed a high-grain diet associated with fungal probiotics

Variables	Whole				Ground			
	RZ	TA	MX	TE	RZ	TA	MX	TE
Coccus	+++	+++	+++	+++	+++	+++	+++	++
Diplococcus	+++	+++	+++	+++	+++	+++	+++	+++
Streptococcus	+	++	+	++	+	++	++	+
Staphylococci	-	+	+	+	+	+	+	+
Spirillum	++	++	++	++	++	+	++	++
Vibrion	+	++	++	+	++	+	+	+
Bacilli	+++	+++	+++	+++	+++	++	+++	+++
Yeast	++	+	+	++	++	++	++	+
Gram positive, %	82.8[a]	69.7[a]	72.5[a]	80.1[a]	86.4[a]	70.1[a]	80.2[a]	76.5[a]
Gram-negative, %	17.2[b]	33.5[b]	30.7[b]	26.5[b]	25.1[b]	25.0[b]	26.3[b]	25.1[b]
Total	100	100	100	100	100	100	100	100

Means followed by different lowercase letters in the column indicate significant differences at 5% significance by the Wilcoxon-Mann-Whitney test. RZ: *Rhizomucor* spp.; AT: *Aspergillus terreus*; MX: mixture of the two fungi (RZ+AT); TE: Control. +++: high concentration; ++: medium concentration; +: low concentration.

In this study, the large protozoan population was almost non-existent in most treatments, except in the treatment with ground corn with mixed *Rhizomucor* spp. and *Aspergillus terreus* fungi (Table 6). We also observed a higher predominance of small protozoa in almost all treatments, except for those with *Aspergillus terreus* regardless of processing (Table 6). Thus, the bigger the protozoa, the lower the protozoan population in HGC

diets.

Some studies confirmed that a ruminal environment characterized by acidosis reduces microbial diversity[3]. According to Nagaraja and Titgemeyer[35], a reduced ruminal protozoan population in animals on HGC, in which the pH is close to 5.5, is considered a good indicator of rumen acidosis. The protozoa are recognized for engulfing the starch and can contribute up to 45% of microbial activity, thus aiding in pH control and serving as a buffer. In addition, very acidic rumen environments present reduced ciliate activity. Therefore, the protozoa should be favored by starch accumulation in the rumen. However, the acidic pH of the rumen prevents the development of protozoa, which may lead to death, especially of large ones[36,37].

No significant difference was observed for Lac+ and Lac- populations within the probiotic factor. In contrast, there was a statistical difference between types of processing ($P < 0.05$) (Table 6 and 7), with a higher population in the ground corn diet (WGC: 1.29×10^6 and GGC: 1.75×10^6; Table: 7). A higher concentration of lactose fermenting bacteria ($P < 0.05$) was also observed in all factors (probiotic x processing) (Table 7). The higher concentration of fermenting bacteria in animals on a ground grain diet can be explained by the greater availability of highly fermentable starch that bacteria can use as an energy source, resulting in lactate release[3,9].

Table 6. Evaluation of the rumen microbiota population of confined sheep fed a high-grain diet associated with fungal probiotics.

Variable	Whole grain corn CFU/mL^{-1}				Mean
	RZ	AT	MX	TE	
Lac+ bacteria	2.6×10^4	2.6×10^4	6.3×10^4	7.4×10^4	3.8×10^4
Lac- bacteria	1.9×10^4	1.8×10^4	6.9×10^3	1.5×10^4	1.4×10^4
Filamentous fungi	1.4×10^3	9.6×10^2	1.3×10^3	8.6×10^2	1.1×10^3
Yeasts	2.2×10^4	7.8×10^3	5.0×10^3	4.8×10^3	4.9×10^3
Small protozoa	2.0×10^7	0	9.0×10^5	1.8×10^5	5.27×10^6
Medium protozoa	5.8×10^5	0	2.7×10^4	2.3×10^3	1.45×10^5
Large protozoa	0	0	0	2.6×10^3	6.50×10^2
	Ground grain corn CFU/mL^{-1}				
Lac+ bacteria	7.6×10^4	2.8×10^4	6.1×10^4	1.1×10^5	6.87×10^4
Lac- bacteria	1.8×10^4	1.1×10^4	1.7×10^4	2.3×10^4	1.72×10^4
Filamentous fungi	2.8×10^3	1.2×10^3	1.5×10^3	1.9×10^3	1.85×10^3
Yeasts	8.3×10^3	2.8×10^3	6.7×10^3	1.7×10^3	4.87×10^3
Small protozoa	2.6×10^4	0	8.7×10^4	2.0×10^5	7.82×10^4
Medium protozoa	60	0	1.0×10^4	2.2×10^2	2.57×10^3
Large protozoa	0	0	1.7×10^4	0	4.25×10^3

RZ: *Rhizomucor* spp.; AT: *Aspergillus terreus*; MX: mixture of the two fungi (RZ+AT); TE: Control; Lac+: lactose fermenting bacteria; Lac-: non-lactose fermenting bacteria.

Table 7. Analyses of the lactose fermenting bacteria population in the fluid of confined sheep fed a high-grain diet associated with fungal probiotics

Treatment	Quantification CFU/mL	Positive cultures (%)		
		Total	Lac+	Lac-
Whole grain corn				
Rhizomucor spp.	2.27×10^5	17.59	57.92^a	42.07^b
Aspergillus terreus	2.22×10^5	17.23	58.36^a	41.64^b
Mixture of fungi	3.52×10^5	27.31	90.12^a	9.87^b
Control	4.89×10^5	37.86	76.29^a	23.70^b
Total WGC	1.29×10^6A	100	-	-
Ground grain corn				
Rhizomucor spp.	4.72×10^5	26.95	80.75^a	19.25^b
Aspergillus terreus	2.38×10^5	13.61	58.26^a	41.74^b
Mix of fungi	3.91×10^5	22.31	78.07^a	21.93^b
Control	6.50×10^5	37.13	88.27^a	11.73^b
Total GGC	1.75×10^6B	100	-	-

Variables followed by different lowercase letters in the rows show significant differences at 5% significance by the Wilcoxon test between Lac+ and Lac-, and different capital letters between types of processing show significant difference by the Kruskal-Wallis test. Lac+: lactose fermenting bacteria; Lac-: non-lactose fermenting bacteria; CFU: colony forming unit; WGC: whole grain corn; GGC: ground grain corn.

Distribution of bacterial genera

The most predominant bacterial genera were, in descending order, *Alcaligenes* (37), *Escherichia coli (*22), *Klebsiella (*11), *Shigella* (10), and *Enterobacter (*6) (Table 8). The lowest *Alcaligenes* level among the treatments was in the WGC control treatment (2.7%), *Aspergillus terreus* treatment (5.4%), and GGC control (8.1%). Bacteria of the genus *Escherichia coli* were absent in the treatments with *Rhizomucor* spp. (0.0%), and less frequent mixture of fungi (4.5%), with ground grain processing and *Aspergillus terreus (*9.1%), and with whole grain processing (Table 11). According to the literature, ruminant animals fed high-concentrate diets have a higher predominance of total *Escherichia coli* bacteria in the rumen[23]. A pH close to 5.5 increases the *Lactobacillus* spp. population replacing *Streptococcus bovis*, which increases lactate concentration and may result in ruminal acidosis[8,10,11], thus reducing bacterial diversity[38].

Vieira et al.[23] evaluated the distribution of the genera of aerobic and facultative anaerobic Gram-

negative bacteria in the ruminal fluid of cattle fed tropical pasture or a high-concentrate diet. They reported that the highest population of bacteria was in animals fed without a source of roughages and that the population of *Escherichia* was significantly higher (71.6% compared to the other treatments) in the treatment for animals on HGC ($P < 0.05$), between treatments and genera. The results of this research corroborate Vieira et al.[23] regarding the predominance of *Escherichia*, *Klebsiella*, and *Enterobacter* in the ruminal fluid of animals fed a high-concentrate diet.

E. coli is more predominant in the ruminal fluid of animals fed a high-grain diet than those fed with a high roughage source. Animals on high-concentrate diets can have subacute ruminal acidosis and a significantly higher *E. coli* population, which characterizes a more favorable environment for this genus, considered the main zoonotic agent in the GIT of ruminant animals[32,39].

Table 8. Distribution of the genera of Gram-negative bacteria present in the ruminal fluid of confined sheep fed a high-grain diet associated with fungal probiotics

Bacteria	Total	Whole								Ground							
		RZ		AT		MX		TE		RZ		AT		MX		TE	
	N*	n	%	N	%	n	%	N	%	n	%	N	%	N	%	n	%
Alcaligenes	37	4	10.8	8	21.6	4	10.8	1	2.7	9	24.3	2	5.4	6	16.2	3	8.1
Escherichia coli	22	4	18.2	2	9.1	3	13.6	3	13.6	-	-	6	27.3	1	4.5	3	13.6
Klebsiella	11	1	9.1	2	18.2	1	9.1	-	-	2	18.2	2	18.2	2	18.2	1	9.1
Shigella	10	1	10.0	-	-	1	10.0	3	30.0	1	10.0	-	-	1	10.0	3	30.0
Enterobacter	6	1	16.7	1	16.7	1	16.7	-	-	-	-	-	-	-	-	3	50.0
Edwardsiella	2	-	-	-	-	-	-	-	-	2	100	-	-	-	-	-	-
Proteus	1	-	-	1	100	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella	1	-	-	-	-	-	-	-	-	1	100	-	-	-	-	-	-
Total	91	11	12.1	14	15.4	10	11.0	7	7.7	15	16.5	10	11.0	10	11.0	13	14.3

RZ: *Rhizomucor* spp.; AT: *Aspergillus terreus*; MX: mixture of the two fungi (RZ+AT); TE: Control; n: number of genus observations. *Refers to the sum of observations between the bacterial genera.

The genera *Salmonella* and *Proteus* were observed less frequently in this study. *Salmonella* and *E. coli* cause food poisoning[23,40]. These two genera produce lactate and grow in the ruminal environment with a high concentration of highly available carbohydrates, decreasing the pH, and causing ruminal acidosis[6,41]. However, in this study, the animals presented no symptoms of food poisoning (diarrhea, loss of appetite, and dehydration, for example) due to these bacteria, which can be explained by the low frequency of these genera in the samples. Furthermore, these results corroborate the histological analyses presented below. Animals with symptoms of acidosis may have ruminal mucosa lesions with damaged ruminal epithelium, which can be observed in the histological examination of the rumen papillae[6,42] (Table 10).

Distribution of facultative anaerobic fungi genera

Six fungal genera were identified in the fifteen samples used to analyze the anaerobic fungal population. *Cladosporium* was the most prevalent genus with seven occurrences, corresponding to 46.66%. *Cladosporium* is a fungus characterized by dark brown and black spots, with a velvety appearance[43]. According to Hankin and Anagnostakis[44], this fungal genus can produce lipase, protease, urease, and chitinase enzymes, aiding the digestion of a diet high in whole corn. They are characterized by slow growth, reaching maturity with 14–21 days, and present effusive or casually punctiform colonies, with a flat surface, circular shape, wrinkled, and the color ranging from olive green to dark brown[45].

The second most prevalent fungus was *Aspergillus* spp., at 26.66% of the total identified fungi. *Aspergillus* spp. contains around 100 species and eleven different teleomorphs (sexual reproduction types). It belongs to the family Trichocomaceae (mold fungi family), and has a white to yellowish color, similar to cotton, with a yellowish base and a darker center[43]. This genus is very common and found globally in adverse environments. It has various actions, causing numerous diseases, and is used in antibiotic production. It is also an important food decomposer being used in the food industry.

Abrão et al.[46–50] reported that this genus has the potential for cellulolytic enzyme production. It can survive the adversities of the ruminal environment in the presence of the main volatile fatty acids and remain viable after a 96-h incubation after being stored for up to two years. Similarly, Mustafa et al.[51] confirmed the efficiency of *Aspergillus terreus* in cellulase enzyme production in filtered fungus culture from in onion seed extract. The activities were higher at 60 °C and pH 5.5, and at 55 °C and pH 6.0 for a production of 15 and 12.5 mg/mL, respectively.

The third most observed group in the analyses was the genus *Absidia* spp. (13.33%), which belongs to the family Mucoraceae, commonly found in decomposing vegetables and occasionally associated with human infection[43]. The genera *Rhizomucor* spp. and *Fusarium* spp. were the least observed,

corresponding to 6.66% of the total genera identified by microculture. *Rhizomucor* spp. also belongs to the family Mucoraceae[430. Bernardes et al.[52] analyzed the potential of *Rhizomucor miehei* for producing stabilized α-amylase enzyme, and confirmed its specific potential in a process requiring a pH of 4.0 to 5.0 and high temperature (70 ºC).

Distribution of ruminal protozoan genera

The quantification of the population of large, medium, and small protozoa is shown in Table 6. The identification of protozoan genera (Table 9) showed *Entodinium* and *Charonina,* but no significant difference was observed between these genera by probiotic and processing type.

Table 9. Distribution of protozoan genera present in rumen fluids of confined sheep fed a high-grain diet associated with fungal probiotics

Protozoan	Treatments - CFU/mL-1										P-value	
	Processing		Whole grain corn				Ground grain corn					
	Whole	Ground	RZ	AT	MX	TE	RZ	AT	MX	TE	Proc	Prob
Entodinium	51.65	23.61	110.6	-	16	80	3	-	2.5	80	0.80	0.63
Charonina	8	-	24	-	2	6	-	-	-	-	0.74	0.84

Variables followed by different lowercase letters in the rows present significant differences at 5% significance by the Kruskal-Wallis test. RZ: *Rhizomucor* spp.; AT: *Aspergillus terreus*; MX: *mix of* the two fungi – RZ+AT; TE: Control. Proc: processing; Prob: probiotic.

Histological analyses of the gastrointestinal tract

The analysis of variations in the histological characteristics of the GIT showed no significant differences regarding the height of the lamina propria-submucosa (LPS) of the rumen of lambs fed the different treatments (Table 10). There was no significant difference in small intestine crypt depth (SCD) regarding the processing factor. However, there was a significant SCD change regarding the fungal probiotic factor ($P < 0.05$), with the RZ treatment being higher.

The width of the papilla base (WPB) showed expressive correlation between the processing and fungal probiotic factors ($P < 0.05$). It was significantly greater in the ground grain diet ($P<0.05$), and the RZ and TE treatments ($P < 0.05$).

The findings regarding *Rhizomucor* spp. may be related to its characteristic of growing well in environments with pH between 4.0 and 5.0 and temperature around 70 ºC[52]. According to Bernardes et al.[52], this fungus showed higher efficiency in alpha-amylase enzyme production at a pH between 4.0 and 5.0. Alpha-amylase acts in the ruminal digestion of starch, the main component in a HGC diet[53].

There was a significant correlation between the factors ($P < 0.05$) for the thickness of the muscular tunica of the ruminal epithelium (TMT), with an expressive difference between the processes and being greater with the whole grain treatments ($P < 0.05$). Among the fungal probiotics, it was higher in the RZ and TE treatments ($P < 0.05$) and lower in the AT treatment. The small intestine villus height (SVH) also showed significant correlations ($P < 0.05$) the treatments. It showed the same trend as TMT regarding the processing factors. However, the trend with the fungal probiotics was different, with the highest values found in the AT and MX treatments.

These results favor the whole grain diet and may be correlated with the higher concentration of larger particles since studies comparing the effect of finely and coarse ground corn have shown better results with coarse ground corn[54]. The presence of particles larger than three millimeters in the rumen improves the peristaltic movement, thus stimulating the development of the ruminal epithelium muscle[42]. RZ showed a more significant effect than TA, which may be correlated with the ability of this fungus to better adapt to GIT conditions, with higher production of enzymes such as α-amylase[52].

Table 10. Histological evaluation of the rumen, small intestine, and large intestine of confined sheep fed a high-grain diet associated with fungal probiotics.

Variables	Processing (µm)		Probiotic (µm)				P-value			CV
	Whole	Ground	RZ	AT	MX	TE	Proc	Prob	Proc:Prob	
LPS	2093	2267	1920	2224	22880	2288	0.297	0.346	0.128	34.84
WPB	771[b]	9774[a]	1080[a]	752[b]	711[b]	949[a]	0.001*	0.001*	0.001	27.67
TMT	1530[a]	1380[b]	1596[a]	1250[c]	1403[b]	1572[a]	0.001*	0.001*	0.001	14.78
SVH	507[a]	385[b]	352[b]	500[a]	511[a]	421[b]	0.001*	0.001*	0.241	27.34
PCD	451[a]	408[a]	574[a]	387[b]	353[b]	403[b]	0.096	0.001*	0.463	28.90
SCD	564[b]	753[a]	520[b]	652[a]	755[a]	706[a]	0.001*	0.001*	0.015	27.62

*Variable followed by different lowercase letters in the row shows significant difference at a 5% probability by the Tukey test. LPS: height of the lamina propria-submucosa of the ruminal epithelium; WPB: width of the base of the rumen papillae; TMT: thickness of the muscular tunica of the ruminal epithelium; SVH: small intestine villus height; SCD: small intestine crypt depth; and LCD: large intestine crypt depth. Proc: processing; Prob: probiotic; Proc:Prob: processing:probiotic.

Gallo et al.[55] performed an experiment with confined sheep subjected to a high WGC diet (80% corn and 20% base mix) compared to control (conventional diet with roughage source) and concluded that the length and width of the rumen papillae were not affected ($P > 0.05$) by the type of diet. Oliveira et al.[56] also evaluated anatomical characteristics of the rumen (number of papillae/cm^2 in each fragment, mean area of papillae, % papillary area, and total absorption surface area per cm^2 of wall) of confined sheep on a HGC diet (WGC, GGC, and moistened corn grain) and reported no difference ($P > 0.05$) in the number of papillae/cm^2 or the mean area of papillae. However, a significant difference was found between the percentage of papillary area and total absorption surface area.

The large intestine crypt depth (LCD) also showed a significant correlation ($P < 0.05$) with the factors, with the GGC diet being the highest. However, TMT, SVH, and SCD were higher in the WGC treatments ($P < 0.05$), and the treatment with Rhizomucor spp. ($P < 0.05$) presented the lowest LCD ($P < 0.05$)

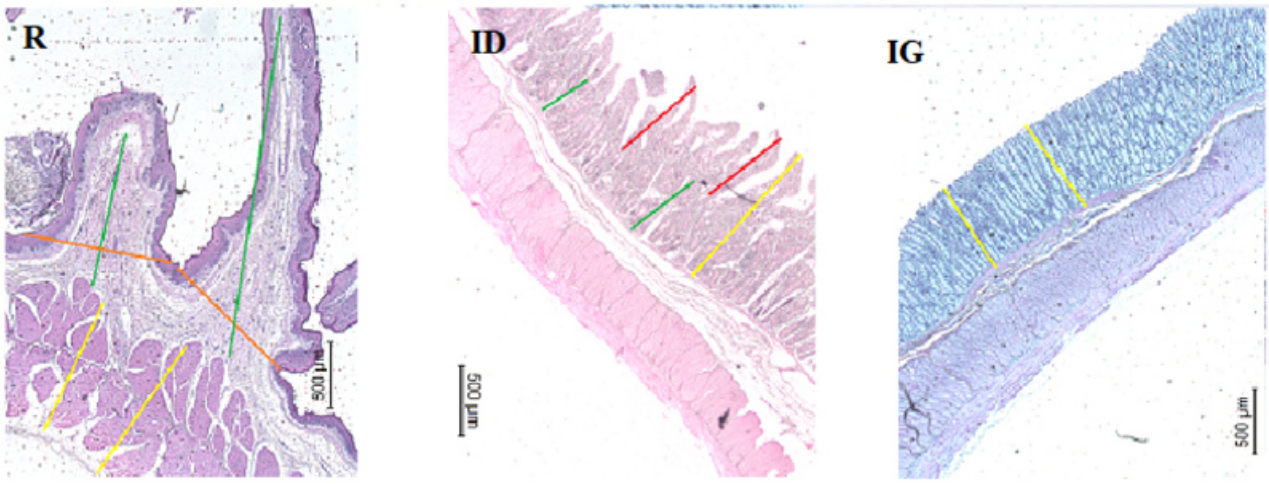

Figure 1. Image of a histological slide (500 µm). In R: ruminal epithelium, the green lines represent the lamina propria-submucosa - LPS, the orange lines the width of the papilla base - WPB, and the yellow line represents the tunica muscularis thickness - TMT; in SI: small intestine epithelium, the red line represents the villus height - SVH, and the green line the crypt depth - SCD; and in LI: large intestine epithelium, the yellow line represents the crypt depth - LCD.

Conclusion

The fungi used in this study showed no probiotic actions influencing the microbiota or the macroscopic characteristics of the rumen environment.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization: R. Fabino Neto, F. O. A. Pessoa, M. M. Godoy; E.S. Miyagi; *Formal analysis*: F. O. A. Pessoa, R. Fabino Neto; *Funding acquisition*: F. O. A. Pessoa; M.M. Godoy; *Investigation*: F.O.A. Pessoa; R. Fabino Neto, T. D. Silva, V. V. Santana Neto, D. K. S. Lima, R. J. M. Silva; *Project administration*: R. Fabino Neto, F. O. A. Pessoa, M. M. Goday, E. S. Miyagi; *Resources*: R. Fabino Neto, F. O. A. Pessoa; *Supervision*: F. O. A. Pessoa, R. Fabino Neto, E. S. Miyagi; *Validation*: F. O. A. Pessoa, E. S. Miyagi; *Visualization*: F.O.A. Pessoa, M.M.A. Brainer, R. Fabino Neto; *Writing (original draft)*: F. O. A. Pessoa, M. M. A. Brainer, R. Fabino Neto; *Writing (review and editing)*: F. O. A. Pessoa, M. M. A. Brainer, R. Fabino Neto.

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