University of São Paulo
"Luiz de Queiroz" College of Agriculture

Effect of length of storage and chemical additives on the nutritive value and starch degradability of reconstituted corn grain silage

Késia da Silva Oliveira

Dissertation presented to obtain the degree of Master in Science. Area: Animal Science and Pastures

Piracicaba
2020

Késia da Silva Oliveira
Animal Scientist

Effect of length of storage and chemical additives on the nutritive value and starch degradability of reconstituted corn grain silage

versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
Prof. Dr. **LUIZ GUSTAVO NUSSIO**

Dissertation presented to obtain the degree of Master in Science. Area: Animal Science and Pastures

Piracicaba
2020

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Oliveira, Késia da Silva

Effect of length of storage and chemical additives on the nutritive value and starch degradability of reconstituted corn grain silage/ Késia da Silva Oliveira. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2020.

37 p.

Dissertação (Mestrado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Silagem de grão de milho reconstituído 2. Tempo de armazenamento 3. Aditivo químico 4. Polissorbato 80 I. Título

To my parents, Zélia and Job, and my sister, Fernanda, for all support and love.
To Filipe, for his patience, companionship and love.
I DEDICATE

ACKNOWLEDGMENTS

First of all, I thank God for the gift of life and for sustaining me on my journey up here.

To my parents, Zélia and Job, for being examples of honesty and not measuring effort to help me. I am thankful for all love.

To my sister, Fernanda, for all supporting and cheer.

To my fiancé, Filipe, for your love, companionship and for being present at all times.

To my grandmother, Maria José, for sustaining me in your prayers.

To my family who always supported and encouraged me to keep following my dreams.

To my friends from the Forage Quality and Conservation Group (QCF), especially, Vivi, Jessiquinha, Gustavo, Willian, Pedro, Larissa, Lorena, Gabriel, Érica and Dannylo for the help and contribution in my work and for the good moments lived.

To Department of Animal Science's employees for all supporting and collaboration while conducting my work.

To my advisor, Professor Luiz Gustavo Nussio, for shared knowledge and for the opportunity and trust during my MSc training.

To "Luiz de Queiroz" College of Agriculture, University of São Paulo and Animal Science and Pastures Graduate Program, for supporting during my MSc training.

To FAPESP and CAPES foundation for my scholarship and supporting.

To Kemin for the donation of Mycoflake™ and financial support.

To Luciana Castelar and Daniel for help and support during conducting nitrogen analysis.

To Alex and Benedito for the help at work and animal management.

To Professor Roberto Sartori and Animal Reproduction Laboratory (LRA) for all help and support at the reproduction management of the dairy cows during my technical training.

To Professor João Luiz Pratti Daniel for the scientific contribution in my project and during its conduction.

To Professor Elisabet Nadeau for her scientific contributions to the interpretation of my results.

To Dissertation Judging Committee for all contributions.

CONTENTS

RESUMO

Efeito do tempo de estocagem e aditivos químicos no valor nutritivo e degradabilidade do amido em silagem de grãos de milho reconstituído

A reidratação e ensilagem do grão de milho seco é um método de processamento capaz de melhorar a digestibilidade do amido pela quebra da matriz proteica que envolve os grânulos de amido permitindo o acesso pelas bactérias ruminais. Esse tipo de processamento do grão pode resultar em maior eficiência alimentar de vacas leiteiras, diminuindo a ingestão de matéria seca sem alterar a produção de leite. O uso de aditivos químicos pode controlar a produção de etanol, por inibir o desenvolvimento de leveduras, melhora a estabilidade aeróbica e diminui as perdas de matéria. Surfactantes sintéticos, tal como polissorbato 80, possuem diversas propriedades funcionais na indústria alimentícia, pois são eficientes em estabilizar substâncias imiscíveis, como interfaces entre água-óleo, interagem com matrizes alimentares e podem modificar sua estrutura através de mecanismos físicos. O objetivo deste estudo foi avaliar o efeito do tratamento com ácido propiônico, polissorbato 80 ou uma mistura de ácido propiônico e polissorbato 80 na silagem reconstituída de grãos de milho armazenados com diferentes tempos de estocagem (15, 30 ou 60 dias) na composição química, degradabilidade ruminal *in situ* e na conservação da silagem. Os grãos de milho foram moídos antes da ensilagem (peneira de 5-mm). O milho moído seco foi misturado manualmente com água destilada para atingir um teor de umidade de 35%. Cento e setenta e sete quilos e duzentos gramas de milho moído seco foram reidratados e posteriormente ensilados em baldes de plástico com capacidade de 5 litros, equipados com tampas de plástico (mini-silo). Quatro pilhas (44,3 kg de grão seco por pilha) foi tratada com polissorbato 80 (2 L / t) (POL), ácido propiônico 28% (2 L / t) (PRO), Mycoflake ™ (2 L / t) (MYC) ou sem aditivo (CON). O efeito de tempo de estocagem (15, 30 ou 60 d), também foi avaliado e combinado em um arranjo fatorial com o efeito de aditivos. Quatro repetições foram usadas por tratamento. Os dados foram analisados usando o procedimento MIXED do SAS. Foram avaliadas composição química, pH, teor de ácido láctico, ácidos graxos voláteis, etanol, nitrogênio amoniacal, proteína solúvel, perdas de matéria seca, estabilidade aeróbica, distribuição de tamanho de partícula do grão, tamanho geométrico médio de partículas (TMP), área superficial do grão de milho e degradabilidade ruminal *in situ* do amido e da matéria seca (MS). O teor de MS foi maior aos 15 e 30 dias do que aos 60 dias de ensilagem. O teor de proteína bruta (PB) diminuiu com o tempo e foi menor nas silagens tratadas com Mycoflake em comparação com os outros tratamentos. Houve interação entre aditivo e tempo de estocagem para o teor de proteína solúvel, onde, aos 15 dias de ensilagem o POL apresentou menor teor de proteínas solúveis que CON e MYC e após 30 dias apresentou menor teor que MYC, e não foram observadas diferenças entre tratamentos após 60 dias de estocagem. O teor de nitrogênio amoniacal foi maior aos 30 e 60 dias de armazenamento que aos 15 dias, 53,2 e 59,4%, respectivamente. O tempo de armazenamento e o tratamento alteraram a distribuição do tamanho de partículas do grão. Houve diminuição do material retido no fundo da peneira aos 60 dias de armazenamento, comparado aos 15 e 30 dias. O MYC apresentou menor porcentagem de grãos retidos nas peneiras de 1,7 e 1,18 mm em comparação aos outros tratamentos. Na peneira de 0,6 mm, o PRO e MYC diminuíram a quantidade de partículas mantidas em relação ao CON e POL. No entanto, a porcentagem de material retido no fundo foi maior para MYC em relação aos outros tratamentos. Observou-se um aumento no TMP nas silagens armazenadas por 60 dias e o MYC reduziu o TMP em relação ao CON e POL. Ocorreu diminuição da área superficial nas silagens armazenadas por 60 dias e o MYC apresentou maior área superficial. Houve interação entre aditivo tempo de estocagem para pH. Após 30 dias de ensilagem, somente o PRO apresentou pH inferior ao CON, mas após 60 dias todas as silagens tratadas com aditivo apresentaram pH inferior ao CON. A concentração de ácido lático foi maior aos 30 dias em comparação aos 15 dias de armazenamento. Aos 30 d, o POL e o PRO apresentaram menor concentração de etanol que o CON, e aos 60 d todos os tratamentos exceto CON, foram eficientes em diminuir o etanol da silagem. A concentração de ácido propiônico foi maior nas silagens que continham ácido propiônico (PRO e MYC) do que no CON e POL em todos os períodos de armazenamento. Todas as silagens armazenadas por 15 dias apresentaram baixa e semelhante concentração de ácido butírico, mas aos 30 e 60 dias de armazenamento, as silagens não tratadas apresentaram maior concentração de ácido butírico do que as silagens tratadas. A estabilidade aeróbica foi maior aos 30 e 60 dias. Aos 15 dias de armazenamento, os tratamentos PRO e MYC foram eficientes em reduzir as perdas de MS e aos 60 dias todas as silagens tratadas apresentaram menor perda de MS que o CON. O tempo de estocagem aumentou a degradabilidade ruminal *in situ* do amido e da MS. O MYC aumentou a degradabilidade do MS em 12 h de incubação em comparação com o POL. Em conclusão, aumentar o tempo de armazenamento de silagens de grãos de milho reconstituídos melhora a degradabilidade ruminal do amido e da MS. O Mycoflake pode aumentar a disponibilidade de nutrientes e o Polissorbato 80 pode ser um potencial agente antimicrobiano em silagens de grãos de milho. Mais estudos são necessários para determinar melhor o efeito de uma combinação de aditivo químico e polissorbatos em silagens de grãos de milho.

Palavras-chave: Silagem de grão de milho reconstituído, Tempo de estocagem, Aditivo químico, Polissorbato 80

ABSTRACT

Effect of length of storage and chemical additives on the nutritive value and starch degradability of reconstituted corn grain silage

The rehydration and ensiling of dry corn grain are processing methods capable to improve the starch digestibility by breakdown of the protein matrix that surrounds starch granules, allowing the access by the ruminal bacteria. This processing method can result in greater feed efficiency of dairy cows by decreasing dry matter intake without altering milk production. Chemical additives can control ethanol production by inhibiting yeasts development, improve aerobic stability and decreasing the dry matter losses in grain silage. Synthetic surfactants, such as polyoxyethylene (20) sorbitan monooleate, have lipophilic (hydrocarbon chain) and hydrophilic (polar head) properties in the same molecule, and are widely applied in the food industry to stabilize immiscible substances, as water-oil interfaces, interacting with food matrices and modifying its structure through physical mechanisms. The objective of this study was to evaluate the effect of treatment with propionic acid, polysorbate 80 or propionic acid and polysorbate 80 mixture on reconstituted corn grain silage stored for 15, 30, or 60 d on the chemical composition, ruminal *in situ* degradability, and silage conservation. Dry ground corn was mixed with distilled water manually to achieve a moisture content of 35%. A total of 177.2 kg of dry ground corn, were rehydrated and then ensiled in 5-L plastic buckets sealed with plastic lids. Four piles (44.3 kg of dry grain per pile) were treated with polysorbate 80 (2 L/t) (POL), propionic acid 28% (2 L/t) (PRO), Mycoflake™ (2 L/t) (MYC) or nothing (CON). The effect of length of storage (15, 30, or 60 d), was also evaluated and combined in a factorial arrangement with the additives effect. Four replicates were used per treatment. Data were analyzed using the MIXED procedure of SAS. We evaluated the chemical composition, pH, lactic acid content, volatile fatty acids, ethanol, ammonia nitrogen, soluble protein, dry matter losses, aerobic stability, kernel fraction particle size distribution, geometric mean particle size (GMPS) and surface area of the corn grain, and ruminal *in situ* starch and dry matter degradability. The DM content was higher at 15 and 30 d than at 60 d of ensiling. The crude protein (CP) content decreased over time and was lower in silages treated with Mycoflake compared to the other treatments. There was an interaction between additive and storage length for soluble protein content, where at 15 d of ensiling, POL had lower soluble protein content than CON and MYC, after 30 d it had lower soluble protein content than MYC, and no differences were observed among treatments after 60 d. Ammonia nitrogen content was higher for 30 and 60 d of storage than 15 d, 53.2 and 59.4%, respectively. The length of storage and treatment changed the kernel particle size distribution. There was a decreased amount of material retained on the bottom pan at 60 d of storage compared with 15 and 30 d. The MYC had lower percentage of grain held at 1.7 and 1.18-mm sieves compared to the other treatments. At 0.6-mm sieve, the PRO and MYC decreased the amount of particles kept regarding CON and POL. However, the percentage of material held at pan was higher for MYC than other treatments. It was observed an increase in GMPS at silages stored for 60 d and with the MYC there was a reduction compared with CON and POL. A decreased in surface area occurred in silages stored for 60 d and the MYC presented a higher surface area. There was an interaction between additive and storage length for pH. After 30 d of ensiling only PRO had lower pH than CON, but after 60 d all silages treated with additives had lower pH than CON. The lactic acid concentration was higher at 30 d in comparison with 15 d of storage. At 30 d, the POL and PRO had lower ethanol concentration than CON, and at 60 d all treatments except CON controlled ethanol concentration. The propionic acid concentration was higher in silages treated with additives that contained propionic acid (PRO and MYC) than for CON and POL at all lengths of storage analyzed. All silages stored for 15 d had low and similar butyric acid concentration, but at 30 and 60 d of storage, the untreated silages had higher butyric acid concentration than treated silages. Aerobic stability increased from 15 to 30 d, but was the same at 30 and 60 d. At 15 d of storage, the treatments PRO and MYC were efficient to decrease the DM losses. At 60 d all treated silages presented lower DM loss than CON. The length of storage increased the ruminal *in situ* degradability of starch and DM. The MYC increased the DM degradability at 12 h of incubation compared to POL. In conclusion, increasing the storage time of reconstituted corn grain silages improved the ruminal degradability of starch and DM. The Mycoflake increased the availability of nutrients and Polysorbate 80 can be a potential antimicrobial agent in corn grain silages. Further studies are needed to better determine the effect of a combination of chemical additive and polysorbates in corn grain silages.

Keywords: Reconstituted corn grain silage, Length of storage, Chemical additive, Polysorbate 80

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

The corn grain is the second most produced grain in Brazil, with a total production of approximately 98.4 million of tons in 2019, behind soybean only (Conab, 2019) and is an important energy source for animal feed. However, mature corn hybrids with high density and vitreousness have low ruminal digestibility of starch (Correa et al., 2002). The protein matrix in the grain is a physicochemical barrier for starch digestion by ruminal microorganisms (Owens et al., 1986). The zein-proteins are present in greater amounts in the vitreous endosperm than in the floury endosperm, encapsulating the starch granules and reducing their accessibility by the rumen microorganisms (Philippeau et al., 2000). The rehydration and ensiling of corn grain are processing methods capable of improving the starch availability (Arcari et al., 2016; Da Silva et al., 2019). It is well reported that extension of fermentation time in silo increases ruminal digestibility of starch by the breaking down of the protein matrix in the grain endosperm (Kung et al., 2018). The rehydration and ensiling of the corn grain can result in greater feed efficiency of dairy cows by decreasing dry matter intake without altering milk production (Ferraretto et al., 2013).

The propionic acid presents high antifungal properties in a pH around 4.8, where 50% of the acid is at undissociated form (COOH) (Kung et al., 2003) and the proportion of undissociated to dissociated form is extremely dependent of the pH (Lambert and Stratford, 1999). Although of the different chemical structure, the weak acid-based additives have a common mode of action, such as antimicrobial agents. According to mechanism presented to Lambert and Stratford (1999), the undissociated acid diffuses through the microbial membrane and in the cytoplasm, with a pH close to neutrality, it is dissociated. For each acid molecule dissociated within the cell, releases a proton of H^+ and the environment becomes more acidic. The pumping of protons out of the cell, by the H^+-ATPase pump, causes a delay and reduction on microbial growth. The accumulation of protons inside the cell can lead inhibition of glycolysis (Krebs et al., 1983), of the active transport (Freese et al., 1973) and intervention on transduction of the signal.

In general, the content of propionic acid in silages is low and can be formed from the reduction of lactic acid by propionic acid bacteria and *Veillonella alcalescens* under low pH. This acid also can be produced from the deamination and decarboxylation of amino acids, such as alanine, by *Clostridium propionicum* (McDonald et al., 1991) and by strains of *Lactobacillus diolivorans*, where the 1,2-propanediol is degraded at equimolar amounts of 1-propanol and propionic acid (Krooneman et al., 2002). Propionic acid and its salts are effective antimicrobial agents used in the food industry (Doores, 2005) and undissociated propionic acid presents antifungal properties able to remain active on the membrane of microorganisms altering its permeability (Kung, 2010). The effectiveness of propionic acid base additives improved with increasing application rate in corn silage (Kung et al., 2000) and in high moisture corn silage (HMC) had good results in the control of the yeasts, ethanol production and aerobic stability (Auerbach et al., 2015).

Surface agents, generally referred to as surfactants, are substances that reduce the surface tension between oil-water or water-oil, thus increasing emulsification and emulsion stability, and are widely used in the food and pharmaceutical industries. Surfactants have lipophilic (hydrocarbon chain) and hydrophilic (polar head) properties in the same molecule (Mahmood and Al-koofee, 2013). Synthesis of polysorbates occurs by the bonding (polymerization) of ethylene oxide to the sorbitan fatty acid esters (Mahungu and Artz, 2001) and are nonionic surfactants. The polysorbate 80 (Tween 80), of chemical name Polyoxyethylene (20) sorbitan monooleate, has the hydrophilic group formed by oxyethylene chains, and the hydrophobic group mainly by oleic acid (Kaur and Mehta, 2017). The number twenty (20) in the chemical nomenclature refers to the approximately number of oxyethylene groups found in the molecule. The surfactants have several functional properties in food industries, like the stabilization of emulsions, humectant, solubilization, softening, among others (Mahungu and Artz, 2001). Synthetic surfactants are included in the food and may influence its structure through physical mechanisms, for instance the interaction with amylose and amylopectin chains of the starch, present in several basic food (Vernon-Carter et al., 2018). In wheat starch, nonionic surfactants demonstrated had ability to form strong surfactant-amylose complex and are difficult to dissociate (Ghiasi et al., 1982). There are few studies approaching about the feed digestibility in the presence of synthetic surfactants and in silage is unknown. In studies investigating the role of surfactants, including polysorbate, on moisture migration in bakery products, showed that the surfactant was adsorbed onto the starch surface and this complexation reduced the rate water migration to crust, delaying firming and keeping characteristic of freshly bread (Stampfli and Nersten, 1995; Mahungu and Artz, 2001).

1.1. Objectives

The objective of this study was to evaluate the effect of treatment with propionic acid, polysorbate 80 or propionic acid and polysorbate 80 mixture on reconstituted corn grain silage stored for 15, 30 or 60 d on chemical composition, ruminal *in situ* degradability and silage conservation.

LITERATURE CITED

Arcari, M. A., C. M. M. R. Martins, T. Tomazi, J. L. Gonçalves, and M. V. Santos. 2016. Effect of substituting dry corn with rehydrated ensiled corn on dairy cow milk yield and nutrient digestibility. Anim. Feed Sci. Technol. 221:167–173. doi:10.1016/j.anifeedsci.2016.08.005.

Auerbach, H., U. Weber, G. Weber, K. Weiss, and P. Theobald. 2015. Effects of different chemical additives on the fermentation and aerobic stability of high-moisture corn ensiled in bags. Pages 542-543 in Proc. 17th International Silage Conference, Piracicaba, SP, Brazil. ESALQ-USP, Piracicaba, Brazil.

Conab (Companhia Nacional de Abastecimento). 2019. Acompanhamento da safra brasileira de grãos, Dezembro 2019. 7:1-106. Acessed Jan. 10, 2020. https://www.conab.gov.br/info-agro/safras/graos/boletim-da-safra-de-graos

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

Da Silva, N. C., C. F. Nascimento, V. M. A. Campos, M. A. P. Alves, F. D. Resende, J. L. P. Daniel, and G. R. Siqueira. 2019. Influence of storage length and inoculation with *Lactobacillus buchneri* on the fermentation, aerobic stability, and ruminal degradability of high-moisture corn and rehydrated corn grain silage. Anim. Feed Sci. Technol. 251:124–133. doi:10.1016/j.anifeedsci.2019.03.003.

Doores, S. 2005. Organic Acids. Pages 91-142 in Antimicrobials in Food, 3rd ed. P. M. Davidson, J. N. Sofos, and A. L. Branen, ed. Taylor and Francis Group, Boca Raton, FL.

Ferraretto, L. F., P. M. Crump, and R. D. Shaver. 2013. Effect of cereal grain type and corn grain harvesting and processing methods on intake, digestion, and milk production by dairy cows through a meta-analysis. J. Dairy Sci. 96:533–550. doi:10.3168/jds.2012-5932.

Freese, E., C. W. Sheu, and E. Galliers. 1973. Function of lipophilic acids as antimicrobial food additives. Nature. 241:321-325.

Ghiasi, K., E. Varriano-Marston, and R. C. Hoseney. 1982. Gelatinization of Wheat Starch. II. Starch-Surfactant Interaction. Cereal Chem. 59:86–88.

Kaur, G., and S. K. Mehta. 2017. Developments of Polysorbate (Tween) based microemulsions: Preclinical drug delivery, toxicity and antimicrobial applications. Int. J. Pharm. 529:134–160. doi:10.1016/j.ijpharm.2017.06.059.

Krebs, H. A., D. Wiggins, M. Stubbs, A. Sols, and F. Bedoya. 1983. Studies on the mechanism of the antifungal action of benzoate. Biochem. J. 214:657-663.

Krooneman, J., F. Faber, A. C. Alderkamp, S. J. H. W. Oude Elferink, F. Driehuis, I. Cleenwerck, J. Swings, J. C. Gottschal, and M. Vancanneyt. 2002. *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. Int. J. Syst. Evol. Micr. 52:639-646. doi:10.1099/ijs.0.01979-0.

Kung, L. 2010. Aerobic stability of silage. Pages 1–14 in Proceedings California Alfalfa & Forage Symposium and Corn/Cereal Silage Conference, Visalia, CA.

Kung, L., J. R. Robinson, N. K. Ranjit, J. H. Chen, C. M. Golt, and J. D. Pesek. 2000. Microbial populations, fermentation end-products, and aerobic stability of corn silage treated with ammonia or a propionic acid-based preservative. J. Dairy Sci. 83:1479–1486. doi:10.3168/jds.S0022-0302(00)75020-X.

Kung, L., M. R. Stokes, and C. J. Lin. 2003. Silage Additives. Pages 305-360 in Silage Science and Technology. R. D. Buxton, R. E. Muck, and J. R. Harrisson, ed. American Society of Agronomy, Madison, WI.

Kung, L., R. D. Shaver, R. J. Grant, and R. J. Schmidt. 2018. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. J. Dairy Sci. 101:4020–4033. doi:10.3168/jds.2017-13909.

Lambert, R. J., and M. Stratford. 1999. Weak-acid preservatives: Modeling microbial inhibition and response. J. Appl. Microbiol. 86:157-164.

Mahmood, M. E., and D. a F. Al-koofee. 2013. Effect of Temperature Changes on Critical Micelle Concentration for Tween Series Surfactant. Global Journal of Science Frontier Research Chemistry 13:1–7.

Mahungu, S. M., and W. E. Artz. 2001. Emulsifiers. Pages in Food Additives, 2nd ed. A. L. Branen, P. M. Davidson, S. Salminen, J. H. Thorngate, ed. Marcel Dekker, Inc., New York, NY.

McDonald, P., N. Henderson, and S. Heron. 1991. The Biochemistry of Silage. 2nd ed. Chalcombe Publications, Marlow, UK.

Owens, F. N., R. A. Zinn, and Y. K. Kim. 1986. Limits to starch digestion in the ruminant small intestine. J. Anim Sci. 63:1634–1648. doi:10.2527/jas1986.6351634x.

Philippeau, C., J. Landry, and B. Michalet-Doreau. 2000. Influence of the protein distribution of maize endosperm on ruminal starch degradability. J. Sci. Food Agric. 80:404–408. doi:10.1002/1097-0010(200002)80:3<404::AID-JSFA541>3.0.CO;2-Z.

Stampfli, L., and B. Nersten. 1995. Emulsifiers in bread making. Food Chem. 52:353–360. doi:10.1016/0308-8146(95)93281-U.

Vernon-Carter, E. J., J. Alvarez-Ramirez, L. A. Bello-Perez, A. Garcia-Hernandez, C. Roldan-Cruz, and S. Garcia-Diaz. 2018. In vitro digestibility of normal and waxy corn starch is modified by the addition of Tween 80. Int. J. Biol. Macromol. 116:715–720. doi:10.1016/j.ijbiomac.2018.05.076.

2. EFFECT OF LENGTH OF STORAGE AND CHEMICAL ADDITIVES ON THE NUTRITIVE VALUE AND STARCH DEGRADABILITY OF RECONSTITUTED CORN GRAIN SILAGE

ABSTRACT

The objective of this trial was to evaluate the nutritive value and ruminal *in situ* starch degradability of reconstituted corn grain silage (RCGS) treated with chemical additives stored for different lengths of time. Dry ground corn (177.2 kg) was rehydrated to 350 g/kg moisture and ensiled in 5-L plastic buckets sealed with plastic lids (mini-silo). The rehydrated grain was treated with polysorbate 80 (2 L/t) (POL), propionic acid 28% (2 L/t) (PRO), Mycoflake™ (2 L/t) (MYC) or nothing (CON). Mycoflake is a blend polysorbate 80 plus propionic acid. The effect of length of storage (15, 30, or 60 d) was also evaluated and combined in a factorial arrangement with the additives. Four replicates were used per treatment. Ammonia nitrogen increased from 15 d of storage from 30 and 60 d, 53.2 and 59.4%, respectively. There was an interaction between additive and storage length for ethanol content, at 60 d of storage all treatments had lower ethanol concentration than CON. A treatment vs. storage length interaction was observed for butyric acid content, at 30 and 60 d of storage, the untreated silages had higher butyric acid concentration than treated silages. Aerobic stability increased from 15 to 30 d, but was the same at 30 and 60 d. At 15 d of storage, the treatments PRO and MYC decreased the DM losses. The length of storage increased the ruminal *in situ* degradability of starch and dry matter (DM) and MYC increased in 3.6% the DM degradability at 12 h of incubation compared with POL. In conclusion, increasing the storage time of RCGS from 15 to 60 d improved starch and DM degradability, Mycoflake increased the availability of nutrients and POL can be a potential antimicrobial agent.

Keywords: Reconstituted corn grain silage; Length of storage; Chemical additive; Polysorbate 80

2.1. INTRODUCTION

In Brazil, corn grain is the main source of grain used in beef cattle feedlots and high moisture corn represents 6.1% of the type of processing chosen by nutritionists, according to Pinto and Millen (2016). The pericarp, present on corn grains, coats the germ and endosperm, preventing the microbial attachment and degradation in the rumen. In the endosperm, which contains the most energy source, starch granules are associated in a protein matrix (Huntington, 1997). Breakdown of the protein matrix that surrounds the starch granules, is an important factor to increase starch digestion (McAllister et al., 1993). Increase grain processing improves starch digestion in ruminants. Corn grain ensiling is a processing method that has been associated with increased ruminal starch digestibility (Owens et al., 1986).

Chemical additives, such as acids, can be applied onto silages to prevents the growth undesirable microorganisms and improve aerobic stability (Muck et al., 2018). The use of propionic acid in high moisture corn prevents the growth of molds and yeasts during the fermentation process and when the silages are exposes to the air (Sebastian et al., 1996) and utilization of buffered propionic acid-base additives decrease yeasts counting in high moisture corn grain silage (Kung et al., 2004).

Non-ionic surfactants, as polyoxyethylene (20) sorbitan monooleate (Tween 80), have wide functional properties in food industries, like the emulsification of water-in-oil (margarine) and oil-in-water (mayonaise), humectant, improved of solubility in drinks, softening in baked products, among others (Mahungu and Artz, 2001). Beyond the use in pharmaceutical formulations to improve permeability by acting on modification hydrogen bonding and ionic forces (Kaur and Mehta, 2017). Therefore, we hypothesized that treatment of RCGS with propionic acid would lead to a higher aerobic stability, whereas the addition of polysorbate 80 might increase permeability of material and allow the absorption of moisture early by grain, increasing the ruminal starch degradability at short storage length.

2.2. MATERIAL AND METHODS

2.2.1. Ensiling Process

The experiment was conducted at the Department of Animal Science of the "Luiz de Queiroz" College of Agriculture/University of São Paulo, São Paulo - Brazil. An unknown corn hybrid used to make the grain silage reconstituted. The corn grains were ground before ensiling, with a hammer mill through a 5-mm screen and dry matter (DM) was determined by oven drying at 105° C for 12 h. Dry ground corn was mixed with distilled water manually for 20 min to achieve a moisture content of 35%. One hundred and seventy seven point two kilograms of silage were prepared and ensiled in forty-eight 5-L plastic buckets fitted with sealing plastic lids (mini-silo). Packing was performed manually, to reach a density of approximately 1000 ± 50 kg/m³ as fed.

The rehydrated corn grain (35% of moisture) was split into 4 piles (44.3 kg per pile) per treatment, each pile was treated with the following: (1) without additive (control); (2) polysorbate 80 (2 L/t) (Tween™ 80); (3) propionic acid 28% (2 L/t); and (4) Mycoflake™ (2 L/t) (Kemin América do Sul, Indaiatuba, SP, Brazil), based on recommendations of the company. The effect of length of storage (15, 30, or 60 d) was also evaluated and combined in a factorial arrangement with the additives. Four replicates were used per treatment. Additives were previously diluted in the distilled water used for reconstitution (88.6 mL) and the control received the same amount of water (88.6 mL). Samples of rehydrated corn grain were collected before ensiling and frozen for further analysis.

2.2.2. Laboratory analysis

Silos were weighed after filling and at the opening of the buckets to determine DM recovery according to (Jobim et al., 2007). After the opening, the silages were mixed and samples were collected for the analysis of the chemical composition, ruminal *in situ* starch degradability, aerobic stability, and fermentation profile.

Silage samples were dried in a forced ventilation oven for 72 h at 55°C and then ground through a 1-mm screen (Wiley mill, Arthur H. Thomas, Philadelphia, PA). Sub-samples were analyzed for DM, ash, and ether extract (EE) (AOAC, 1990; methods 934.01, 942.05 and 920.39, respectively). The ash free neutral detergent fiber (NDF) was analyzed by filtration in porous crucible with sodium sulfite and heat stable amylase (Mertens, 2002). The ash free acid detergent fiber (ADF) was analyzed by filtration in porous crucible (Mertens, 2002). Starch content was determined by an enzymatic method according to (Hall, 2009). The crude protein (CP) was measured by the Dumas method (Leco® FP-2000A nitrogen analyzer; Leco corp., St. Joseph, MI). Soluble protein (% of CP) was estimated by submitting the samples to a borate phosphate buffer bath at 39°C for 1 h and filtered on Whatman™ N° 541 filter paper (Krishnamoorthy et al., 1982). Soluble protein content was estimated from the difference between the silage sample CP content and the residual CP content of the filtered sample.

Table 1. Dry matter content and chemical composition of fresh reconstituted corn grain (n = 4).

Item	Mean	SD[1]
Dry matter, % as fed	63.62	0.16
Crude protein, % of DM	8.68	0.42
Neutral detergent fiber, % of DM	9.39	1.24
Acid detergent fiber, % of DM	2.57	0.31
Ether extract, % of DM	3.14	0.15
Ash, % of DM	1.20	0.06
Starch, % of DM	66.8	2.89
Soluble protein, % of CP	16.8	3.39

[1]Standard deviation

A fresh subsample of silage (25 g) was mixed with 225 mL of distilled water in a stomacher (Lab-Blender Stomacher®, Nova Ética Produtos e Equipamentos Científicos Ltda., Vargem Grande Paulista, Brazil) for 4 min. The pH of the extract was measured using a pH meter (TEC-7, Tecnal Equipamentos, Piracicaba, Brazil). Aqueous extract was filtered through 2 layers of cheesecloth and centrifuged at 10,000 x g for 15 min. The supernatant was used to the measured lactic acid content (Pryce, 1969) and the ammonia nitrogen content by the method of Chaney and Marbach (1962), adapted by (Weatherburn, 1967). Concentrations of volatile fatty acids, alcohols and esters were determined by gas chromatography with a mass spectrophotometer detector (GCMS QP2010 Plus; Shimadzu, Kyoto, Japan) using a capillary column (Stabilwax; Restek, Bellefonte, PA; 60 m length, 0.25 mm outside diameter, 0.25 μm film thickness). The silage DM content was corrected for volatile compounds (Weissbach, 2009).

Aerobic stability test was determined at each silo opening time and approximately 2 kg of samples of RCGS, were placed in buckets without compaction and exposed to air in the laboratory (21.5 ± 2.3°C). The ambient and silages temperatures were measured every 30 min for 240 h using a data logger (Tag Temp, NOVUS Produtos Eletrônicos Ltda., Canoas, Brazil) placed in the geometric center of the mass. Aerobic stability was calculated as the number of hours the silage temperature kept stable before rose 2°C above the ambient (Kung et al., 2000).

The kernel particle size distribution was measured using approximately 400 g of dried sample in a Ro-Tap Shaker (Bertel® Ltda., Caieiras, Brazil) equipped with five screens of 4.75, 3.35, 2.36, 1.70, 1.18, 0.59 mm and bottom pan. Geometric mean particle size (GMPS; μm) and surface area ($cm^2.g^{-1}$) were determined by log normal distribution (Baker and Herrman, 2002) as reported by Dias Junior et al. (2016).

To determine the ruminal *in situ* starch and DM degradability, 6 g of dried sample were placed in nylon bags (10 cm x 20 cm) with 50 ± 10 μm of porosity (model R1020, Ankon, Macedon, NY). Bags were incubated in 2 rumen-cannulated Holstein dairy cows fed a total mix ration (TMR) containing corn silage (59.1% of DM), citrus pulp (12.6% of DM), dry ground corn (11.2% of DM), soybean meal (13.9% of DM), mineral premix (2.5% of DM) and urea (0.7% of DM). Two replicate from each silo were incubated in each cow for 12 h. After incubation, the bags were removed and put in ice-cold water to stop the fermentation and then washed machine. Bags were dried at 55°C for 72 h in a forced ventilation oven and weighed. After drying the residues obtained from duplicate samples (two rumen-cannulated cows) were ground through a 1-mm screen (Wiley mill, Arthur H. Thomas, Philadelphia, PA) to determine starch content. Blank bags (without sample) were also incubated, to allow for the correction for particle contamination. The weights of the blank bags were subtracted of the bags with sample. The ruminal *in situ* DM degradability was calculated based on initial DM and residual DM and starch degradability base on initial starch and residual starch. The experimental procedures were approved by the ethic committee of USP/ESALQ, Piracicaba (protocol: 2018.5.1093.11.4).

2.2.3. Statistical analyses

Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC), as a completely randomized design with a factorial arrangement of treatments, 4 additives x 3 times of storage, and 4 replicates per treatment. The model was following: $Y_{ij} = \mu + A_i + T_j + AT_{ij} + e_{ij}$, where μ = overall mean, A_i = fixed effect of additive (i = CON, POL, PRO or MYC), T_j = fixed effect of storage length (j = 15, 30, or 60 days) as repeated measure, AT_{ij} = interaction between additive and length of storage, and e_{ij} = error. A mini-silo was represented as the experimental unit. The covariance structure for the repeated measured was chosen based on Akaike's information criterion among variance components (VC), compound symmetry (CS), first-order autoregressive (AR(1)) or unstructured covariance structure (UN). For the ruminal *in situ* degradability assay at 12 h, two cannulated cows were included as random effect of blocks, the model was following: $Y_{ij} = \mu + A_i + T_j + B_k + A_iT_j + e_{ijk}$, where μ = overall mean, A_i = fixed effect of additive (i = CON, POL, PRO or MYC), T_j = fixed effect of storage length (j = 15, 30 or 60 days) as repeated measure, B_k = random effect of block (k = 1 and 2), A_iT_j = interaction between additive and length of storage, and e_{ijk} = error . All means were compared using the Tukey's test at 5% probability. Statistical significance was considered at $P \leq 0.05$.

2.3. RESULTS

2.3.1. Chemical composition

Table 1 shows the DM content and chemical composition of reconstituted corn grain before ensiling. The DM content was higher ($P < 0.05$) at 15 and 30 d than at 60 d of ensiling (Table 2). The CP content decreased ($P < 0.01$) over time and was lower in silages treated with Mycoflake compared to the other treatments ($P < 0.05$). We observed that the ash content increased ($P < 0.01$) over time and silages treated with propionic acid had lower ($P < 0.01$) ash content than the other treatments.

There was an interaction ($P < 0.01$) between additive and storage length for soluble protein content. At 15 d, POL had lower ($P < 0.05$) soluble protein content than CON and MYC, and after 30 d it had lower ($P < 0.05$) soluble protein content than MYC. After 60 d of ensiling, no differences were observed among treatments (Figure 1). Ammonia nitrogen increased ($P < 0.05$) from 15 d of storage from 30 and 60 d, 53.2 and 59.4%, respectively.

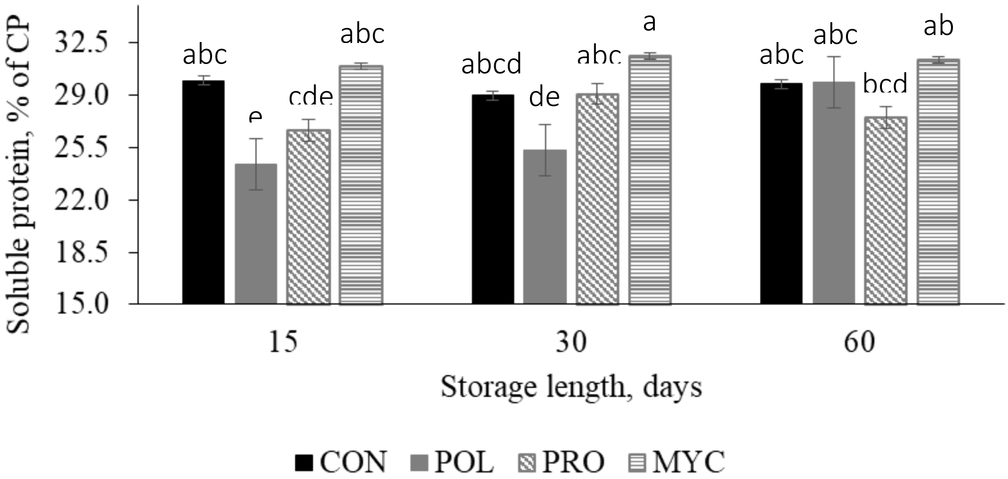

Figure 1. Soluble protein content of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON) or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: $P < 0.01$; effect of storage length: $P = 0.03$; effect of the interaction between additive and storage length: $P < 0.01$; SEM = 0.81.

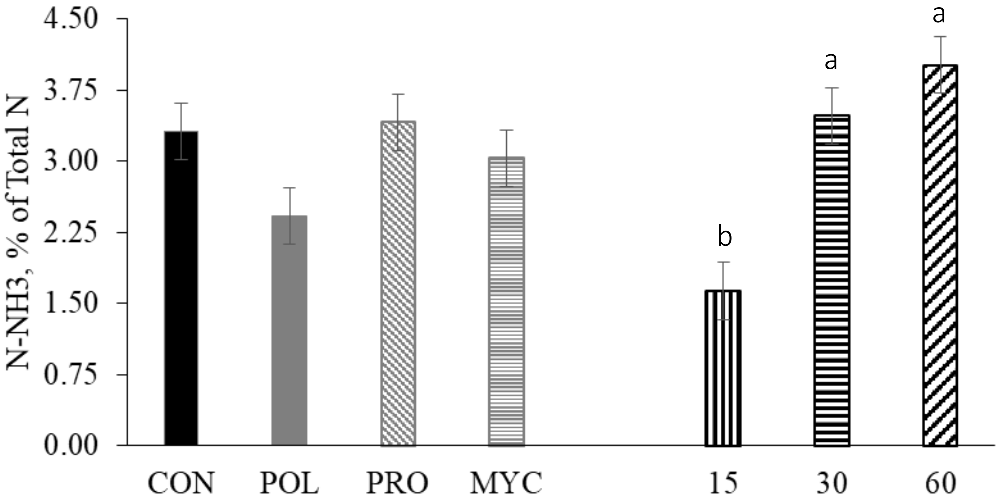

Figure 2. NH_3-N of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: $P = 0.06$, SEM = 0.27; effect of storage length: $P < 0.01$, SEM = 0.28; effect of the interaction between additive and storage length: $P = 0.08$.

The length of storage and treatment changed the kernel particle size distribution (Table 3). No particle of grain retained at 4.75 and 3.35-mm sieves. Percentage of particles retained at 2.36 and 1.7-mm sieves increased ($P < 0.05$) for 30 and 60 d of storage. At 1.18-mm sieve there was an increasing of particles retained by 2.0 percentage units, for 15 d of storage compared with 60 d. There was a rise of the percentage of particles at 0.59-mm sieve for 60 d of storing in comparison with 15 and 30 d ($P < 0.05$). At pan there was decreased of material retained at 60 d compared with 15 d of storage ($P < 0.05$). The MYC had lower percentage of grain held at 1.7 and 1.18-mm sieves compared to the other treatments. At 0.59-mm sieve, the PRO and MYC decreased amount of particles kept regarding CON and POL. However, the percentage of material held at pan was higher ($P < 0.05$) for MYC than other treatments. It was observed an increase ($P < 0.05$) in GMPS at silages stored for 60 d and with the MYC there was a reduction ($P < 0.05$) compared with CON and POL. A decreased ($P > 0.05$) in surface area occurred in silages stored for 60 d and the MYC presented a higher surface area.

2.3.2. Fermentation profile

There was an interaction ($P < 0.01$) between additive and storage length for pH (Figure 3). At 30 d of ensiling only PRO had lower ($P < 0.05$) pH than CON, but after 60 d all silages treated with additives had lower ($P < 0.05$) pH than CON. The lactic acid concentration was higher ($P < 0.05$) at 15 d in comparison with 30 d of storage (Figure 4). In the Figure 5, the POL had lower, PRO had the same and MYC had higher concentration of acetic acid compared to CON ($P < 0.05$). There was an interaction ($P < 0.01$) between additive and storage length for ethanol content (Figure 6). At 30 d, only POL and PRO had lower ($P < 0.05$) ethanol concentration than CON, but at 60 d all treatments including MYC had lower ($P < 0.05$) ethanol concentration than CON.

Table 2. Chemical composition of reconstituted corn grain ensiled for 15, 30 or 60 d, without additive treatment (CON), or treated with polysorbate 80 (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC).

	Treatment					Storage length				*P-value*[3]		
Item	CON	POL	PRO	MYC	SEM	15	30	60	SEM	A	S	A x S
DM,[1] % as fed	63.1	63.2	63.6	63.3	0.14	63.7[a]	63.4[a]	62.9[b]	0.12	0.12	<0.01	0.65
CP,[2] % of DM	8.46[a]	8.40[a]	8.40[a]	7.88[b]	0.05	8.53[a]	8.27[b]	8.06[c]	0.04	<0.01	<0.01	0.15
Ash, % of DM	1.16[a]	1.08[ab]	0.92[c]	1.05[b]	0.03	0.98[b]	1.06[a]	1.12[a]	0.02	<0.01	<0.01	0.09
Starch, % of DM	68.2	68.2	67.5	68.4	1.54	70.7	66.3	67.3	1.32	0.97	0.06	0.79

[1]Dry matter; [2]Crude protein; [3]Effect of additive (A), storage length (S), and the interaction between additive and storage length (A x S). [a-c]Rows with unlike letters differ by the Tukey's test ($P \leq 0.05$).

Table 3. Kernel fraction particle size distribution, geometric mean particle size (GMPS) and surface area of reconstituted corn grain ensiled for 15, 30 or 60 d, without additive treatment (CON), or treated with polysorbate 80 (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC).

	Treatment					Storage length				*P-value*[2]		
Item	CON	POL	PRO	MYC	SEM	15	30	60	SEM	A	S	A x S
Sieve,[1] mm												
2.36	1.7	1.7	1.7	1.6	0.07	1.5[b]	1.8[a]	1.8[a]	0.06	0.56	<0.01	0.18
1.7	7.0[a]	7.1[a]	7.3[a]	6.2[b]	0.21	6.3[b]	7.0[a]	7.4[a]	0.18	<0.01	<0.01	0.28
1.18	18.9[a]	19.1[a]	18.4[a]	16.5[b]	0.45	17.2[b]	18.3[ab]	19.2[a]	0.39	<0.01	<0.01	0.15
0.59	44.9[a]	44.8[a]	43.3[b]	42.4[b]	0.37	43.2[b]	43.4[b]	44.9[a]	0.32	<0.01	<0.01	0.40
Pan	27.2[b]	26.8[b]	29.4[b]	33.3[a]	0.92	31.6[a]	29.3[ab]	26.6[b]	0.80	<0.01	<0.01	0.15
GMPS, µm	94.5[a]	93.9[a]	80.3[ab]	60.8[b]	5.64	69.1[b]	79.5[b]	98.5[a]	4.89	<0.01	<0.01	0.09
Surface area, cm^2/g	106.2[b]	106.2[b]	110.5[ab]	116.5[a]	1.59	113.8[a]	110.6[a]	105.1[b]	1.38	<0.01	<0.01	0.07

[1]Percentage of particles retained on each sieve (DM basis); [2]Effect of additive (A), storage length (S), and the interaction between additive and storage length (A x S). [a-b]Rows with unlike letters differ by the Tukey's test ($P \leq 0.05$).

As expected the propionic acid concentration was higher (*P* < 0.05) in silages treated with additives that contained propionic acid (PRO and MYC) than for CON and POL at all lengths of storage analyzed (Figure 7). A treatment vs. storage length interaction (P < 0.01) was observed for butyric acid content (Figure 8). All silages stored for 15 d had lower and similar butyric acid concentration, but at 30 and 60 d of storage, the untreated silages had higher butyric acid concentration than treated silages. Aerobic stability of RCGS increased (*P* < 0.05) from 15 to 30 d, but was the same at 30 and 60 d (Figure 9). At 15 d of storage, the treatments PRO and MYC decreased the DM losses (Figure 10). At 30 d, the propionic acid was more efficient to control the losses in the silo and at 60 d all treated silages presented lower (*P* < 0.05) DM loss than CON.

2.3.3. Ruminal *in situ* degradabitily

Silages stored for 30 and 60 d had 6.98 and 12.5% higher ruminal *in situ* degradability of starch (*P* < 0.01), respectively, compared to silages stored for 15 d (Figure 11). The MYC increased (*P* = 0.02) in 3.6% the DM degradability in 12 h of incubation compared with POL. There was an increase in the DM degradability (*P* < 0.01) over time, silages ensiled for 30 and 60 d had 5.7 and 16.8% higher DM degradability than silages ensiled for 15 d, respectively (Figure 12).

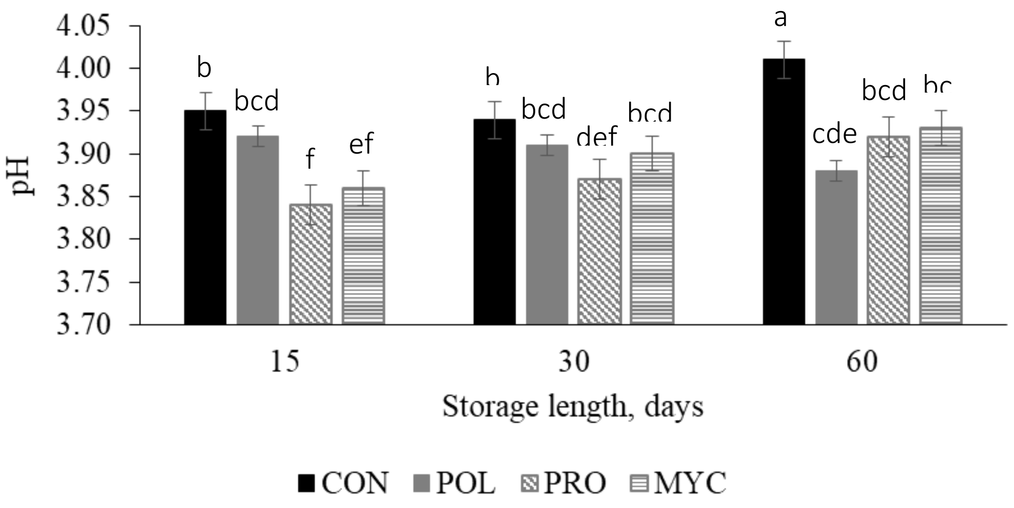

Figure 3. pH of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: *P* < 0.01; effect of storage length: *P* < 0.01; effect of the interaction between additive and storage length: *P* < 0.01, SEM = 0.01.

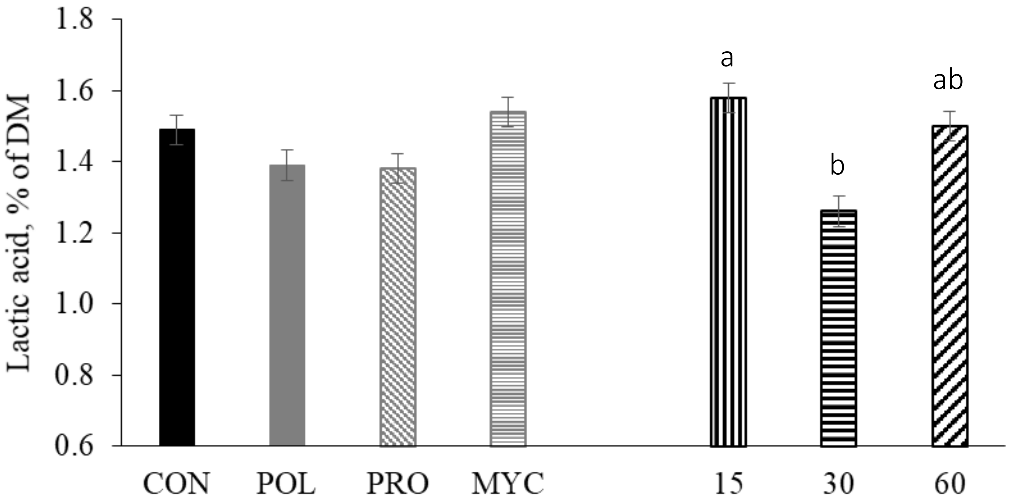

Figure 4. Lactic acid content of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: P = 0.56, SEM = 0.09; effect of storage length: P = 0.02, SEM = 0.08; effect of the interaction between additive and storage length: P = 0.33.

2.4. DISCUSSION

The data of CP, NDF, EE, ash, and starch of unfermented reconstituted corn grain had approximate values to those found in unfermented dry ground corn by Ferraretto et al. (2015). Silage fermentation depends on available moisture to enable microbial growth (Pahlow et al., 2003). The moisture content, by itself, is an important aspect to increase extension of fermentation and to improve the dry matter and starch degradability of grain silages (Goodrich et al., 1975; Benton et al., 2005). In the present study, corn kernels were reconstituted to achieve 35% of moisture, based on suggested literature data. The DM content decreased across the storage time due to the typical increase in dry matter losses. According to Morais et al. (2017), the chemical additives added to the grain silage, can alter the CP and ash content due its own composition. In general, the Mycoflake reduced particle size and increased surface area of the RCGS, while the length of storage increased the GMPS and therefore affected the surface area. However, we did not find in the literature an explanation about the chemical strategy and storage length changing the physical parameters of grain silage and it is a great finding that deserves to be explored.

In mature corn grains there are low soluble carbohydrates content because the starch formation in kernel endosperm is completed. The lactic acid bacteria (BAL) ferment soluble sugars, such as hexoses and pentoses, and yields lactic acid (McDonald et al., 1991), a strong acid able to drop the pH of silages. In the present study, silages were well fermented and pH variations were relatively small. According to Kung et al. (2018), the lengthening of the storage time may cause a decline in the pH of HMC due to a gradual increase in the concentrations of lactate and acetate throughout storage. Silages containing propionic acid improved fermentation over CON. The use of propionic acid was also effective in reducing the pH of high moisture ear corn at 40 d of ensiling compared to untreated silage and silage that contained microbial inoculants in a study done by Sebastian et al. (1996).

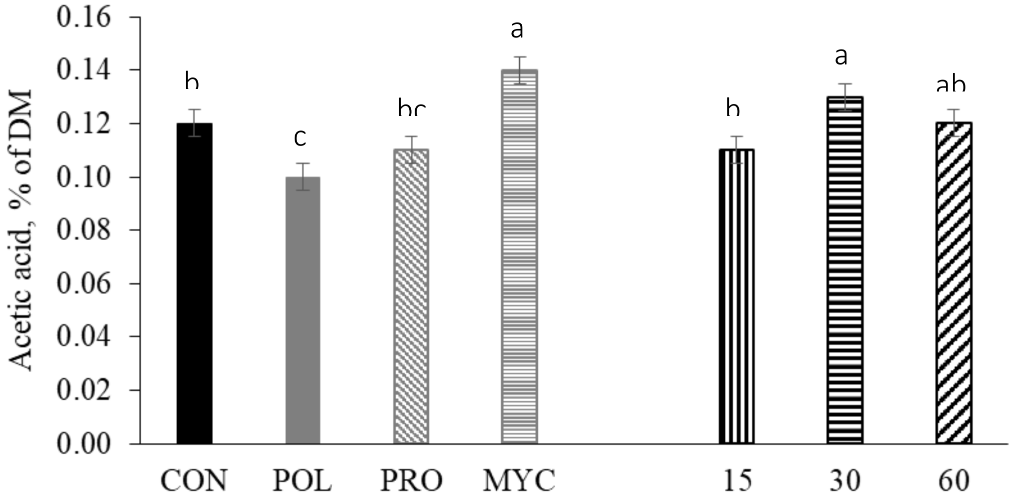

Figure 5. Acetic acid content of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: $P < 0.01$, SEM = 0.004; effect of storage length: $P < 0.01$, SEM = 0.003; effect of the interaction between additive and storage length: $P = 1.00$.

The main contributors of the proteolysis in reconstituted corn grain silage are bacteria and kernel enzymes, in approximately 60% and 30%, respectively (Junges et al., 2017). Several factors affect the breakdown of the protein matrix surrounding the starch granule in the grain endosperm, such as processing degree, moisture content, and ensiling time (Benton et al., 2005; Hoffman et al., 2011; Ferraretto et al., 2015). In the present study, results of soluble protein content were inconsistent, making it difficult to explain proteolytic activity only with this trait. However, NH_3-N is also an indicator of deamination during fermentation process (Ohshima and McDonald, 1978). In the current trial, the length of storage increased NH_3-N at first days of ensiling, probably allowing microorganisms to access to starch granules, making starch more digestible. Hoffman et al. (2011) observed a steadily increases in NH_3-N up to 240 d of storage in high moisture corn silage, an indicator of endosperm protein degradation. Conversely, it was observed a reduction in prolamin-zein subunits at 240 d of ensiling. Zein-proteins are strongly adhered to starch granules, forming a starch-protein matrix, where the extended ensiling time (240 d) degraded hydrophobic zein-proteins. In the present trial was not observed treatment effect in the concentration of NH_3-N and probably it had no influence on proteolytic activity. In a study evaluating different treatments with organic acids and storage length on high moisture corn grain silage, showed that ground corn treated with 0.2% acetic:propionic acid (20:80) did not prevent soluble nitrogen fraction formation compared to untreated silage (Baron et al., 1986). Prigge et al. (1976), did not found influence of a mixture of 80:20 propionic to acetic acid (1.5%) on the soluble nitrogen in high moisture ground corn stored for 28 d, but they observed an increased on soluble nitrogen in high moisture whole corn stored for 28 and 56 d compared to untreated silage. Proteolytic microorganisms, such as certain bacteria of the genus clostridia, *Clostridium sporogens* and *Clostridium bifermentans*, can develop in the early days of the fermentation process and deaminate amino acids by releasing ammonia. However, the development of these microorganisms depends on the rate of lactic acid production and pH fall, and its activity can be suppressed (Ohshima and McDonald, 1978). In addition to clostridia, enterobacteria are important ammonia-producing microorganisms in silages, through degradation of proteins and nitrate reduction, producing NH_3 (Pahlow et al., 2003). Taking to account the silages presented low pH in all treatments, the growth of these microorganisms probably was inhibited.

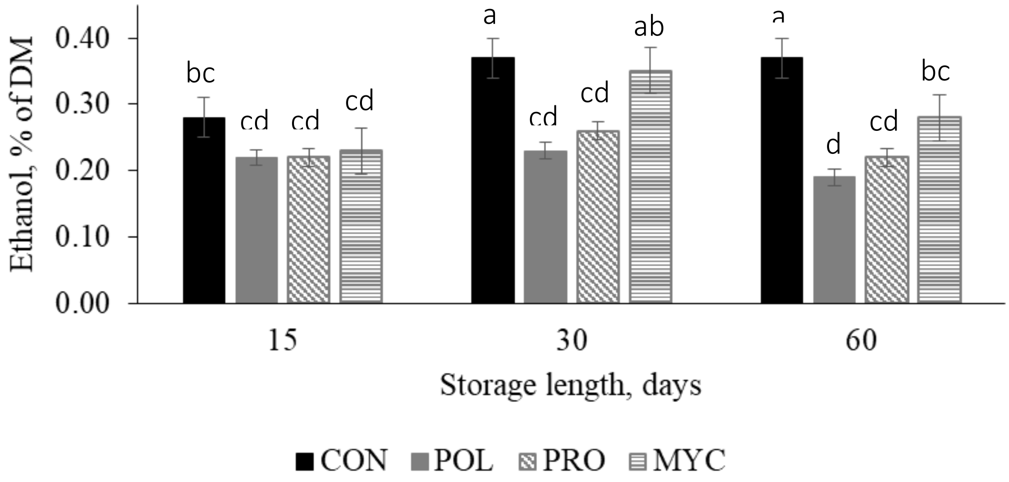

Figure 6. Ethanol content of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: $P < 0.01$; effect of storage length: $P < 0.01$; effect of the interaction between additive and storage length: $P < 0.01$, SEM = 0.02.

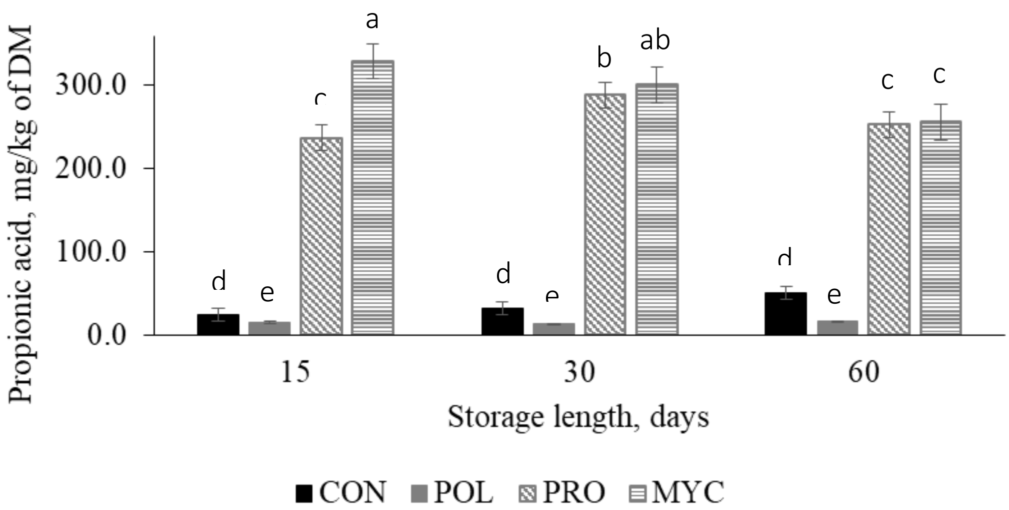

Figure 7. Propionic acid content of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: $P < 0.01$; effect of storage length: $P < 0.01$; effect of the interaction between additive and storage length: $P < 0.01$, SEM = 6.11.

Although the acetic acid content was low, it is in agreement with the values of high moisture corn silages, <0.5% of DM, according to Kung et al. (2018). The addition of *Lb. buchneri* at silages is capable to reduce lactic acid to acetic acid and 1,2-propanediol via anaerobic conversion (Oude Elferink et al., 2001) and consequently increase of acetic acid concentration in rehydrated corn grain silage improves the aerobic stability in comparison with untreated silages (Da Silva et al., 2018), because its strong antifungal characteristics. In the present study the increase of acetic acid content at 30 d of storage coincided to the reduced lactic acid content at the same ensiling time, demonstrating a mechanism of action that might have reduced lactic acid to acetic acid. This can be a defense mechanism of heterofermentative bacteria against a low pH, degrading lactic acid into a fatty acid with a higher pK_a (Oude Elferink et al., 2001). According to data of Da Silva et al. (2019), observed that there was a gradual accumulation of acetic acid with the increasing of storing in high moisture and rehydrated corn grain silage.

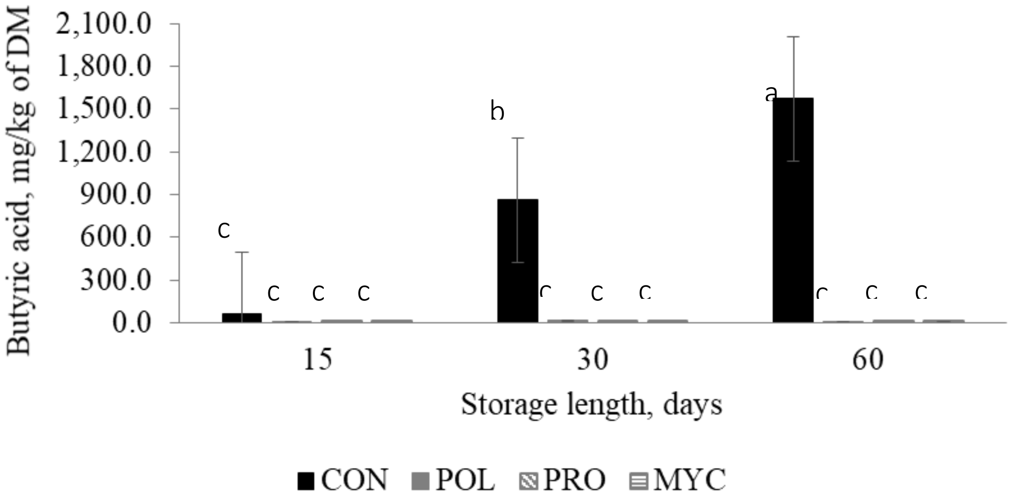

Figure 8. Butyric acid content of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: $P < 0.01$; effect of storage length: $P < 0.01$; effect of the interaction between additive and storage length: $P < 0.01$, SEM = 133.4.

The reduction of ethanol concentration from 30 d of storage in treated silages was most likely due to the effect of the propionic acid and polysorbate on the yeasts metabolism, which under anaerobic conditions can ferment sugars and produce ethanol as main fermentation product in silage (Pahlow et al., 2003). In environment with low pH (4.5), propionic acid (7.6 mmol/L) reduced the growth efficiency and growth rate of yeast (Moon, 1983). Corn silage treated with propionic acid base additives at an application rate of 0.3% (3 L/t), had lower ethanol content than untreated silage (Kung et al., 2000). A food-grade study evaluating a microemulsion composed of glycerol monolaurate (GML), propionic acid, Tween 80 and water (3:9:8:12), evaluated antimicrobial activity under *Escherichia coli* and *Staphylococcus aureus*, and showed that bacterial cells were completely killed after addition of the microemulsion. Even utilizing diluted microemulsion by 10-fold and 100-fold, it demonstrated loss of cell viability. The experiment proved that bacterial membrane hydrophobicity was altered in the presence of the microemulsion and there was release cytoplasmic material. According to the authors, the interaction between microbial membranes and antimicrobial microemulsions altered the phospholipid bilayer, affecting membrane permeability and leading to its rupture (Zhang et al., 2009). The same microemulsion with sodium benzoate combination was evaluated for anti-yeast activity against *Candida albicans* and *Saccharomyces cerevisiae*. The results showed that 1.2 mg/mL (minimum fungicidal concentration determined) of the microemulsion was able to inhibit growth of *C. albicans* and *S. cerevisiae* in liquid medium by broth dilution method (Zhang et al., 2010). However, anti-yeast activity of each microemulsion component was evaluated and observed that inhibition was mainly attributed to the combination of propionic acid, GML and sodium benzoate. The Tween 80 (polysorbate 80) did not demonstrate any microbial inhibition. Addition of Tween 80 is important in microemulsions as it provides stability of the components up to thousand-fold dilution (Kaur and Mehta, 2017). Although the application rate have been lower than the rate applied to corn silage, according to Kung et al. (2000), propionic acid was effective to control ethanol production after 30 d of ensiling. Despite the scarcity of studies of surfactant molecules in feed conservation, the opportunity to further investigate their role in inhibiting microorganisms in silages is evident, as its effect on the decrease of ethanol concentration in RCGS is clear on the present study.

The propionic acid presents high antifungal properties in a pH around 4.8, where 50% of the acid is at undissociated form (COOH). Undissociated acid can keep at surface of microorganisms and bind to active sites of enzymes or altering the membrane permeability (Kung et al., 2003; Kung, 2010). Propionic acid can be found in greater concentrations (>0.3-0.5%) in silages that likely had fermentations by *Clostridium propionicum* or with the use of chemical additives, that is effective to increase propionic acid concentration by about 0.15-0.30% of DM, but can range with the proportion of the acid (Kung et al., 2018). As expected, treatments that contained propionic acid had greater concentration of this acid in all storage times. However, there is no reasonable explanation for the difference of concentration between PRO and MYC silages at 15 d of storage, since they had the same application rate and showed the same concentrations on days 30 and 60.

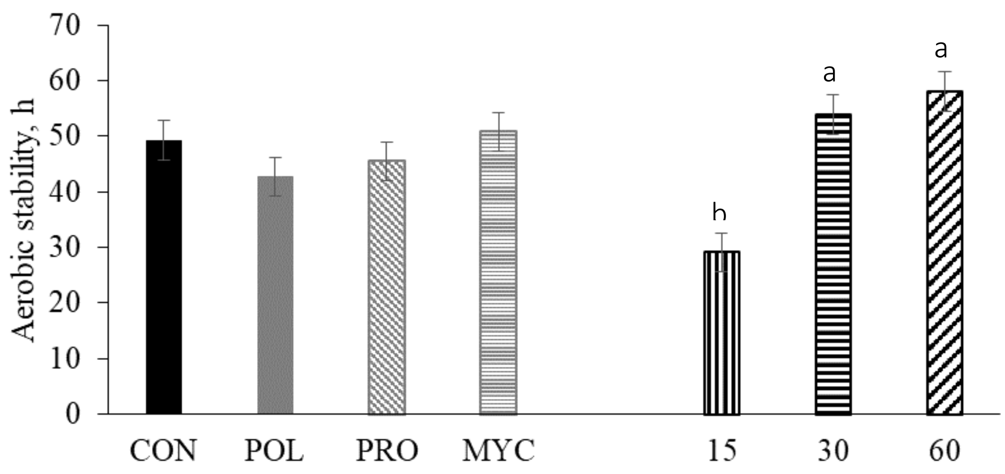

Figure 9. Aerobic stability of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: P = 0.10, SEM = 2.35; effect of storage length: P < 0.01, SEM = 2.04; effect of the interaction between additive and storage length: P = 0.42.

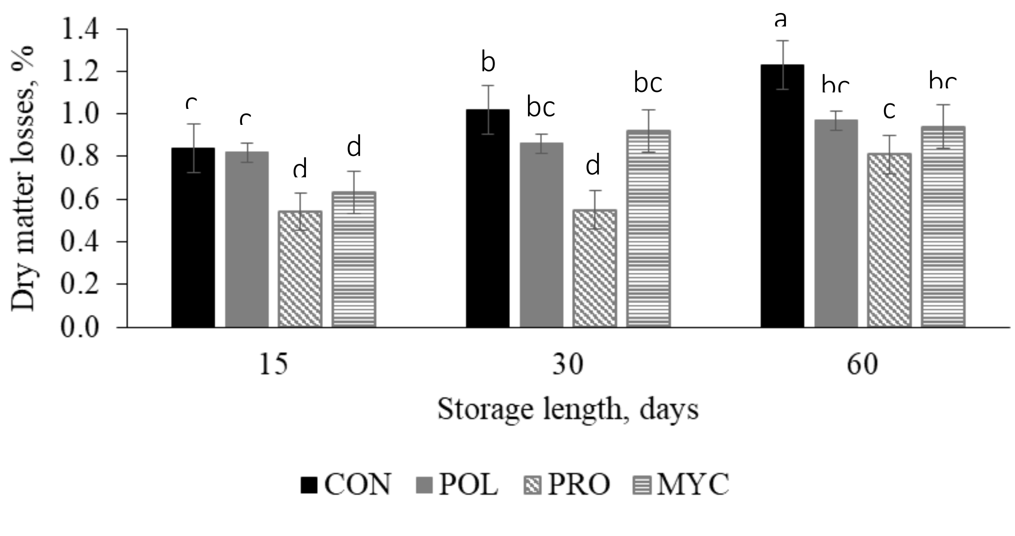

Figure 10. Dry matter losses of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: P < 0.01; effect of storage length: P < 0.01; effect of the interaction between additive and storage length: P < 0.01; SEM = 0.04.

A significant increase of butyric acid was observed in untreated silage stored for 30 and 60 d. Butyric acid is a product of the mainly *Clostridium* fermentation when there are low DM and water-soluble carbohydrate concentration (McDonald et al., 1991). The fermentation pathway is characterized by saccharolytic clostridia (Rooke and Hatfield, 2003). They are spore formers microorganisms and most of the species are obligatory anaerobic. Low water activity (a_w) is inhibitory factor to development of microorganisms. The lactic acid bacteria (LAB) can growth at a_w 0.98 but some genera are able to develop at a_w 0.94 (Woolford and Sawczyc, 1984), whereas *Clostridium* decrease your growth rate at a_w < 0.97 (Leibensperger and Pitt, 1987). During the field maturation process of crop, the moisture reduction is associated with the reduction of water activity. However, as mentioned the *Clostridium* has the advantage to keep sporulated when the environment is not suitable for their development. Therefore, it is unlikely that the moisture reduction of forage on the field affects the *Clostridium* population (McDonald et al., 1991). Besides that, a minimum LAB count come from epiphytic microflora or inoculants (100,000 cfu/g^{-1} of fresh matter) is needed to decrease *Clostridium* growth; and products of nitrate degradation, such as nitrite and nitric oxide, which can inhibit the activity these microorganisms (Pahlow et al., 2003). Fernandes (2014), performed a study evaluating different hybrids, maturation and storage length of corn grains silage, and found high levels of butyric acid as the storage increased from 0 to 120 d in reconstituted corn grain silages. The microbial profile demonstrated *Clostridium* population was already high compared to the *Lactobacillus* at the ensiling moment (19.5 e 9.1%, respectively) and at 120 d of storage represented about 40% of the microbial population (Carvalho et al., 2015). Dried grains suffer more stress during the maturation period in the field and this may interfere with the plant epiphytic population, favoring the occurrence these microorganisms in relation to the competing strains. Low production of butyric acid was observed in rehydrated corn gran silages (Da Silva et al., 2018; Da Silva et al., 2019) contrary to what we found in this study. The PRO, MYC and POL may have controlled *Clostridium* growth, since the butyric acid concentration was lower than the CON after 15 d of ensiling. Propionic acid in pH around 5 and 6 can prevent the endospore-forming bacteria (McDonald et al., 1991). Evaluating the minimum inhibitory concentration of straight chain fatty acids in several silage microorganisms, Woolford (1975) demonstrated that < 8mmol/L of propionic acid was necessary to inhibit clostridia growth at pH 6 and 5, while at pH between 3 to 4, this microorganism was inhibited by pH.

The addition of propionic acid in corn silage with 44% DM resulted in lower temperature during fermentation and feeding, controlled the development of fungi and deterioration compared to untreated silages, showing that the treated silages were more stable. The authors observed the same effects in silage with 36% DM, but were more subtle (Huber and Soejono, 1976). In a study evaluating different chemical additives, it showed that treatment of high moisture corn silage (HMCS) with a product containing propionic acid, ammonium propionate, sodium benzoate, and potassium sorbate (3 L/t) decreased yeast count resulting in higher aerobic stability than control silage (Auerbach et al., 2015). The use of chemical additives in HMCS has been shown to enhance aerobic stability due to efficient control of yeasts development and, according to data of literature review, Morais et al. (2017) showed that there was a linear increasing on the aerobic stability according to the dose of chemical additive, in the range of 1 to 4 g/kg^{-1} of fresh matter. Using the regression model for estimate stability in a best application rate (P < 0.01; R^2 = 0.81), indicated that 2 g/kg^{-1} of fresh matter obtained approximately 80 h of stability (Morais et al., 2017). However, propionic acid treatments were expected to increase the aerobic stability, but no difference was observed between treatments in our study. To control fungal growth and spoilage in feed with high concentration of DM, Collins (1995) suggested a propionic acid concentration range between 12.5 to 30.0 g/kg^{-1} of water and, as the water concentration increases, more acid is needed to inhibit molds development. In the present study, the rate of application based on recommendations was 2 L/t (28%). According to propionic acid concentration presents in the results, the content was equivalent to 862 mg/kg of DM (approximately 0.22% of DM). The lack of response in aerobic stability can be attributed to the concentration of the acid in the product or the application rate. Mycoflake was developed to treat grains before the flocculation process and, this type of processing requires less moisture (Sindt et al., 2006) than RCGS. The use of the Mycoflake in feed conservation opens doors for future studies with higher acid concentrations or application rates, seeking to increase the stability of ensiled grains. The improve in aerobic stability after 15 d of storage was probably due to increase of fermentation end products, as acetic acid, which has antifungal properties (McDonald et al., 1991). An increase in aerobic stability with increase of length of storage was also observed by Da Silva et al. (2019) in high moisture corn and rehydrated corn grain silage, with a parallel increase in the concentration of acetic acid.

The fermentation pathway depends on the substrates and microbial population and can lead to losses of DM and gross energy, by the production of carbon dioxide and hydrogen, respectively (Borreani et al., 2018). The addition of propionic acid was efficient to reduce dry matter losses when compared to CON, probably as a result of decreased ethanol content after 15 d of ensiling, because of the inhibition fermentation of sugars by yeasts, which produces ethanol and CO2 as main fermentation products (Pahlow et al., 2003), and results in loss of DM. In a meta-analysis, Goeser et al. (2015) developed a model to predict DM losses in forage, based on parameters related to forage and fermentation. The authors noted that the application of acids to silage, including propionic acid, improved DM recovery and appeared to be related to lower losses as DM increased. In the current study although POL and CON have had the same DM loss values, at 60 d of storage the untreated silage had higher value of DM losses and POL, PRO and MYC had lower suggesting the inhibition of yeasts.

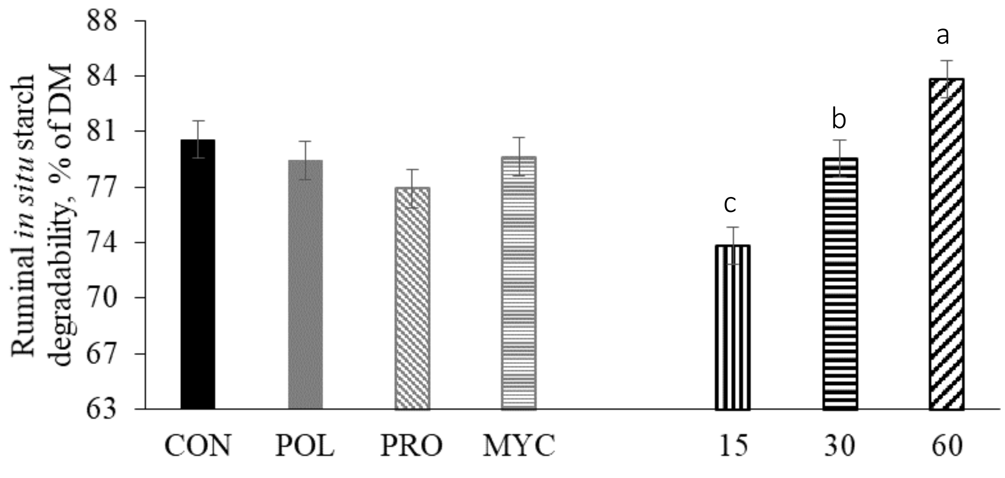

Figure 11. Ruminal *in situ* starch degradability in 12 h of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: P = 0.07, SEM = 1.03; effect of storage length: P < 0.01, SEM = 0.94; effect of the interaction between additive and storage length: P = 0.71.

The proteolysis indicators, such as soluble protein and ammonia nitrogen content in grain silage, are positively related to in vitro starch degradability (Ferraretto et al., 2014). In the current study, higher DM and starch degradability was observed over time possibly resulted from increased proteolytic activity of silages stored for 30 and 60 d. Although many factors influence starch digestibility, breakdown of the protein matrix is an important reason that facilitates the access of enzymes to starch granules (Giuberti et al., 2014). The RCGS stored for 15, 30 and 60 d showed similar trend of the DM degradability at 12 h, with approximately 75%, 79% and 83%, respectively, at values found to Da Silva et al. (2019). Although the degradability values were higher than in the current trial, an increase of NH_3-N was observed during the storage up to 300 d of ensiling by these authors. The data of the DM degradability at 0 d of storage was 51.7%.

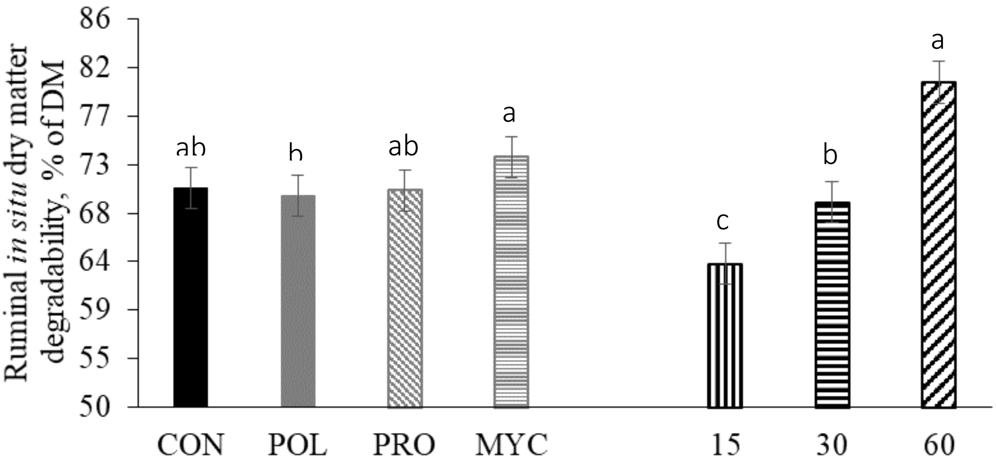

Figure 12. Ruminal *in situ* dry matter degradability in 12 h of reconstituted corn grain silage ensiled for 15, 30 or 60 d, without additives treatment (CON), or treated with polysorbate (POL), propionic acid (PRO) or polysorbate and propionic acid (MYC). Effect of additive: P = 0.02, SEM = 0.89; effect of storage length: P < 0.01, SEM = 0.78; effect of the interaction between additive and storage length: P = 0.60.

The ruminal in situ DM degradability is highly correlated to ruminal starch degradability (Philippeau et al., 1999) and may be a parameter to predict starch digestibility, since more than 70% of corn grain is composed of starch (Huntington, 1997). The treatment with Mycoflake increased the DM degradability with a parallel increase in the proportion of particle retained in bottom pan, a reduction in GMPS and an increase in surface area. Dias Junior et al. (2016), showed a negative quadratic relationship between GMPS and ruminal DM digestibility in different points of incubation (3, 6, 12, and 24 h) in unfermented kernels; at 12 h of incubation they observed a R^2 = 0.97. The authors also found a positive quadratic relationship between surface area and ruminal DM degradability at the same points of incubation. The fragmentation of corn and barley grains increased enzymatic digestion of starch granules by rumen microorganisms (McAllister et al., 1993). These data suggest that there was a fragmentation of particles in silage treated with Mycoflake resulting in greater ruminal DM degradability.

Conversely, silages stored for 60 d presented higher GMPS and a decrease in surface area and yet had greater ruminal DM and starch degradability. These data suggest that the parameters used to measure particle size not fully explain the ruminal degradation. According to Hoffman et al. (2012), the GMPS alone explained only 50% of fermentation potential of dry corn and HMCS, because the GMPS alone cannot account differences in chemical composition, as zein-protein present in corn, or the nutrient composition, as ammonia-N and soluble protein. Then a model of the ruminal and total-tract starch digestibility for dry corn and HMCS was proposed, utilizing GMPS in combination with zein-protein (dry corn) or NH_3-N (HMCS) and the authors showed that this model explained 84% of the variation of fermentation potential these type of grains.

In a study evaluating the *in vitro* digestibility of corn starch gelatinized (normal and waxy starch) with different rates of polysorbate 80 addition, showed by optical microscopy that the surfactant molecule was adsorbed on the particles surface of starch and possibly formed a surfactant-starch chain complexes. The *in vitro* digestibility analysis demonstrated that the fractions rapidly digestible starch (RDS) and resistant starch (RS) tented to increase at the expense of the decrease of fraction slowly digestible starch (SDS), as the rate of polysorbate increased. The effect was greater in the normal corn starch, that have higher proportion of amylose than the waxy starch, suggesting that amylose may be related to the formation of these indigestible complexes. According to the authors, the increase of the fraction RS can be due to starch chain-surfactant complexes formation (Vernon-Carter et al., 2018). A study investigating the interaction between starch-surfactant in wheat starch demonstrated that nonionic surfactants had ability to form strong surfactant-amylose complex and are difficult to dissociate (Ghiasi et al., 1982). However, it is worth mentioning that these studies were carried out with starch submitted to high temperatures (approximately 95 ° C). Gelatinization is a processing method that makes starch available for digestion different from the ensilage process that does not use heat. There are few studies approaching about the feed digestibility in the presence of synthetic surfactants and in silage is unknown.

Research evaluating the *in vitro* effect of Tween 80 and Monensin on the ruminal fermentation of diets based on barley grain and barley silage, showed that Tween 80 applied alone (at 0.5 μl/ml) increased DM digestibility and the accumulation of reducing sugars at 4 h of incubation. Besides that, it was observed the effect of association between Monensin and Tween 80 on the increase of the accumulation of reducing sugars and in the acetate:propionate ratio. According to the authors the Tween 80 may have acted as an emulsifier, improving the solubility of Monensin and enhance the action of inophore (Wang et al., 2004). Considering the results of ruminal *in situ* DM degradability in the present study, the association of Tween 80 and propionic acid in the Mycoflake can have a positive effect on the nutrient availability, but it is still not clear.

Chemical additives are good options for grain silages; however, it is necessary to know the objectives for using the additive, as the initial cost can be a barrier to the adoption of these products. A future exploration would be to add polysorbate 80 to a formulation with sodium benzoate to explore the effect on yeast control and the effectiveness of reducing benzoate doses. Moreover, further studies are needed to better determine the effect of combinations of others chemical additives and polysorbates in corn grain silages.

2.5. CONCLUSIONS

Increasing storage length of reconstituted corn grain silage from 15 to 60 d increased starch and dry matter degradability and Mycoflake increased the availability of nutrients. Although Mycoflake was no efficient to increase the aerobic stability of the RCGS, it controlled the ethanol production and reduced DM losses in silages with more advanced storage.

LITERATURE CITED

AOAC (Association of Official Analytical Chemists). 1990. Official Methods of Analysis. 15th ed. AOAC, Airlington, VA.

Auerbach, H., U. Weber, G. Weber, K. Weiss, and P. Theobald. 2015. Effects of different chemical additives on the fermentation and aerobic stability of high-moisture corn ensiled in bags. Pages 542-543 in Proc. 17th International Silage Conference, Piracicaba, SP, Brazil. ESALQ-USP, Piracicaba, Brazil.

Baker, S., and T. Herrman. 2002. Evaluating Particle Size. MF-2051. Kansas State Uni., Manhattan.

Baron, V. S., K. R. Stevenson, and J. G. Buchanan-Smith. 1986. Proteolysis and fermentation of grain-corn ensiled at several moisture levels and under several simulated storage methods. Can. J. Anim. Sci. 66:451-461.

Benton, J. R., T. J. Klopfenstein, and G. E. Erickson. 2005. Effects of corn moisture and length of ensiling on dry matter digestibility and rumen degradable protein. Pages 31-33 in Nebraska Beef Cattle Reports. University of Nebraska, Lincoln.

Borreani, G., E. Tabacco, R. J. Schmidt, B. J. Holmes, and R. E. Muck. 2018. Silage review: Factors affecting dry matter and quality losses in silages. J. Dairy Sci. 101:3952–3979. doi:10.3168/jds.2017-13837.

Carvalho, P. A., A. D. Bigaton, J. Fernandes, M. C. Santos, J. L. P. Daniel, A. P. Duarte, and L. G. Nussio. 2015. Shifts on bacterial population of high moisture corn silages and its correlation with fermentation end-products. Pages 556-557 in Proc. 17th Int. Silage Conf., Piracicaba, SP, Brazil, ESALQ-USP, Piracicaba, Brazil.

Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia.. Clin. Chem. 8:130–132.

Collins, M. 1995. Hay preservation effects on yield and quality. Pages 67-89 in Post-harvest physiology and preservation of forages: Proceedings of a symposium sponsored by C-6 of the Crop Science Society of America. K. J. Moore, and M. A. Peterson, Madison, WI.

Da Silva, N. C., C. F. Nascimento, F. A. Nascimento, F. D. Resende, J. L. P. Daniel, and G. R. Siqueira. 2018. Fermentation and aerobic stability of rehydrated corn grain silage treated with different doses of *Lactobacillus buchneri* or a combination of *Lactobacillus plantarum* and *Pediococcus acidilactici*. J. Dairy Sci. 101:4158–4167. doi:10.3168/jds.2017-13797.

Da Silva, N. C., C. F. Nascimento, V. M. A. Campos, M. A. P. Alves, F. D. Resende, J. L. P. Daniel, and G. R. Siqueira. 2019. Influence of storage length and inoculation with *Lactobacillus buchneri* on the fermentation, aerobic stability, and ruminal degradability of high-moisture corn and rehydrated corn grain silage. Anim. Feed Sci. Tech. 251:124–133. doi:10.1016/j.anifeedsci.2019.03.003.

Dias Junior, G. S., L. F. Ferraretto, G. G. S. Salvati, L. C. de Resende, P. C. Hoffman, M. N. Pereira, and R. D. Shaver. 2016. Relationship between processing score and kernel-fraction particle size in whole-plant corn silage. J. Dairy Sci. 99:2719–2729. doi:10.3168/jds.2015-10411.

Fernandes, J. 2014. Influência de genótipo, maturidade e tempo de armazenamento na qualidade de silagens de grãos de milho com alta umidade. Pages 1-98, Master Dissertation, University of São Paulo. ESAL-USP, Piracicaba, Brazil.

Ferraretto, L. F., S. M. Fredin, and R. D. Shaver. 2015. Influence of ensiling, exogenous protease addition, and bacterial inoculation on fermentation profile, nitrogen fractions, and ruminal in vitro starch digestibility in rehydrated and high-moisture corn. J. Dairy Sci. 98:7318–7327. doi:10.3168/jds.2015-9891.

Ferraretto, L. F., K. Taysom, D. M. Taysom, R. D. Shaver, and P. C. Hoffman. 2014. Relationships between dry matter content, ensiling, ammonia-nitrogen, and ruminal in vitro starch digestibility in high-moisture corn samples. J. Dairy Sci. 97:3221–3227. doi:10.3168/jds.2013-7680.

Ghiasi, K., E. Varriano-Marston, and R. C. Hoseney. 1982. Gelatinization of Wheat Starch. II. Starch-Surfactant Interaction. Cereal Chem. 59:86–88.

Giuberti, G., A. Gallo, F. Masoero, L. F. Ferraretto, P. C. Hoffman, and R. D. Shaver. 2014. Factors affecting starch utilization in large animal food production system: A review. Starch/Staerke. 66:72–90. doi:10.1002/star.201300177.

Goeser, J. P., C. R. Heuer, and P. M. Crump. 2015. Forage fermentation product measures are related to dry matter loss through meta-analysis. Prof. Anim. Sci. 31:137-145. doi:10.15232/pas.2014-01356.

Goodrich, R. D., F. M. Byers, and J. C. Meiske. 1975. Influence of Moisture Content, Processing and Reconstitution on the Fermentation of Corn Grain. J. Anim. Sci. 41:876–881. doi:10.2527/jas1975.413876x.

Hall, M.B. 2009. Analysis of Starch, including maltooligosaccharides, in animal feeds: A comparison of methods and a method recommended for AOAC collaborative study. J. AOAC Int. 92:42–49.

Hoffman, P. C., N. M. Esser, R. D. Shaver, W. K. Coblentz, M. P. Scott, A. L. Bodnar, R. J. Schmidt, and R. C. Charley. 2011. Influence of ensiling time and inoculation on alteration of the starch-protein matrix in high-moisture corn. J. Dairy Sci. 94:2465–2474. doi:10.3168/jds.2010-3562.

Hoffman, P. C., D. R. Mertens, J. Larson, W. K. Coblentz, and R. D. Shaver. 2012. A query for effective mean particle size in dry and high-moisture corns. J. Dairy Sci. 95:3467–3477. doi:10.3168/jds.2011-5126.

Huber, J. T., and M. Soejono. 1976. Organic acid treatment of high dry matter corn silage fed lactating dairy cows. J. Dairy Sci. 59:2063-2070.

Huntington, G. B. 1997. Starch Utilization by Ruminants: From Basics to the Bunk. J. Anim. Sci. 75:852–867. doi:10.2527/1997.753852x.

Jobim, C. C., L. G. Nussio, R. A. Reis, and P. Schmidt. 2007. Avanços metodológicos na avaliação da qualidade da forragem conservada. Rev. Bras. Zootec. 36:101–119. doi:10.1590/s1516-35982007001000013.

Junges, D., G. Morais, M. H. F. Spoto, P. S. Santos, A. T. Adesogan, L. G. Nussio, and J. L. P. Daniel. 2017. Short communication: Influence of various proteolytic sources during fermentation of reconstituted corn grain silages. J. Dairy Sci. 100:9048–9051. doi:10.3168/jds.2017-12943.

Kaur, G., and S. K. Mehta. 2017. Developments of Polysorbate (Tween) based microemulsions: Preclinical drug delivery, toxicity and antimicrobial applications. Int. J. Pharm. 529:134–160. doi:10.1016/j.ijpharm.2017.06.059.

Krishnamoorthy, U., T. V. Muscato, C. J. Sniffen, and P. J. Van Soest. 1982. Nitrogen Fractions in Selected Feedstuffs. J. Dairy Sci. 65:217–225. doi:10.3168/jds.S0022-0302(82)82180-2.

Kung, L. 2010. Aerobic stability of silage. Pages 1–14 in Proceedings California Alfalfa & Forage Symposium and Corn/Cereal Silage Conference, Visalia, CA.

Kung, L., C. L. Myers, J. M. Neylon, C. C. Taylor, J. Lazartic, J. A. Mills, and A. G. Whiter. 2004. The effects of buffered propionic acid-based additives alone or combined with microbial inoculation on the fermentation of high moisture corn and whole-crop barley. J. Dairy Sci. 87:1310–1316. doi:10.3168/jds.S0022-0302(04)73280-4.

Kung, L., J. R. Robinson, N. K. Ranjit, J. H. Chen, C. M. Golt, and J. D. Pesek. 2000. Microbial populations, fermentation end-products, and aerobic stability of corn silage treated with ammonia or a propionic acid-based preservative. J. Dairy Sci. 83:1479–1486. doi:10.3168/jds.S0022-0302(00)75020-X.

Kung, L., R. D. Shaver, R. J. Grant, and R. J. Schmidt. 2018. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. J. Dairy Sci. 101:4020–4033. doi:10.3168/jds.2017-13909.

Kung, L., M. R. Stokes, and C. J. Lin. 2003. Silage Additives. Pages 305-360 in Silage Science and Technology. R. D. Buxton, R. E. Muck, and J. R. Harrisson, ed. American Society of Agronomy, Madison, WI.

Leibensperger, R. Y., and R. E. Pitt. 1987. A model of clostridial dominance in ensilage. Grass Forage Sci. 42:297–317. doi:10.1111/j.1365-2494.1987.tb02118.x.

Mahungu, S. M., and W. E. Artz. 2001. Emulsifiers. Pages in Food Additives, 2nd ed. A. L. Branen, P. M. Davidson, S. Salminen, J. H. Thorngate, ed. Marcel Dekker, Inc., New York, NY.

McAllister, T. A., R. C. Phillippe, L. M. Rode, and K. J. Cheng. 1993. Effect of the protein matrix on the digestion of cereal grains by ruminal microorganisms.. J. Anim. Sci. 71:205–212. doi:10.2527/1993.711205x.

McDonald, P., N. Henderson, and S. Heron. 1991. The Biochemistry of Silage. 2nd ed. Chalcombe Publications, Marlow, UK.

Mertens, D. R. 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborativy study. J. AOAC Int. 85:1217-1240.

Moon, N. J. 1983. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. J. Appl. Bacteriol. 55:453–460. doi:10.1111/j.1365-2672.1983.tb01685.x.

Morais, G., J. L. P. Daniel, C. Kleinshmitt, P. A. Carvalho, J. Fernandes, and L. G. Nussio. 2017. Additives for grain silages: A review. Slovak J. Anim. Sci. 50:42–54.

Muck, R. E., E. M. G. Nadeau, T. A. McAllister, F. E. Contreras-Govea, M. C. Santos, and L. Kung. 2018. Silage review: Recent advances and future uses of silage additives. J. Dairy Sci. 101:3980–4000. doi:10.3168/jds.2017-13839.

Ohshima, M., and P. McDonald. 1978. A review of the changes in nitrogenous compounds of herbage during ensilage. J. Sci. Food Agric. 29:497–505. doi:10.1002/jsfa.2740290602.

Oude Elferink, S. J., J. Krooneman, J. C. Gottschal, S. F. Spoelstra, F. Faber, and F. Driehuis. 2001. Anaerobic conversion of aactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. Appl. Environ. Microbiol. 67:125–132. doi:10.1128/AEM.67.1.125.

Owens, F. N., R. A. Zinn, and Y. K. Kim. 1986. Limits to starch digestion in the ruminant small intestine. J. Anim. Sci. 63:1634–1648. doi:10.2527/jas1986.6351634x.

Pahlow, G., R. E. Muck, F. Driehuis, S. J. W. H. Oude Elferink, and S. F. Spoelstra. 2003. Microbiology of Ensiling. Pages 31-93 in Silage Science and Technology. R. D. Buxton, R. E. Muck, and J. R. Harrison, ed. American Society of Agronomy, Madison, WI.

Philippeau, C., F. Le Deschault De Monredon, and B. Michalet-Doreau. 1999. Relationship between ruminal starch degradation and the physical characteristics of corn grain. J. Anim. Sci. 77:238–243. doi:10.2527/1999.771238x.

Pinto, A. C. J., and D. D. Millen. 2016. Situação atual da engorda de bovinos em confinamento e modelos nutricionais em uso. Proc. X Simpósio de Produção de Gado de Corte (Simcorte). (In Portuguese). Federal University of Viçosa, Viçosa-MG, Brazil. 103-120.

Prigge, E. C., R. R. Johnson, F. N. Owens, and D. Williams. 1976. Soluble nitrogen and acid production of high moisture corn. J. Anim. Sci. 42:490-496.

Pryce, J. D. 1969. A modification of the Barker-Summerson method for the determination of lactic acid. Analyst. 94:1151–1152. doi:10.1039/AN969940115125.

Rooke, J. A., and R. D. Hatfield. 2003. Biochemistry of Ensiling. Pages 95-140 in Silage Science and Technology. R. D. Buxton, R. E. Muck, and J. R. Harrison, ed. American Society of Agronomy, Madison, WI.

Sebastian, S., L. E. Phillip, V. Fellner, and E. S. Idziak. 1996. Comparative assessment of bacterial inoculation and propionic acid treatment on aerobic stability and microbial populations of ensiled high-moisture ear corn. J. Anim. Sci. 74:447–456. doi:10.2527/1996.742447x.

Sindt, J. J., J. S. Drouillard, E. C. Titgemeyer, S. P. Montgomery, E. R. Loe, B. E. Depenbusch, and P. H. Walz. 2006. Influence of steam-flaked corn moisture level and density on the site and extent of digestibility and feeding value for finishing cattle. J. Anim. Sci. 84:424-432.

Vernon-Carter, E. J., J. Alvarez-Ramirez, L. A. Bello-Perez, A. Garcia-Hernandez, C. Roldan-Cruz, and S. Garcia-Diaz. 2018. In vitro digestibility of normal and waxy corn starch is modified by the addition of Tween 80. Int. J. Biol. Macromol. 116:715–720. doi:10.1016/j.ijbiomac.2018.05.076.

Wang, Y., T. W. Alexander, and T. A. McAllister. 2004. In vitro effects of Monensin and Tween 80 on ruminal fermentation of barley grain:barley silage-based diets for beef cattle. Anim. Feed Sci. Tech. 116:197–209. doi:10.1016/j.anifeedsci.2004.06.006.

Weatherburn, M. W. 1967. Phenol-Hypochlorite reaction for determination of ammonia. Anal. Chem. 39:971–974. doi:10.1021/ac60252a045.

Weissbach, F. 2009. Correction of dry matter content of silages used as substrate for biogas production. Pages 483-484 in Proc. 15th International Silage Conference, US Dairy Forage Research Center, Madison, WI. College of Agriculture and Life Sciences, University of Wisconsin, Madison.

Woolford, M. K. 1975. Microbiological screening of the straight chain fatty acids (c1-c12) as potential silage additives. J. Sci. Food Agric. 26:219–228. doi:10.1002/jsfa.2740260213.

Woolford, M. K., and M. K. Sawczyc. 1984. An investigation into the effect of cultures of lactic acid bacteria on fermentation in silage: 1. Strain selection. Grass Forage Sci. 39:139–148. doi:10.1111/j.1365-2494.1984.tb01675.x.

Zhang, H., Y. Shen, P. Weng, G. Zhao, F. Feng, and X. Zheng. 2009. Antimicrobial activity of a food-grade fully dilutable microemulsion against Escherichia coli and Staphylococcus aureus. Int. J. Food Microbiol. 135:211–215. doi:10.1016/j.ijfoodmicro.2009.08.015.

Zhang, H., Y. Xu, L. Wu, X. Zheng, S. Zhu, F. Feng, and L. Shen. 2010. Anti-yeast activity of a food-grade dilution-stable microemulsion. Appl. Microbiol. Biotechnol. 87:1101–1108. doi:10.1007/s00253-010-2623-2.