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Full Length Article

Treatment with Enzyme Extracts of *Phanerochaete chrysosporium* and *Penicillium chrysogenum* to Improve Silage Quality of Alfalfa and Bermuda Grass

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Abstract

The effectiveness of a control and two treatments, using extracts from lignolytic fungi, namely *Phanerochaete chrysosporium* and *Penicillium chrysogenum*, was examined to enhance the silage quality and nutritional value of alfalfa *(Medicago sativa* L.*)* and Bermuda grass Treated substrates, containing approximately 150 g of dry matter (DM), were ensiled in 300 mL microsilos over 90 days. The treatments and the substrates significantly influenced various parameters, including pH, buffering capacity, lactic acid content, butyric acid content, lignin content, cellulose content, and digestibility. The treatment with *P. chrysosporium* extract led to a significant degradation of lignin (18.37 ± 1.30%), a remarkable increase in cellulose content (19.10 ± 1.20%), and a notable mean increase in lactic acid production (18.21 ± 0.3 g/Kg DM), surpassing the effects of *P. chrysogenum* extract and the control. Bermuda grass was more responsive to both treatments than alfalfa. Lactic acid content was positively correlated to pH in the treated substrates. The pH levels were slightly higher in the treated substrates compared to the control, because of the higher buffering capacity, ranging from 142 to 242 mEq/kg DM. In conclusion, the enzymatic treatment, using extracts from lignolytic fungi significantly improves the fermentation quality and nutritional value of the ensiled forages.

Keywords: Silage quality; Alfalfa; Bermuda grass; Treatment; Ligninolytic fungi; Enzyme extract

Introduction

Recently, the increasing feeding costs, resulting from the scarcity of fodder resources and irregular seasonal supply, encompassing prolonged droughts and brief rainy periods, constitute a significant obstacle to the swift development of ruminant breeding in Morocco. Moroccan farmers produce a substantial quantity of ruminant feed, including alfalfa and grass, which they carefully preserve as hay or straw through sun-drying to overcome the lack of forage during prolonged droughts.

To overcome the limitations of sun-dried hay, ensiling offers an alternative and effective method of preserving forage. However, practical results frequently reveal that the quality of silage often falls below standard or proves unsatisfactory. Ensiling alfalfa presents difficulties primarily due to its heightened buffering capacity and the restricted concentration of water-soluble carbohydrates (WSC) and dry matter (DM) (Wang *et al*. 2019). Moreover, the ensiling of grasses is very difficult due to their low WSC and high lignocellulosic content (Desta *et al.* 2016).

To enhance the quality of silage fermentation and reduce the structural complexity of the cell wall components, various additives have been employed, encompassing chemical agents like sugars (e.g. molasses) and organic acids (Tao *et al.* 2021), as well as biological agents such as lactic acid bacteria (LAB) and fibrolytic enzymes (Desta *et al.* 2016). Ju *et al.* (2023) demonstrated that the addition of *Lactiplantibacillus plantarum* and cellulase to *Caragana korshinskii* (native to sandy grass) improved the fermentation quality of silage by reducing the NH_3-N, DM, neutral detergent fiber (NDF), and acid detergent fiber (ADF) contents and by increasing lactic

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acid (LA) content. Tao *et al.* (2021) found that the addition of a silage bacterial inoculant improved the fermentation quality of wilted tropical grass silage. Desta *et al.* (2016) reported that the utilization of fibrolytic enzymes as additives increased LA, WSC, and decreased pH and all the fiber components except acid detergent lignin (ADL). Moreover, Several studies have demonstrated that the addition of enzymes like laccase during the ensiling process facilitates delignification, thus partially hydrolyzing cellulose and hemicellulose into soluble sugars that are necessary for the formation of lactic acid (Fabiszewska *et al.* 2019; Guo *et al.* 2020; Bao *et al.* 2022). Despite this, the use of individual enzymes as additives in the ensiling process has unfortunately not yielded the desired results in improving fermentation quality. An alternative approach that shows more promising prospects lies in the utilization of enzymatic extracts from fungi. These enzymatic extracts have the advantage of containing a variety of enzymes, thus forming a synergy of catalytic activities.

Fungi, such as *Phanerochaete chrysosporium*, and *Penicillium chrysogenum*, are well-documented for their ability to break down high-lignin materials. They are known for their highly efficient ligninolytic enzymes, notably lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Liu *et al.* 2014; Benaddou *et al.* 2023a; van der Made *et al.* 2023), which possess oxidative potential for both phenolic and non-phenolic lignin units. *P. chrysogenum*, for example, is commonly used as a feed additive in ruminant diets to improve forage fiber digestibility (Hansen *et al.* 2015; Karpe *et al.* 2015).

The direct use of *P. chrysosporium* and *P. chrysogenum* as additives in silage is hindered by the fact that these two fungi are not anaerobic-ligninolytic. Therefore, using enzyme extracts of these fungi as additives may act synergistically, contributing to a more efficient breakdown of complex cellular constituents and enhancing the nutritional value of silage. Furthermore, treatment with enzyme extract may improve buffering capacity and can in turn contribute to keeping the pH level within a range that is ideal for the action of LAB.

While prior research has extensively investigated the pretreatment of lignocellulosic biomass with individual enzymes or enzymatic extracts from fungi to enhance silage quality, there's a noticeable gap in studies focusing on applying these enzymes during ensilage, especially when using alfalfa or Bermuda grass as substrate. This research was designed to address this gap and evaluate the impact of enzymatic treatment during ensiling. The objective was to assess to what extent treatment with *P. chrysosporium* or *P. chrysogenum* enzyme extract can improve the nutritional value and overall quality of alfalfa and Bermuda grass silages.

Material and Methods

Overview of silage preparation and sampling

Alfalfa (*Medicago sativa* L.) and Bermuda grass (*Cynodon dactylon*) underwent treatment just before ensiling by adding *P. chrysosporumrium* or *P. chrysogenum* enzymatic extract or a similar solution without extract. Substrates, containing 150 g DM, were packed into 300 mL microsilos. Four replicates were prepared for each treatment, resulting in a total of 24 microsilos for each of the two experiments (3 treatments × 2 substrates × 4 replicates).

After 90 days of ensiling, the microsilos were opened, and the silage was assessed for levels of lactic acid, butyric acid, pH, fiber (ADF and ADL), and *in vitro* true digestibility (IVTD).

Strain cultivation and enzymatic extract preparation

The initial culture of *P. chrysosporium* and *P. chrysogenum* was carried out in Czapek's agar (De León-Medina *et al.* 2023). After 10 days of incubation at 28°C, enzymatic extracts were carried out according to Rodrigues method with some modifications (Rodrigues *et al.* 2008; Fernandes *et al.* 2023). Extracts were obtained from a liquid culture medium containing 4.5 g of milled maize (dried whole maize with cobs, particle size 1mm) with 99 mL of citrate buffer 50 mM, adjusted to pH 4.5, and 1 mL of nutrient solution prepared with: 5 g glucose, 2.2 g ammonium tartrate, 1 g KH_2PO_4, 0.26 g NaH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 2.9 g 2,2-dimethyl succinic acid, 10 mg $CuSo_4.5H_2O$, 74 mg $CaCl_2.2H_2O$, 6 mg $Z_NSO_4.7H_2O$, 5 mg $FeSO_4.7H_2O$, 5 mg $MnSO_4.4H_2O$, 1 mg $CoCl_2.2H_2O$, 5 mL of vitamin solution prepared with: 0.5 g thiamine–HCl, 0.5 g yeast extract, 0.16 g pyridoxine–HCl, 0.08 g calcium pantothenate, and 0.001 g biotin (per 100 mL).

Targeted ligninase activities were measured as follows: Laccase activity was determined using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as substrate, lignin peroxidase (LiP) was assayed using the dye azure B as a substrate (Srinivasan *et al.* 1995) (Hermosilla *et al.* 2018), and manganese peroxidase (MnP) activity was measured by monitoring the oxidation of Mn^{2+} to Mn^{3+} in 0.11 M of sodium lactate. Cellulase activity was determined as described by Ghose (1987). All enzyme activities were expressed in IU/mL.

Enzymatic treatment and ensiling

Bermuda grass (*Cynodon dactylon*) was collected from the lawn of the Faculty of Science in Meknes, Morocco. Alfalfa *(Medicago sativa* L.*)* was harvested at the early bloom stage from a field in Fez, Morocco. The two substrates were collected in two periods (July and March). Fresh substrates were immediately chopped into a length of 1.5–3.5 cm and ensiled with a 3.6% (v/w) enzyme extract solution on a DM basis. The two substrates were subjected to treatment using extracts from *P. chrysosporium* (E-sporium) or *P. chrysogenum* (E-genum). The control was treated by adding the solution prepared above without enzyme. Then, approximately 150 g DM equivalent of treated substrates

(alfalfa and Bermuda grass) were packed into 300 mL laboratory microsilos, with a length of 9.7 cm and a diameter of 8 cm, and stored at ambient temperature (25–32ºC) after being sealed.

Organic acids analysis and buffering capacity measurement

After 90 days of ensiling, a 15 g DM sample from each silage was mixed with 60 mL of distilled water. Afterward, the mixture was filtered through three layers of cheesecloth and Whatman filter paper. Immediately, pH was measured, and the filtrate was stored at -20°C for subsequent determination of organic acids (lactic acid and butyric acid). The filtrate obtained after the initial filtration was centrifuged at 10^4 × g for 10 min. Subsequently, the supernatant was passed through a microfilter with a pore size of 0.45 µm for the determination of organic acids. This analysis was performed using an Agilent 1260 HPLC system (Agilent Technologies, Inc., Waldbronn, Germany), which was equipped with a refractive index detector (RI (55 °C)). The HPLC column used was Agilent Hi-Plex H, 7.7 x 300 mm, 8 µm (p/n PL1170-6830), and the eluent used was 0.005M H_2SO_4 flowing at a rate of 0.7 mL/min. The system was maintained under 60°C and 4.6 MPa during analysis (Tao *et al.* 2020; Mgamat *et al.* 2023).

The buffering capacity was determined using electro-metric titration with a pH meter. The filtrate obtained earlier was titrated first to pH 3 using 0.1 N HCl which caused the release of bicarbonate as carbon dioxide. Afterward, the filtrate was titrated to pH 6 using 0.1 N NaOH. The buffering capacity was expressed as milliequivalents (mEq) of alkali required to change the pH from 4 to 6 per 100 g of DM after adjusting for the titration value obtained from a 250 mL of water blank (Playne and McDonald 1966).

Fiber and *In vitro* true digestibility determination

After 90 days of ensiling, and after removing the 15 g of DM for the above-mentioned analyses, the remaining substrate was dried at 60°C for 72 h. Fiber analysis was performed using the method of Van Soest *et al.* (1991). Lignin (L) content was measured as acid detergent lignin (ADL), while cellulose (C) was determined as the difference between acid detergent fiber (ADF) and ADL.

To compare the two substrates, percentage change in fiber content, indicating a positive direct or indirect effect of the treatment, was calculated using the following formulae:

$$(1): Cellulose_{-imp} = (C_f - C_i)/C_i) \times 100)$$

$$(2): \text{Lignin\_loss } (L_i - L_f)/L_i) \times 100)$$

Where: Cellulose_imp and lignin_loss stand for cellulose improvement (increase) and lignin loss (decrease), respectively after 90 days of ensiling; C_i and L_i stand for the initial cellulose and lignin content just before ensiling, respectively; C_f and L_f represent the cellulose and lignin contents after 90 days of ensiling, respectively.

IVTD was measured as described by Gulecyuz (2017). IVTD change was calculated using the following formula:

$$(3): Digestibility\_imp = \frac{IVTD_f - IVTD_i}{IVTD_i} \times 100$$

Where: Digestibility_imp: Percentage of improvement in IVTD; $IVTD_i$: *In vitro* true digestibility at the beginning of the trial; $IVTD_f$*: In vitro* true digestibility at the end of the 90-day ensiling.

Statistical analysis

Data were collected according to a 3*2 factorial experiment (3 treatments *2 substrates) in a randomized complete block (the two seasons), with four repetitions of each treatment in each block. A variety of suitable statistical tests were applied to the data. R and OriginPro software were used to analyze variance (ANOVA) and Tukey's mean comparison to find significant differences between treatments (at a significance threshold of 0.01) (Alkarkhi and Alqaraghuli 2020). R software was used to do Principal Component Analysis (PCA).

Results

Lignocellulolytic activities and substrates characteristics

Both strains revealed cellulolytic and ligninolytic profiles. However, they differed in the type and efficiency of enzymes (Table 1).

The characteristics and chemical composition of alfalfa and Bermuda grass before ensiling are presented in Table 2. Bermuda grass exhibited a higher fiber content compared to alfalfa. In contrast, alfalfa exhibited significantly different levels of ash, crude protein, and total nitrogen. Additionally, alfalfa displayed a higher percentage of IVTD. The concentration of organic acids was low in both substrates.

Effect of enzymatic treatment on fermentation quality

Significant effects ($P<0.001$) of enzyme extract, substrate type, and their interaction were observed on lactic acid content, butyric acid content, pH, and buffering capacity, as shown in Fig. 1.

Effect of enzymatic treatment on fiber and digestibility change

The treatments and substrates showed significant differences in fiber change (cellulose_imp and lignin_loss) and digestibility_imp ($p<0.001$) (Fig. 1d). After 90 days of ensiling, the addition of enzyme extracts as an additive resulted in an increase in cellulose content, degradation of lignin, and an improvement in digestibility compared with the control. These effects were more pronounced in Bermuda grass silage than in alfalfa silage. Among the

Table 1: Enzyme activities measured after 12 days of incubation in submerged fermentation. The results were presented as means±standard deviation. ND refers to the non-detected activities

Enzyme	Strains	
	P.chrysosporium	*P. chrysogenum*
Endoglucanase (IU.mL^{-1})	ND	ND
β-glucosidase (IU.mL^{-1})	2.94 ± 0.3	0.69 ± 0.1
Laccase (IU.mL^{-1})	ND	2.4 ± 0.2
Lignin peroxidase (IU.mL^{-1})	6.48 ± 0.2	ND
Manganese peroxidase (IU.mL^{-1})	ND	3.1 ± 0.3

Table 2: Chemical characteristics and composition of alfalfa and Bermuda grass before ensiling

Chemical characteristics and composition	Alfalfa (Mean ± SD)	Grass (Mean ± SD), n=3	p-value of the difference
DM (g/Kg wet weight)	370± 1	290.45 ± 2.4	<0.001
NDF (g/Kg DM)	362.45 ± 2.4	660.15 ± 3.1	<0.001
ADF (g/Kg DM)	340.55 ± 1.4	410.25 ± 2.7	<0.001
ADL (g/Kg DM)	50.12 ± 2.4	90.56 ± 1.6	<0.001
Ash (g/Kg DM)	92.45 ± 1.6	42.54 ± 0.7	<0.001
CP (g/Kg DM)	219.45 ± 3.1	110.30 ± 1.4	<0.001
NH_3-N (g/Kg TN)	6.80 ± 0.4	1.02 ± 0.01	<0.001
pH	6.32 ± 0.4	6.45 ± 0.3	0.91
IVTD (%)	73.45 ± 2.4	52.75 ± 1.7	<0.001
Lactic acid (g/Kg DM)	3.10 ± 1.1	5.3 ± 1.2	0.051
Butyric acid (g/Kg DM)	0.00 ± 0.0	0.00 ± 0.0	01.00
Buffering capacity (mEq/kg DM)	26.80 ± 1.3	12.60 ± 0.7	<0.001

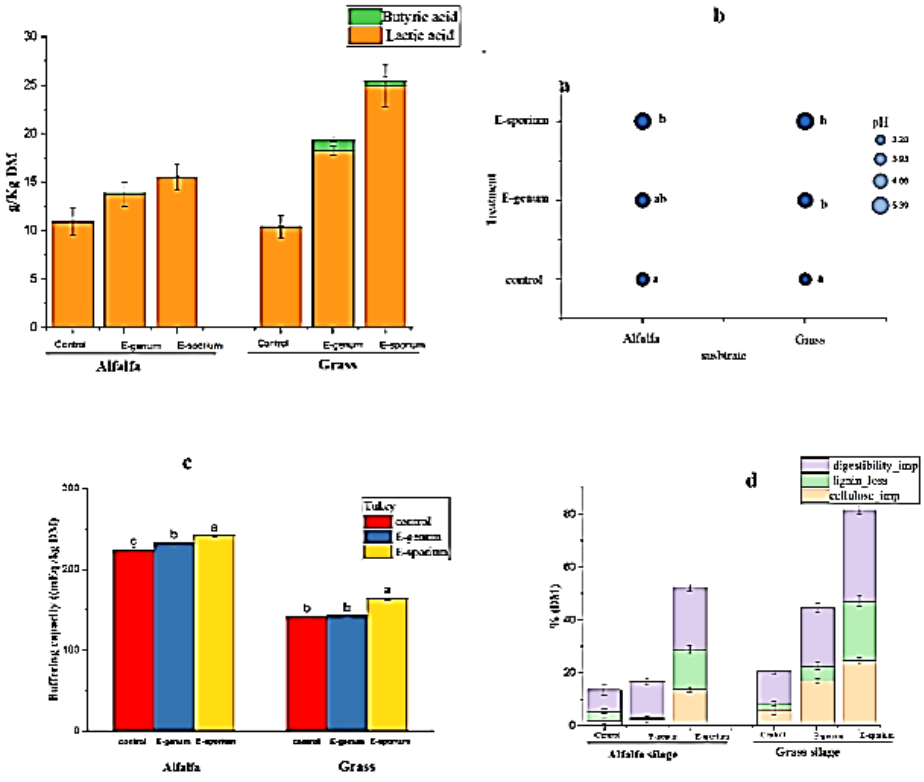

Fig 1: Effect of *P. chrysosporium* and *P. chrysogenum* enzymatic extract treatments on lactic acid and butyric acid (a), pH (b), buffering capacity (c), fiber and digestibility change (d) of treated and untreated alfalfa and Bermuda grass after ensiling for 90 days. Error bars represent standard deviations

treatments, E-sporium resulted in greater increases in cellulose, greater degradation of lignin, and greater improvement in digestibility compared with E-*genum.*

Variables correlation

Correlations were observed among different studied variables

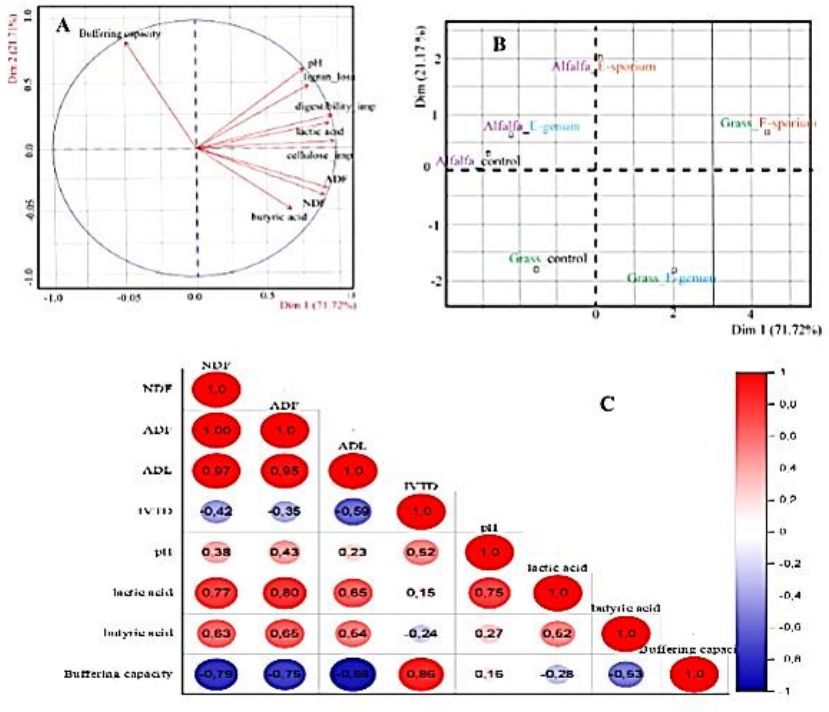

Fig 2: Principal Component Analysis (PCA) (A), qualitative factor map (B), and Pearson correlation matrix (C)

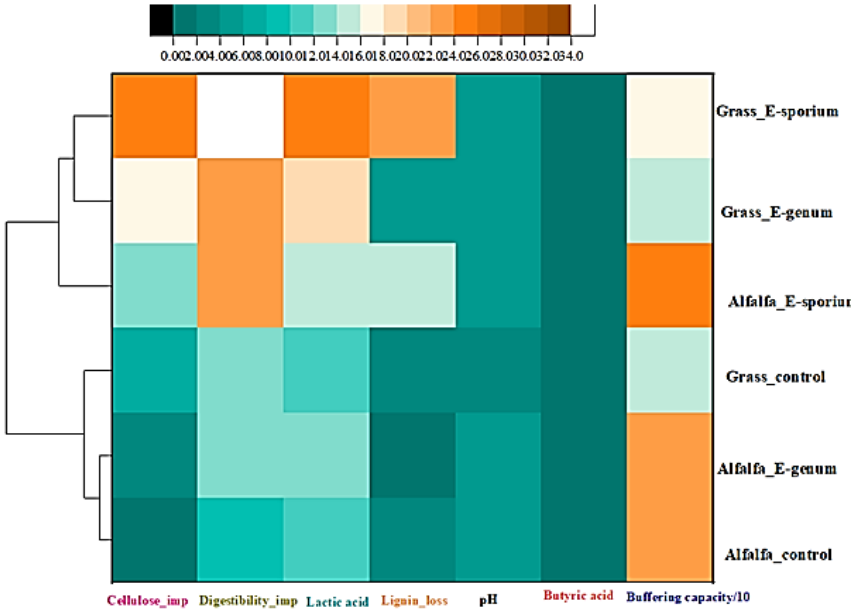

Fig 3: Pearson correlation heatmap for illustrating the relationships between ensiling parameter and fiber change (lignin_loss, cellulose improvement (cellulose_imp)), *in vitro* true digestibility improvement (Digestibility_imp), treatment with enzymatic extract from *P. chrysogenum* (E-genum) of alfalfa silage (alfa_E-genum) and Bermuda grass silage (Grass_E-genum), treatment with enzymatic extract from *P. chrysosporium* (E-sporium) of alfalfa silage (Alfalfa_E-sporium) and Bermuda grass silage (Grass_E-sporium), and their control (Alfalfa-control, Grass-control). Heatmap colors denote the Pearson correlation coefficient

(Fig. 2,3). Notable correlations were particularly observed for the couples: pH vs lactic acid (r=0.75), lactic acid vs ADF (r=0.8), and buffering capacity vs IVTD (r=0.86).

Discussion

The objective of this study was to enhance the fermentation

quality of both Alfalfa and Bermuda grass, both of which are considered challenging for ensiling (Desta *et al.* 2016; Wang *et al.* 2021). pH and organic acids, especially lactic acid (LA) and butyric acid (BA) are crucial indicators for determining the fermentation quality of silage.

The results of this study indicated that the addition of enzyme extract significantly ($p<0.05$) improved the fermentation quality by decreasing the pH and BA and increasing LA, indicating successful preservation of the forage. The reduction in pH value in the additive-treated silages corresponds well to the increase in lactic acid production because the final pH of silage mainly depends on the concentration of lactic acid (Bao *et al.* 2023). Lactic acid is known to have stronger acidity compared with other major acids such as acetic (pK_a of 4.75) and propionic acids (pK_a of 4.87) found in silages (Kung *et al.* 2018; Bao *et al.* 2023). According to Bao *et al.* (2023), alfalfa stems silage treated with laccase had a pH of 4.7, which was higher than the pH of alfalfa silage treated with E-*genum* (pH 4.3) in the present study, but lower than alfalfa silage treated with E-*sporium* (pH 4.84). Additionally, Rinne *et al.* (2020) reported that the pH of Bermuda grass treated with a fibrolytic enzyme containing cellulase and hemicellulase was 4.24, which was lower than the pH values in the current study after treatment with E-*genum* (pH 4.75) and E-*sporium* (pH 5.14). While treatment with enzyme extracts has contributed to the acidification of the silage, the extent of pH reduction remains within a favorable range (4.31–5.14), thanks to the increased buffering capacity of the silage (Fig. 1c). Both extracts demonstrated an increase in buffering capacity, which aligns with previous studies (Norris 1980; Tassone *et al.* 2019; Dong *et al.* 2022; Arwenyo *et al.* 2023; Tian *et al.* 2023). Maintaining a pH that is not excessively low is crucial because overly acidic conditions can hinder the growth of beneficial lactic acid bacteria responsible for the desired fermentation process. Such inhibition could lead to the proliferation of undesirable microorganisms, as observed in alfalfa treated with E-*genum*, where the production of butyric acid increased, ultimately resulting in a decline in the silage quality. Striking the right balance in pH levels during ensilage is essential to promote the growth of beneficial bacteria while suppressing the activity of undesirable microorganisms, ensuring the preservation of high-quality silage for optimal animal nutrition (Li *et al.* 2020). Alfalfa was less responsive to treatment than grass, probably because of the former's high initial buffering capacity (Fig. 1c).

The use of enzyme extract, especially E-sporium, has demonstrated a significant reduction in butyric acid levels. This reduction strongly suggests a significant decrease in the presence of undesirable microbes during the ensiling process (Hristov *et al.* 2020; Chen *et al.* 2021; Aloba *et al.* 2022; Sadhasivam *et al.* 2022; Sun *et al.* 2022; Wang *et al.* 2022).

Our results highlight the potential of enzymatic treatment, especially with E-sporium, as an effective alternative to traditional bacterial inoculation for improving silage production and preservation.

Improving silage nutritional value was one of the objectives of this study. The outcomes demonstrated that enzymatic treatment significantly ($p<0.05$) contributed to improving the nutritional value of Bermuda grass and alfalfa silage by reducing lignin content, increasing cellulose content, and enhancing digestibility (Fig. 1d). While High-quality alfalfa and grass silages are difficult to produce due to their high buffering capacity (Fig. 1c) and low WSC and DM concentrations (Tao *et al.* 2021; Wang *et al.* 2021), the enzymatic treatment overcame this challenge. Enzymes extracts possess properties such as cellulase and ligninase (Table 2), which enable them to directly hydrolyze lignocellulosic contents during ensiling (Dehghani *et al.* 2012).

Lignin has been identified as responsible for limiting digestibility (r (ADL vs IVTD) = -0.56) (Fig. 2c) and used as a marker in digestibility studies (Diouri and Wiedmeier 2000; Kanani *et al.* 2014). The observed high lignin loss of silage treated with E-*sporium* may be explained by the high LiP activity of E-*sporium* (Table 2). The presence of MnP in E-*genum* extract did not have a high impact on lignin, either because of its low concentration or because of some inhibitors that are secreted during ensiling (Franco *et al.* 2018; Luo *et al.* 2023). However, this extract was more promising on grass than on alfalfa.

Regarding cellulose content, E-*sporium* treatment resulted in an increased percentage of cellulose both in Bermuda grass and alfalfa silage, like the treatment of wheat straw with fungi during solid-state fermentation over 12 weeks (van Kuijk *et al.* 2016). The same effect was observed for Bermuda grass treated with E-*genum* extract. This outcome suggests that the enzymatic activities of both extracts likely facilitated the breakdown of various cell components other than cellulose. As these components were removed, the proportion of cellulose in the silage increased. This cellulose will be available for the rumen bacteria during digestion (Benaddou *et al.* 2023a, b), especially after the breakdown of the lignin barrier.

The enzymatic treatments, especially with E-*sporium*, exhibited a noteworthy increase in digestibility compared to the control treatment. Therefore, lignin breakdown not only optimized the efficiency of the silage preservation process, as highlighted in previous studies (Nolan *et al.* 2018) but also directly elevated the nutritional value of the silage.

The enzymatic treatments had a more pronounced positive effect on the digestibility of Bermuda grass as compared to alfalfa. This difference may be attributed to more than one factor. Firstly, the enzymatic treatments effectively reduced more lignin in Bermuda grass silage, as previously mentioned. Lignin is a known barrier to nutrient accessibility and microbial degradation in plant cell walls, so its reduction likely increased the availability of other nutrients in Bermuda grass for microbial fermentation and digestion in the rumen (Benaddou *et al.* 2023a, b). in alfalfa,

even less lignin was degraded with E-genum than in the control. Furthermore, the increased cellulose content in the treated Bermuda grass likely contributed to the improved digestibility. This may mean that the extracts attacked less cellulose in grass than in alfalfa, because of the different fiber profiles in the two substrates. The combined effects of lignin degradation and cellulose increase made Bermuda grass cell walls more susceptible to microbial degradation and nutrient release, resulting in a higher digestibility.

In this study, the pH and nutrient digestibility data revealed a moderate positive correlation (r=0.52) between pH and IVTD. The pH levels of silages observed ranged from 5.14 to 3.61, and this correlation suggests that higher pH values within this range are associated with improved nutrient digestibility in the ensiled material. This finding is consistent with the work of Yan *et al.* (2022) and Fazzino *et al.* (2021), who reported similar positive correlations between pH and nutrient digestibility in their studies. The alignment of these results across studies suggests that maintaining an optimal pH level within the observed range during ensiling is crucial for enhancing the availability of nutrients in silage, making it more suitable for livestock consumption (Yang *et al.* 2006).

The relationship between pH and lactic acid production is a noteworthy aspect of our study. When considering all samples, including fresh substrates, a negative correlation between pH and lactic acid was observed, a finding consistent with the results of Bao *et al.* (2023). However, when focusing solely on treated and ensiled substrates, a positive correlation (r=0.75) emerged between pH and lactic acid concentration. Within our pH range, higher values are associated with increased lactic acid production. Interestingly, this correlation challenges the previous notion that LAB thrives in low pH conditions. Extremely high acidity can inhibit their growth and activity. Our study suggests that acidity levels beyond a certain threshold may surpass the bacteria's tolerance levels, impairing their ability to effectively ferment sugars and produce lactic acid (Hartinger *et al.* 2019).

In our study, a notable positive correlation between lactic acid and both ADL (r=0.54) and ADF (r=0.8) was uncovered. The lactic acid concentrations observed ranged from 10.88 to 24.88 g/kg DM, indicating that the production of lactic acid is influenced by the presence of plant cell wall-less digestible components falling within this range. These data align with the findings of previous research (Hristov *et al.* 2020; Bao *et al.* 2023), where positive correlations between lactic acid and plant cell wall components were reported. The consistent presence of these correlations across multiple studies underscores the significant role of lactic acid bacteria during the fermentation process.

The results regarding buffering capacity and nutrient digestibility showed a strong positive correlation (r=0.86) between buffering capacity and IVTD. The buffering capacity levels measured ranged from 142 to 242 mEq/kg DM, indicating that silages with higher buffering capacity within this range tend to have improved nutrient digestibility. This finding is in line with several studies (Playne and McDonald 1966; Dong *et al.* 2022; Arwenyo *et al.* 2023; Tian *et al.* 2023) who reported similar positive correlations between buffering capacity and nutrient digestibility. The convergence of these results within the observed range emphasizes the importance of buffering capacity in maintaining a stable pH environment during fermentation, thereby enhancing nutrient preservation and availability in the silage.

Pearson correlation heat map (Fig. 3) demonstrated the classification of enzymatic treatment results on silage. The success of different combinations of substrate and enzymatic extract treatment was characterized by specific changes in lactic acid concentration, pH levels, buffering capacity, as well as lignin_loss, cellulose_imp, and digestibility_imp. A successful combination of silage and enzymatic extract treatment was indicated by an increase in lactic acid concentration, reflecting enhanced fermentation and conversion of sugars into lactic acid by the action of LAB. Additionally, there was an increase in pH levels within the range of 3 to 4, which suggested the establishment of an optimal acidic environment for preserving the silage. Moreover, an increase in buffering capacity was observed, indicating the ability of the silage to resist changes in pH during fermentation. This finding highlighted the importance of stable pH conditions in preserving the nutritional integrity of the silage.

Furthermore, there was an increase in lignin_loss, cellulose_imp, and digestibility_imp, which are indicative of the breakdown of plant cell wall components and improved nutrient availability in the ensiled material. This suggested that the enzymatic extract treatment facilitated the degradation of complex carbohydrates, releasing nutrients and increasing their digestibility.

Based on these correlations, the treatment of Bermuda grass with E-*sporium* was identified as the most successful combination, displaying favorable changes in lactic acid concentration, pH levels, buffering capacity, and nutrient digestibility. The closely following combinations were Bermuda grass treated with E-*genum* and alfalfa treated with E-*sporium*.

Conclusion

The results highlight the significant impact of enzymatic treatments using lignolytic fungi extracts on the fermentation quality and nutritional value of ensiled crops (alfalfa and Bermuda grass), presenting a promising alternative to conventional methods in animal feed preparation. Particularly, extracts from *P. chrysogenum* and *P. chrysosporium* demonstrated efficient lignin breakdown, increased cellulose content, and elevated lactic acid production, collectively enhancing forage digestibility and nutrient availability. These effects were more pronounced with *P. chrysosporium* extract and on Bermuda grass. This

innovative, eco-friendly approach not only provides a dynamic solution aligned with sustainable agricultural practices but also opens new possibilities for more efficient feed production, reduces crop wastage, and improves animal performance. Further research and development in this field could help unlock the full potential of enzymatic treatments in modern agriculture, offering a brighter and more sustainable future for both crop preservation and livestock nutrition.

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Author Contributions

MB, HH, and MD conceptualized the experiments. MB, FM, MJ, and SA collected and curated the data. AB, AB, MD, HH, and MB interpreted the results and statistically analyzed the data. MJ, SA, MD, and MB made the write up.

Conflicts of Interest

All other authors declare no conflicts of interest.

Data Availability

Not applicable.

Ethics Approval

Not applicable

References

Alkarkhi AFM, WAA Alqaraghuli (2020). R Statistical Software. In: *Applied Statistics for Environmental Science with R*, pp:11–27. Elseveir, Amsterdam, The Netherlands

Aloba TA, EE Corea, M Mendoza, U Dickhoefer, J Castro-Montoya (2022). Effects of ensiling length and storage temperature on the nutritive value and fibre-bound protein of three tropical legumes ensiled alone or combined with sorghum. *Anim Feed Sci Technol* 283:115172

Arwenyo B, JJ Varco, A Dygert, S Brown, CU Pittman, T Mlsna (2023). Contribution of modified P-enriched biochar on pH buffering capacity of acidic soil. *J Environ Manage* 339:117863

Bao X, H Feng, G Guo, W Huo, Q Li, Q Xu, Q Liu, C Wang, L Chen (2022). Effects of laccase and lactic acid bacteria on the fermentation quality, nutrient composition, enzymatic hydrolysis, and bacterial community of alfalfa silage. *Front Microbiol* 13:1035942

Bao X, G Guo, W Huo, Q Li, Q Xu, L Chen (2023). Ensiling pretreatment fortified with laccase and microbial inoculants enhances biomass preservation and bioethanol production of alfalfa stems. *Sci Total Environ* 857:159442

Benaddou M, H Hajjaj, M Diouri (2023a). Eco-friendly utilisation of agricultural coproducts – enhancing ruminant feed digestibility through synergistic fungal co-inoculation with *Fusarium solani*, *Fusarium oxysporum*, and *Penicillium chrysogenum*. *Ecol Eng Environ Technol* 24:120–32

Benaddou M, H Hajjaj, M Diouri (2023b). Fungal treatment and wheat straw blend for enhanced animal feed from olive pulp. *J Ecol Eng* 24:187–200

Chen L, X Bao, G Guo, W Huo, Q Xu, C Wang, Q Liu (2021). Treatment of alfalfa silage with tannin acid at different levels modulates ensiling characteristics, methane mitigation, ruminal fermentation patterns and microbiota. *Anim Feed Sci Technol* 278:114997

Dehghani MR, MR Weisbjerg, T Hvelplund, NB Kristensen (2012). Effect of enzyme addition to forage at ensiling on silage chemical composition and ndf degradation characteristics. *Livest Sci* 150:51–58

Desta ST, XJ Yuan, J Li, T Shao (2016). Ensiling characteristics, structural and nonstructural carbohydrate composition and enzymatic digestibility of napier grass ensiled with additives. *Bioresour Technol* 221:447–454

Diouri M, RD Wiedmeier (2000). Nutritional metabolism of hydrogen peroxide/anhydrous ammonia treated barley straw in ewe lambs. *Ann Zootech* 49:329–333

Dong Z, J Li, S Wang, J Zhao, D Dong, T Shao (2022). Characterization of bacterial community and fermentation parameters during ensiling of napier grasses harvested at various times within a day. *Anim Feed Sci Technol* 291:115380

Fabiszewska AU, KJ Zielińska, B Wróbel (2019). Trends in designing microbial silage quality by biotechnological methods using lactic acid bacteria inoculants: A minireview. *World J Microbiol Biotechnol* 35:76

Fazzino F, F Mauriello, E Paone, R Sidari, PS Calabrò (2021). Integral valorization of orange peel waste through optimized ensiling: Lactic acid and bioethanol production. *Chemosphere* 271:129602

Fernandes CG, SC Sawant, TA Mule, VS Khadye, AA Odaneth (2023). Synergistic β-glucosidases for improving cellulases recyclability and biomass enzymatic saccharification in wheat straw. *Biomass Bioenergy* 175:106881

Franco RT, P Buffière, R Bayard (2018). Co-ensiling of cattle manure before biogas production: Effects of fermentation stimulants and inhibitors on biomass and methane preservation. *Renewable Energy* 121:315–323

Ghose TK (1987). Measurement of cellulase activities. *Pure Appl Chem* 59:257–68

Gulecyuz E (2017). Effects of some additives on *in vitro* true digestibility of wheat and soybean straw pellets. *Open Life Sci* 12:206–213

Guo L, D Yao, D Li, Y Lin, S Bureenok, K Ni, F Yang (2020). Effects of lactic acid bacteria isolated from rumen fluid and feces of dairy cows on fermentation quality, microbial community, and *in vitro* digestibility of alfalfa silage. *Front Microbiol* 10:2998

Hansen GH, M Lübeck, JC Frisvad, PS Lübeck, B Andersen (2015). Production of cellulolytic enzymes from ascomycetes: Comparison of solid state and submerged fermentation. *Proc Biochem* 50:1327–1341

Hartinger T, N Gresner, KH Südekum (2019). *In vitro* ruminal fermentation characteristics of alfalfa silages in response to different pre-ensiling treatments. *Anim Feed Sci Technol* 258:114306

Hermosilla E, O Rubilar, H Schalchli, AS Ana da Silva, V Ferreira-Leitao, MC Diez (2018). Sequential white-rot and brown-rot fungal pretreatment of wheat straw as a promising alternative for complementary mild treatments. *Waste Manage* 79:240–250

Hristov AN, MT Harper, G Roth, C Canale, P Huhtanen, TL Richard, K DiMarco (2020). Effects of ensiling time on corn silage neutral detergent fiber degradability and relationship between laboratory fiber analyses and *in vivo* digestibility. *J Dairy Sci* 103:2333–2346

Ju J, G Zhang, M Xiao, C Dong, R Zhang, L Du, Y Zheng, M Wei, M Wei, B Wu (2023). Effects of cellulase and *Lactiplantibacillus plantarum* on the fermentation quality, microbial diversity, gene function prediction, and *in vitro* rumen fermentation parameters of *Caragana korshinskii* silage. *Front Food Sci Technol* 2:1108043

Kanani J, D Philipp, KP Coffey, EB Kegley, CP West, S Gadberry, J Jennings, AN Young, RT Rhein (2014). comparison of acid-detergent lignin, insoluble ash as internal markers for predicting fecal output and digestibility by cattle offered bermudagrass hays of varying nutrient composition. *J Anim Sci Biotechnol* 5:7

Karpe AV, DJ Beale, NB Godhani, PD Morrison, IH Harding, EA Palombo (2015). Untargeted metabolic profiling of winery-derived biomass waste degradation by *Penicillium chrysogenum*. *J Agric Food Chem* 63:10696–10704

van Kuijk, JA Sandra, ASM Sonnenberg, JJP Baars, WH Hendriks, JW Cone (2016). The effect of particle size and amount of inoculum on fungal treatment of wheat straw and wood chips. *J Anim Sci Biotechnol* 7:39

Kung L, RD Shaver, RJ Grant, RJ Schmidt (2018). Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *J Dairy Sci* 101:4020–4033

De León-Medina, C José, JJ Buenrostro-Figueroa, L Sepúlveda, CN Aguilar, JA Ascacio-Valdés (2023). Fungal biodegradation of ellagitannins extracted from rambutan peel. *Food Bioprod Proc* 141:81–90

Li F, W Ke, Z Ding, J Bai, Y Zhang, D Xu, Z Li, X Guo (2020). Pretreatment of Pennisetum sinese silages with ferulic acid esterase-producing lactic acid bacteria and cellulase at two dry matter contents: Fermentation characteristics, carbohydrates composition and enzymatic saccharification. *Bioresour Technol* 295

Liu S, X Li, S Wu, J He, C Pang, Y Deng, R Dong (2014). Fungal pretreatment by *Phanerochaete chrysosporium* for enhancement of biogas production from corn stover silage. *Appl Biochem Biotechnol* 174:1907–1918

Luo X, Y Liu, L Lei, J Shen, Q Zhang, Y Wang, R Ruan, X Cui (2023). Co-ensiling of rice straw and distillers grains to increase methane production and maximise energy output. *Bioresour Technol* 386:129496

van der M, JA Julian, EA Landis, GT Deans, RA Lai, K Chandran (2023). Synergistic lignin degradation between *Phanerochaete chrysosporium* and Fenton chemistry is mediated through iron cycling and ligninolytic enzyme induction. *Sci Total Environ* 905:166767

Mgamat F, M Benaddou, S Haida, A Oubihi, HE Ibaoui, R Zouhair, A Kribii, RE Ayadi, D Hmouni (2023). *Phytochemical Evaluation, Antioxidant, and Antibacterial Activity of Extracts of Ammi visnaga* Lam. *Chem Data Collect* 48:101096

Nolan P, EM Doyle, J Grant, P O'Kiely (2018). Upgrading grass biomass during ensiling with contrasting fibrolytic enzyme additives for enhanced methane production. *Renewable Energy* 115:462–473

Norris DM (1980). Degradation of C-labeled lignins and C-labeled aromatic acids by *Fusarium solani*. *Appl Environ Microbiol* 40:376–380

Playne MJ, P McDonald (1966). The buffering constituents of herbage and of silage. *J Sci Food Agric* 17:264–268

Rinne M, E Winquist, V Pihlajaniemi, P Niemi, A Seppälä, M Siika-aho (2020). Fibrolytic enzyme treatment prior to ensiling increased press-juice and crude protein yield from grass silage. *Bioresour Technol* 299:122572

Rodrigues MAM, P Pinto, RMF Bezerra, AA Dias, CVM Guedes, VMG Cardoso, JW Cone, LMM Ferreira, J Colaço, CA Sequeira (2008). Effect of enzyme extracts isolated from white-rot fungi on chemical composition and *in vitro* digestibility of wheat straw. *Anim Feed Sci Technol* 141:326–338

Sadhasivam S, R Marshi, O Barda, V Zakin, M Britzi, A Gamliel, E Sionov (2022). Ensiling process and pomegranate peel extract as a natural additive in potential prevention of fungal and mycotoxin contamination in silage. *Toxicol Rep* 9:1557–1565

Van Soest PJ, JB Robertson, BA Lewis (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* 74:3583–3597

Srinivasan C, TM D'Souza, K Boominathan, CA Reddy (1995). Demonstration of laccase in the white rot basidiomycete *Phanerochaete chrysosporium* BKM-F1767. *Appl Environ Microbiol* 61:4274–4277

Sun H, R Li, E Wang, J Guo, Y Zhou, R Dong (2022). Coupling biorefinery and biogas production from maize stover by enhancing the ensiling process: Role of the carbon/nitrogen ratio and buffer capacity. *J Cleaner Prod* 339:130770

Tao X, S Chen, J Zhao, S Wang, J Li, F Sun, T Shao (2021). Fermentation and aerobic stability of napier grass silage treated with different levels of citric acid residue. *Grassl Sci* 67:139–147

Tao X, S Wang, J Zhao, Z Dong, J Li, Q Liu, F Sun, T Shao (2020). Effect of ensiling alfalfa with citric acid residue on fermentation quality and aerobic stability. *Anim Feed Sci Technol* 269:114622

Tassone S, M Renna, S Barbera, E Valle, R Fortina (2019). *In vitro* digestibility measurement of feedstuffs in donkeys using the daisy ii incubator. *J Equine Vet Sci* 75:122–126

Tian J, L Huang, R Tian, J Wu, R Tang, J Zhang (2023). Fermentation quality and bacterial community of delayed filling stylo silage in response to inoculating lactic acid bacteria strains and inoculating time. *Chem Biol Technol Agric* 10:44

Wang M, M Chen, J Bai, J Zhang, R Su, M Franco, Z Ding, X Zhang, Y Zhang, X Guo (2022). Ensiling characteristics, *in vitro* rumen fermentation profile, methane emission and archaeal and protozoal community of silage prepared with alfalfa, sainfoin and their mixture. *Anim Feed Sci Technol* 284:115154

Wang M, R Gao, M Franco, DB Hannaway, W Ke, Z Ding, Z Yu, X Guo (2021). Effect of mixing alfalfa with whole-plant corn in different proportions on fermentation characteristics and bacterial community of silage. *Agriculture* 11:174

Wang, Z Yu (2019). Fermentation dynamics and bacterial diversity of mixed lucerne and sweet corn stalk silage ensiled at six ratios. *Grass Forage Sci* 74:264–273

Yan J, Y Sun, Y Kang, X Meng, H Zhang, Y Cai, W Zhu, X Yuan, Z Cui (2022). An innovative strategy to enhance the ensiling quality and methane production of excessively wilted wheat straw: Using acetic acid or hetero-fermentative lactic acid bacterial community as additives. *Waste Manage* 149:11–20

Yang HY, XF Wang, JB Liu, LJ Gao, M Ishii, Y Igarashi, ZJ Cui (2006). Effects of water-soluble carbohydrate content on silage fermentation of wheat straw. *J Biosci Bioeng* 101:232–237