STATE UNIVERSITY OF MARINGA
AGRICULTURAL SCIENCES CENTER

EFFECTS OF LIGNOCELLULOLYTIC ENZYMES PRODUCED BY *PLEUROTUS OSTREATUS* ON THE NUTRITIVE VALUE OF WHOLE-PLANT CORN SILAGE

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State of Parana
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TITULAÇÃO: Doutora em Zootecnia - Área de Concentração Produção Animal

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“The size of your dreams must always exceed your current capacity to achieve them.
If your dreams do not scare you, they are not big enough.”
Ellen Johnson Sirleaf

To my mother, Marta Calvo,

who always support me and believe in my dreams.

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BIOGRAFY

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In December of 2020, she submitted her Ph.D. dissertation.

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ABSTRACT

Three experiments were carried out to examine the effect of lignocellulolytic enzymes on the nutritive value of whole-plant corn silage (WPCS). In **experiment 1,** we examined the effect of spent substrate from *Pleurotus* ostreatus cultivation (SSPO) on the chemical composition, antioxidant capacity, lignin monomers, and *in vitro* digestibility of WPCS. In **experiment 2,** we evaluated the performance of lactating goats fed WPCS treated with different levels of SSPO. In **experiment 3,** we verified the activity of an enzymatic complex at different pH and the effects of adding increasing levels of that enzymatic complex produced by *Pleurotus ostreatus* on the fermentative profile, chemical composition, and ruminal digestibility along of the days on the onset of fermentation of WPCS. In **experiment 1**, four levels of lignocellulolytic enzymes from spent substrate of *Pleurotus ostreatus* were tested in a completely randomized design: 0, 10, 20, and 30 mg of lignocellulosic enzymes/kg of fresh matter, and four replicates per treatment (vacuum-sealed bags). The bags were opened 60 d after ensiling. The NDF, ADF, lignin, and cellulose concentration decreased quadratically. At the nadir point, SSPO decreased NDF by 14.1%, ADF by 19.5%, lignin by 9.07%, and cellulose by 22.1% compared to the untreated silage. Therefore, SSPO led to a quadratic increase in IVDMD of WPCS (+10.3 % at vertex). In **experiment 2**, WPCS treated with three enzyme levels (0, 10, or 30 mg/kg fresh matter, chosen based on experiment 1) were fed to lactating goats as part of TMR. Nine lactating Saanen goats (62.68±7.62 kg BW; 44±8 days in milk; 2.91±0.81 kg of milk/day, mean±SD) were assigned to three 3 × 3 Latin squares. Intake and digestibility of dry matter and nutrients, microbial protein syntheses, and milk production and composition were examined. The SSPO increase

the *in vivo* total-tract ADF digestibility quadratically. Additionally, the concentration of polyphenols in milk increased linearly with the addition of SSPO in WPCS; however, no other differences were detected among treatments. In **experiment 3**, the lignocellulolytic enzymatic complex was obtained through *in vitro* cultivation of *Pleurotus ostreatus*, and the activities of laccase, lignin peroxidase, manganese peroxidase, endo and exoglucanase, xylanase, and mannanase were determined at pH 3, 4, 5, and 6. Following, five different enzymatic complex levels were tested in a completely randomized block design: 0; 9; 18; 27, and 36 mg of lignocellulosic enzymes/kg of fresh matter (FM) of whole-plant corn, with four replicates per treatment (vacuum-sealed bags). The bags were opened after 1, 2, 3, and 7 d of ensiling to evaluate the onset of fermentation and after 30 d of storage to evaluate the fermentation, chemical composition, and *in situ* digestibility of WPCS. Laccase showed highest activity at pH 5 ($P < 0.01$), whereas manganese peroxidase and lignin peroxidase had a higher activity at pH 4 ($P < 0.01$;< 0.01, respectively). There was no interaction between the enzymatic complex and days of fermentation ($P > 0.11$). The concentration of WSC decreased quadratically at the onset of fermentation ($P = 0.02$) due to its consumption that led to a quadratic increase of lactic acid ($P = 0.01$) and a linear increase of acetic acid ($P = 0.02$). As a result of increasing those organic acid concentrations, pH decreased quadratically ($P = 0.01$). Lignin concentration decreased linearly ($P = 0.04$) with the enzymatic complex at 30 d of storage. The collective interpretation of these results leads to the conclusion that 10 mg of lignocellulolytic enzymes from SSPO per kg of FM of WPC presented the best effect in silage production due to more evident reduction in NDF, ADF, and lignin concentration and increased ADF digestibility of lactation goats.

Keywords: fiber, fibrolytic enzyme, laccase, lignin, white-rot fungi

I. INTRODUCTION

Fiber is one of the most predominant fractions that constitute ruminant diets (Van Soest, 1994). Its concentration can vary according to the ingredients that compose the diet, and its main function is related to provide effective physical capacity and energy through its digestion (NRC, 2001).

However, the extension of fiber digestion has a huge variation between the forage sources, and it is strongly related to three factors: total cell wall content, variation in structural arrangement associated with plant anatomy, and cell wall composition (Wilson, 1994), as reviewed below.

1. Literature Review

1.1. Plant cell wall

Plant cell walls are complex structures vital to plant survival, providing structural integrity and flexibility (Houston et al., 2016) and defense against pathogens and stress as a physical barrier (Underwood, 2012). The type of polysaccharides in the cell wall varies according to plant species, location in the wall (primary or secondary), plant tissue, maturity, and environment (Doblin et al., 2010).

Plant cell walls are divided into primary and secondary walls and middle lamella (Figure 1). The primary wall consists of a cellulosic network encapsulated in a matrix of other polysaccharides, such as xylan and pectins (O'Neill and York, 2003). Polysaccharides (cellulose and hemicellulose) represent approximately 90% of the primary wall, whereas structural glycoproteins (2-10%), phenolic esters (<2%), and minerals (1-5%) comprise a minor proportion (O'Neill and York, 2003).

The secondary wall contains celluloses, hemicelluloses (xylan and glucomannan), and lignin, with a low proportion of structural protein amount. Three layers compose the secondary wall (S1, S2, and S3). In S1, the cellulose microfibrils present crossed in each other; in the S2, they are oriented nearly parallel to the cell elongation axis; in the S3, they are oriented in a flat helix (Zhong et al., 2019). Between cell walls are present the middle lamella, which cements two cells together and is constituted of pectin (Srivastava, 2001).

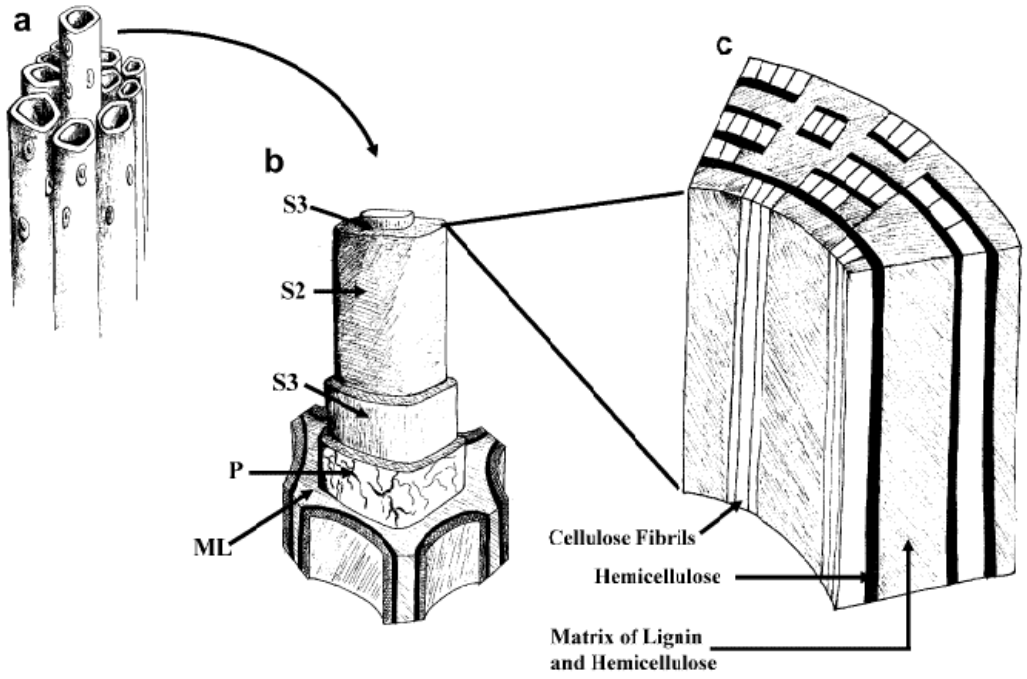

Figure 1. Configuration of the plant cell wall. a) adjacent cells, b) cell wall layers, c) distribution of lignin and polysaccharides (from Pérez et al., 2002, adapted from Kirk and Cullen (1998)).
Abbreviation: ML: middle lamella; P: primary cell wall; S3, S2, and S3: layers in the secondary cell wall.

Cellulose is the most abundant carbohydrate in the cell wall, and it is formed by glucose molecules linked by β-1,4 bonds, whose chain length can present variation between 100 and 14000 residues (Béguin and Unitd, 1994). The chains are highly ordered, presenting a crystalline structure with amorphous regions. The degree of crystallinity varies according to the material, between 0% and 100% (Béguin and Unitd, 1994), and the crystallinity degree of cellulose is associated with a linear reduction of degradation (Hall et al., 2010).

On the other hand, hemicellulose is a heterogeneous group composed of polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids. Hemicelluloses are strongly associated with lignin in the secondary wall and are classified according to the main sugar residue in the backbone (Wyman et al., 2005). The main residues are xylose, mannose, galactose, glucose, arabinose, and glucuronic acid (Zhou et al., 2017). The linkage usually is β-1,4- and occasionally β-1,3-glycosidic bonds (Pérez et al., 2002). Xylans are the most common hemicellulose group, with the backbone of β-1,4-linkage xylose residues (Scheller and Ulvskov, 2010), and it has a critical function to link lignin to hemicellulose (Wyman et al., 2005). Mannan is another relevant group; its structure presents β-1,4-linkage between mannose residue and backbone. The mannan is divided into glucomannan, galactomannan, and galactoglucomanan, according to the composition of the residues (Ebringerova et al., 2005).

Another important cell wall component is the lignin. Unlike cellulose and hemicellulose, lignin is not a polysaccharide. Lignin is a complex structure formed from oxidative-coupling of monolignols that are *p*-coumaryl, coniferyl, and sinapyl alcohols, where the respective phenylpropanoid units are *p*-hydrophenyl (H), guaiacyl (G), and syringyl (S) (Wong, 2009). Lignin is a heterogeneous group due to the extensive possibility of monolignols combination (Hatakeyama and Hatakeyama, 2010). For instance, grasses present G:S:H ratio, with proportions around 22:44:34, respectively (Van Soest, 1994).

Lignin affects forage digestibility because it acts as a physical barrier. Cell wall digestibility and plant maturity are negatively related since the plant lignification increases with maturity. Its functions are related to the rigidity of the cell wall, reducing water loss, and avoiding plant disease (Moore and Jung, 2001).

1.2. Fiber in ruminant nutrition

Forage is commonly used in ruminants diets, and in general, has high fiber concentration. Fiber plays an essential role in maintaining ruminal function by stimulating chewing and salivation, mat formation, and rumen motility. Therefore, a minimal inclusion of roughage in ruminant diets is vital to avoid acidosis (Allen, 1997).

The minimum required level of physically effective neutral detergent fiber (peNDF) to maintain animal health corresponds to approximately 31.2% peNDF

inclusive of particles >1.18 mm or 18.5% peNDF inclusive of particles >8 mm (Zebeli et al., 2012). The peNDF is provided through forage addition in the diets and is altered by forage proportion or particle length of forages (Yang and Beauchemin, 2007).

On the other hand, fiber is a significant energy source by microbial degradation that produces volatile fatty acids (Tamminga, 1993). Fiber usually is provided to the animal as grass pasture and conserved forage.

1.3.Silage in ruminant nutrition

Forage conservation is the process of preserving the forage to fed the animals posteriorly. It is the key to feed cattle when there is no forage enough due to temperature and rainfall (Gallaher and Pitman, 2000), or the animals stay the most of the life inside the barn (Mayne et al., 2011). Therefore, forage conservation is a technique essential to produce dairy cows and beef cattle efficiently. The two techniques most used in the world to conserve forage are silage and hay. These techniques offer benefits over the rest of the techniques, such as minimizing losses and keeping forage with high quality during a long period, preserving the original feed composition (Muck and Shinners, 2001). Moreover, the ensilage process has the advantage over hay production because it is less dependent on climate conditions (Wilkinson and Rinne, 2017).

Silage is produced on spontaneous fermentation of water-soluble carbohydrates (WSC) in compounds, such as organic acids and alcohols by epiphytic lactic acid bacterias from the plant, under anaerobic conditions (Kung et al., 2017). Organic acids are responsible for decreasing pH, where lactic and acetic acid are those organic acids with higher concentrations in most silages. Lactic acid is the strongest organic acid produced during the fermentation process; consequently, it is the one that contributes the most to decreasing and maintaining acidic pH (Kung et al., 2017). While, acetic acid works to improve aerobic stability because it inhibits molds and yeast growth at opening time, whose silage is exposed to oxygen (Danner et al., 2003).

Corn (*Zea mays L.*) is the most common feedstuff used to produce silage and is highly used as a forage source in dairy diets. Corn has a high yield per area and provides fiber and energy in dairy diets (Grant and Ferraretto, 2018). To produce high-quality corn silage is essential to pay attention to some factors, such as hybrid choice, dry matter control, speed of packing, particle size, covering, and utilization of additives

(Allen et al., 2003). The hybrid choice is the first point to consider in silage production. Among the various types of hybrids, there are conventional or dual-purpose hybrids; silage-specific hybrids, which produce silage with better nutritive value; brown midrib mutant (BMR) hybrids, which present less lignin and a higher fiber digestibility; and leafy hybrids, which produce more leaves above the ear, and consequently more yield per area (Ferraretto and Shaver, 2015). Dry matter content is another crucial factor, and, ideally, the whole-plant corn (WPC) must have approximately 35% of dry matter. When the dry matter is higher than 40%, the material could have a problem during compaction that hampers removing oxygen, leading to heat damage by Maillard Reaction, and spoilage. However, a low dry matter content in the WPC (less than 30%) also can lead to losses of nutrients due to the leaching process that impair the nutritional value (Muck and Shinners, 2001). Packing speed must be done as quickly as possible to avoid losses by fermentation of the water-soluble carbohydrates in aerobic conditions and cell respiration (Moser, 1995). The recommendation to chop length of the WPC must provide enough physically effective fiber to the animal and good silage compaction to avoid problems in the storage process. Regarding silage covering film, it is essential to prevent damage from precipitation, animal attack, sunlight exposure, and the most crucial point, to ensure anaerobiosis conditions (Bernardes, 2016). The utilization of additives at ensiling is a key factor in producing good silage.

The additives can improve corn silage fermentation, avoid nutrient losses, and increase digestibility. Among those additives are inoculants, chemical additives, and enzymes, such as proteolytic, cellulolytic, and hemicellulolytic (Muck et al., 2018). Enzymatic additives is a class of additive which can improve silage composition and digestibility. Different enzymatic additives have been tested and used in silages, including fibrolytic and proteolytic enzymes.

The proteases improve starch digestibility in whole-plant corn silage since they can hydrolyze prolamin in the matrix covering the starch (Young et al., 2012; Der Bedrosian and Kung, 2019). Fibrolytic enzymes aim to break down the fibrous carbohydrates and consequently increase dry matter digestibility. Fibrolytic enzymes usually are used as a mix of cellulase and hemicellulase, and have been applied in combination with bacterial inoculants (Muck et al., 2018).

1.4.Fibrolytic enzymes

Enzymes are complex three-dimensional proteins whose structure comprises amino acids linked via an amide bond (Blanco and Blanco, 2017). Enzyme names are based on the substrate that it acts, followed by the suffix –ase. For instance, cellulase is the name of the enzyme group that degrades cellulose (Blanco and Blanco, 2017). Cellulases hydrolyze β-1,4 linkages in celluloses chains, and they are classified in endoglucanases, exoglucanases, and β-glucosidases (Gupta et al., 2013). Endoglucanases hydrolyze internal cellulose bonds at the amorphous regions in oligomers releasing new terminal ends, while exoglucanases, also known as cellobiohydrolases, hydrolyze long-chain oligosaccharides (Pérez et al., 2002). Exoglucanases are divided into two types, and they both act unidirectionally on the long oligomers chain, one from reducing and the other one from nonreducing ends, releasing cellobiose (Liu and Kokare, 2017), while β-glucosidases release D-glucose from cellobiose and soluble cellodextrins (Wyman et al., 2005).

Hemicellulase is an extensive group of enzymes that hydrolyze hemicellulose, whose main enzymes are xylanase, mannanase, α-arabinofuranosidase (Shallom and Shoham, 2003). Xylanases degrade β-1,4 linkages, converting linear polysaccharide xylan into xylose (Liu and Kokare, 2017), and it is the most studied hemicellulase given that xylan is the predominant hemicellulose (Kormelink and Voragen, 1993). Another important hemicellulose is endo-β-mannanases that release mannose from polysaccharides (Freiesleben et al., 2018).

In general, commercial enzymes are complex and are comprised of a combination of various enzymes. A commonly used enzyme combination is cellulase and hemicellulase (McAllister et al., 2009), which act synergistically to intensify the substrate degradation (Song et al., 2016). Most commercial products are derived from fungi cultivation, mainly from *Trichoderma longibrachiatum*, *Aspergillus niger* and *A. oryzae* (Pendleton 2000, cited in Beauchemin et al., 2004). Although there are many cellulolytic and hemicellulolytic enzymes, there are no commercial enzymes that breakdown lignin in animal nutrition. The development of a commercial enzyme that prioritizes lignin breakdown can become a key method to increasing fiber digestibility.

Enzymes capable of lignin breakdown have the ability to oxidize a huge variety of organic and inorganic compounds (Wong, 2009), which act by cross-linking phenylpropanoid units with the aromatic ring, cleaving aromatic compounds (Liu and Kokare, 2017); this effect has shown to work synergistically (Wong, 2009).

Laccases are multicopper enzymes consisting of four copper atoms per molecule in the catalytic center that are one Cu atom type 1 (T1), one Cu atom type 2 (T2), and two Cu atoms type 3 (T3) (Wong, 2009). In the enzymatic resting form, all atoms of cooper are $Cu^{2+,}$ which corresponds to the oxidated state. The first step of catalysis is reducing the Cu^{2+} T1 to Cu^{1+} as an electron acceptor from the substrate. After this step, the next electrons extracted from the reducing substrate are transferred to T2/T3 site, and in this time, the enzyme is transformed to the fully reduced (Giardina et al., 2010). The cycle of laccase is O_2 dependent, which due to the high molecular mass (~60-90 kDa) (Shekher et al., 2011) and the low redox potential; this enzyme requires a mediator to oxidize non-phenolic compounds (Pollegioni et al., 2015a).

Lignin peroxidase (LiP) and manganese peroxidase (MnP) are also enzymes that break down lignin. These enzymes are dependent on H_2O_2 or another organic peroxide to act as an electron acceptor during catalysis (Pollegioni et al., 2015b). LiP is a glycoprotein with a heme group in the active center, is nonspecific to the substrate, and is known to oxidize phenolic and non-phenolic compounds (Falade et al., 2017). The LiP-I (first active form) is formed from the reaction between LiP and H_2O_2, and it is pH-independent. However, the reaction of LiP-I to oxidate the substrate and form LiP-II is pH-dependent, and it usually decreases when the pH increases. The optimum pH in this part of the cycle is around 3 (Tien and Kirk, 1984). The next reaction where LiP-II is converted to LiP-III (inactive form) is pH-dependent and occurs when there is excess of H_2O_2 (Wong, 2009).

The enzyme MnP catalyzes the oxidation of Mn (II) to Mn (III). This Mg (III) is chelated into organic acids, as oxalate, malonate, and fumarate, and it favors the capacity to penetrate small molecular pores between cellulose microfibrils to breakdown lignin (Makela et al., 2015). The MnP oxidizes a massive variety of monomeric phenols during the Mn oxidation, catalyzing a molecule of H_2O_2 to form MgP-I and the release of H_2O. Following, Mg^{2+} is oxidized, forming MnP-II and Mg^{3+} (Vrsanska et al., 2016). The Mn-P-II is oxidized and produces MnP-III, which in turn oxidizes another Mg^{2+} to Mg^{3+}. In the trivalent state, magnesium can oxidize and break down phenolic compounds in the presence of a second mediator, such as glutathione. Mg^{3+} is highly reactive and subsequently oxidizes non-phenolic substrates, attributing to electron abstraction from aromatic rings (Wong, 2009).

1.5.Fibrolytic enzymes in ruminant nutrition

Ruminants can use fiber as a source of energy due to their mutualism with ruminal microorganisms. Fiber degradation yields volatile fatty acids, the main source of metabolizable energy for ruminants (Bergman, 1990). However, part of the fiber cannot be degraded by the microorganisms due to its recalcitrance, which is caused by its organization as well as the association between lignin and carbohydrates. Thus, some alternatives to increase fiber utilization have been developed, such as mechanical processing, genetic improvement, alkali and acid treatments, bacterial inoculants, and exogenous fibrolytic enzymes (Adesogan et al., 2019).

Exogenous fibrolytic enzymes release sugars and oligomers when applied to the substrate before feeding animals, thus improving de fiber digestibility. Some studies with these types of enzymes in dairy cow nutrition have shown an increase in fiber digestibility and milk production (Arriola et al., 2017). However, the results are inconsistent (Beauchemin et al., 2003) since there were improvements in animal performance in some experiments, while there were no differences between the treatments in other researches. These variations can be attributed to variable factors among the experiments, such as diet chemical composition, doses, pH and temperature, cofactors, animal category, and application methods (Adesogan et al., 2019).

1.6.Pleurotus ostreatus

The white-rot fungi belong to the group basidiomycetous fungi, and it is known to produce enzymes that can hydrolyze cellulose and hemicellulose, and break down lignin (Chandra and Madakka, 2019). *Pleurotus ostreatus* is one of those white-rot fungi species and produces carboxymethylcellulose, xylanase, laccase, manganese peroxidase, and lignin peroxidase (Elisashvili et al., 2003; Membrillo et al., 2008).

Researchers have been developing with *Pleurotus ostreatus* in agriculture wastes, such as rice straw, wheat straw, with the goal to enhance digestibility and viability for animal nutrition. Fazaeli et al. (2004) observed that diets with wheat straw previously treated with *Pleurotus ostreatus* presented greater dry matter digestibility compared to untreated straw. Kholif et al. (2014) observed that rice crop residue treated with *Pleurotus ostreatus* allowed to clover replacement (*Trifolium alexandrinum*) by 50% without reducing the digestibility of neutral fiber detergent.

Recently, Machado et al. (2020) evaluated an enzymatic complex produced by *Pleurotus ostreatus* and used it to treat whole-plant corn and sugarcane at ensiling. They reported a reduction in the concentration of lignin, cellulose, and hemicellulose, as well as greater *in vitro* digestibility, and antioxidant capacity in both corn and sugarcane silages treated with enzyme complex.

Moreover, *Pleurotus ostreatus* is one of the most cultivated fungi to produce mushrooms in the world. The cultivation is performed in lignocellulosic material, as palm heart waste, sugarcane bagasse, and hay. The fungi produce lignocellulolytic enzymes that can degrade cellulose and hemicellulose, leading to the release of glucose (Mikiashvili et al., 2006). After mushroom harvest, the cultivation medium becomes low in nutrients and is named as spent mushroom substrate. The spent mushroom substrate of *Pleurotus ostreatus* (SSPO) has been used to fertilize the soil, fed animals, or burned; however, it presents a high concentration of lignocellulolytic enzymes and can be used as an alternative enzymatic source (Phan and Sabaratnam, 2012). Although the SSPO has the potential to improve the digestibility of feedstuffs through the enzymatic action. Therefore, it has just been tested as a feed and not as an enzyme source to improve the chemical composition of the feeds. Therefore, this dissertation aimed to evaluate the addition of sources of lignocellulolytic enzymes from *Pleurotus ostreatus* (spent substrate and enzymatic complex produced in laboratory) in whole-plant corn at ensiling, and performance of goats fed with silage treated with lignocellulolytic enzymes.

2. Reference

Adesogan, A.T., K.G. Arriola, Y. Jiang, A. Oyebade, E.M. Paula, J.J. Romero, L.F. Ferraretto, and D. Vyas. 2019. Symposium review : Technologies for improving fiber utilization. J. Dairy Sci. 102:5726–5755. doi:10.3168/jds.2018-15334.

Allen, M.S. 1997. Relationship between fermentation acid production in the rumen and the requirement for physically effective fiber. J. Dairy Sci. 80:1447–1462. doi:10.3168/jds.S0022-0302(97)76074-0.

Allen, M.S., J.G. Coors, and G.W. Roth. 2003. Corn Silage. Silage Sci. Technol. 547–608.

Arriola, K.G., A.S. Oliveira, Z.X. Ma, I.J. Lean, M.C. Giurcanu, and A.T. Adesogan. 2017. A meta-analysis on the effect of dietary application of exogenous fibrolytic

335 enzymes on the performance of dairy cows. J. Dairy Sci. 100:4513–4527.
336 doi:10.3168/jds.2016-12103.

337 Beauchemin, K.A., D. Colombatto, and D.P. Morgavi. 2004. A rationale for the
338 development of feed enzyme products for ruminants. Can. J. Anim. Sci. 84:23–36.
339 doi:10.4141/A02-103.

340 Beauchemin, K.A., D. Colombatto, D.P. Morgavi, and W.Z. Yang. 2003. Use of
341 Exogenous Fibrolytic Enzymes to Improve Feed Utilization by Use of Exogenous
342 Fibrolytic Enzymes to Improve. J. Anim. Sci. 81:E37–E47.

343 Der Bedrosian, M.C., and L. Kung. 2019. The effect of various doses of an exogenous
344 acid protease on the fermentation and nutritive value of corn silage. J. Dairy Sci.
345 102:10925–10933. doi:10.3168/jds.2019-16436.

346 Béguin, P., and J.-P.A. Unitd. 1994. The biological degradation of cellulose. FEMS
347 Microbiol. Rev. 13:25–58.

348 Bernardes, T.F. 2016. Advances in silage production and utilization: Advances in silage
349 sealing. BoD-Books on Demand.

350 Blanco, G., and A. Blanco. 2017. Medical biochemistry: Enzymes. Academic Press.

351 Chandra, M.R.G.S., and M. Madakka. 2019. Recent Developments in Applied
352 Microbiology and Biochemistry: Comparative Biochemistry and Kinetics of Microbial
353 Lignocellulolytic Enzymes. Elsevier Inc.

354 Danner, H., M. Holzer, E. Mayrhuber, and R. Braun. 2003. Acetic Acid Increases
355 Stability of Silage under Aerobic Conditions. Appl. Environ. Microbiol. 69:562–567.
356 doi:10.1128/AEM.69.1.562.

357 Doblin, M.S., F. Pettolino, and A. Bacic. 2010. Evans Review : Plant cell walls : the
358 skeleton of the plant world. Funct. Plant Biol. 37:357–381. doi:10.1071/FP09279.

359 Ebringerova, A., Z. Hromadkova, and T. Heinze. 2005. Hemicellulose. In:
360 Polysaccharides I, Structure, characterization and use. Adv Polym Sci. Springer, ed.
361 New York.

362 Elisashvili, V., D. Chichua, E. Kachlishvili, N. Tsiklauri, and T. Khardziani. 2003.
363 Lignocellulolytic Enzyme Activity During Growth and Fruiting of the Edible and
364 Medicinal Mushroom Pleurotus ostreatus (Jacq.:Fr.) Kumm. (Agaricomycetideae). Int.
365 J. Med. Mushrooms 5:6. doi:10.1615/interjmedicmush.v5.i2.80.

366 Falade, A.O., U.U. Nwodo, B.C. Iweriebor, E. Green, L. V. Mabinya, and A.I. Okoh.
367 2017. Lignin peroxidase functionalities and prospective applications. Microbiologyopen
368 6:1–14. doi:10.1002/mbo3.394.

369 Fazaeli, H., H. Mahmodzadeh, Z.A. Jelan, Y. Rouzbehan, J.B. Liang, and A. Azizi.
370 2004. Utilization of fungal treated wheat straw in the diet of late lactating cow. Asian-
371 australasian J. Anim. Sci. 17:467–472. doi:10.5713/ajas.2004.467.

372 Ferraretto, L.F., and R.D. Shaver. 2015. Effects of whole-plant corn silage hybrid type

373 on intake , digestion , ruminal fermentation , and lactation performance by dairy cows
374 through a meta-analysis. J. Dairy Sci. 98:2662–2675. doi:10.3168/jds.2014-9045.

375 Freiesleben, P. Von, N. Spodsberg, A. Stenbæk, H. Stålbrand, K.B.R.M. Krogh, and
376 A.S. Meyer. 2018. Biotechnology for Biofuels Boosting of enzymatic softwood
377 saccharification by fungal GH5 and GH26 endomannanases. Biotechnol. Biofuels 1–14.
378 doi:10.1186/s13068-018-1184-y.

379 Gallaher, R.N., and W.D. Pitman. 2000. Conservation of forages in the tropics and
380 subtropics. 1st editio. C. Press, ed. Boca Raton.

381 Giardina, P., V. Faraco, C. Pezzella, A. Piscitelli, S. Vanhulle, and G. Sannia. 2010.
382 Laccases: A never-ending story. Cell. Mol. Life Sci. 67:369–385. doi:10.1007/s00018-
383 009-0169-1.

384 Grant, R.J., and L.F. Ferraretto. 2018. Silage review: Silage feeding management:
385 Silage characteristics and dairy cow feeding behavior. J. Dairy Sci. 101:4111–4121.
386 doi:10.3168/jds.2017-13729.

387 Gupta, R., G. Mehta, D. Deswal, S. Sharma, K.K. Jain, R.C. Kuhad, and A. Singh.
388 2013. Cellulases and their biotechnological applications. Springer, ed. India.

389 Hall, M., P. Bansal, J.H. Lee, M.J. Realff, and A.S. Bommarius. 2010. Cellulose
390 crystallinity - A key predictor of the enzymatic hydrolysis rate. FEBS J. 277:1571–
391 1582. doi:10.1111/j.1742-4658.2010.07585.x.

392 Hatakeyama, H., and T. Hatakeyama. 2010. Lignin structure, properties, and
393 applications. Adv. Polym. Sci. 232:1–63. doi:10.1007/12_2009_12.

394 Houston, K., M.R. Tucker, J. Chowdhury, N. Shirley, A. Little, and K. Houston. 2016.
395 The Plant Cell Wall : A Complex and Dynamic Structure As Revealed by the Responses
396 of Genes under Stress Conditions 7:1–18. doi:10.3389/fpls.2016.00984.

397 Kholif, A.E., H.M. Khattab, A.Z.M. Salem, and U. Autónoma. 2014. Nutrient
398 digestibility, ruminal fermentation activities, serum parameters and milk production and
399 composition of lactating goats fed diets containing rice straw treated with Pleurotus
400 ostreatus. Asian-Australasian J. Anim. Sci. 27:357–364. doi:10.5713/ajas.2013.13405.

401 Kormelink, F.J.M., and A.G.J. Voragen. 1993. Degradation of different
402 [(glucurono)arabino]xylans by a combination of purified xylan-degrading enzymes.
403 Appl. Microbiol. Biotechnol. 38:688–695. doi:10.1007/BF00182811.

404 Kung, L.J., R.D. Shaver, R.J. Grant, and R.J. Schmidt. 2017. Silage review :
405 Interpretation of chemical , microbial , and organoleptic components of silages. J. Dairy
406 Sci. 101:4020–4033. doi:10.3168/jds.2017-13909.

407 Liu, X., and C. Kokare. 2017. Biotechnology of Microbial Enzymes: Microbial
408 Enzymes of Use in Industry. Academic Press, India.

409 Machado, E., P. Toshimi Pintro Matumoto, L.C. Vinhas, B.C. Agustinho, P. Daniel,
410 N.W. Santos, M. Zeoula, and M. Ribeiro. 2020. Reduction in lignin content and
411 increase in the antioxidant capacity of corn and sugarcane silages treated with an

412 enzymatic complex produced by white rot fungus 1–15.
413 doi:10.1371/journal.pone.0229141.

414 Makela, M.R., M. Marinovic, P. Nousiainen, A.J.M. Liwanag, I. Benoit, J. Sipila, A.
415 Hatakka, R.P. de Vries, and K.S. Hildén. 2015. Aromatic Metabolism of Filamentous
416 Fungi in Relation to the Presence of Aromatic Compounds in Plant Biomass.
417 doi:10.1016/bs.aambs.2014.12.001.

418 Mayne, S., J. McCaughey, and C. Ferris. 2011. Dairy Farm Management Systems: Non-
419 Seasonal, Pasture-Based Milk Production Systems in Western Europe. Encycl. Dairy
420 Sci. Second Ed. 44–51. doi:10.1016/B978-0-12-374407-4.00116-3.

421 McAllister, T.A., A.N. Hristov, K.A. Beauchemin, L.M. Rode, and K.J. Cheng. 2009.
422 Enzymes in ruminant diets.. Enzym. farm Anim. Nutr. 273–298.
423 doi:10.1079/9780851993935.0273.

424 Membrillo, I., Sanchez, C., Meneses, M., Favela, E., Loera, O., 2008. Effect of substrate
425 particle size and additional nitrogen source on production of lignocellulolytic enzymes
426 by Pleurotus ostreatus strains. Bioresour. Technol. 99, 7842–7847.
427 https://doi.org/10.1016/j.biortech.2008.01.083

428 Mikiashvili, N., S.P. Wasser, E. Nevo, and V. Elisashvili. 2006. Effects of carbon and
429 nitrogen sources on Pleurotus ostreatus ligninolytic enzyme activity 999–1002.
430 doi:10.1007/s11274-006-9132-6.

431 Moore, K.J., and H.G. Jung. 2001. Lignin and fiber digestion. J. Range Manag. 54:420–
432 430.

433 Moser, L.E. 1995. Post-harvest physiological changes in forage plants. CSSA Spec.
434 Publ., ed. Madison.

435 Muck, R.E., E.M.G. Nadeau, T.A. Mcallister, M.C. Santos, and L.K. Jr. 2018. Silage
436 review : Recent advances and future uses of silage additives 1. J. Dairy Sci. 101:3980–
437 4000. doi:10.3168/jds.2017-13839.

438 Muck, R.E., and K.J. Shinners. 2001. Conserved forage (silage and hay): progress and
439 priorities. Pages 753–762 in International Grassland Congress.

440 NRC, 2001. Nutrient Requirements of Dairy Cattle, 7th Revised. National Academic
441 Science, Washington, DC, USA.

442 O'Neill, M.A., and W.S. York. 2003. Annual Plant Reviews: The composition and
443 structure of plant primary cell walls. First. Blackwell Publishing Ltd.

444 Pérez, J., J. Muñoz-Dorado, T. de la Rubia, and J. Martínez. 2002. Biodegradation and
445 biological treatments of cellulose, hemicellulose and lignin: an overview. Int.
446 Microbiol. 5:53–63. doi:10.1007/s10123-002-0062-3.

447 Phan, C., and V. Sabaratnam. 2012. Potential uses of spent mushroom substrate and its
448 associated lignocellulosic enzymes. Appl. Microbiol. Biotechnol. 96:863–873.
449 doi:10.1007/s00253-012-4446-9.

Pollegioni, L., F. Tonin, and E. Rosini. 2015a. Lignin-degrading enzymes. FEBS J. 282:1190–1213. doi:10.1111/febs.13224.

Pollegioni, L., F. Tonin, and E. Rosini. 2015b. Lignin-degrading enzymes 282:1190–1213. doi:10.1111/febs.13224.

Scheller, H.V., and P. Ulvskov. 2010. Hemicelluloses. Annu. Rev. Plant Biol. 61. doi:10.1146/annurev-arplant-042809-112315.

Shallom, D., and Y. Shoham. 2003. Microbial hemicellulases. Curr. Opin. Microbiol. 6:219–228. doi:10.1016/S1369-5274(03)00056-0.

Shekher, R., S. Sehgal, M. Kamthania, and A. Kumar. 2011. Laccase : Microbial Sources , Production , Purification , and Potential Biotechnological Applications 2011. doi:10.4061/2011/217861.

Van Soest, P.J. 1994. Nutritional Ecology of the Ruminant. 2nd. ED. Cornell Univ. Press, ed. Ithaca, NY.

Song, H., Y. Gao, Y. Yang, W. Xiao, S. Liu, W. Xia, Z. Liu, L. Yi, and Z. Jiang. 2016. Bioresource Technology Synergistic effect of cellulase and xylanase during hydrolysis of natural lignocellulosic substrates 219:710–715. doi:10.1016/j.biortech.2016.08.035.

Srivastava, L.M. 2001. Cell wall, cell division, and cell growth. A. Press, ed. San Diego.

Tamminga, S. 1993. Chapter 22 Influence of Feeding Management on Ruminant Fiber Digestibility 571–602.

Tien, M., and T.K. Kirk. 1984. Lignin-degrading enzyme from Phanerochaete chrysosporium: purification, characterization, and catalytic properties of a unique H2O2-requiring oxygenase. Proc. Natl. Acad. Sci. 81:2280–2284. doi:10.1073/pnas.81.8.2280.

Underwood, W. 2012. The plant cell wall : a dynamic barrier against pathogen invasion 3:1–6. doi:10.3389/fpls.2012.00085.

Vrsanska, M., S. Voberkova, V. Langer, D. Palovcikova, A. Moulick, V. Adam, and P. Kopel. 2016. Induction of laccase, lignin peroxidase and manganese peroxidase activities in white-rot fungi using copper complexes. Molecules 21:1–15. doi:10.3390/molecules21111553.

Wilkinson, J.M., and M. Rinne. 2017. Highlights of progress in silage conservation and future perspectives. Grass Forage Sci. 73:40–52. doi:10.1111/gfs.12327.

Wilson, J.R. 1994. Cell Wall Characteristics in Relation to Forage Digestion by Ruminants. J. Agric. Sci. 122:173–182. doi:10.1017/S0021859600087347.

Wong, D.W.S. 2009. Structure and action mechanism of ligninolytic enzymes. Appl. Biochem. Biotechnol. 157:174–209. doi:10.1007/s12010-008-8279-z.

Wyman, C.E., S.R. Decker, M.E. Himmel, J.W. Brady, and C.E. Skopec. 2005. Hydrolysis of Cellulose and Hemicellulose.

487 Young, K.M., J.M. Lim, M.C. Der Bedrosian, and L. Kung. 2012. Effect of exogenous
488 protease enzymes on the fermentation and nutritive value of corn silage. J. Dairy Sci.
489 95:6687–6694. doi:10.3168/jds.2012-5628.

490 Zhong, R., D. Cui, and Z. Ye. 2019. Tansley review Secondary cell wall biosynthesis
491 1703–1723. doi:10.1111/nph.15537.

492 Zhou, X., W. Li, R. Mabon, and L.J. Broadbelt. 2017. A critical review on
493 hemicellulose pyrolysis. Energy Technol. 5:52–79. doi:10.1002/ente.201600327.
494

II. OBJECTIVE

The objective of this study was to examine the effect of two sources of lignocellulolytic enzymes from *Pleurotus ostreatus* (spent mushroom substrate and enzymatic complex) in whole-plant corn silage and the performance of lactating goats fed corn silage treated with different levels of spent mushroom substrate.

III. Effect of the spent substrate from *Pleurotus ostreatus* cultivation as fibrolytic enzyme source on the composition of corn silage and performance of lactating goats

(Manuscript style and form consistent with the Instructions for Authors of the Journal of Dairy Science)

RUNNING TITLE: SPENT SUBSTRATE IN CORN

INTERPRETIVE SUMMARY:

Enzymes have been widely researched to improve fiber digestibility in roughages; however, the fibrolytic effect of the spent substrate of mushroom cultivation as a source of lignocellulolytic enzymes is unknown. In this study, we evaluated the addition of the spent substrate levels from *Pleurotus ostreatus* cultivation (SSPO) at the ensiling of whole-plant corn on silage quality and animal performance. The SSPO decreased silage concentrations of lignin and cellulose and increased ADF digestibility in goats fed treated corn silage. The SSPO has the potential to serve as a source of enzymes in whole-plant corn silage production.

ABSTRACT

The objectives of this study were to examine the effect of the spent substrate from *Pleurotus* ostreatus cultivation (SSPO) on corn silage chemical composition, antioxidant capacity, lignin monomers, and *in vitro* digestibility, as well as the performance of lactating goats fed corn silage treated with different levels of SSPO. In experiment 1, four levels of lignocellulolytic enzymes were tested in a completely randomized design: 0, 10, 20, and 30 mg of lignocellulosic enzymes/kg of fresh matter, and four replicates per treatment (vacuum-sealed bags). The bags were opened 60 d after ensiling. In experiment 2, corn silage treated with three enzyme levels (0, 10, or 30 mg/kg fresh matter) was fed to lactating goats as part of TMR. Nine lactating Saanen goats (62.68±7.62 kg BW; 44±8 days in milk; 2.91±0.81 kg of milk/day, mean±SD) were assigned to three 3 × 3 Latin squares. Data were analyzed using the GLIMMIX procedure of SAS, and the means were compared by linear and quadratic orthogonal contrast. In experiment 1, NDF, ADF, lignin, and cellulose decreased quadratically. At the nadir point, SSPO decreased NDF by 14.1%, ADF by 19.5%, lignin by 9.07%, and cellulose by 22.1% compared to the untreated silage. Therefore, SSPO led to a quadratic increase in IVDMD of corn silage (+10.3 % at the vertex). In experiment 2, SSPO increased quadratically the in vivo total-tract ADF digestibility. Also, the concentration of polyphenols in the milk of goats increased linearly with the addition of SSPO in corn silage, but no other differences were detected among treatments. In summary, adding 10 mg of lignocellulolytic enzymes from SSPO per kg fresh matter of corn at ensiling presented the best effect in silage production, with a more evident reduction in ADF and NDF concentration and increased ADF digestibility *in vivo*.

Keywords: fibrolytic enzyme, laccase, lignin, white-rot mushroom

INTRODUCTION

Some technologies have been studied to increase fiber digestibility, such as exogenous fibrolytic enzymes produced by fungi or bacteria (Beauchemin et al., 2003), and these enzymes are directly provided to the animal or used to treat feedstuffs prior to feeding (Adesogan et al., 2019).

Treating feedstuffs with fibrolytic enzymes at ensiling is an alternative to decrease fiber concentration, change fermentation profile, and increase fiber digestibility, as observed in some research with cellulolytic and hemicellulolytic enzymes (Colombatto et al., 2004a; Dean et al., 2005). However, few studies aimed to degrade lignin present in forage with exogenous enzymes (Machado et al., 2020; Lynch et al., 2014; Pech-Cervantes et al., 2019).

Lignolytic enzymes, such as laccase, manganese peroxidase, and lignin peroxidase can be used to break down lignin (Pérez et al., 2002) and possibly increase fiber digestibility. These enzymes, as well as several cellulolytic and hemicellulolytic enzymes, are produced by white-rot mushrooms, such as *Pleurotus ostreatus* (Bánfi et al., 2015), as a mechanism to obtain energy and nutrients from substrates (Phan and Sabaratnam, 2012).

Previous studies had used *Pleurotus ostreatus* to treat straws, increased their digestibility (Fazaeli et al., 2004), and replaced conventional ingredients in ruminant diets (Khattab et al., 2013; Kholif et al., 2014). Recently, Machado et al. (2020) examined the effects of enzymes isolated from *Pleurotus ostreatus* on the conservation and nutritive value of whole-plant corn and sugarcane silages. In these studies, the enzymes reduced the concentration of lignin, cellulose, and hemicellulose and increased *in vitro* digestibility and antioxidant capacity in corn and sugarcane silages (Machado et al., 2020). However, there is no study on the effects of residue from mushroom cultivation as a potential source of lignocellulolytic enzymes to treat feedstuffs.

Thus, the objectives of this study were to evaluate the effect of the spent substrate from *Pleurotus ostreatus* cultivation (SSPO) on corn silage chemical composition, antioxidant capacity, lignin monomers, and *in vitro* digestibility, as well as to evaluate the intake, digestibility, microbial protein synthesis, and milk yield and composition of lactating goats fed with corn silage treated with different levels of SSPO. We hypothesized that the addition of SSPO at ensiling could decrease fiber concentration of the corn plant through lignin degradation and consequently increase fiber digestibility in lactating goats.

MATERIAL AND METHODS

This study consisted of two experiments. In experiment 1, we evaluated the composition and *in vitro* digestibility of whole-plant corn silage treated with different doses of SSPO. Based on the results of the first trial, in experiment 2, we examined the effects of treating corn silage with different doses of SSPO on the performance of lactating goats.

Enzyme source characterization

The spent substrate from *Pleurotus ostreatus* (Jacq.) P. Kumm cultivation (SSPO) was obtained from a mushroom producer in Maringa, PR, Brazil, after the commercial harvest. The spent substrate was originated from mushroom cultivation carried out in plastic bags, in which the initial substrate consisted of a mix of tropical grass hay and palm kernel waste.

The SSPO was freeze-dried, ground to pass a 1 mm screen (Marconi MA340, Piracicaba, SP, Brazil), and homogenized. A subsample was collected for measuring the

activities of laccase, manganese peroxidase, lignin peroxidase, carboxymethylcellulase, mannanase, and xylanase. The same batch of SSPO was used in both trials.

Laccase activity was determined in UV-Vis spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) at 420 nm, through the 2,2´-azino-bis(3-etilbenzotiazolina-6-sulfonato) (ABTS) oxidation, where we used 0.2 mL of ABTS solution (20mM), 0.2 mL of enzymes solution, and 1.6 mL of McIlvaine Buffer (pH 4.0) with a reaction time of 6 min at 25°C. Laccase activity was expressed in units (U), and one U was defined as μmol of ABTS oxidized per min (Li and Xu, 1999).

Manganese peroxidase activity was determined in UV-Vis spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) at 270 nm according to Wariishi Method (Wariishi et al., 1992) where 0.6 mL of sodium malonate buffer (50mM, pH 4.5), 1.2 mL of enzymes solution, 0.6 mL of $MnSO_4$ (4.5 mM), and 0.3mL of H_2O_2 (9 mM) with a reaction time of 5 min at room temperature was used. Manganese peroxidase activity was expressed in U, and one U was defined as 1 μmol $MnSO_4$ oxidized per min.

Lignin peroxidase activity was determined in UV-Vis spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) at 310 nm according to Tien and Kirk (1984) through veratryl alcohol oxidation to veratrilaldehyde (3,4 dimethoxybenzaldehyde), where 0.75 mL of sodium tartrate buffer (10 mM, pH 3.0), 0.5 mL of enzyme solution, 0.25 mL of veratryl alcohol (3mM), and 0.10 mL of H_2O_2 (5 mM), with a reaction time of 5 min at room temperature was used. Lignin peroxidase activity was expressed in U, and one U was defined as 1 μmol veratryl alcohol oxidized per min.

Carboxymethylcellulase activity was determined in UV-Vis spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) at 540 nm, according to Ghose (1987), where 0.25 mL of carboxymethylcellulose (0.5% in 50 mL sodium acetate buffer, pH 5.8), as the substrate, and 0.25 mL of enzyme solution for the reaction was used. The

reaction time was 30 min at 50° C, and posteriorly the amount of released reducing sugar was marked with dinitrosalicylic acid, according to Miller (1959). Glucose was used to generate a standard curve. Carboxymethylcellulase activity was expressed in U, and one U was defined as 1 μmol of glucose released per min.

Xylanase activity was determined in UV-Vis spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) at 540 nm, according to Damiano et al. (2003), where 0.9 mL of Birchwood-Sigma xylan (1% in 50 mM sodium acetate buffer, pH 5.4), as the substrate, and 0.1 mL of enzyme solution for the reaction was used. The reaction time was 30 min at 50° C, and posteriorly the amount of released reducing sugar was marked with dinitrosalicylic acid, according to Miller (1959). Xylose was used to generate a standard curve. Xylanase activity was expressed in U, and one U was defined as 1 μmol of xylose released per min.

Mannanase activity was determined in UV-Vis spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) at 540 nm, according to Rättö and Poutanen (1988), where 0.9 mL of galactoglucomannan (0.5% in 50 mM sodium acetate buffer, pH 5.4), as the substrate, and 0.1 mL of enzyme solution for the reaction was used. The reaction time was 30 min at 50° C, and posteriorly the amount of released reducing sugar was marked with dinitrosalicylic acid, according to Miller (1959). Mannose was used as standard. Mannanase activity was expressed in U, and one U was defined as 1 μmol of mannose released per min.

Protein concentration was measured using the Bradford Protein Assay (Sigma-Aldrich Co., St. Louis, MO), and BSA was used as standard, according to Bradford (1976), and one mg of protein in this method was defined as one mg of the enzyme.

Experiment 1:

Silage production and treatment

Whole-plant corn hybrid Biomatrix BM 3061 (Biomatrix, Rio Claro, SP, Brazil) was harvested with 29.3 % dry matter (DM) by manual cutting and chopped into approximately 2 cm length particles in a stationary forage harvester. A sample was collected to determine the plant chemical composition before ensiling. Approximately 500 g of forage was weighed, homogenized, treated with the respective levels of SSPO, placed into nylon-polyethylene vacuum bags, and heat-sealed using a vacuum machine (TecMaq, TM-250, Sao Paulo, Brazil) to ensure an anaerobic environment. The silage bags were stored for 60 d after ensiling, in a dark environment at room temperature.

This trial consisted of a completely randomized design with four treatments (levels of enzyme) and four replicates per treatment (vacuum-sealed bags). The tested levels were 0, 10, 20, and 30 mg of lignocellulolytic enzymes/kg of fresh matter, which correspond to 0; 1,100; 2,200, and 3,300 U/min/kg of fresh matter, respectively (sum of enzymatic activities of laccase, manganese peroxidase, lignin peroxidase, carboxymethylcellulase). The levels (mg/kg of fresh matter) were based on a previous study carried out with lignocellulolytic enzyme from *Pleurotus ostreatus* produced in our laboratory (Machado et al., 2020).

Chemical composition

Mini silos were opened 60 d after ensiling, weighed to estimate the recovery of nutrients, and samples of silages were collected to determine pH, chemical composition, antioxidant capacity, and *in vitro* digestibility. Approximately 9 g of undried silage was diluted with 60 mL of distilled water, manually mixed, and left to stand for 30 mins before measuring pH, and it was measured in duplicate using a digital pH meter. Two

samples from each mini silo were dried in a forced-air oven at 55°C for 72 h and then ground to pass through a 1 mm screen in a Wiley mill (Marconi MA340, Piracicaba, SP, Brazil).

The absolute DM content was determined at 105°C using an oven according to method No. 924.01 (AOAC, 1990). Ash was determined by combustion at 600°C for 6 h in a furnace, according to method No. 924.05 (AOAC, 1990). Crude protein was determined by the Kjeldahl method (Method No. 990.03, AOAC, 1990). Neutral detergent fiber (NDF) was determined, according to Mertens (2002), using thermostable α-amylase without sodium sulfite. Acid detergent fiber (ADF) was determined according to method No. 973.18 (AOAC, 1990). Ether extract (EE) was determined according to method No. 7.060 (AOAC, 1990). The non-fibrous carbohydrates (NFC) concentration was calculated, according to Weiss (1999), using the following equation: NFC = 100 − (% NDF + % CP + % EE + % ash). The concentration of lignin was determined using the acid detergent lignin methodology, according to Van Soest and Wine (1968), by submitting the material to sulfuric acid (72/28, v/v in DI water) sequentially following ADF analysis. The cellulose concentration was obtained by the difference between the ADF and lignin, whereas hemicellulose concentration was obtained by the difference between NDF and ADF. Acid detergent insoluble nitrogen (ADIN) and neutral detergent insoluble nitrogen (NDIN) were obtained through nitrogen determination according to AOAC (AOAC, 1990) method No. 990.03 using the residue from ADF, and NDF analyses, respectively.

The DM recovery was estimated using the following equation: DMR (%) = 100 × ((FMop × DMop)/(FMen × DMen)), where FMop = fresh matter mass at opening; DMop = dry matter content at opening; FMen = fresh matter mass at ensiling; DMen =

730 dry matter content at ensiling. Nutrient recovery was estimated as the mass of a given
731 nutrient at silo opening as a proportion of the mass of that nutrient at ensiling.
732 The fractionation of carbohydrates was carried out according to System CNCPS
733 equations (Sniffen et al., 1992): Fraction A + B1 = 100 – (C + B2); Fraction B2 = 100 ×
734 ((NDF (%DM)) – NDIN (%CP) × 0.01 × CP(%DM) – NDF(%DM) × 0.01 ×
735 Lignin(%NDF) × (Lignin × 2.4))/Total carbohydrates (%DM); Fraction C = (100 ×
736 NDF(%DM) × 0.01 × Lignin(%NDF) × (Lignin × 2.4)/Total carbohydrates(%DM)),
737 where: NDIN = neutral detergent insoluble nitrogen.
738 Lignin monomer composition was determined by oxidation with nitrobenzene,
739 according to Bubna et al. (2011). The material was purified to obtain a protein-free cell
740 wall. Approximately 0.3 g of sample was homogenized with sodium, potassium
741 phosphate buffer (7 mL, 50 mM, pH 7.0), centrifuged at 1,400 g for 2 min supernatant
742 was discarded. This process was repeated four more times with sodium and potassium
743 phosphate buffer, three times with Triton (7 mL, 1/99, v/v in phosphate buffer, pH 7.0),
744 and three times with NaCl (7 mL, 1 M in pH phosphate buffer, pH 7.0). Subsequently,
745 the sample was washed 3 times with distilled water (7 mL) and 2 times with acetone (5
746 mL). The precipitated material was kept in an oven at 60°C for 24 h for drying, cooled
747 in a desiccator, and weighed to determine the cell wall concentration.
748 Approximately 50 mg of the purified cell wall, 1 mL de NaOH 2 M e 100 µL de
749 nitrobenzeno were placed in ampoules and then sealed under temperature. The
750 ampoules were kept at 170°C for 150 min and stirred manually when they reached 75
751 min. The samples were cooled after the reaction period, washed with chloroform twice,
752 acidified with HCl 5M, and submitted again to chloroform twice to promote the
753 extraction. The extracted sample was dried in rotavapor (IKA rotary evaporator RV10
754 digital V, IKA, Wilmington, NC), diluted in methanol, filtered through a 0.45 µm filter,

and analyzed by High-performance liquid chromatography (HPLC) (Agilent 1100 Series, Massy, France), using column ACE C18-AR, 150 mm × 4.6 mm × 5μm, Aberdeen, Scotland, using 4% methanol/acetic acid in water (20/80, v/v) as a mobile phase and flow 1.2 mL/min for isocratic analysis and 20 mins for isocratic analysis. Quantification of *p*-hydroxybenzaldehyde (H), guaiacyl (G), and syringaldehyde (S) at 290 nm. The results were expressed in mg/g of the cell wall. The S/G ratio was calculated with the data obtained from the quantification.

Antioxidants

The silage extract to determine the total antioxidant capacity (TAC), polyphenols, flavonoids, and reducing power were performed with a 0.5 g of sample and a 4.5 mL of 90% methanol-water (90/10, v/v). The extract was stirred on a rotary shaker overnight, centrifuged at 2,500 g for 10 min, and the supernatant was used in the analyzes. The reducing power was determined according to Zhu et al. (2002), with Santos et al. (2014) modifications. The absorbance was determined in a UV-Vis spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) and expressed as gallic acid equivalents (GAE; mg/100 g of silage, in DM basis). TAC was determined as described by Rufino et al. (2007) with the addition of radical $ABTS^{+-}$ (2,2-azinobis-(3-ethyl-benzothiazolin-6-sulfonic acid)) to the extract. The absorbance was determined in a UV-Vis spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) and expressed in Trolox equivalent (mM Trolox/100 g of silage, in DM basis). Total polyphenols were determined according to Singleton and Rossi (1965). The absorbance was determined in a UV-Vis spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) and expressed as gallic acid equivalents (EAG; mg/100 g of silage in, DM basis). According to Woisky and Salatino (1998), Flavonoids were determined with the modifications of

780 Sanchéz et al. (2010). The absorbance was determined in a UV-Vis spectrophotometer
781 (PC 300 ThermoScientific, Waltham, MA, USA) and expressed as quercetin equivalent
782 (QE; mg/100 g of silage, in DM basis).
783
784 ***In vitro Digestibility***
785 All animal procedures were approved by the Committee for Use of Animals in
786 Experimentation from the State University of Maringá. The *in vitro* dry matter
787 digestibility (IVDMD) was performed according to Tilley and Terry (1963). Ruminal
788 content was taken from two non-lactating water buffaloes through ruminal cannula.
789 Buffaloes were fed a diet (corn silage, ground corn, soybean meal, wheat meal, and a
790 vitamin-mineral supplement) formulated for the maintenance of nutritional requirement
791 (CP = 10%; TDN = 65%), according to Paul and Lal (2010). The liquid and solid phase
792 of the ruminal contents were collected manually prior to the morning feeding from the
793 ventral, central, and dorsal areas of the rumen. Ruminal contents were mixed in a
794 blender, filtered into four layers of cheesecloth, stored in thermo bottle. The bottle was
795 flushed with CO_2, and maintained at 39°C until incubations.
796 Corn silage sample (0.5 g), artificial saliva (40 mL), and ruminal fluid (10 mL)
797 were added to each tube, and tubes were saturated with CO_2 to maintain an anaerobic
798 environment. Incubations were performed in a water bath for 48 h at 39°C with constant
799 stirring. Duplicate samples were incubated in three separate *in vitro* runs. In each
800 incubation, two additional tubes containing only artificial saliva and ruminal fluid, and
801 another two tubes with a forage with a known IVDMD were included as blank and
802 standard, respectively. After incubation, residues were filtered in analytical filter paper
803 N° 40 and dried to calculate the DM disappearance.

804 The determination of *in vitro* NDF digestibility (IVNDFD) was performed
805 according to the methodology proposed by Tilley and Terry (1963) using the artificial
806 rumen Daisy II Fermenter® (Ankom Technology, Macedon, NY, USA), modified by
807 Holden (1999) (Holden, 1999). Approximately 0.5 g of silage was weighed into a non-
808 woven textile bag (100 g/m²). The ruminal fluid was collected as previously mentioned
809 in the methodology of IVDMD. The samples, 1600 mL of buffer solution, and 400 mL
810 of ruminal fluid were added to the jars. Subsequently, the jars were saturated with CO_2,
811 and constantly rotated at 39 °C for 48 h. The bags were drained, washed with water
812 until the water remained clean, and subsequently, the bags were frozen. The bags were
813 analyzed for NDF concentration in the fiber determiner (TE-149, Tecnal, Piracicaba,
814 SP, Brazil; Mertens et al., 2002). Duplicate samples were incubated in three separate *in*
815 *vitro* runs. In each incubation, two additional empties bags and another two bags with a
816 sample with known IVNDFD were included as blank and standard, respectively.
817
818 ***Experiment 2***
819 ***Ensiling and treatments***
820 The whole-plant corn hybrid DKB 290 Pro3 (Dekalb, Brazil) was harvested at a
821 half-milk line stage, chopped (theoretical length of cut of 10 mm), and the material was
822 manually ensiled in 200-L plastic drums (600 kg/m³ of FM). The corn was mixed with
823 SSPO at the compaction process. The silages were stored for 5 months before the
824 feeding trial. The tested levels were 0, 10, and 30 mg of lignocellulolytic enzymes/kg of
825 fresh matter, and those were chosen based on the results from experiment 1.
826

827 ***Animals and experimental diets***

828 The experiment was approved by the Committee for Use of Animals in
829 Experimentation of the State University of Maringá, PR (protocol 4361101018), and the
830 study fully complied with the ethical principles of animal experimentation prepared by
831 the Brazilian College of Animal Experimentation. The experiment was conducted at the
832 Iguatemi Experimental Farm, PR, Brazil.

833 Nine lactating Saanen goats (62.68±7.62 kg of BW 44±8 days in milk; 2.91±0.81
834 kg/d of milk mean±SD) were used in a triplicated 3 × 3 Latin square design, with three
835 periods and three treatments. The animals were maintained in individual stalls with free
836 access to water and diet.

837 Experimental diets were formulated to meet the nutritional requirements of
838 lactating goats weighing 60 kg and a milk yield of 3 kg/day of milk, according to NRC
839 (2007) (Table 5). The same concentrate was used in all the experimental diets, and those
840 diets differed according to the enzymatic levels added in the whole-plant corn at
841 ensiling.

842 Each experimental period lasted 22 d, with 17 d for adaptation to the treatments and
843 5 d of sampling. The animals were fed *ad libitum* with total mixed rations, and diets
844 were offered daily at 8:30 and 16:00 h, allowing 5 to 10% of refusal. The goats were
845 milked twice daily at 8:00 and 16:00 h.

846

847 ***Sampling***

848 The amount of feed provided and refusals from each animal were weighed and
849 recorded daily during the whole experiment. Feed was sampled from d 18 to d 21, and
850 refusals and feces from d 19 to d 22 of each experimental period. Fecal samples were
851 collected directly from the rectum at 8:30 and 16:30 hrs. The samples were stored at -

852 20°C, posteriorly thawed, dried in a convection oven at 55°C for 72 h, ground in Wiley
853 mill (Marconi MA340, Piracicaba, Brazil) to pass through a 2 mm sieve for indigestible
854 neutral detergent fiber (iNDF) determination and a 1 mm sieve for chemical
855 composition analysis. Fecal and refusal samples were pooled for each goat to obtain a
856 composite sample per animal per period.
857 Between d 19 and d 22 of each period, the milk yield was recorded daily, and the
858 energy-corrected (ECM) and fat-corrected milk (FCM) yield were calculated according
859 to Tyrrell and Reid (1965) and NRC (2001), respectively. Milk samples were obtained
860 from six consecutive milkings between d 20 and 22 of each period and divided into two
861 aliquots. The first aliquot, approximately 50 mL of the milk, was kept at room
862 temperature and preserved with 2-bromo-2-nitropropane-1,3-diol (Bronopol, San
863 Ramon, CA, USA) for determination of milk fat, protein, lactose, and milk urea
864 nitrogen (MUN). The second aliquot (2 mL of milk), without preservative, was frozen
865 at -80°C to determine antioxidants capacity.
866 Spot urine samples were collected on d 21 of each period 4 h after feeding to
867 estimate the efficiency of microbial protein synthesis, according to Chen and Gomes
868 (1992).
869
870 ***Chemical composition and antioxidants***
871 The chemical composition was determined on samples ground to pass a 1 mm
872 sieve, according to the methodologies previously described in experiment 1.
873 The iNDF was used as an intern marker to estimate the fecal production (Huhtanen
874 et al., 1994); thus the concentration of iNDF in feces, feed and refusals were performed
875 to determine the apparent total tract digestibility of DM, and nutrients. Samples were
876 incubated for 288 h in ruminal cannulated dairy cows fed a diet composed with 60% of

877 corn silage and 40% of grain mix (DM basis). After removal from the rumen, the bags
878 were drained, washed with water until the water remained clean, and analyzed for NDF.
879 The milk extract to determine TAC, polyphenols, and reducing power were
880 performed with the addition of 1 mL of milk and 9 ml of methanol. Posteriorly, samples
881 were vortexed for 5 min and centrifuged at 2,500 g × 10 min, and the supernatant was
882 used in the following analysis.
883 The reducing power was determined according to Zhu et al. (2002), with the
884 modifications of Santos et al. (2014). The absorbance was determined in a UV-Vis
885 spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) and expressed as
886 gallic acid equivalents (GAE; mg/L of milk).
887 Total antioxidant capacity (TCA) was determined as described by Rufino et al.
888 (2007) with the addition of radical $ABTS^{+-}$ (2,2-azinobis-(3-ethyl-benzothiazolin-6-
889 sulfonic acid)) to the extract. The absorbance was determined in a UV-Vis
890 spectrophotometer (PC 300 ThermoScientific, Waltham, MA, USA) and expressed in %
891 of $ABTS^{+-}$ degradation (% of ABTS degradation).
892 Total polyphenols were determined according to Singleton and Rossi (1965). The
893 absorbance was determined in a UV-Vis spectrophotometer (PC 300 ThermoScientific,
894 Waltham, MA, USA) and expressed as gallic acid equivalents (EAG; g/L of milk).
895
896 ***Milk and urine analyses***
897 The fat, protein, and lactose concentrations in milk were determined by infrared
898 spectroscopy (Bentley model 2000, Chaska, MN. USA) following procedure 972.16 of
899 AOAC (1990). MUN concentration was determined by a colorimetric method with the
900 Berthelot reaction (Chemspec 150, Chaska, MN, USA).

Creatinine concentration was analyzed in a semi-automatic biochemistry analyzer (Bio 2000–Bioplus, São Paulo, Brazil) using a commercial kit (Gold Analisa, Belo Horizonte, Brazil). Urine volume (L/d) was estimated by dividing the average daily creatinine excretion rate reported for goats of 26.05 mg/kg BW, according to Da Fonseca et al. (2006). Diluted urine was analyzed for allantoin, uric acid, xanthine and hypoxanthine concentrations according to methods of Chen and Gomes (1992). The purines derivatives (PD) absorbed (mmol/d) and intestinal flow of microbial nitrogen were calculated based on the following equations described by Chen and Gomes (1992): $PD = 0.84 \times + (0.150BW^{0.75}e^{-0.25X})$, and MN (g/d) = 0.727 × PD, where X = purines derivatives excreted.

Statistical analysis

The outcomes from experiment 1 were analyzed using the GLIMMIX procedure of SAS (Statistical Analysis System, 9.4, Cary, NC, USA) with the following model: $Yij = \mu + Ei + eij$, where Yij = dependent variable; μ = overall mean; Ei = fixed effect of enzyme levels (i = 0 to 30 mg of enzyme/kg of FM); and eij = residue (j = 1 to 4). Data from IVDMD and IVNDFD were analyzed using the same model but including a random effect of the run (1 to 3). Means were compared by linear and quadratic orthogonal contrasts.

Data from experiment 2 were analyzed using the GLIMMIX procedure of SAS, with the following model: $Yijk = \mu + Ei + Sj + Ak(j) + Pl + eijklm$, where Yi = dependent variable; μ = overall mean; Ei = fixed effect of enzyme levels (i = 0 to 30 mg of enzyme/kg of FM); Sj = fixed effect of square (j = 1 or 3); Ak(j) random effect of goat nested within square (j = 1 to 9); Pl = fixed effect of period (l = 1 to 3); and eijklm = residue. Means were compared by linear and quadratic orthogonal contrasts. The

coeficients for orthogonal contrasts were determined using the IML procedure of SAS. Differences were declared significant at $P \leq 0.05$ and tendencies if $P > 0.05$ and ≤ 0.10.

RESULTS

Experiment 1

Spent substrate from *Pleurotus ostreatus* cultivation showed enzymatic activity for three enzymes that degrade lignin. These enzymes were laccase (390 U/g/min), lignin peroxidase (176 U/g/min), and manganese peroxidase (12.6 U/g/min; Table 1). Enzymatic activity was also observed for carboxymethylcellulase (2,894 U/g/min); however, no activity was observed for xylanase and mannanase, which are enzymes capable of degrading hemicellulose.

The chemical composition of the whole-plant corn used in experiment 1 is described in Table 2. There was no effect of SSPO ($P > 0.11$) on the recovery of hemicellulose and lignin, while NFC recovery increased linearly ($P = 0.01$) (Table 3) with the addition of SSPO. The NDF recovery tended to decrease quadratically ($P = 0.09$), which was associated with a quadratic reduction ($P < 0.01$) on cellulose recovery.

The addition of SSPO linearly decreased ($P = 0.02$) silage pH, while DM concentration increased quadratically ($P = 0.03$; Table 4). The concentrations of NDF and ADF decreased quadratically ($P = 0.02$; and < 0.01, respectively). Due to the enzymatic activities from SSPO, NDF decreased by 14.1%, and ADF decreased by 19.5% at the nadir point achieved with 10 mg/kg FM of enzymes compared to the control treatment. Enzyme concentration also affected carbohydrate fractionation of the corn silage. Fraction A+B1 increased quadratically ($P = 0.04$), whereas fraction C and B2 decreased quadratically ($P < 0.01$; 0.01, respectively). The concentration of NFC decreased linearly ($P = 0.01$) with the addition of SSPO at ensiling. The concentrations

951 of lignin (P = 0.03), cellulose (P < 0.01) and ADIN (P < 0.02) decreased quadratically
952 with higher doses of SSPO. Compared with the control, lignin concentration decreased
953 by 9.07% and cellulose by 22.1% at the nadir point achieved with 20 and 10 mg of
954 enzyme/kg FM respectively. Minimum values of ADIN were also achieved with 10 mg
955 of enzymes/kg of FM. The addition of SSPO at ensiling did not change ($P > 0.42$) the
956 concentrations of EE and CP in corn silage.

957 The addition of SSPO at ensiling increased IVDMD quadratically ($P < 0.01$), and
958 the IVNDFD increased linearly ($P < 0.01$). Regarding the antioxidants, the polyphenols
959 and reducing power were reduced linearly ($P < 0.01$; and < 0.01 respectively) as SSPO
960 levels increased. Other antioxidant parameters did not differ among SSPO levels ($P >$
961 0.05).

962 Lignin monomers were also affected by SSPO. *p*-Hydroxyphenyl tended to
963 decrease quadratically ($P = 0.06$), while syringyl tended to decrease linearly ($P = 0.07$)
964 and consequently decreased the syringyl:guaiacyl ratio linearly ($P = 0.01$) in the cell
965 wall of corn silage.

966

967 ***Experiment 2***

968 The intake of DM and nutrients were not affected ($P > 0.24$) by treatments (Table
969 6). Apparent total tract digestibility of ADF increased quadratically ($P < 0.01$), and
970 NDF digestibility tended to increase quadratically ($P = 0.10$) with SSPO levels. The
971 apparent digestibility of other nutrients was not affected by treatments ($P > 0.15$). The
972 absorption of purine derivatives and intestinal flow of microbial nitrogen were not
973 affected by SSPO levels ($P > 0.65$; Table 7).

974 The concentration of polyphenols in the milk increased linearly with SSPO levels
975 ($P < 0.01$; Table 8). Milk yield, composition, and the other antioxidants parameters did
976 not differ among SSPO levels ($P > 0.66$; 0.30; 0.13, respectively).
977

978 **DISCUSSION**

979 The fungi *Pleurotus ostreatus* produces enzymes capable of breaking down cell
980 wall polymers (Isikhuemhen and Mikiashvilli,2009), consequently reducing NDF and
981 ADF and increasing NFC concentration in silage, as shown in experiment 1.
982 Interestingly, ADF reduction was more pronounced than NDF because the fractions that
983 compose ADF were decreased (i.e., lignin and cellulose), while hemicellulose, which
984 represented approximately 43% in NDF, did not change. These results corroborate with
985 the observed by Colombatto et al. (2004), who reported a reduction in NDF and ADF
986 concentration in corn silage treated with a mixture of fibrolytic enzymes from different
987 fungi.

988 As expected, in this study, a decrease of cellulose was paired with a simultaneous
989 increase in NFC concentration. Cellulose degradation can explain an increase of NFC
990 concentration when forages are treated with fibrolytic enzymes to glucose and
991 oligosaccharides (that constitute NFC) through cellulase activity. The NFC can be
992 rapidly assimilated by ruminal microorganisms and positively correlates with volatile
993 fatty acid syntheses (Getachew et al., 2004). Carbohydrate A+B1 fraction corresponds
994 to the sugars and starch (Sniffen et al., 1992); therefore, the increase in this fraction by
995 SSPO is correlated to the increase in NFC concentration.

996 The reduction of lignin concentration in the corn silage observed in experiment 1,
997 was certainly a result of lignocellulolytic enzymes present in SSPO (laccase, lignin
998 peroxidase, and manganese peroxidase) capable of degrading phenolic and non-phenolic

compounds (Wong, 2009). Machado et al.(2020) observed a 33% reduction in lignin concentration in corn silage treated at ensiling with a complex of lignocellulolytic enzymes produced in the laboratory by *Pleurotus ostreatus.* However, this reduction observed by Machado et al. (2020) was approximately 3 times greater than in the current study. The difference could have potentially been caused by different proportions of enzymatic activities, lignin concentration, and arrangement of the plant cell wall components between studies. In the current study, the carboxymethylcellulase activity in SSPO was predominant, and consequently, there were a lower proportion of enzymes that degrade lignin.

Khattab et al. (2013) also reported a reduction of cellulose and lignin when applied *Pleurotus ostreatus* on the rice harvest wastes. However, the proportion of reduction in lignin in this residue was around 50%, while in the present study, it was 11.3%. The reduction of cellulose concentration using SSPO in our study was 22.2%, whereas the authors reported a decrease of 3.7% when treating rice wastes. The different magnitude of reductions of fiber components may have occurred for several reasons, such as the anaerobic environment of silage production since oxygen is required for laccase cycling (Wong, 2009); the difference of *Pleurotus ostreatus* strains, as each strain can produce enzymes in different proportions and amounts (Membrillo et al., 2008); differences in chemical composition between the rice residue and the corn plant, due enzyme-feed specificity (Beauchemin et al., 2003); as well as, the way that the feeds were treated, since in the silage the enzymes were added through SSPO, and in the rice residue the fungus was placed to grow directly in the material.

The reduction in ADIN concentration using the enzymes from SSPO follows the results observed with the reduction of lignin and might be likely through the cleavage of the linkage between N and lignin. The ADIN is recognized by its very low ruminal

degradation and intestinal digestibility (Lanzas et al., 2008); thus, releasing nitrogen associated with lignin may increase the metabolizable protein for the animal. The reduction of carbohydrate fraction C was a consequence of lignin degradation.

Although white-rot mushroom, as *Pleurotus ostreatus*, produces enzymes capable of degrading hemicellulose, such as xylanase and mannanase (Machado et al., 2020), in our study, the SSPO did not present hemicellulase activity (xylanase and mannanase). The absence of hemicellulase activity may be occurred due to culture conditions (Qinnghe et al., 2004; Membrillo et al., 2008) or be a characteristic of the strain used in our study (Membrillo et al., 2008).

Applying SSPO to corn silage also altered lignin monomers. The effect observed in *p*-hydroxyphenyl and syringyl concentration is likely a result of cleavage provided by ligninolytic enzymes (Wong, 2009). Machado et al. (2020) reported the opposite for the concentration of polyphenols and lignin monomers in corn silage, since polyphenol concentration increased with the addition of lignocellulolytic enzymes, accompanied by an increase in the concentration of lignin monomers.

The increase in IVDMD was likely due to a modification in corn silage chemical composition due to the reduction of lignin and cellulose concentration simultaneous to an increase of NFC. This increase in IVDMD corroborates with Fazaeli et al. (2004), who fed cows at the end of lactation with 30% wheat straw in the diet and observed that diet with wheat straw previously treated with *Pleurotus ostreatus* showed greater DM digestibility compared to the diet with untreated wheat straw.

In experiment 2, alterations in corn silage composition were not consistent with experiment 1 that may be due to differences in corn hybrids and silo type. No difference was observed between the enzymatic levels in the silage chemical composition (data are not shown). However, the addition of SSPO increased quadratically total tract ADF and

NDF digestibility that may be explained due by the loosening effect in the lignin-hemicellulose-cellulose complex. This loosening effect was previously reported by Taniguchi et al. (2005); it might favor the access to fiber carbohydrate and became easier the degradation of fiber by rumen microbial for the goats.

Rojo et al. (2015) observed that goats fed with a similar concentration of CP, and NDF, and proportion of forage:concentrate in the diet presented a higher NDF digestibility when the TMR was treated with a cellulase, that corroborates with the tendency observed in our study. However, the authors also observed that the enzymes increased OM and DM digestibility of the diets, which was not observed in our study.

The SSPO did not affect milk yield and nutrient concentration. However, the results of those variables in this study were similar to those related by Ferro et al. (2017) in Saanen goats milk, where the milk composition of goats was characterized.

Despite that the concentration of total polyphenols in the silage was similar in both treatments (477 ± 6.09 mg/100 g of diet, DM basis), the concentration of polyphenols in milk increased with the addition of SSPO. The total polyphenol combines free, soluble conjugated, and insoluble-bound phenolic compounds (Madhujith and Shahidi, 2009). Therefore, the POSS may be released free phenolic compounds by the cleavage of insoluble-bound phenolic compounds present in the lignin, favoring the absorption and the transfer for the milk. The total polyphenols analysis determines polyphenols concentration. It does not distinguish the three categories in the quantification, and this justifies the absence of difference among polyphenols concentration in the diets, leading to a similar concentration.

CONCLUSION

Therefore, we concluded that adding 10 mg of lignocellulolytic enzymes from SSPO per kg fresh matter of whole-plant corn at ensiling presented the greatest effect on animal performance due to increasing total tract ADF digestibility. Thus, POSS may be an option at ensiling to improve fiber digestibility of lactating animals. Future studies should investigate the effect of those enzymes in WPCS on other lactating species, such as dairy cows, which may show an increase in milk yield.

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REFERENCES

Adesogan, A.T., K.G. Arriola, Y. Jiang, A. Oyebade, E.M. Paula, J.J. Romero, L.F. Ferraretto, and D. Vyas. 2019. Symposium review : Technologies for improving fiber utilization. J. Dairy Sci. 102:5726–5755. doi:10.3168/jds.2018-15334.

AOAC. 1990. Official Methods of Analysis. 15th ed. Arlington, VA, USA.

Bánfi, R., Z. Pohner, J. Kovács, S. Luzics, A. Nagy, M. Dudás, P. Tanos, K. Márialigeti, and B. Vajna. 2015. Characterisation of the large-scale production process of oyster mushroom (Pleurotus ostreatus) with the analysis of succession and spatial heterogeneity of lignocellulolytic enzyme activities Ren a Bal a. Fungal Biol. 9:1354–1363. doi:10.1016/j.funbio.2015.10.003.

Beauchemin, K.A., D. Colombatto, D.P. Morgavi, and W.Z. Yang. 2003. Use of Exogenous Fibrolytic Enzymes to Improve Feed Utilization by Use of Exogenous Fibrolytic Enzymes to Improve. J. Anim. Sci. 81:E37–E47.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 254:248–254. doi:10.1016/0003-2697(76)90527-3.

Bubna, G.A., R.B. Lima, D. Yara, L. Zanardo, W. Dantas, M. De Lourdes, L. Ferrarese, and O. Ferrarese-filho. 2011. Exogenous caffeic acid inhibits the growth and enhances the lignification of the roots of soybean (Glycine max). J. Plant Physiol.

168:1627–1633. doi:10.1016/j.jplph.2011.03.005.

Chen, X.B., and M.J. Gomes. 1992. Estimation of microbial protein supply to sheep and cattle based on urinary excretion of purine derivatives -an overview of the technical details.

Colombatto, D., F.L. Mould, M.K. Bhat, R.H. Phipps, and E. Owen. 2004. In vitro evaluation of fibrolytic enzymes as additives for maize (Zea mays L.) silage III . Comparison of enzymes derived from psychrophilic, mesophilic or thermophilic sources. Anim. Feed Sci. Technol. 111:111–128. doi:10.1016/j.anifeedsci.2003.08.012.

Damiano, V.B., D.A. Bocchini, E. Gomes, and R. Da Silva. 2003. Application of crude xylanase from Bacillus licheniformis 77-2 to the bleaching of eucalyptus Kraft pulp. World J. Microbiol. Biotechnol. 19:139–144. doi:10.1023/A:1023244911314.

Dean, D.B., A.T. Adesogan, N. Krueger, and R.C. Littell. 2005. Effect of fibrolytic enzymes on the fermentation characteristics, aerobic stability, and digestibility of bermudagrass silage. J. Dairy Sci. 88:994–1003. doi:10.3168/jds.S0022-0302(05)72767-3.

Fazaeli, H., H. Mahmodzadeh, Z.A. Jelan, Y. Rouzbehan, J.B. Liang, and A. Azizi. 2004. Utilization of fungal treated wheat straw in the diet of late lactating cow. Asian-australasian J. Anim. Sci. 17:467–472. doi:10.5713/ajas.2004.467.

Ferro, M.M., L.O. Tedeschi, and A.S. Atzori. 2017. The comparison of the lactation and milk yield and composition of selected breeds of sheep and goats. Transl. Anim. Sci. 1:498–506. doi:10.2527/tas2017.0056.

Da Fonseca, C.E.M., R.F.D. Valadares, S.D.C. Valadares Filho, M.I. Leão, P.R. Cecon, M.T. Rodrigues, D.D.S. Pina, M.I. Marcondes, M.L. Paixão, and A.M. Araújo.

2006. Microbial protein synthesis in lactating goats fed diets with increasing levels of dietary protein. Rev. Bras. Zootec. 35:1169–1177. doi:10.1590/s1516-35982006000400031.

Getachew, G., P.H. Robinson, E.J. Depeters, and S.J. Taylor. 2004. Relationships between chemical composition , dry matter degradation and in vitro gas production of several ruminant feeds. Anim. Feed Sci. Technol. 111:57–71. doi:10.1016/S0377-8401(03)00217-7.

Ghose, T.K. 1987. Measurement of cellulase activities. Pure Appl. Chem. 59:257–268. doi:10.1351/pac198759020257.

Holden, L.A. 1999. Comparison of methods of in vitro dry matter digestibility for ten feeds. J. Dairy Sci. 82:1791–1794. doi:10.3168/jds.S0022-0302(99)75409-3.

Huhtanen, P., K. Kaustell, and S. Jaakkola. 1994. The use of internal markers to predict total digestibility and duodenal flow of nutrients in cattle given six different diets. Anim. Feed Sci. Technol. 48:211–227. doi:10.1016/0377-8401(94)90173-2.

Isikhuemhen, O.S., and Æ.N.A. Mikiashvilli. 2009. Lignocellulolytic enzyme activity , substrate utilization , and mushroom yield by Pleurotus ostreatus cultivated on substrate containing anaerobic digester solids 1353–1362. doi:10.1007/s10295-009-0620-1.

Khattab, H.M., H.M. Gado, L.M. Camacho, A.M. Kholif, and A.E. Kholif. 2013. Chemical composition and in vitro digestibility of Pleurotus ostreatus spent rice straw. Anim. Nutr. Feed Technol. 13:507–516.

Kholif, A.E., H.M. Khattab, A.Z.M. Salem, and U. Autónoma. 2014. Nutrient digestibility, ruminal fermentation activities, serum parameters and milk production and composition of lactating goats fed diets containing rice straw treated with Pleurotus ostreatus. Asian-Australasian J. Anim. Sci. 27:357–364.

1173 doi:10.5713/ajas.2013.13405.
1174 Lanzas, C., G.A. Broderick, and D.G. Fox. 2008. Improved feed protein fractionation
1175 schemes for formulating rations with the cornell net carbohydrate and protein
1176 system. J. Dairy Sci. 91:4881–4891. doi:10.3168/jds.2008-1440.
1177 Li, K., and F. Xu. 1999. Comparison of fungal laccases and redox mediators in
1178 oxidation of a nonphenolic lignin model compound. Appl. Environ. Microbiol.
1179 65:2654–2660. doi:10.1128/AEM.65.6.2654-2660.1999.
1180 Lynch, J.P., L. Jin, E.C. Lara, J. Baah, and K.A. Beauchemin. 2014. The effect of
1181 exogenous fibrolytic enzymes and a ferulic acid esterase-producing inoculant on
1182 the fibre degradability , chemical composition and conservation characteristics of
1183 alfalfa silage. Anim. Feed Sci. Technol. 193:21–31.
1184 doi:10.1016/j.anifeedsci.2014.03.013.
1185 Machado, E., P. Toshimi Pintro Matumoto, L.C. Vinhas, B.C. Agustinho, P. Daniel,
1186 N.W. Santos, M. Zeoula, and M. Ribeiro. 2020. Reduction in lignin content and
1187 increase in the antioxidant capacity of corn and sugarcane silages treated with an
1188 enzymatic complex produced by white rot fungus 1–15.
1189 doi:10.1371/journal.pone.0229141.
1190 Madhujith, T., and F. Shahidi. 2009. Antioxidant potential of barley as affected by
1191 alkaline hydrolysis and release of insoluble-bound phenolics. Food Chem.
1192 117:615–620. doi:10.1016/j.foodchem.2009.04.055.
1193 Membrillo, I., C. Sanchez, M. Meneses, E. Favela, and O. Loera. 2008. Effect of
1194 substrate particle size and additional nitrogen source on production of
1195 lignocellulolytic enzymes by Pleurotus ostreatus strains. Bioresour. Technol.
1196 99:7842–7847. doi:10.1016/j.biortech.2008.01.083.
1197 Mertens, D.R., M. Allen, J. Carmany, J. Clegg, A. Davidowicz, M. Drouches, K. Frank,

D. Gambin, M. Garkie, B. Gildemeister, D. Jeffress, C.S. Jeon, D. Jones, D. Kaplan, G.N. Kim, S. Kobata, D. Main, X. Moua, B. Paul, J. Robertson, D. Taysom, N. Thiex, J. Williams, and M. Wolf. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: Collaborative study. J. AOAC Int. 85:1217–1240.

Miller, G.L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar Use. Anal. Chem. 31:426–428. doi:10.1021/ac60147a030.

NRC, 2001. Nutrient Requirements of Dairy Cattle, 7th Revised. National Academic Press, Washington, DC, USA.

NRC, 2007. Nutrient requirements of small ruminants: sheep, goats, cervids, and new world camelids, 6th Revised. National Academic Press, Washington, DC, USA.

Paul, S.S., and D. Lal. 2010. Nutrient requeriments of buffaloes. Satish Ser. Publ. house 81.

Pech-Cervantes, A.A., I. Muhammad, I.M. Ogunade, Y. Jiang, D.H. Kim, C.F. Gonzalez, T.J. Hackmann, A.S. Oliveira, D. Vyas, and A.T. Adesogan. 2019. Exogenous fibrolytic enzymes and recombinant bacterial expansins synergistically improve hydrolysis and in vitro digestibility of bermudagrass haylage. J. Dairy Sci. 102:8059–8073. doi:10.3168/jds.2019-16339.

Pérez, J., J. Muñoz-Dorado, T. de la Rubia, and J. Martínez. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int. Microbiol. 5:53–63. doi:10.1007/s10123-002-0062-3.

Phan, C., and V. Sabaratnam. 2012. Potential uses of spent mushroom substrate and its associated lignocellulosic enzymes. Appl. Microbiol. Biotechnol. 96:863–873. doi:10.1007/s00253-012-4446-9.

Qinnghe, C., Y. Xiaoyu, N. Tiangui, J. Cheng, and M. Qiugang. 2004. The screening of

culture condition and properties of xylanase by white-rot fungus Pleurotus ostreatus 39:1561–1566. doi:10.1016/S0032-9592(03)00290-5.

Rättö, M., and K. Poutanen. 1988. Production of mannan-degrading enzymes. Vol, Biotechnol. Lett. I:661–664. doi:10.1007/BF01024721.

NRC, 2001. Nutrient Requirements of Dairy Cattle, 7th Revised. National Academic National Academic Press, Washington, DC, USA.

Rojo, R., A.E. Kholif, A.Z.M. Salem, M.M.Y. Elghandour, N.E. Odongo, R. Montes De Oca, N. Rivero, and M.U. Alonso. 2015. Influence of cellulase addition to dairy goat diets on digestion and fermentation, milk production and fatty acid content. J. Agric. Sci. 153:1514–1523. doi:10.1017/S0021859615000775.

Rufino, M. do S.M., R.E. Alves, E.S. de Brito, S.M. de Morais, C. de G. Sampaio, J. Pérez-Jiménez, and F.D.S. Calixto. 2007. Metodologia Científica: Determinação da Atividade Antioxidante Total em Frutas pela Captura do Radical Livre ABTS+ [Determination of total antioxidant activity in fruits by capturing the free radical ABTS+.]. Embrapa Agroindústria Trop. Técnico 128:1–4.

Sánchez, N., S. Miranda, P. Vit, and A.J. Rodríguez-malaver. 2010. Propolis protects against oxidative stress in human saliva. J. apiproduct apimedical Sci. 2:72–76. doi:10.3896/IBRA.4.02.2.02.

Santos, N.W., G.T.D. Santos, D.C. Silva-Kazama, P.A. Grande, P.M. Pintro, F.E. de Marchi, C.C. Jobim, and H. V. Petit. 2014. Production, composition and antioxidants in milk of dairy cows fed diets containing soybean oil and grape residue silage. Livest. Sci. 159:37–45. doi:10.1016/j.livsci.2013.11.015.

Shahidi, F., and J.D. Yeo. 2016. Insoluble-bound phenolics in food. Molecules 21. doi:10.3390/molecules21091216.

Singleton, V.L., and J.A. Rossi. 1965. Colorimetry of total phenolics with

1248 phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16:144–158.
1249 Sniffen, C.J., J.D. O'Connor, P.J. Van Soest, D.G. Fox, and J. Russell. 1992. A net
1250 carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and
1251 protein availability. J. Anim. Sci. 70:3562–3577. doi:/1992.70113562x.
1252 Van Soest, P.J., and R.H. Wine. 1968. Determination of lignin and cellulose in acid-
1253 detergen fiber with permanganate determination of lignin and cellulose in acid-
1254 detergent fiber with permanganate. J. Assoc. Off. Anal. Chem. 51:780–785.
1255 doi:10.1093/jaoac/51.4.780.
1256 Taniguchi, M., H. Suzuki, D. Watanabe, K. Sakai, K. Hoshino, and T. Tanaka. 2005.
1257 Evaluation of pretreatment with Pleurotus ostreatus for enzymatic hydrolysis of
1258 rice straw. J. Biosci. Bioeng. 100:637–643. doi:10.1263/jbb.100.637.
1259 Tien, M., and T.K. Kirk. 1984. Lignin-degrading enzyme from Phanerochaete
1260 chrysosporium: purification, characterization, and catalytic properties of a unique
1261 H2O2-requiring oxygenase. Proc. Natl. Acad. Sci. 81:2280–2284.
1262 doi:10.1073/pnas.81.8.2280.
1263 Tilley, J.M.A., and R.A. Terry. 1963. Two stage technique for the in vitro digestion of
1264 forage crops. Grass Forage Sci. 18:104–111. doi:10.1111/j.1365-
1265 2494.1963.tb00335.x.
1266 Tyrrell, H.F., and J.T. Reid. 1965. Prediction of the energy value of cow's milk. J. Dairy
1267 Sci. 48:1215–1223. doi:10.3168/jds.S0022-0302(65)88430-2.
1268 Wariishi, H., K. Valli, and M.H. Gold. 1992. Manganese (II) oxidation by manganese
1269 peroxidase from the basidiomycete Phanerochaete chrysosporium. Kinetic
1270 mechanism and role of chelators. J. Biol. Chem. 267:23688–23695.
1271 Woisky, R.G., and A. Salatino. 1998. Analysis of propolis: some parameters and
1272 procedures for chemical quality control. J. Apic. Res. 37:99–105.

doi:10.1080/00218839.1998.11100961.

Wong, D.W.S. 2009. Structure and action mechanism of ligninolytic enzymes. Appl. Biochem. Biotechnol. 157:174–209. doi:10.1007/s12010-008-8279-z.

Zhu, Q.Y., R.M. Hackman, J.L. Ensunsa, R.R. Holt, and C.L. Keen. 2002. Antioxidative activities of oolong tea. J. Agric. Food Chem. 50:6929–6934. doi:10.1021/jf0206163.

TABLES

Table 1. Characterization of enzymatic activity of spent substrate from *Pleurotus ostreatus* cultivation (SSPO)

Enzymes	Enzymatic activity (U/g/min)[1]
Laccase	390
Lignin peroxidase	176
Manganese peroxidase	12.6
Carboxymethylcellulase	2,894

[1] The enzymatic activities were identified based on the final product obtained in the reaction. Enzymatic activity was expressed in U/g of SSPO/min.

1303 Table 2. Chemical composition (% of dry matter) of whole-plant corn at ensiling
1304 (Experiment 1)

	Chemical composition (%DM, unless otherwise stated)
Dry matter (g/100 g)	29.3
Organic matter	96.4
NDF	47.9
ADF	27.4
Lignin	5.64
Crude protein	7.43
EE	2.40
NFC	38.7

1305 NDF= neutral detergent fiber; ADF= acid detergent fiber; Lignin = acid detergent lignin; EE=
1306 ether extract; NFC= non-fiber carbohydrates.

1307 Table 3. Recovery of fiber fractions (%) in corn silage treated with spent substrate from
1308 *Pleurotus ostreatus* cultivation, after 60 d of storage (Experiment 1)

	Treatments (mg enzymes/kg FM)[1]				SEM	*P*-value	
Recovery (%)	0	10	20	30		Linear	Quadratic
Dry matter	86.7	93.3	88.6	92.7	1.83	0.14	0.51
NDF	90.1	83.0	84.7	86.8	2.50	0.48	0.09
NFC	78.4	103.3	89.1	98.4	3.56	0.01	0.05
Cellulose	98.9	81.1	85.4	86.0	2.21	0.01	<0.01
Hemicellulose	85.1	85.5	85.7	89.7	4.22	0.47	0.68
Lignin	83.1	81.2	75.5	79.2	2.27	0.11	0.24

1309 FM= fresh matter; NDF= neutral detergent fiber; EE= ether extract; NFC= non-fiber
1310 carbohydrates [1] = treatments corresponded to the following enzymatic activities: 0 (0 U/min/kg
1311 FM), 10 (1,100 U/min/kg FM), 20 (2,200 U/min/kg FM), 30 (3,300 U/min/kg FM).

Table 4. Chemical composition, carbohydrate fractionation (% of DM), antioxidant capacity and lignin monomers (ug/mg protein-free cell wall) in corn silage treated with spent substrate from *Pleurotus ostreatus* cultivation, after 60 d of storage (Experiment 1)

	Treatments (mg enzymes/kg FM)[1]				SEM	*P*-value	
Parameters (%)	0	10	20	30		Linear	Quadratic
pH	3.61	3.63	3.59	3.56	0.014	0.02	0.12
Chemical composition							
Dry matter	26.3	29.6	27.8	28.5	0.508	0.05	0.03
Organic matter	94.7	95.5	94.8	95.6	0.292	0.04	0.86
NDF	49.7	42.7	45.7	44.8	1.53	0.03	0.02
ADF	29.7	23.9	26.0	25.1	0.627	<0.01	<0.01
Lignin	5.40	4.91	4.79	4.82	0.105	<0.01	0.03
Hemicellulose	20.0	18.8	19.7	19.7	0.819	0.96	0.47
Cellulose	24.4	19.0	21.0	20.2	0.570	<0.01	<0.01
ADIN	5.25	4.54	4.92	4.96	0.113	0.38	<0.01
Crude protein	7.41	7.36	7.53	7.21	0.161	0.55	0.42
EE	2.61	2.65	2.68	2.57	0.087	0.78	0.43
NFC	34.9	40.6	38.9	41.2	0.871	<0.01	0.07
Fraction B2	50.54	46.0	47.5	46.0	0.932	0.01	0.13
Fraction C	8.11	6.37	6.44	6.03	0.274	<0.01	0.03
Fraction A+B1	41.3	47.6	46.1	48.0	0.954	<0.01	0.04
In vitro Digestibility (%)							
*IV*DMD	56.2	62.0	58.4	58.3	5.27	<0.01	<0.01
*IV*NDFD	48.8	55.1	48.2	53.4	4.66	<0.01	0.24
Antioxidant capacity							
TAC[2]	758	757	756	756	0.479	0.05	0.74
Flavonoids[3]	120	110	104	109	1.78	0.22	0.31
Reducing power[4]	267	252	241	194	13.3	<0.01	0.25
Poliphenols[5]	674	581	532	475	22.4	<0.01	0.45
Monomers lignin							
p-Hydroxyphenyl	12.8	6.23	12.8	20.1	3.11	0.07	0.06
Guaiacyl	118	92.1	116	118	9.81	0.59	0.18
Syringyl	1123	76.7	86.8	80.0	9.24	0.07	0.15
Syringyl:guaiacyl	0.958	0.832	0.754	0.677	0.063	0.01	0.71

FM= fresh matter; NDF= neutral detergent fiber; ADF= acid detergent fiber; EE= ether extract; NFC= non-fiber carbohydrates, TAC= total antioxidant capacity; [1] = treatments corresponded to the following enzymatic activities: 0 (0 U/min/kg FM), 10 (1,100 U/min/kg FM), 20 (2,200 U/min/kg FM), 30 (3,300 U/min/kg FM); [2]= expressed in mM Trolox/100 g of silage (DM basis); [3=]= expressed in mg of equivalente quercetin/100 g of silage (DM basis); = expressed in mg of gallic acid/100 g of silage (DM basis); [5]= expressed in mg of gallic acid/100 g of silage (DM basis).

1323 Table 5. Ingredient and chemical composition of the experimental diets (% DM, unless
1324 otherwise stated, Experiment 2)

	Treatments (mg enzymes/kg FM)[1]		
Item	0	10	30
Ingredients			
Corn silage	60.0	60.0	60.0
Ground corn	19.3	19.3	19.3
Soybean meal	19.1	19.1	19.1
Mineral and vitamin supplement	1.0	1.0	1.0
Dicalcium phosphate	0.6	0.6	0.6
Chemical composition			
DM (% of fresh matter)	53.0	53.0	53.0
Organic matter	94.4	94.7	95.1
Crude protein	15.3	15.3	15.3
Neutral detergent fiber	34.9	35.9	35.2
Acid detergent fiber	19.0	20.1	19.2
Lignin	3.16	3.05	2.78
Ether extract	2.76	2.72	2.64
ADIN, % of ADF	5.20	4.55	4.96

1325 ADIN = Acid detergent insoluble nitrogen; [1] = treatments corresponded to the following
1326 enzymatic activities: 0 (0 U/min/kg FM), 10 (1,100 U/min/kg FM), 30 (3,300 U/min/kg FM).

Table 6. Dry matter (DM) and nutrient intake (kg/d) and total tract apparent digestibility (%) in lactating goats fed diets containing corn silage treated with spent substrate from *Pleurotus ostreatus* cultivation (Experiment 2)

	Treatments (mg enzymes/kg FM)[1]			SEM	*P*-value	
Items	0	10	30		Linear	Quadratic
Intake						
Dry matter	2.09	2.04	2.06	0.106	0.76	0.62
Organic matter	1.93	1.92	2.01	0.103	0.30	0.57
Crude protein	0.322	0.315	0.330	0.0177	0.47	0.43
NDF	0.688	0.703	0.720	0.0353	0.31	0.87
ADF	0.372	0.394	0.391	0.0202	0.37	0.31
Ether extract	0.0574	0.0557	0.0573	0.00502	0.96	0.68
NFC	0.865	0.833	0.885	0.0511	0.43	0.24
TND	1.483	1.420	1.478	0.0732	0.89	0.28
Total-tract digestibility						
Dry matter	72.1	72.3	71.6	0.966	0.52	0.71
Organic matter	72.7	73.3	73.8	0.83	0.26	0.76
Crude protein	72.8	74.0	75.1	1.36	0.15	0.73
NDF	50.5	53.5	53.3	1.38	0.09	0.10
ADF	50.2	54.4	52.9	1.36	0.10	<0.01
Ether extract	82.1	81.5	82.1	1.66	0.95	0.77
NFC	89.6	88.7	88.8	0.815	0.50	0.53
TDN	73.1	73.5	73.8	0.853	0.42	0.84

NDF= neutral detergent fiber; ADF= acid detergent fiber; NFC= non-fiber carbohydrates; TDN= total digestible nutrient; [1] = treatments corresponded to the following enzymatic activities: 0 (0 U/min/kg FM), 10 (1,100 U/min/kg FM), 30 (3,300 U/min/kg FM).

Table 7. Efficiency of protein synthesis in lactating goats fed diets containing corn silage treated with spent substrate from *Pleurotus ostreatus* cultivation (Experiment 2)

Items	Treatments (mg enzymes/kg FM)[1]			SEM	*P*-value	
	0	10	30		Linear	Quadratic
Purine derivatives[2]	17.4	18.2	17.6	1.55	0.97	0.65
Microbial nitrogen[3]	12.6	13.2	12.8	1.13	0.97	0.65

[2]= purines derivatives absorbed was expressed in mmol/d; [3]= microbial nitrogen was expressed in g/d

Table 8. Milk yield and composition and concentration of antioxidants in milk of goats fed diets containing corn silage treated with spent substrate from *Pleurotus ostreatus* cultivation (Experiment 2)

	Treatments (mg enzymes/kg FM)[1]			SEM	*P*-value	
Items	0	10	30		Linear	Quadratic
Milk Yield, kg/d						
Actual	2.92	2.88	2.92	0.380	0.95	0.76
Fat-corrected[2]	2.54	2.49	2.52	0.350	0.94	0.66
Energy-corrected[3]	2.76	2.70	2.75	0.380	0.97	0.61
Yield, g/d						
Fat	91.6	89.2	90.0	13.2	0.85	0.56
Protein	86.2	84.0	87.3	12.0	0.73	0.55
Lactose	124	123	123	16.7	0.99	0.85
Total Solids	324	316	319	49.9	0.81	0.67
Concentration, % *unless otherwise stated*						
Fat	3.09	3.09	3.06	0.136	0.76	0.94
Protein	2.95	2.93	3.00	0.073	0.31	0.39
Lactose	4.22	4.26	4.22	0.0654	0.86	0.30
Total Solids	11.0	10.9	10.9	0.386	0.44	0.72
MUN, mg/dL	12.2	12.6	11.9	1.24	0.41	0.37
Antioxidant						
TAC[4]	19.7	19.2	21.5	3.20	0.13	0.36
Polyphenols[5]	1.00	1.03	1.23	0.222	<0.01	0.51
Reducing power[6]	145	145	154	17.9	0.48	0.86

TAC = total antioxidant capacity; [1] = treatments corresponded to the following enzymatic activities: 0 (0 U/min/kg FM), 10 (1,100 U/min/kg FM), 30 (3,300 U/min/kg FM); [2] = FMC (kg/d) = 4% fat-corrected (kg/d) = (0.4 × milk yield (kg/d)) + (15.0 × fat yield (kg/d)) [15]; [3] ECM (kg/d) = (0.327 × kg of milk) + (12.95 × kg of fat) + (7.2 × kg of protein) [33];[4] = expressed in % of ABTS degradation; [5] = expressed in g of equivalent gallic acid/L of milk; [6] = expressed in mg of gallic acid/L of milk.

IV. Effects of lignocellulolytic enzymes on the fermentation profile, chemical composition, and in situ ruminal disappearance of whole-plant corn silage

(Manuscript style and form consistent with the Instructions for Authors of the Journal Animal Feed Science and Technology)

Abbreviations: ADF, acid detergent fiber; aNDF, neutral detergent fiber assayed with a heat-stable alpha-amylase and inclusive of residual ash; DM, dry matter; FM, fresh matter; uNDF, undigested neutral detergent fiber; U, unit of enzyme activity; WPC, whole-plant corn; WPCS, whole-plant corn silage; WSC, water-soluble carbohydrates

ABSTRACT: The objective of this study was to examine the enzymatic activity of an enzymatic complex produced by *Pleurotus ostreatus* in differents pH; and the effects of adding increasing levels of this enzymatic complex on the fermentation profile, chemical composition, and *in situ* ruminal disappearance of whole-plant corn silage (WPCS) at the onset of fermentation and after 30 d of ensiling. The lignocellulolytic enzymatic complex was obtained through *in vitro* cultivation of *Pleurotus ostreatus.* The activities of laccase, lignin peroxidase, manganese peroxidase, endo- and exo-glucanase, xylanase, and mannanase were determined at pH 3, 4, 5, and 6. Afterward, five enzymatic complex levels were tested in a completely randomized block design in the levels of 0; 9; 18; 27; and 36 mg of lignocellulosic enzymes/kg of fresh matter (FM) of whole-plant corn. There were four replicates per treatment (vacuum-sealed bags) per opening time. The bags were opened after 1, 2, 3, and 7 d of ensiling (onset of fermentation period) and after 30 d of storage to evaluate the fermentation, chemical

composition, and *in situ* disappearance of WPCS. Laccase showed highest activity at pH 5 ($P < 0.01$), whereas manganese peroxidase and lignin peroxidase had a higher activity at pH 4 ($P < 0.01$;< 0.01, respectively). There was no interaction between the enzymatic complex and days of fermentation ($P > 0.11$). Also, there was no effect of enzymatic complex addition ($P > 0.27$) on the fermentation profile and chemical composition at the onset of fermentation. The concentration of WSC decreased quadratically at the onset of fermentation ($P = 0.02$) through the fermentation, leading to a quadratic increase of lactic acid ($P = 0.01$) and a linear increase of acetic acid ($P = 0.02$). As a consequence of increasing those organic acid concentrations, pH decreased quadratically ($P = 0.01$). Lignin concentration decreased linearly ($P = 0.04$) with the enzymatic complex levels at 30 d of storage; however, other nutrients ($P > 0.12$) and fermentation profile ($P > 0.11$, Table 5) did not change. The addition of lignocellulolytic enzymatic complex from *Pleurotus ostreatus* cultivation decreased lignin concentration in WPCS after 30 d of ensiling; however, it was not enough to affect the *in situ* disappearance.

Keywords: fibrolytic enzyme, laccase, lignin, *Pleurotus* ostreatus, white-rot fungi

1. INTRODUCTION

Whole-plant corn silage (WPCS) is the main forage source in dairy diets (Grant and Ferraretto, 2018). However, just approximately 560 g/kg of neutral fiber detergent (NDF) in the WPCS is digestible (Ferraretto and Shaver, 2015), and it is highly related to some factors, such as lignin. Lignin is a phenolic polymer that constitutes plant cell walls and negatively affects fiber degradability (Jung and Allen, 1995) due to the cross-linking of lignin to arabinoxylans (Hatfield et al., 2017). Meanwhile, reducing lignin

concentration by changing genotypes (Oba and Allen, 2000) or cleaving lignin linkage are strategies to potentially increase fiber degradability (Machado et al., 2020).

Some organisms, such as white-rot fungi, are known to produce lignocellulolytic enzymes as a mechanism to obtain energy and nutrients from fibrous substrates (Manavalan et al., 2015). *Pleurotus ostreatus*, a white-rot fungus, produces enzymes that degrade cellulose (endo- and exo-glucanase), hemicellulose (xylanase and mannanase), and lignin, such as laccase, lignin peroxidase, and manganese peroxidase (Leonowicz et al., 1999). Although these enzymes have the potential to break down lignin and possibly increase fiber degradability, few research trials have focused on evaluating the effects of adding these enzymes to whole-plant corn (WPC) at ensiling.

Recently we reported the potential of lignocellulolytic enzymes produced by *Pleurotus ostreatus* in reducing the concentration of lignin, cellulose, and hemicellulose and increasing *in vitro* degradability and antioxidant capacity of WPCS (Machado et al., 2020). However, no study on the fermentation profile after 30 d of fermentation; and fermentation profile and chemical composition of the WPC at the onset of fermentation period with lignocellulolytic enzymatic complex from *Pleurotus ostreatus* cultivation.

Therefore, the objective of this study was to evaluate the enzymatic activity of each fibrolytic enzyme present in the enzymatic complex at different pH and examine the effects of adding increasing levels of the enzymatic complex produced in the cultivation of *Pleurotus ostreatus* on the fermentative profile, chemical composition, and ruminal disappearance of WPCS along of the days of fermentation. We hypothesized that treating WPCS with the lignocellulolytic enzymatic complex from *Pleurotus ostreatus* would decrease the concentration of aNDF and lignin in the material and consequently increase *in situ* dry matter disappearance.

2. MATERIAL AND METHODS

2.1. Production of the enzymatic complex and their activities

A sample of *Pleurotus ostreatus* (strain number 1833) was purchased from the company DSMZ® (Leibniz Institute DSMZ, Braunschweig, Germany) and propagated in Petri dishes using Potato Dextrose Agar (Sigma-Aldrich Co., St. Louis, MO) as culture medium and incubated for 10 d at room temperature. After grown, 10 mm-diameter disk of *Pleurotus ostreatus* was incubated in 125 mL flasks with 25 mL of liquid culture medium (0.5% sugarcane diluted in distilled water) and 0.5 g of Coastal bermudagrass hay (*Cynodon dactylon* [L.] Pers) ground at 2 mm as a carbon source. The flasks were placed in a platform shaker, incubated at 28°C in constant agitation for 8 d. The liquid culture medium, proportion of carbon source, and incubation length were previously tested and chosen based on a pre-trial enzymatic activity. After each incubation, the material was frozen at -80° C, freeze-dried, and stored at 4°C to prevent possible enzymatic denaturation or degradation.

The activities of lignocellulolytic enzymes were determined for laccase, manganese peroxidase, lignin peroxidase, endoglucanase, exoglucanase, xylanase, and mannanase at pH 3, 4, 5, and 6. The liquid extracts of the enzymatic complex to determine the enzymatic activities were carried out using the respective buffers adding 1 g of the enzymatic complex to 19 mL of buffer in a 50 mL tube. The extracts were vortexed for 1 min, filtered in 2 layers of cheesecloth, centrifuged at 2,500 g for 10 min at 4°C, and the supernatants were used to determine the enzymatic activities.

Laccase activity was determined in a spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA, USA) at 420 nm, through the 2,2´-azino-bis(3- etilbenzotiazolina-6-sulfonato) (ABTS) oxidation, where 35 µL of ABTS solution (20mM), 35 µL of enzymes extract, and 280 µL of McIlvaine Buffer (corrected

to each pH evaluated) were loaded in a 96-well UV microplate, incubated at 25°C for 5 min. Laccase activity was expressed in units (U), and one U was defined as μmol of ABTS oxidized per min (Li and Xu, 1999).

Manganese peroxidase activity was determined in a UV-Vis spectrophotometer (Jasco V-530, Jasco, Easton, MD, USA) at 270 nm according to Wariishi (1992), where 0.6 mL of sodium malonate buffer (50mM, corrected to each pH evaluated), 1.2 mL of enzymes solution, 0.6 mL of $MnSO_4$ (4.5 mM), and 0.3 mL of H_2O_2 (9 mM) reacted for 5 mins at room temperature. Manganese peroxidase activity was expressed in U, and one U was defined as 1 μmol $MnSO_4$ oxidized per min.

Lignin peroxidase activity was determined in a UV-Vis spectrophotometer (Jasco V-530, Jasco, Easton, MD, USA) at 310 nm according to Tien and Kirk (1984) through veratryl alcohol oxidation to veratrilaldehyde (3,4 dimethoxybenzaldehyde). Specifically, 0.75 mL of sodium tartrate buffer (10 mM, corrected to each pH evaluated), 0.5 mL of enzyme solution, 0.25 mL of veratryl alcohol (3mM), and 0.10 mL of H_2O_2 (5 mM), with a reaction time of 5 min at room temperature was used. Lignin peroxidase activity was expressed in U, and one U was defined as 1 μmol veratryl alcohol oxidized per min.

Endoglucanase and exoglucanase activities were determined in a spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA, USA) at 540 nm, adapted from Wood and Bhat (1988). In the endoglucanase assay, 1 mL of carboxymethylcellulose (1%, w/v), as substrate, and 0.9 mL of citrate phosphate buffer (0.1 M, corrected to each pH evaluated) were added into a 19 mL borosilicate glass tube and incubated for 10 min at 39°C. Posteriorly, 0.1 mL of enzyme extract was added and incubated at 39° C for 5 min. Specifically, 3 mL of dinitrosalicylic acid was added into the tube and boiled for 5 min to mark the released reducing sugar, according to Miller

(1959). Glucose was used as the standard. Endoglucanase activity was expressed in U, and one U was defined as 1 μmol of glucose released per min. The exoglucanase activity assay was similar, except the substrate was replaced with microcrystalline cellulose (1%, w/v).

Xylanase activity was determined in a spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA, USA) at 540 nm, adapted from Bailey et al. (1992). In the xylanase assay, 1 mL of xylan (1%, w/v) as substrate and 0.9 mL of citrate phosphate buffer (0.1 M, corrected to each pH evaluated) were added to a 19 mL borosilicate glass tube and incubated for 10 min at 39°C. Posteriorly, 0.1 mL of enzyme extract was added and incubated at 39° C for 5 min. Released reducing sugar was market as described previously, according to Miller (1959). Xylose was used as the standard. Xylanase activity was expressed in U, and one U was defined as 1 μmol of xylose released per min.

Mannanase activity was determined in a spectrophotometer (Spectra Max 340 PC, Molecular Devices Corporation, Sunnyvale, CA, USA) at 540 nm, adapted from Rättö and Poutanen (1988), where 1 mL of galactoglucomannan (0.5%, w/v), as the substrate, as substrate, and 0.9 mL of citrate phosphate buffer (0.1 M, corrected to each pH evaluated) were added into a 19 mL borosilicate glass tube and incubated for 10 min at 39°C. Posteriorly, 0.1 mL of enzyme extract was added and incubated at 39°C for 5 min. Released reducing sugar was market as described previously, according to Miller (1959). Mannose was used as the standard. Mannanase activity was expressed in U, and one U was defined as 1 μmol of mannose released per min.

Protein concentration was measured using the Bradford Protein Kit Assay (Sigma-Aldrich Co., St. Louis, MO) according to Bradford (1976). BSA was used as standard, and one mg of protein was determined as one mg of enzyme.

2.2. Silage preparation and experimental design

The WPC was harvested, and the ensiling process was carried out at the Plant Science Research and Education Unit (Citra, FL). The *in situ* incubation was carried out at the University of Florida Dairy Research Unit (Gainesville, FL). The procedures for animal care and handling required were approved by the Institutional Animal Use and Care Committee of the University of Florida.

The WPC hybrid Syngenta NK1694-3111 (Syngenta International AG, Basel, Switzerland) was manually harvested at 378 g/mg of DM from 4 different locations in the same field. The average chemical composition is shown in Table 1.

The experiment evaluated WPCS during the onset of fermentation stage and after 30 d of ensiling. The onset of fermentation was carried out in a completely randomized block design using a 5 × 4 factorial split-plot arrangement of the treatments (5 enzymatic levels and 4 opening times), totaling 20 treatments with 4 replicates per treatment. Each replicate originated from a different location in the field, named as plot. The enzymatic levels were 0, 9, 18, 27, and 36 mg of lignocellulosic enzymes/kg of WPC (FM basis), corresponding to 0, 0.587, 1.156, 1.734, and 2.312 g of enzymatic complex/kg of FM, and the opening times were 1, 2, 3, and 7 d after ensiling. Silage stored for 30 d of ensiling was performed in a completely randomized block design, with the same five enzymatic levels and four replicates. The enzymatic levels (mg of enzyme/kg FM) were based on a previous study carried out with lignocellulolytic enzyme from *Pleurotus ostreatus* produced in our research group (Machado et al., 2020).

The WPC was chopped at 2 cm of the theoretical length of cut with a single line combine harvester Cibus S (Wintersteiger inc., Salt Lake City, USA) and immediately

homogenized with the respective levels of lignocellulosic enzymes. Treated WPC (600 g/bag) was immediately placed into nylon-polyethylene vacuum bags (89µm thickness, 25.4 × 35.6 cm; Doug Care Equipment Inc., Springville, USA) and heat-sealed using a vacuum machine (Bestvac; distributed by Doug Care Equipment Inc.). The silage bags were stored in a dark environment at room temperature.

2.3. Sample collection and analyses

The bags were weighted to estimate the dry matter (DM) losses and opened on the respectively day of fermentation. Two sub-samples from each bag were dried in a forced-air oven at 55°C for 72 h. The two replicates were combined and ground in a Wiley mill to pass a 4 mm screen to determine the *in situ* DM and NDF disappearance and to pass a 1 mm screen to determine chemical composition and uNDF.

An aqueous extract was prepared by mixing 20 g of the fresh sample plus 200 mL of double-distilled water in a Stomacher (Lab-Blender 400, Tekmar Company, Cincinnati, USA) at high speed for 30 seconds and filtered through two layers of cheesecloth. The pH was determined using a digital pH meter (Accumet XL25, Thermo Fisher Scientific Inc., Waltham, USA). Forty-mL aliquot of each extract was acidified with 0.4 mL of sulfuric acid in water (50/50, v/v), centrifuged at 7000 × g for 15 min at 4°C, and stored at -20°C to determine organic acids and NH_3-N.

Organic acid concentrations were determined as described by Muck and Dickerson (1988) using HPLC (Merck Hitachi Elite La-Chrome; Hitachi L2400, Tokyo, Japan). A Bio-Rad Aminex HPX-87H ion exclusion column (300 × 7.8-mm i.d.; Bio-Rad Laboratories, Hercules, CA) was used in an isocratic elution system containing 0.015 M sulfuric acid in the mobile phase of HPLC with a UV detector (wavelength 210 nm; L-2400, Hitachi), using a flow rate of 0.7 mL/min at 45°C.

The NH_3-N concentration was determined according to Broderich and Kang (1980) and adapted to a plate reader by using 2 µL of the sample, 100 µL of phenol, and 80 µL of hypochlorite in each well of the microplate. The plate was incubated at 95°C for 10 min and maintained at room temperature for 10 min for cooling. Absorbance readings were done utilizing a UV-Vis spectrophotometer at 620 nm (Spectra Max 340 197 PC, Molecular Devices Corporation, Sunnyvale, USA).

To determine the NDF *in situ* disappearance, approximately 5 g of ground material at 6 mm was weighed into filter bags (R1020, 10 x 20 cm, 50 ± 10-micron porosity; Ankom Technology, Macedon, USA) in duplicate for each sample. Bags were incubated for 30 h in two rumen-cannulated lactating Holstein cows (1 bag per sample per cow). Those animals used to the incubation were fed a diet consisting of 38% corn silage, 19% ground corn, 13% soybean meal, 11% cottonseed, 9% citrus pulp, 8.5% mineral premix, and 1.5% palmitic acid supplement.

To determine the *in situ* undigested NDF (uNDF), approximately 0.5 g of ground material (1 mm) was weighed into fiber filter bags (F57, 25-micron porosity; Ankom,Technology, Macedon, USA) in duplicate for each sample. Bags were incubated for 240 h in two rumen-cannulated lactating Holstein cows (1 bag per sample per cow).

All the bags were dried in a forced-air oven at 60°C for 48 h. The bags from 30 h of incubation were weighed to determine the DM disappearance, and the replication was combined and ground to pass a 1 mm sieve, and samples were placed into fiber filter bags (F57, 25 micron porosity; Ankom,Technology, Macedon, USA) for aNDF analysis. The aNDF was determined according to Mertens et al. (2002), using thermostable α-amylase and sodium sulfite in Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, USA).

The DM content was determined at 105°C using an oven according to method No. 924.01 (AOAC, 1990). Ash was determined by combustion at 600°C for 6 h in a furnace, according to method No. 924.05 (AOAC, 1990). Neutral detergent fiber (aNDF) was determined, according to Mertens (2002), using thermostable α-amylase and sodium sulfite. Acid detergent fiber (ADF) was determined according to method No. 973.18 (AOAC, 1990). The concentration of lignin was determined using the acid detergent lignin methodology, according to Van Soest and Wine (1968), by submitting the material to sulfuric acid (72/28, v/v in DI water) sequentially following ADF analysis. The cellulose concentration was obtained by the difference between the ADF and lignin, whereas hemicellulose concentration was obtained by the difference between aNDF and ADF. Water-soluble carbohydrate (WSC) was determined using the anthrone reaction test (Weiss et al., 1990) and the starch concentration, according to Hall et al. (2015), by colorimetric method.

2.4. Statistical analyses

All analyses were carried out using the GLIMMIX procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Differences were declared significant at $P \leq 0.05$ and tendencies if $P > 0.05$ and ≤ 0.10.

For the **enzymatic activity**, data were analyzed in a completely randomized design using the following model:

$$Y_{ij} = \mu + H_i + \varepsilon_{ij},$$

where Y_{ij} = dependent variable; μ = overall mean; H_i = fixed effect of the pH (i = 3 to 6); and ε_{ij} = random error. Enzymatic activity means were compared using the Bonferroni t-test option whenever differences were observed.

Data from the onset of fermentation stage were analyzed in a completely randomized block design using a 5 × 4 factorial slip-plot arrangement of treatments (5 enzymatic levels and 4 ensiling time), using the following model:

$$Y_{ijkl} = \mu + P_i + E_j + P_i \times E_j + F_k + E_j \times F_k + \varepsilon_{ijkl},$$

where Y_{ijkl} = dependent variable; μ = overall mean; P_i = random effect of plot (i = 1to 4); E_j = fixed effect of enzymatic level (j = 0 to 36 mg of enzymes/kg of FM); $P_i \times E_j$ = main-plot error; F_k = fixed effect of days of fermentation (k = 1 to 4); $E_j \times F_k$ = fixed effect of interaction; and ε_{ijkl} = random error. Linear and quadratic effects were tested for the enzymatic levels and fermentation length by using orthogonal contrasts. The coeficients for orthogonal contrasts were determined using the IML procedure of SAS.

Data at 30 d of fermentation was analyzed in a completely randomized block design, containing 5 enzymatic levels, using the following model:

$$Y_{ijk} = \mu + E_i + P_j + \varepsilon_{ijk},$$

where Y_{ijk} = dependent variable; μ = overall mean; E_i = fixed effect of enzymatic levels (i = 0 to 36 mg of enzymes/kg of FM); P_j = random effect of plot (j = 1 to 4); and ε_{ijk} = random error. Linear and quadratic effects were tested for the enzymatic levels by using orthogonal contrasts.

3. RESULTS

The characterization of the enzymatic complex produced by *Pleurotus ostreatus* is presented in Table 2. Laccase showed the highest activity ($P < 0.01$) at pH 5, which corresponded to 97.8 U/g of enzymatic complex per min. Laccase activity decreased at pH 4 and 6 that corresponded to a reduction of approximately 23% when both were

compared with the activity at pH 5. The lowest laccase activity was observed at pH 3, whose reduction was 40.2% compared with the activity observed at pH 5.

Manganese peroxidase and lignin peroxidase had the highest activity at pH 4 ($P < 0.01$). Manganese peroxidase had no activity at pH 5 and 6 and low activity at pH 3. Lignin peroxidase activity decreased by approximately 68% at pH 3 and 98.7% at pH 5 compared to the highest activity observed at pH 4. Mannanase had the highest activity at pH 5 ($P < 0.04$); however, it did not differ from the activities observed at pH 6 and 4. Activities of xylanase, endo- and exo-glucanase did not change from pH 3 to 6.

The *P*-values for the effects of enzymatic complex, days on the onset of fermentation, and their interactions at the onset of fermentation are in Table 3. There was no interaction between enzymatic complex and days on the onset of fermentation ($P > 0.11$), also enzymatic complex did not affect fermentation profile and chemical composition at the onset of fermentation ($P > 0.21$). Differences observed for the main effect of days of fermentation are presented in Table 4. The pH decreased quadratically ($P = 0.01$), and the concentration of lactic acid increased quadratically ($P = 0.01$) as fermentation progressed. From 1 to 7 d, acetic acid ($P = 0.02$) and the total organic acids ($P < 0.01$) increased linearly. The concentrations of lignin, uNDF, and WSC decreased quadratically with days of fermentation ($P \leq 0.02$).

After 30 d of storage, the fermentation profile was not affected by lignocellulolytic enzymes (Table 5). Ash concentration increased quadratically ($P = 0.05$), whereas organic matter decreased quadratically ($P = 0.05$) with the addition of enzymes. The lignin concentration decreased linearly ($P = 0.04$) with the addition of the enzymatic complex (Table 6). Other nutrients were not affected by enzymatic levels ($P > 0.11$).

4. DISCUSSION

White-rot fungi, such as *Pleurotus ostreatus*, have been reported in the literature to produce enzymes that break down lignin and degrade cellulose and hemicellulose (Bánfi et al., 2015). In this study, we observed that *Pleurotus ostreatus* produced three enzymes that break down lignin (laccase, lignin peroxidase, and manganese peroxidase), two types of cellulases (endoglucanase and exoglucanase), and two hemicellulases (xylanase and mannanase).

The enzymatic activity is recognized to be affected by temperature, substrate concentration, and pH (Beauchemin et al., 2003). Therefore, the activity of enzymes from *Pleurotus ostreatus* cultivation was tested at different pH. As expected, the optimum pH varied among enzymes. The optimum pH observed for laccase was at 5 in our study, previous studies (Manole et al., 2008; El-Batal et al., 2015). Laccase activity was likely impaired during the onset of fermentation due to the pH drop, once silage pH decreased to almost 4 on the first day of fermentation, and also to anaerobiosis establishment in silages. As demonstrated, at pH 4 the activity of this enzyme was suppressed by approximately 23% compared to the optimum pH (pH 5). Anaerobiosis also may affect laccase activity negatively, as O_2 is an essential cofactor for its catalytic cycle (Shekher et al., 2011). In the silo, the residual oxygen is consumed by plant cell respiration and aerobic or facultative microorganisms shortly after silo sealing (Elferink et al., 2000), allowing a short time of optimal conditions for proper enzyme function.

The optimum pH to lignin peroxidase (LiP) was at 4, which is related to the pH required to LiP Compound I oxidize a non-phenolic aromatic and convert to LiP Compound II (Wong, 2009; Datta et al., 2017), being essential to lignin peroxidase cycle. The optimum pH favored the activity of this enzyme along the fermentation when the pH range was around 4.

The higher activities of manganese peroxidase and lignin peroxidase at low pH may have allowed these enzymes to act during the whole fermentation. Also, manganese peroxidase and LiP do not require aerobic conditions for the cycle activation (Wong, 2009); however, manganese peroxidase does require Mn^{+2}, as a cofactor, to donate an electron converting to Mn^{+3} that oxidize and consequently cleave the lignin (Hofrichter et al., 2010). This requirement is convenient in materials as whole-plant corn because it has approximately 914 mg/kg DM of Mn (NRC, 2001), contributing to manganese peroxidase activity. Therefore, the reduction in lignin concentration in the corn silage at 30 d after ensiling with the addition of enzymatic complex may have resulted from lignin cleavage mainly by manganese and lignin peroxidases along silage fermentation. We expected to observe the effect of the enzymatic levels in the lignin concentration along the onset of fermentation, however, this was not observed. We attributed this lack of effect to not enough accumulation of lignin cleavage until 7 d of fermentation.

Lignin works as a barrier that hinders the rumen microorganisms from degrading the fiber due to its high association with hemicellulose in the cell wall by cross-linking of lignin to arabinoxylans (Hatfield et al., 2017). Thus, degradation of lignin would be a way to increase the access of the microorganisms to hemicellulose and cellulose and possibly increase fiber degradability (van Kuijk et al., 2015). However, despite reducing the lignin concentration, *in situ* DM and NDF disappearance did not increase by adding the enzymatic complex.

The reduction of lignin concentration with crescent levels of enzymatic complex was similar to that observed by Machado et al. (2020). However, in this previous study, the reduction in lignin led to an increase of *in vitro* DM and NDF digestibility that was not observed in the present study. Differences among studies may have occurred due to

different lignin concentrations and the cell wall arrangement in the WPC, since in the current study, lignin concentration in WPC was quite lower than that observed by Machado et al. (2020) (16.3 *vs.* 66.4 g/kg of DM). Therefore, these factors related to lignin might favor the magnitude of its reduction by enzyme treatment (8.59% in the present study *vs.* 44.4% in Machado et al., 2020), and consequently, the enzymes benefited in NDF and DM degradability.

Another factor that may be played a role in the lignin concentration and the degradability was the range in lignocellulolytic activities. Machado et al. (2020) observed higher activity of laccase, cellulases, and hemicellulases than in the present study. Differences in enzymatic activities are attributed to fungal strain, and culture medium conditions since Machado et al. (2020) used KIRK medium (Kirk et al., 1986), and the *Pleurotus ostreatus* was not described. In the current study, the culture medium was prepared with Bermuda grass (*Cynodon dactylon*) hay, sugarcane and water, chosen from a pre-trial to replace KIRK medium to decrease the production cost. Membrillo et al. (2008) also observed that two different strains of *Pleurotus ostreatus* produced different proportions of enzymes when the fungi were exposed to different medium conditions, which supported our results.

Although the enzymatic complex presented activities of cellulases and hemicellulases, its addition onto WPC at ensiling did not affect the concentration of aNDF and ADF at the onset of fermentation or 30 d after ensiling. Colombatto et al. (2004) and Lynch et al. (2015) observed a reduction in the concentration of aNDF and ADF, that differed from the present study, using fibrolytic enzymes (a combination of cellulolytic and hemicellulolytic) in WPC at ensiling. This divergence may be attributed to the levels of enzymatic activities and/or the concentration and arrangement of NDF and ADF in the unfermented WSC. In those studies, the enzymatic activity per kg of

WPC and the concentration of NDF and ADF in WPC was much higher than in our study.

The concentrations of lactic acid, acetic acid, propionic acid, and NH_3-N, the pH, and the absence of butyric acid in the corn silage after 30 days of ensiling were similar to the values described in the literature to a good fermentation, evidencing that the WPCS showed a satisfactory fermentation (Kung et al., 2017).

Our suggestion for future studies is to test the enzymatic complex from the *Pleurotus ostreatus* cultivation in WPC with high lignin and NDF concentration, usually observed in silage production in tropical areas (Correa et al., 2002), where the enzymes may express more effect.

5. CONCLUSIONS

The addition of lignocellulolytic enzymatic complex from *Pleurotus ostreatus* cultivation decreased lignin concentration in the WPCS after 30 d of ensiling; however, this did not affect *in situ* disappearance of dry matter and neutral detergent fiber. This reduction is associated with the activities of lignin peroxidase and manganese peroxidase, which are capable of degrading lignin anaerobioses and low pH. No difference in the fermentation profile and chemical composition was observed at onset fermentation.

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review. Matheus R. Pupo: Formal analysis. Lucas G. Ghizzi: Formal analysis, Writing - review. Mariele C. N. Agarussi: Formal analysis. Celso Heinzen Junior: Formal analysis, Data curation. Richard R. Lobo: Formal analysis. Antonio P. Faciola: Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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REFERENCES

AOAC, 1990. Official Methods of Analysis, 15th ed. Arlington, VA, USA.

Bailey, M.J., Biely, P., Poutanen, K., 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23, 257–270. https://doi.org/10.1016/0168-1656(92)90074-J

Bánfi, R., Pohner, Z., Kovács, J., Luzics, S., Nagy, A., Dudás, M., Tanos, P., Márialigeti, K., Vajna, B., 2015. Characterisation of the large-scale production process of oyster mushroom (Pleurotus ostreatus) with the analysis of succession and spatial heterogeneity of lignocellulolytic enzyme activities Ren a Bal a. Fungal Biol. 9, 1354–1363. https://doi.org/10.1016/j.funbio.2015.10.003

Beauchemin, K.A., Colombatto, D., Morgavi, D.P., Yang, W.Z., 2003. Use of Exogenous Fibrolytic Enzymes to Improve Feed Utilization by Use of Exogenous Fibrolytic Enzymes to Improve. J. Anim. Sci. 81, E37–E47.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 254, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Broderick, G.A., Kang, J.H., 1980. Automated Simultaneous Determination of Ammonia and Total Amino Acids in Ruminal Fluid and In Vitro Media 1 64–75. https://doi.org/10.3168/jds.S0022-0302(80)82888-8

Colombatto, D., Mould, F.L., Bhat, M.K., Phipps, R.H., Owen, E., 2004. In vitro evaluation of fibrolytic enzymes as additives for maize (Zea mays L.) silage. I. Effects of ensiling temperature, enzyme source and addition level. Anim. Feed Sci. Technol. 111, 111–128. https://doi.org/10.1016/j.anifeedsci.2003.08.010

Correa, C.E.S., Shaver, R.D., Pereira, M.N., Lauer, J.G., Kohn, K., 2002. Relationship between corn vitreousness and ruminal in situ starch degradability. J. Dairy Sci. 85, 3008–3012. https://doi.org/10.3168/jds.S0022-0302(02)74386-5

Datta, R., Kelkar, A., Baraniya, D., Molaei, A., Moulick, A., Meena, R.S., Formanek, P., 2017. Enzymatic degradation of lignin in soil: A review. Sustain. 9. https://doi.org/10.3390/su9071163

El-Batal, A.I., Elkenawy, N.M., Yassin, A.S., Amin, M.A., 2015. Laccase production by Pleurotus ostreatus and its application in synthesis of gold nanoparticles. Biotechnol. Reports 5, 31–39. https://doi.org/10.1016/j.btre.2014.11.001

Elferink, S.J.W.H.O., Driehuis, F., Gottschal, J.C., Spoelstra, S.F., 2000. Silage fermentation processes and their manipulation. FAO Plant Prod. Prot. Pap. 17–30.

Ferraretto, L.F., Shaver, R.D., 2015. Effects of whole-plant corn silage hybrid type on intake , digestion , ruminal fermentation , and lactation performance by dairy cows through a meta-analysis. J. Dairy Sci. 98, 2662–2675. https://doi.org/10.3168/jds.2014-9045

Grant, R.J., Ferraretto, L.F., 2018. Silage review: Silage feeding management: Silage characteristics and dairy cow feeding behavior. J. Dairy Sci. 101, 4111–4121. https://doi.org/10.3168/jds.2017-13729

Hall, M.B., Arbaugh, J., Binkerd, K., Carlson, A., Thi Doan, T., Grant, T., Heuer, C., Inerowicz, H.D., Jean-Louis, B., Johnson, R., Jordan, J., Kondratko, D., Maciel, E., McCallum, K., Meyer, D., Odijk, C.A., Parganlija-Ramic, A., Potts, T., Ruiz, L., Snodgrass, S., Taysom, D., Trupia, S., Steinlicht, B., Welch, D., 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: Collaborative study. J. AOAC Int. 98, 397–409. https://doi.org/10.5740/jaoacint.15-012

Hatfield, R.D., Rancour, D.M., Marita, J.M., 2017. Grass cell walls: A story of cross-linking. Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.02056

Hofrichter, M., Ullrich, R., Pecyna, M.J., Liers, C., Lundell, T., 2010. New and classic families of secreted fungal heme peroxidases. Appl. Microbiol. Biotechnol. 87, 871–897. https://doi.org/10.1007/s00253-010-2633-0

Jung, H.G., Allen, M.S., 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. J. Anim. Sci. 73, 2774–2790. https://doi.org/10.2527/1995.7392774x

Kirk, T.K., Croan, S., Tien, M., Murtagh, K.E., Farrell, R.L., 1986. Production of multiple ligninases by Phanerochaete chrysosporium: effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. 8, 27–32. https://doi.org/10.1016/0141-0229(86)90006-2

Kung, L.J., Shaver, R.D., Grant, R.J., Schmidt, R.J., 2017. Silage review : Interpretation of chemical , microbial , and organoleptic components of silages. J. Dairy Sci. 101, 4020–4033. https://doi.org/10.3168/jds.2017-13909

Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Wojtaś-Wasilewska, M., Cho, N.S., Hofrichter, M., Rogalski, J., 1999. Biodegradation of lignin by white rot fungi. Fungal Genet. Biol. 27, 175–185. https://doi.org/10.1006/fgbi.1999.1150

Li, K., Xu, F., 1999. Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound. Appl. Environ. Microbiol. 65, 2654–2660. https://doi.org/10.1128/AEM.65.6.2654-2660.1999

Lynch, J.P., Baah, J., Beauchemin, K.A., 2015. Conservation, fiber digestibility, and nutritive value of corn harvested at 2 cutting heights and ensiled with fibrolytic enzymes, either alone or with a ferulic acid esterase-producing inoculant. J. Dairy Sci. 98, 1214–1224. https://doi.org/10.3168/jds.2014-8768

Machado, E., Toshimi Pintro Matumoto, P., Vinhas, L.C., Agustinho, B.C., Daniel, P., Santos, N.W., Zeoula, M., Ribeiro, M., 2020. Reduction in lignin content and increase in the antioxidant capacity of corn and sugarcane silages treated with an enzymatic complex produced by white rot fungus 1–15. https://doi.org/10.1371/journal.pone.0229141

Manavalan, T., Manavalan, A., Heese, K., 2015. Characterization of Lignocellulolytic Enzymes from White-Rot Fungi. Curr. Microbiol. 70, 485–498. https://doi.org/10.1007/s00284-014-0743-0

Manole, A., Herea, D., Chiriac, H., Melnig, V., 2008. Laccase Activity Determination. Biomater. Biophys. Med. Phys. Ecol. 17–24.

Membrillo, I., Sanchez, C., Meneses, M., Favela, E., Loera, O., 2008. Effect of substrate particle size and additional nitrogen source on production of lignocellulolytic enzymes by Pleurotus ostreatus strains. Bioresour. Technol. 99, 7842–7847. https://doi.org/10.1016/j.biortech.2008.01.083

Mertens, D.R., Allen, M., Carmany, J., Clegg, J., Davidowicz, A., Drouches, M., Frank, K., Gambin, D., Garkie, M., Gildemeister, B., Jeffress, D., Jeon, C.S., Jones, D., Kaplan, D., Kim, G.N., Kobata, S., Main, D., Moua, X., Paul, B., Robertson, J., Taysom, D., Thiex, N., Williams, J., Wolf, M., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: Collaborative study. J. AOAC Int. 85, 1217–1240.

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar Use. Anal. Chem. 31, 426–428. https://doi.org/10.1021/ac60147a030

Muck, R.E., Dickerson, J.T., 1988. Storage Temperature Effects on Proteolysis in Alfalfa Silage. Trans. Am. Soc. Agric. Eng. 31, 1005–1009. https://doi.org/10.13031/2013.30813

Oba, M., Allen, M.S., 2000. Effects of brown midrib 3 mutation in corn silage on productivity of dairy cows fed two concentrations of dietary neutral detergent fiber: 2. Chewing activities. J. Dairy Sci. 83, 1342–1349. https://doi.org/10.3168/jds.S0022-0302(00)75001-6

Rättö, M., Poutanen, K., 1988. Production of mannan-degrading enzymes. Vol, Biotechnol. Lett. I, 661–664. https://doi.org/10.1007/BF01024721

Shekher, R., Sehgal, S., Kamthania, M., Kumar, A., 2011. Laccase : Microbial Sources , Production , Purification , and Potential Biotechnological Applications 2011. https://doi.org/10.4061/2011/217861

Tien, M., Kirk, T.K., 1984. Lignin-degrading enzyme from Phanerochaete chrysosporium: purification, characterization, and catalytic properties of a unique H2O2-requiring oxygenase. Proc. Natl. Acad. Sci. 81, 2280–2284. https://doi.org/10.1073/pnas.81.8.2280

van Kuijk, S.J.A., Sonnenberg, A.S.M., Baars, J.J.P., Hendriks, W.H., Cone, J.W., 2015. Fungal treated lignocellulosic biomass as ruminant feed ingredient: A review. Biotechnol. Adv. 33, 191–202. https://doi.org/10.1016/j.biotechadv.2014.10.014

Van Soest, P.J., Wine, R.H., 1968. Determination of lignin and cellulose in acid-detergen fiber with permanganate determination of lignin and cellulose in acid-detergent fiber with permanganate. J. Assoc. Off. Anal. Chem. 51, 780–785. https://doi.org/10.1093/jaoac/51.4.780

Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese (II) oxidation by manganese peroxidase from the basidiomycete Phanerochaete chrysosporium. Kinetic mechanism and role of chelators. J. Biol. Chem. 267, 23688–23695.

Weiss, W.P., Hogan, J.S., Smith, K.L., Hoblet, K.H., 1990. Relationships among selenium, vitamin E, and mammary gland health in commercial dairy herds. J. Dairy Sci. 73, 381–90. https://doi.org/10.3168/jds.S0022-0302(90)78684-5

Wong, D.W.S., 2009. Structure and action mechanism of ligninolytic enzymes. Appl. Biochem. Biotechnol. 157, 174–209. https://doi.org/10.1007/s12010-008-8279-z

Wood, T.M., Bhat, K.M., 1988. Methods for measuring cellulase activities. Methods Enzymol. 160, 87–112. https://doi.org/10.1016/0076-6879(88)60109-1

Tables

Table 1. Chemical composition of unfermented whole-plant corn (WPC)

Item	Mean	SD
pH	5.84	0.16
DM, g/kg as-fed	378	27.7
aNDF, g/kg of DM	357	24.4
ADF, g/kg of DM	185	12.1
Cellulose, g/kg of DM	166	13.9
Hemicellulose, g/kg of DM	172	13.2
Lignin, g/kg of DM	18.5	2.35
Starch, g/kg of DM	325	20.7
WSC, g/kg of DM	75.4	2.06
Ash, g/kg of DM	24	0.89

SD = standard deviation; DM = dry matter; aNDF = neutral detergent fiber determined with heat-stable alpha-amylase and inclusive of residual ash; ADF = acid detergent fiber; WSC = water soluble carbohydrates.

1907 Table 2. Characterization of enzymatic complex produced by *Pleurotus ostreatus*

Enzymatic activity, U/g/min[1]	pH				SEM	*P*-value
	3	4	5	6		
Laccase	58.5^{c}	72.9^{b}	97.8^{a}	78.6^{b}	2.01	<0.01
Mn Peroxidase[2]	3.79^{b}	22.2^{a}	0.00^{b}	0.00^{b}	1.15	<0.01
Lignin Peroxidase	26.1^{b}	81.8^{a}	1.03^{c}	0.0^{c}	48.1	<0.01
Endoglucanase	16.3	14.3	19.3	14.1	0.17	0.61
Exoglucanase	23.4	27.9	35.4	28.8	1.82	0.64
Xylanase	26.6	28.2	28.6	27.1	0.07	0.42
Mannanase	17.0^{b}	18.5^{ab}	19.0^{a}	17.9^{ab}	0.03	0.04

1908 [a,b, c] Means with different superscript letters differed ($P \leq 0.05$).

1909 [1]The enzymatic activities were identified based on the final product obtained in the reaction.

1910 Enzymatic activity was expressed in U/g of SSPO/min. Abbreviations: Mn peroxidase =

1911 Manganese peroxidase.

Table 3. *P*-values for enzymatic complex addition, days of fermentation, and their interaction at the onset of fermentation (1 to 7 d) in whole-plant corn silage treated with lignocellulolytic enzymes from *Pleurotus ostreatus*

	P-value		
Item	Enzyme	Time	Enzyme*time
pH	0.62	<0.01	0.17
Total acids	0.26	<0.01	0.11
Lactic acid	0.43	<0.01	0.11
Acetic acid	0.21	<0.01	0.14
Propionic acid	0.57	0.02	0.46
N-NH3	0.29	<0.01	0.15
DM	0.36	0.11	0.70
Ash	0.96	0.41	0.99
OM	0.96	0.41	0.99
aNDF	0.57	0.48	0.82
ADF	0.52	0.19	0.71
Cellulose	0.43	0.22	0.66
Hemicellulose	0.58	0.13	0.57
Lignin	0.62	<0.01	0.30
WSC	0.37	<0.01	0.97
DMD	0.68	0.76	0.31
NDFD	0.57	0.34	0.12
uNDF	0.65	0.01	0.38
DM loss	0.91	0.64	0.31

Abbreviations: DM = dry matter; aNDF = neutral detergent fiber determined with heat-stable alpha-amylase and inclusive of residual ash; ADF = acid detergent fiber; WSC = water soluble carbohydrates; uNDF = in situ undigested neutral detergent fiber; DMD = in situ dry matter digestibility; NDFD = in situ neutral detergent fiber digestibility; DM loss = dry matter loss.

1919 Table 4. Fermentation profile and chemical composition of whole-plant corn silage at
1920 the onset of fermentation, when averaged over lignocellulolytic enzymes from
1921 *Pleurotus ostreatus*

	Day of ensiling					*P*-value	
Item	1	2	3	7	SEM	Linear	Quadratic
Fermentation profile							
pH	4.25	4.28	3.87	3.85	0.0413	<0.01	<0.01
Lactic acid[1]	18.2	21.8	33.7	44.3	2.53	<0.01	0.02
Acetic acid[1]	11.9	10.6	15.0	14.3	1.07	0.02	0.16
Propionic acid[1]	8.96	6.51	7.07	7.61	0.580	0.54	0.01
Total acids[1]	39.1	38.9	56.5	66.2	4.14	<0.01	0.09
NH_3-N[1]	29.0	33.4	35.8	40.9	1.77	<0.01	<0.01
Chemical composition, g/kg of dry matter							
Lignin	18.5	16.3	16.6	16.9	0.424	0.10	<0.01
WSC	42.2	36.3	28.6	17.1	2.09	<0.01	<0.01
uNDF	134	122	126	125	2.77	0.16	0.02

1922 Abbreviations: WSC = water soluble carbohydrates; uNDF = undigested neutral detergent fiber.

1923 [1] = g/kg of dry matter.

Table 5. Fermentation profile and dry matter loss of whole-plant corn silage treated with lignocellulolytic enzymes from *Pleurotus ostreatus*, after 30 d of fermentation

	Treatments (mg enzymes/kg FM)[1]						*P*-value	
Item	0	9	18	27	36	SEM	Linear	Quadratic
pH	3.64	3.60	3.71	3.59	3.64	0.024	0.91	0.35
Total acids[2]	71.84	69.1	64.4	83.6	64.6	7.05	0.99	0.67
Lactic acid[2]	58.2	53.3	50.8	61.7	53.5	5.56	0.92	0.65
Acetic acid[2]	8.38	8.44	8.19	12.4	7.76	1.77	0.53	0.34
Propionic acid[2]	5.24	7.33	5.45	9.91	3.35	1.75	0.81	0.12
Butyric acid[2]	ND	ND	ND	ND	ND	-	-	-
NH_3-N[2]	48.9	47.9	50.8	45.4	46.7	3.18	0.16	0.52
DM Loss[3]	133	144	131	125	127	19.5	0.33	0.74

ND = not detected.; [1] = treatments corresponded to the following enzymatic activities: 0 (0 g of enzymatic complex/kg of FM), 9 (0.587 g of enzymatic complex/kg of FM), 18 (1.156 g of enzymatic complex/kg of FM), 27 (1.734 g of enzymatic complex/kg of FM), 36 (2.312 g of enzymatic complex/kg of FM); [2] = g/kg of dry matter; [3] = DM loss = dry matter loss.

Table 6. Chemical composition and *in situ* disappearance of whole-plant corn silage treated with lignocellulolytic enzymes from *Pleurotus ostreatus*, after 30 d of fermentation

	Treatments (mg enzymes/kg FM)[1]						*P*-value	
Item, g/kg of dry matter	0	9	18	27	36	SEM	Linear	Quadratic
Dry matter, g/kg	354	349	354	358	357	12.8	0.28	0.63
Ash	23.7	26.6	27.0	25.0	25.6	0.874	0.44	0.05
Organic matter	976	973	973	975	974	0.874	0.44	0.05
NDF	364	391	392	371	358	20.6	0.57	0.15
ADF	190	209	211	205	196	11.5	0.78	0.12
Lignin	16.3	17.3	15.0	15.0	14.9	0.701	0.04	0.99
Cellulose	173	191	196	190	181	11.2	0.65	0.11
Hemicellulose	174	182	181	166	162	9.91	0.13	0.22
WSC	8.82	8.42	9.07	9.82	9.25	1.10	0.43	0.94
uNDF	116	126	129	123	121	5.33	0.73	0.12
DMD	661	667	663	679	692	14.2	0.15	0.55
NDFD	236	286	287	288	268	27.3	0.39	0.13

[1] = treatments corresponded to the following enzymatic activities: 0 (0 g of enzymatic complex/kg of FM), 9 (0.587 g of enzymatic complex/kg of FM), 18 (1.156 g of enzymatic complex/kg of FM), 27 (1.734 g of enzymatic complex/kg of FM), 36 (2.312 g of enzymatic complex/kg of FM); Abbreviations: DM = dry matter; aNDF = neutral detergent fiber determined with heat-stable alpha-amylase and inclusive of residual ash; ADF = acid detergent fiber; WSC = water soluble carbohydrates; uNDF = in situ undigested neutral detergent fiber; DMD = in situ dry matter degradability; NDFD = in situ neutral detergent fiber degradability.