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The addition of anaerobic fungi isolates from buffalo rumen to increase fiber digestibility, fermentation, and microbial population in ruminants

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Abstract. *Agustina S, Wiryawan KG, Suharti S, Meryandini A. 2024. The addition of anaerobic fungi isolates from buffalo rumen to increase fiber digestibility, fermentation, and microbial population in ruminants. Biodiversitas 25: 107-115.* Rumen microbes have an important role in the rumen. Anaerobic fungi are microbes needed in the forage digestion process in the rumen. The addition of microbes, particularly anaerobic fungi is essential to increase the digestibility of forage within rumen. Therefore, this study aimed to evaluate the addition of anaerobic fungi isolates from buffalo rumen to increase fiber digestibility, fermentation, and microbial population in sheep rumen. The in vitro tests were carried out using the Tilley and Terry method, using elephant grass and rice straw as tested forage. *Piromyces* sp. (F1, and F3), *Caecomyces* sp. (F2, and F5), and *Neocallimastix frontalis* (F4) isolates from buffalo rumen were used as tested anaerobic fungi. The result showed that the addition of anaerobic fungi isolates from buffalo rumen significantly affected fiber digestibility (Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), cellulose and hemicellulose) except lignin digestibility. The addition of *N. frontalis* had higher fiber digestibility which is 49.02% NDF digestibility, 42.11% ADF digestibility, 44.28% hemicellulose digestibility, and 38.60% cellulose digestibility. Furthermore, *N. frontalis* also significantly increased In vitro Dry Matter Digestibility (IVDMD), In vitro Organic Matter Digestibility (IVOMD), ammonium (NH_3) production, total Volatile Fatty Acid (VFA) production, and microbial population compared to *Piromyces* sp., and *Caecomyces* sp. In conclusion, anaerobic fungus type *N. frontalis* showed promising potential to be used as a ruminant probiotic due to its superior effect on fiber digestibility, fermentation, and microbial population compared to *Caecomyces* sp. and *Piromyces* sp.

Keywords: Anaerobic fungi, fermentation, fiber digestibility, microbial population, probiotics

INTRODUCTION

The forage given to ruminants has high fiber content and low digestibility due to the presence of lignin. Lankiewicz et al. (2023) stated that lignin is often found in plant cell walls. Forage digestibility is influenced by several factors such as the crystallinity of cellulose, hemicellulose composition, ferulic acid bonds, lignin content and lignin monomer composition (Zhong et al. 2021). The lignocellulose digestibility improvement can be done mechanically or biologically. However, Stabel et al. (2022) stated that mechanical treatment requires higher energy costs compared to biological treatment. Biological treatment can be done by adding direct-fed microbial or exogenous enzymes (Abdel-Aziz et al. 2015). Thareja et al. (2006) stated that an effective strategy for enhancing the digestibility of fiber is through the addition of lignocellulolytic microbes. Puniya et al. (2015) stated that livestock performance can also be improved by supplementing ruminants with microbes, known as direct-fed microbials. Anaerobic fungi are microbes that can be used as direct-fed microbials (Krol et al. 2023). Gruninger et al. (2014) and Pratt et al. (2023) stated that anaerobic fungi play a role in the feed fiber degradation process and have the potential as probiotics to increase fiber digestibility. It produced lignocellulolytic enzymes which are needed to digest lignocellulose in feed (Andlar et al. 2018; Guo et al. 2020). According to Puniya et al. (2015) and Solomon et al. (2016), anaerobic fungi also produced enzymes that could hydrolyze lignified parts of the cell wall and physically damage feed particles through the use of rhizoids. Anaerobic fungi attach to the feed particles and produce extensive rhizoids to penetrate the feed particles (Jimenez et al. 2020). Anaerobic fungi also could degrade fiber and colonize feed particles better than bacteria (Haghen et al. 2021; Wunderlich et al. 2023). Table 1 showed the other advantages of adding anaerobic fungi to increase lignocellulose digestibility.

Several studies showed that the addition of fungi can increase the digestibility of dietary fiber. Kumar et al. (2018) stated that incorporating anaerobic fungi type *Piromyces* sp. WNG-12 increased the digestibility of NDF in wheat straw. According to Wang et al. (2019), the addition of anaerobic fungi type *Piromyces* sp. isolates from Xinong Saanen dairy goat rumen reduced the NDF and ADF content of maize silage. Thareja et al. (2006) also showed that *Neocallimastix* sp. isolates from sheep rumen had higher in vitro fiber digestibility compared to the control. Another advantage of the addition of anaerobic fungi extends to increase the population of other microbes in the rumen. The penetration process of fungal rhizoids into feed particles increases the surface area available for colonization of another microbe such as bacteria and protozoa (Eckart et al. 2010). Puniya et al. (2015) also stated that the addition of anaerobic fungi can increase the other microbe population in the rumen, particularly the population of cellulolytic bacteria. The existence of several advantages possessed by anaerobic fungi indicates their ability to be used as one of the microbial candidates supplemented for ruminants to increase forage digestibility.

Buffalo is a ruminant that has the potential to be used as a source of anaerobic fungal isolates because it could degrade fiber and lignin more efficiently compared to cattle (Xu et al. 2021; Zhong et al. 2021). Wang et al. (2021) stated that buffalo have microbes such as bacteria and fungi which can degrade fiber in forage better than other ruminants. The results of the study conducted by Agustina et al. (2022a) showed that anaerobic fungi isolated from Indonesian buffalo rumen had high cellulase activity. However, there is a lack of information regarding the use of anaerobic fungi from Indonesian buffalo as probiotics to improve fiber digestion. This study aimed to evaluate the effect of adding 5 anaerobic fungi isolates from buffalo rumen as probiotics to improve in vitro fiber digestibility, fermentation, and rumen microbial population.

MATERIALS AND METHODS

Isolates and fungi preparation

The types of anaerobic fungi used were *Piromyces* sp. (F1, and F3), *Caecomyces* sp. (F2, and F5), and *Neocallimastix frontalis* (F4) from buffalo rumen isolated by Agustina et al. (2022b). The anaerobic fungi were isolated from rumen Badegur buffalo (*Bubalus bubalis*) (Agustina et al. 2022b) using dot method (Ed-har et al. 2017). The fungi isolates were incubated at 39ºC and maintained every 5 days using Orpin media liquid containing antibiotics after the pure fungal isolate was obtained. The molecular analysis process of all anaerobic fungi isolates used in this study was completed by Agustina et al. (2022a). The molecular identification was carried out using the method of Vaidya et al. (2018) which ITS1 and ITS2 as the target (Dagar et al. 2015). The nitrogenous bases DNA of anaerobic fungi were BLAST using NCBI data and aligned using MEGA11.

Nutrient, and fiber content analysis

The nutrient content of elephant grass and rice straw was assessed using the proximate method (AOAC 2005). The analysis of fiber composition, including NDF, ADF, lignin, and silica content was carried out using the method of Van Soest et al. (1991). The Hemicellulose content was calculated using the difference between NDF and ADF while cellulose content was estimated as the variation between ADF and ADL.

In vitro rumen fermentation procedure

In vitro fermentation test was carried out by using the method as per Tilley and Terry (1963) with elephant grass and rice straw as the tested substrates. The fermentation process was carried out for 48 hours at 39° C. A total of 500 mg samples of elephant grass and rice straw were weighed and put into a fermenter tube. Subsequently, the tubes were added with 40 mL McDougall solution, 10 mL sheep rumen fluid, and 5 mL of fungal isolate with a population of 10^4 CFU/mL. The negative control was added with 5 mL liquid sterile Orpin medium and 5 mL sheep rumen fluid was added to the positive control. The tube was filled with CO_2 gas for 30 seconds and closed with a rubber cap. The fermenter tube was put into a water bath with a shaker and incubated at 39° C for 4 hours for analysis of the microbial population, pH, NH_3, and total VFA concentration. The sample was incubated for 48 hours to analyze the digestibility of dry matter, organic matter, and fiber. Fiber digestibility was calculated based on the difference in residual and forage fiber content.

Table 1. The potential of rumen anaerobic fungi to increase feed lignocellulose degradation in several recent studies

Strain	Advantages	Reference
Orpinomyces sp.	Produces GH1 β-glucosidase, GH6 cellobiohydrolase, GH9 endoglucanase, GH10 xylanase, GH11 xylanase, GH43 β-xylosidase, and GH45 endoglucanase which effectively degrades lignocellulosic biomass.	Couger et al. (2015)
Neocallimastix frontalis	Produces xylanase, Fpase, Ferulic Acid Esterase (FAE), p-coumaric Acid Esterase (CAE) and Acetyl Esterase which are required to degrade lignocellulose.	Wei et al. (2016)
Orpinomyces sp.	Increases the degradation of xylan, glucan, and lignocellulose.	Morrison et al. (2016)
Piromyces sp. + *Methanobrevibacter*	Increases acetate production, and xylose utilization.	Li et al. (2017)
Orpinomyces joyonii	Reduces cellulose and hemicellulose from rice straw without pretreatment.	Shetty et al. (2020)
Anaeromyces robustus	Produces xylanase (GH10)	Wen et al. (2021)
Neocallimastix frontalis + *Methanobrevibacter gottschalkii*	Degrades 59.0-68.1% DM, and 49.5-59.7% NDF of corn stalk, wheat straw, oat straw, rice straw, and sorghum straw	Wei et al. (2022)
Neocallimastix cameroonii	Hydrolyzes un-preteated lignocellulosic biomass and produces high hydrogen	Stabel et al. (2022)
Oontomyces sp.	Degrades Dry Matter, NDF, NDS, ADF, Cellulose, Hemicellulose and Lignin in forage significantly.	Xue et al. (2022)

Note: DM: Dry Matter, NDF: Neutral Detergent Fiber, NDS: Neutral Detergent Soluble, and ADF: Acid Detergent Fiber

Analytical procedures

The pH was measured by using a digital pH meter, specifically the type pHep HI98107 (Hanna Instruments Indotama, North Jakarta). Dry matter and organic matter digestibility were assessed using the Tilley and Terry (1963) method. The concentration of NH_3 produced was measured using the Conway micro diffusion method (General Laboratory Procedure 1966) and the total VFA concentration was carried out using steam distillation (AOAC 2005). Furthermore, the rumen microbial population was calculated using the methods of Ogimoto and Imai (1981) and Hungate (1969).

Statistical analysis

A factorial randomized block design 2x7 with 5 replications was used in the in vitro evaluation. The first factor was forage (rice straw and elephant grass) and the second factor was the addition of supplementation, including sterile medium, 5 fungi isolates and sheep rumen liquor. The results of in vitro analysis were analyzed through ANOVA followed by the Duncan test using SPSS 22.

RESULTS AND DISCUSSION

Nutrient and fiber composition

The results of proximate and fiber analysis are presented in Table 2 which showed that elephant grass had better nutritional content, possessing a higher crude protein and lower crude fiber compared to rice straw. Peripolli et al. (2016) stated that the low nutritional quality of rice straw was caused by its high silica and low protein content. In this study, the rice straw contained 9.81% crude protein (CP), which was higher than the 3.63-4.82% obtained by Peripolli et al. (2016). The differences in protein content were caused by several factors such as climate, cultivation management, harvesting time, post-harvest storage and soil fertility (Peripolli et al. 2016). The results showed that rice straw had higher NDF, ADF, cellulose, lignin, and silica content compared to elephant grass. This was following Yanuartono et al. (2017) that rice straw had a higher NDF and ADF content compared to grasses. The high content of silica and lignin in rice straw were limiting factors for its use as an energy source for livestock because these components bind to hemicellulose and cellulose in plant tissues (Yanuartono et al. 2017).

Table 2. Nutrient and fiber composition of elephant grass and rice straw

Variable	Elephant grass	Rice straw
Nutrient Composition (100% DM)		
Ash	12.93	17.84
Crude Fat	1.46	0.99
Crude Protein	14.49	9.81
Crude Fiber	29.69	35.16
Fiber Composition (%)		
NDF	65.59	71.38
ADF	42.41	54.35
Hemicellulose	23.18	17.03
Cellulose	33.55	30.65
Lignin	6.41	10.78
Silica	2.45	12.92

Note: DM: Dry Matter, NDF: Neutral Detergent Fiber, and ADF: Acid Detergent Fiber

Fiber digestibility

The results showed that forage, and type of supplementation significantly affected ($P<0.05$) the digestibility of dry matter, organic matter, NDF, ADF, hemicellulose, and cellulose but did not affect lignin digestibility, as presented in Figure 1. Furthermore, the interaction between forage and fungi supplementation significantly ($P<0.01$) increased fiber digestibility except for lignin. In this study, elephant grass and rice straw had low dry matter and organic matter digestibility due to the high content of complex fiber in the forage. The proximate analysis showed that rice straw contained 35.16% crude fiber while elephant grass comprised 29.69% crude fiber (Table 2). The high crude fiber content indicated that the feed contained higher NDF, ADF, and lignin, making it difficult for rumen microbes to degrade this component (Trisnadewi and Cakra 2020). Elephant grass also had higher NDF, ADF, hemicellulose, and cellulose digestibility, with proportions of 52.12, 43.83, 46.77, and 41.67% compared to rice straw at 36.21, 32.05, 32.89, and 30.52%, respectively. This was in line with the results of Jayanegara et al. (2019) that elephant grass had a higher degradation rate than rice straw. The low digestibility of rice straw was caused by the high lignin content. Zhong et al. (2021) stated that lignin content in plant cell walls affected the level of fiber degradation in rumen. The type of lignin and phenolic acid bonds were negatively correlated with the digestibility of feed due to the resistance to bacterial and fungal degradation in rumen (Raffrenato et al. 2016). Consequently, increasing the lignin content in feed can reduce the digestibility of fiber, as it inhibits the activity of microbial enzymes in rumen (Zhong et al. 2021).

The results showed that forages supplemented with anaerobic fungi F1, F2, F3, F4, and F5 had higher digestibility of dry matter, organic matter and fiber compared to forages with sterile media (control), as shown in Figure 1. This indicated that the addition of rumen anaerobic fungi effectively increased the digestibility of low-quality feed (Gruninger et al. 2014; Puniya et al. 2015). Similarly, Sirohi et al. (2013) reported that the addition of anaerobic fungi increased the digestibility of fiber in wheat straw. This was also agreed with Xue et al. (2022) that anaerobic fungi had high fiber digestibility (Dry Matter, NDF, NDS, ADF, Cellulose, Hemicellulose and Lignin). The feed digestibility in the rumen increased because anaerobic fungi produced cellulase enzymes and penetrated feed cells (Gruninger et al. 2014; Solomon et al. 2016; Henske et al. 2018; Rabee et al. 2019). Anaerobic fungi also could produce rhizoids which could break down feed particles mechanically and enzymatically (Dollhofer et al. 2018). Raffrenato et al. (2016), and Panahi et al. (2022) stated that the penetration of rhizoids into feed particles also could break the lignin bonds and increase the digestibility of hemicellulose and cellulose. Generally, lignin in plant cell walls binds to hemicellulose and cellulose (Kang et al. 2019). The digestibility of hemicellulose and cellulose increased in forages supplemented with rumen anaerobic fungi do to the production of hemicellulase and cellulase enzymes. Based on the report by Comlekcioglu et al. (2017) and Rabee et al. (2019), rumen anaerobic fungi can produce xylanase enzymes, while Ma et al. (2022) stated that the hemicellulases produced by these fungi included xylanase, mannanase and galactanase.

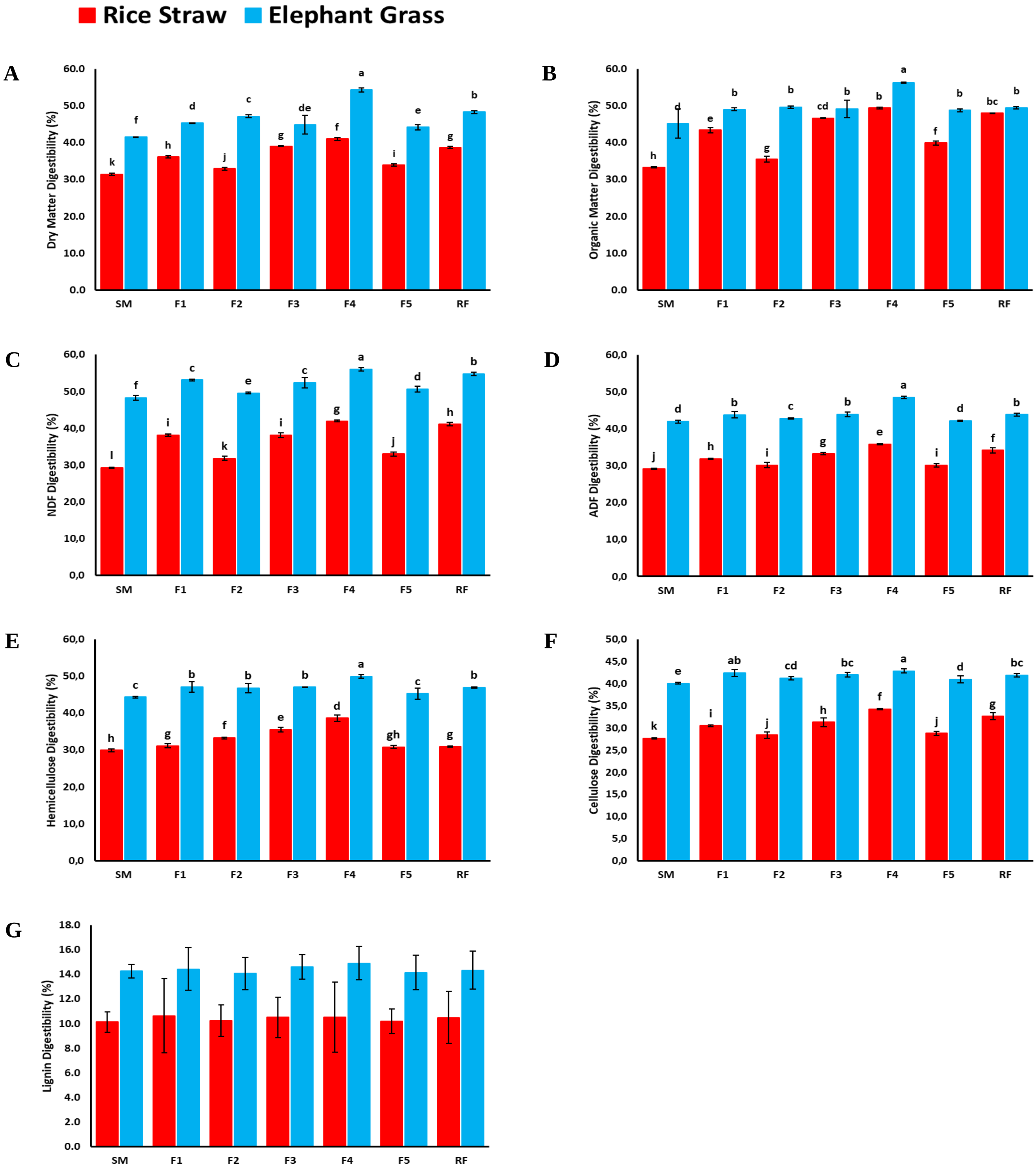

Figure 1. The effect of anaerobic fungi addition on in vitro fiber digestibility. The figure includes Dry Matter Digestibility (A), Organic Matter Digestibility (B), NDF Digestibility (C), ADF Digestibility (D), Hemicellulose Digestibility (E), Cellulose Digestibility (F), Lignin Digestibility (G). SM: Sterile Medium, F1 and F3: *Piromyces* sp., F2 and F5: *Caecomyces* sp., F4: *Neocallimastix frontalis*, RF: Rumen Fluid, NDF: Neutral Detergent Fiber, ADF: Acid Detergent Fiber. The error bars represent standard deviation (n=5), and different letters in the same column showed significantly different interactions of forage x supplementation (P<0.01)

Anaerobic fungi also produce high cellulase enzymes such as endoglucanase, exoglucanase, and β-glucosidase that are important in the process of feed fiber degradation (Dollhofer et al. 2015; Rabee et al. 2019; Agustina et al. 2022a). The endoglucanase enzyme initiated the amorphous part in cellulose, released the oligosaccharides, and formed a new free chain that was further cleaved by exoglucanase (Dollhofer et al. 2015). Subsequently, exoglucanase will produce cellobiose from oligosaccharides, which is then hydrolyzed by β-glucosidase to produce glucose (Dollhofer et al. 2015). Youssef et al. (2013) showed that the enzymes produced by anaerobic fungi, with mechanisms of the endoglucanase included GH5, GH8, GH9, and GH45. Meanwhile enzymes belonging to the GH6 and GH48 groups have mechanisms of action as exoglucanase enzymes (Youssef et al. 2013). According to Wang et al. (2014), the enzymes that acted as endoglucanase in *N. frontalis* belonged to the GH5 group, while exoglucanase were GH6 and GH48. As reported by Chen et al. (2012), anaerobic fungi produced enzymes from the GH3 group acting as efficient β-glucosidase that converted cellobiose into glucose. These characteristics facilitate the reduction of cellobiose limiting the performance of endoglucanase and exoglucanase enzymes and increasing cellulase enzyme activity (Chen et al. 2012). The high digestibility of hemicelluloses and celluloses in the addition of anaerobic fungi was due to the production of cellulosomes. Lillington et al. (2021) stated that cellulosome was an enzyme complex hemicellulase/cellulase produced by anaerobic fungi, playing an important role in the degradation process of feed fiber.

As shown in Table 3, the increased digestibility of hemicellulose and cellulose in forages added with anaerobic fungi was due to a rise in the rumen microbial population. This was in accordance with Puniya et al. (2015), where the population of cellulolytic bacteria in rumen increased with the addition of anaerobic fungi. The results presented in Figure 1 also showed that the forage added with anaerobic fungi type *N. frontalis* had higher digestibility of dry matter, organic matter, NDF, ADF, hemicellulose, and cellulose compared to *Caecomyces* sp. and *Piromyces* sp. This was in line with Shelke et al. (2009), where *Neocallimastix* spp. showed higher dry matter digestibility than *Piromyces*. Nagpal et al. (2010) also reported that wheat straw added with *Neocallimastix* sp. had higher digestibility than wheat straw added with *Caecomyces* sp. This was because *N. frontalis* produced cellulase enzyme and rhizoid higher than *Piromyces* sp., and *Caecomyces* sp. (Agustina et al. 2022a). Fungal fiber degradation can be enhanced by doing consortia with other microbes such as bacteria (Vu et al. 2023). Li et al. (2017) and Li et al. (2021) also said that the co-culture of anaerobic fungi with methanogens produces higher energy, fermentation products, and lignocellulose degradation than monoculture.

Rumen fermentation

Based on the results of the in vitro test presented in Figure 2, the type of forage, supplementation and their interactions did not affect rumen pH but significantly influence ($P<0.01$) NH_3 and total VFA production. The stable pH values were attributed to the main fermentation product of anaerobic fungi was acetic acid (Li et al. 2016; Agustina et al. 2022a). According to Juniawati et al. (2017), acetic acid had a higher pKa value than lactic acid, resulting in low changes on pH value. The greater pKa value indicated a lower acidity degree (Limo et al. 2015). The forages added with *N. frontalis* (F4) produced higher NH_3 than *Piromyces* sp. (F1 and F3), *Caecomyces* sp. (F2 and F5), and control. Puniya et al. (2015) stated that adding rumen anaerobic fungi could increase the concentration of NH_3 in rumen. Anaerobic fungi were able to produce enzymes to degrade feed protein (Hess et al. 2020) and produced NH_3 (Agustina et al. 2022b). Compared to bacteria, anaerobic fungi could produce protease and penetrate the protein coating on feed particles (Hess et al. 2020). This showed that the addition of *N. frontalis* improved protein degradation in forages.

The fermentation of elephant grass produced a higher total VFA (151.26 mM) compared to rice straw, which yielded 103.99 mM. Total VFA production was affected by the type of feed (Ghimire et al. 2014; Bharanidharan et al. 2018), where a higher proportion of forage fiber in the feed caused lower total VFA production (Ramaiyulis et al. 2018; Wang et al. 2019; Zhao et al. 2020). This reduction in VFA production in rice straw was attributed to its lower nutrient content and digestibility. Similarly, Zhao et al. (2020) stated that feed fiber content affected the fermentation characteristics and VFA production. The results also showed that forage added with *N. frontalis* produced a higher total VFA compared to *Piromyces* sp., *Caecomyces* sp., and control. According to Shelke et al. (2009) and Nagpal et al. (2010), the addition of rumen anaerobic fungi increased the fermentation process and VFA production in rumen. The addition of *N. frontalis* increased the total VFA production due to a rise in the digestibility of dry matter, organic matter, and fiber in forages as shown in Figure 1. Zhao et al. (2020) stated that VFA concentration can be used as an indicator to show feed fermentation activity by rumen microbes, with an increase in total VFA concentration indicating an elevated fermentation process in rumen was increased.

Rumen microbial ecosystem

The microbial ecosystem in the rumen plays an important role and influences the fermentation process. This ecosystem comprises bacteria, archaea, protozoa, and anaerobic fungi (Vaidya et al. 2018), which work together to degrade feed particles and produce VFA as a source of energy for livestock (Wei et al. 2016; Vaidya et al. 2018; Beckett et al. 2021). Rumen microbial population including anaerobic fungi, protozoa, and bacteria was influenced by the type and quality of forage (Zhang et al. 2014; Liu et al. 2016; Zhang et al. 2020). The results showed that the type of forage had a significant effect ($P<0.05$) on the rumen microbial population as shown in Table 3. Furthermore, NDF and lignin content in rice straw induced a low rumen microbial population. The differences in the type and chemical composition of the feed affected rumen microbial colonization (Liu et al. 2016; Vahidi et al. 2021). Wang et al. (2017) and Zhong et al. (2021) stated that lignin content in feed reduced enzyme activity, microbial populations, and the level of fiber degradation in rumen. According to Lobo et al. (2021), lignin has antibacterial and antifungal properties affecting the microbial population. The phenolic hydroxyl content in lignin inhibits bacterial growth (Alzagameem et al. 2019; Yun et al. 2021). Based on the result, forage added with *N. frontalis* (F4) had a higher microbial population to other treatments. This was in line with Puniya et al. (2015), which reported that the addition of anaerobic fungi increased the cellulolytic bacteria population in the rumen. The penetration process of fungi into feed cells expanded the area, facilitating the colonization of other microbes, particularly cellulolytic bacteria (Eckart et al. 2010; Puniya et al. 2015).

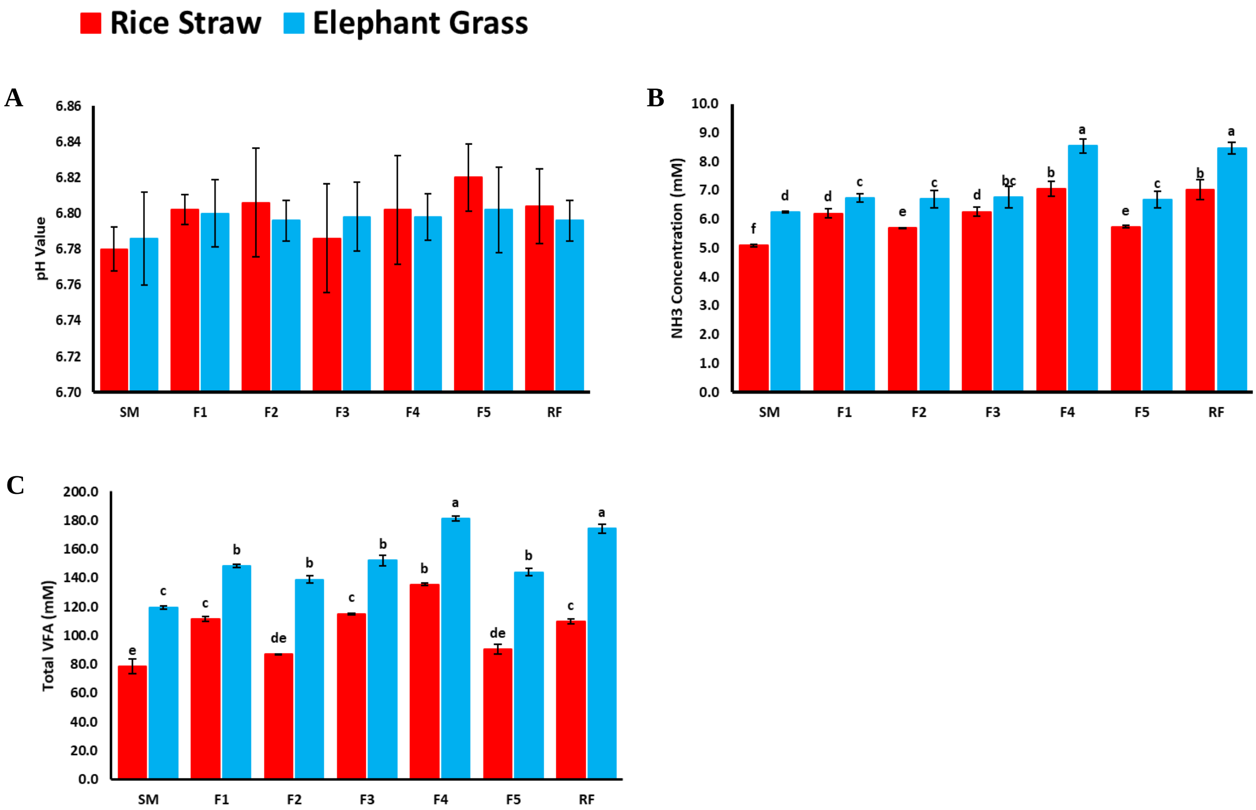

Figure 2. The effect of anaerobic fungi addition on in vitro fermentation. The figure includes Rumen pH Value (A), NH_3 Concentration (B), and Total VFA Concentration (C). SM: Sterile Medium, F1 and F3: *Piromyces* sp., F2 and F5: *Caecomyces* sp., F4: *Neocallimastix frontalis*, RF: Rumen Fluid, NH_3: ammonia, VFA: Volatile Fatty Acid. The error bars represent standard deviation (n=5), and different letters in the same column showed significantly different interactions of forage x supplementation (P<0.01)

Table 3. The effect of anaerobic fungi addition on rumen microbial population

Variable	Forage	Supplementations							Mean	SEM
		SM	F1	F2	F3	F4	F5	RF		
Rumen Microbial Population (Log/mL)										
Total bacteria	RS	9.11^{d}	9.19^{d}	9.17^{d}	9.20^{d}	9.23^{d}	9.18^{d}	9.24^{cd}	9.19^{B}	0.021
	EG	9.25^{cd}	9.48^{b}	9.29^{cd}	9.41^{bc}	10.11^{a}	9.27^{cd}	10.09^{a}	9.56^{A}	0.101
	Mean	9.18^{C}	9.34^{B}	9.23^{BC}	9.31^{B}	9.67^{A}	9.23^{BC}	9.67^{A}		
	SEM	0.030	0.045	0.030	0.038	0.149	0.025	0.143		
Protozoa	RS	5.31	5.39	5.31	5.40	5.50	5.38	5.54	5.40^{B}	0.021
	EG	5.55	5.66	5.65	5.69	5.78	5.65	5.77	5.68^{A}	0.018
	Mean	5.43^{C}	5.52^{ABC}	5.48^{C}	5.54^{ABC}	5.64^{AB}	5.51^{BC}	5.66^{A}		
	SEM	0.042	0.054	0.060	0.059	0.048	0.055	0.042		
Anaerobic fungi	RS	4.02	4.13	4.10	4.13	4.16	4.11	4.11	4.11^{B}	0.010
	EG	4.19	4.26	4.27	4.25	4.28	4.22	4.20	4.24^{A}	0.007
	Mean	4.11^{C}	4.19^{AB}	4.18^{AB}	$4.19A^{B}$	4.22^{A}	4.16^{B}	4.15^{BC}		
	SEM	0.028	0.023	0.028	0.022	0.020	0.019	0.255		

Note: ^{a,b} Different letters in the same bar showed significantly different interactions of forage x supplementation (P<0.01). ^{A,B} Different letters in the same bar showed significantly different (P<0.05). RS: Rice Straw, EG: Elephant Grass, SM: Sterile Medium, F1 and F3: *Piromyces* sp., F2 and F5: *Caecomyces* sp., F4: *Neocallimastix frontalis*, RF: Rumen Fluid, and SEM: standard error of the mean

In conclusion, this study showed that *N. frontalis* isolates from buffalo rumen indicated promising potential as a probiotic candidate for enhancing the digestibility of forage in ruminants due to the addition of *N. frontalis* increased IVDMD, IVOMD, fiber digestibility, production of NH_3, total VFA production, and rumen microbial population in sheep higher compared to *Caecomyces* sp. and *Piromyces* sp.

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