MARIELE CRISTINA NASCIMENTO AGARUSSI

NOVEL LACTIC ACID BACTERIA STRAINS AS INOCULANT FOR ALFALFA AND CORN SILAGES AND MICROBIOME OF REHYDRATED CORN AND SORGHUM GRAIN SILAGES

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of Doctor Scientiae.

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APPROVED: February 28, 2019.

Eliana dos Santos Leandro

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BIOGRAPHY

Mariele Cristina Nascimento Agarussi, daughter of Ana Maria do Nascimento Agarussi and Alvaro Tadeu Agarussi, was born in Itu – SP, Brazil on May 9, 1990.

In 2008 she started the undergrad in Animal Science at Universidade Federal de Viçosa, obtained a Bachelor of Science degree in Animal Science in 2013.

In the same year she started the Master´s program at the same university, concluding on February 25, 2015. In March 2015 she continued in the same program as a doctorate student and defended her thesis on February 28, 2019.

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ABSTRACT

AGARUSSI, Mariele Cristina Nascimento, D.Sc., Universidade Federal de Viçosa, February, 2019. **Novel lactic acid bacteria strains as inoculant for alfalfa and corn silages and microbiome of rehydrated corn and sorghum grain silages.** Adviser: Odilon Gomes Pereira.

This study was divided into five chapters

Chapter 1 - The experiment was carried out under a completely randomized design with three replicates based on a 6 × 6 factorial arrangement, with 6 inoculants: T1- control (CTRL), T2- commercial inoculant containing Lactobacillus plantarum + Pediococcus pentosaceus (CI), T3- Lactobacillus pentosus 14.7SE (LPE), T4- Lactobacillus plantarum 3.7E (LP), T5- Pediococcus pentosaceus 14.15SE (PP), T6- Lactobacillus plantarum 3.7E + Pediococcus pentosaceus 14.15SE (LP+PP); and six fermentation periods: 1, 3, 7, 14, 28 and 56 days. Alfalfa was wilted for 6 h and increased the dry matter (DM) content to 368 g/kg as fed. The crude protein (CP) and yeast population decreased during the fermentation process. Highest pH decline rates in the first week of fermentation were observed for inoculated silages. Among inoculants, the PP strain resulted in lowest pH values from 14 d of fermentation and lowest acetic acid concentration in the last day of fermentation. Enterobacteria and molds populations were more efficiently controlled by new strains at day 56 and 28, respectively. The in vitro dry matter digestibility was higher in PP than LP silages (64.45 vs. 61.18% DM). Adding of P. pentosaceus alone resulted in positive influence on all evaluated parameters, thus providing better silage quality. **Chapter 2 –** We evaluated the effects of wild strains of Lactobacillus buchneri on chemical composition, fermentative profile and aerobic stability of corn silages after 90 days of fermentation. The experiment was carried out under a completely randomized design with three replicates and 13 treatments consisted in 1- water (CRTL), 2- commercial L. buchneri strain (CI), and 11 wild strains of L. buchneri: 3- strain **56.1**, 4- strain **56.2**, 5- strain **56.4**, 6- strain **56.7**, 7- strain **56.8**, 8- strain **56.9**, 9- strain **56.21**, 10- strain **56.22**, 11- strain **56.25**, 12- strain **56.26**, and 13- strain **56.27**. A treatment effect ($P < 0.05$) was observed on pH, WSC, NH_3-N, lactic, acetic and propionic acids, ethanol and 1,2-propanediol concentrations. The lowest pH was observed in CTRL silages, contrary, 56.1, 56.4, and 56.9 silages had the highest values (3.65 vs. 3.84). CTRL silages had higher residual WSC than CI, 56.2 and 56.7 silages and higher lactic acid concentration than CI and 56.4. The lowest NH_3-N concentrations were observed in 56.1 and 56.7 silages conversely the highest concentrations were found in 56.8 and 56.21 (7.11 vs. 10.01% of total nitrogen). Inoculated silages with 56.1 strain had the highest acetic and

propionic acids concentrations and higher ethanol production than CI, 56.7, 56.9, 56.22, 56.25, and 56.26 silages. The populations of enterobacteria and yeasts & molds, DM, CP, neutral detergent fiber, acid detergent fiber contents and DM recovery after 90 d of fermentation were not affected ($P > 0.05$) by treatment. Silage treated with 56.1 strain had higher aerobic stability than non-inoculated silages (68.25 vs. 36 h). The L. buchneri strain 56.1 has the potential to be used as microbial inoculant in corn silage. **Chapters 3 and 4** - We explored the succession of bacterial and fungal populations, and evaluated the impacts caused by Lactobacillus plantarum + Propionibacterium acidipropionici and Lactobacillus buchneri inoculants on those communities of rehydrated corn and sorghum grains and their silages by next-generation sequencing after 0, 3, 7, 21, 90 and 360 days of fermentation. Proteobacteria was predominantly in both grains at the beginning of the fermentation and Firmicutes phylum throughout the fermentation periods. Species of Lactobacillus and Weissella were the main bacteria involved in the fermentation of rehydrated corn and sorghum grain silages. Aspergillus spp. molds were predominant in corn grain fermentation while the yeast Wickerhamomyces anomalus was the major fungal in sorghum grain silages. The inoculant containing L. plantarum and P. acidipropionici was more efficient in promoting a sharply growth of Lactobacillus spp. and maintaining greater stability of the bacterial community during longer periods of storage in both grains silages. The addition of inoculant did not have an influencial effect on fungal population of rehydrated sorghum grain silages. **Chapter 5** - It was evaluated the effect of ensiling on the fermentation profile, corn silage processing score (CSPS) and long-chain fatty acids (LCFA) profile of whole-plant corn. Eleven corn hybrids were obtained at harvest. Each of the 11 samples was homogenized manually and allocated into 4 samples of approximately 600 g each. Each of the 4 samples was randomly assigned to 1 of 2 treatments (0 or 120 d of ensiling) and vacuum-sealed in nylon-polyethylene standard barrier vacuum pouches. Concentration of DM was unaffected ($P > 0.10$) by ensiling and averaged 36.2% as fed. The effects on pH is likely attributed to 7.7%-, 1.0%- and 1.2%-units greater ($P < 0.02$) lactic, acetic and isobutyric acids concentrations, respectively, for 120 d compared with 0 d. Concentrations of NH_3-N increased ($P = 0.001$) with ensiling, as expected. Starch concentrations and CSPS was unaffected ($P > 0.10$) by ensiling and averaged 31.2% of DM and 28.8%, respectively. No effects of ensiling were observed on LCFA profile of major FA including C16:0, C18:0, C18:1, C18:2, and C18:3 FA ($P > 0.10$). Further research is warranted to elucidate under which conditions ensiling time enhances the CSPS.

RESUMO

AGARUSSI, Mariele Cristina Nascimento, D.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Uso de novas estirpes de bactérias láticas como inoculantes para silagens de alfafa e milho e microbioma de silagens de grãos de milho e sorgo reidratados.** Orientador: Odilon Gomes Pereira.

Este estudo foi dividido em cinco capítulos

Capítulo 1 - O experimento foi conduzido em um delineamento inteiramente casualizado com três repetições, baseado em um arranjo fatorial 6 × 6, com 6 inoculantes: T1-Controle (**CTRL**), T2- Inoculante comercial contendo Lactobacillus plantarum + Pediococcus pentosaceus - (**CI**), T3- Lactobacillus pentosus 14.7SE (**LPE**), T4- Lactobacillus plantarum 3.7E (**LP**), T5- Pediococcus pentosaceus 14.15SE (**PP**), T6- Lactobacillus plantarum 3.7E + Pediococcus pentosaceus 14.15SE (**LP + PP**); e seis períodos de fermentação: 1, 3, 7, 14, 28 e 56 dias. A alfafa foi emurchecida por 6 h e aumentou o teor de matéria seca (MS) para 368 g/kg. A população de levedura e o teor de proteína bruta (PB) reduziram durante o processo de fermentação. Maiores taxas de declínio de pH na primeira semana de fermentação foram observadas para as silagens inoculadas. Entre os inoculantes, a estirpe PP resultou em menores valores de pH a partir de 14 d de fermentação e menor concentração de ácido acético no último dia de fermentação. As populações de enterobactérias e mofos foram mais eficientemente controladas pelas novas estirpes nos dias 56 e 28, respectivamente. A digestibilidade in vitro da matéria seca foi maior nas silagens inoculadas com PP do que LP (64.45 vs. 61.18% MS). A adição de P. pentosaceus influenciou positivamente todos os parâmetros avaliados, resultando em silagem de melhor qualidade. **Capítulo 2** – Avaliamos os efeitos de estirpes selvagens de Lactobacillus buchneri sobre a composição química, perfil fermentativo e estabilidade aeróbia de silagens de milho após 90 dias de fermentação. O experimento foi conduzido em um delineamento inteiramente casualizado, com três repetições e 13 tratamentos constituídos por 1-água (**CRTL**), 2 - estirpe comercial de L. buchneri (**CI**) e 11 estirpes selvagens de L. buchneri: 3- estirpe **56.1**, 4- estirpe **56.2**, 5- estirpe **56.4**, 6- estirpe **56.7**, 7- estirpe **56.8**, 8- estirpe **56.9**, 9- estirpe **56.21**, 10- estirpe **56.22**, 11- estirpe **56.25**, 12- estirpe **56.26** e 13- estirpe **56.27**. Observou-se efeito de tratamento ($P < 0.05$) no pH e nos teores de WSC, N-NH_3, ácido lático, acético e propiônico, etanol e 1,2-propanodiol. O menor pH foi encontrado nas silagens CTRL e os maiores valores foram observados nas silagens 56.1, 56.4 e 56.9 (3.65 vs. 3.84). A silagem CTRL apresentou maior carboidrato solúvel em água residual do que as silagens CI, 56.2 e 56.7 e maior concentração de ácido lático do que CI e 56.4. As concentrações mais baixas de N-NH_3 foram observadas nas silagens 56.1 e 56.7,

ao contrário, as maiores concentrações foram encontradas nas silagens 56.8 e 56.21 (7.11 vs. 10.01% do nitrogênio total). A silagem 56.1 apresentou mais altas concentrações de ácidos acético e propiônico. Nesta silagem também foi observado maior produção de etanol em relação às silagens CI, 56.7, 56.9, 56.22, 56.25 e 56.26. As populações de enterobactérias, mofos & leveduras, os teores de MS, PB, fibra insolúvel em detergente neutro, fibra insolúvel em detergente ácido e a recuperação de MS após 90 dias de fermentação não foram afetadas ($P > 0.05$) pelos tratamentos. A silagem inoculada com 56.1 apresentou maior estabilidade aeróbia do que a silagem não inoculada (68.25 vs. 36 h). A cepa de L. buchneri 56.1 tem potencial para ser utilizada como inoculante microbiano em silagem de milho. **Capítulos 3 e 4 -** Exploramos as sucessões das populações bacterianas e fúngicas e avaliamos os impactos causados por inoculantes contendo Lactobacillus plantarum + Propionibacterium acidipropionici e Lactobacillus buchneri nessas populações em grãos de milho e sorgo reidratados e suas silagens por sequenciamento de nova geração após 0, 3, 7, 21, 90 e 360 dias de fermentação. Proteobacteria e Firmicutes foram predominantemente encontrados no início e durante os períodos intermediários de fermentação, respectivamente, em ambos os grãos. Espécies de Lactobacillus e Weissella foram as principais bactérias envolvidas na fermentação de silagens de grãos de milho e sorgo reidratados. Aspergillus spp. predominaram na fermentação de grãos de milho, enquanto a levedura Wickerhamomyces anomalus foi o principal fungo encontrado nas silagens de grãos de sorgo. O inoculante contendo L. plantarum e P. acidipropionici foi mais eficiente em promover crescimento de Lactobacillus spp. e manter maior estabilidade da comunidade bacteriana durante períodos mais longos de armazenamento em ambas as silagens, já a adição de inoculante não resultou em grandes alterações na população fúngica de silagens de grãos de sorgo reidratado. **Capítulo 5** - Avaliou-se o efeito da ensilagem no perfil fermentativo, no corn silage processing score (CSPS) e no perfil de ácidos graxos de cadeia longa (AGCL) em planta inteira de milho. Onze híbridos de milho foram colhidos e cada uma das 11 amostras foi homogeneizada manualmente e alocada em 4 amostras de aproximadamente 600 g cada. Cada uma das 4 amostras foi aleatoriamente designada para 1 de 2 tratamentos (0 ou 120 d de ensilagem) e seladas a vácuo em bags. A concentração de MS não foi afetada ($P > 0.10$) pela ensilagem e teve média de 36.2% as fed. Os efeitos sobre o pH são provavelmente atribuídos a 7.7% -, 1.0% - e 1.2% - maiores concentrações de ácido lático, acético e isobutírico, respectivamente, para 120 d comparado com 0 d. As concentrações de $N\text{-}NH_3$ aumentaram ($P = 0.001$) com a ensilagem, como esperado. As concentrações de amido e CSPS não foram

afetadas (P > 0.10) pela ensilagem e tiveram média de 31.2% MS e 28.8%, respectivamente. Não foram observados efeitos de ensilagem no perfil de AGCL na maioria dos ácidos graxos incluindo C16:0, C18:0, C18:1, C18:2 e C18:3 (P > 0.10). Mais pesquisas são necessárias para elucidar sob quais condições o tempo de ensilagem aumenta o CSPS.

SUMMARY

GENERAL INTRODUCTION

Forage preservation by ensiling has become a global practice because it provides consistent, reliable, and predictable feed supply for ruminants production systems. The process is characterized by spontaneous lactic acid fermentation in an anaerobic environment, where main fermenting agents, the lactic acid bacteria (LAB), metabolize water soluble carbohydrates (WSC) and produce lactic acid. Therefore, the maintenance of anaerobiosis and the decrease of pH are the factors responsible for the preservation of the forage stored (Driehuis et al., 1999; Pahlow et al., 2003).

Corn silage is the most widely used silage worldly (Wilkins et al., 1999), due to the easily corn cultivation and adaptability, high mass production, adequate fermentation and energy value and high consumption by animals (Godoi & Silva, 2010). Even though corn silage contains relatively low levels of total fatty acids (FA), the presence of 70% of unsaturated FA (UFA) in total FA (Mir, 2004), makes it the major source of UFA in ruminant diets. Therefore, better understanding of potential changes in the long chain fatty acid (LCFA) profile of whole-plant corn silage would aid nutritionists to better formulate and manipulate the FA profile of ruminant diets and thereby improve FA utilization.

In spite of the potentialities, the aerobic deterioration of corn silage is its main limitation, because the high residual WSC together with high lactic acid concentration present in these silages favor more intensely the growth and activity of microorganisms that decompose the ensiled material. Changes in the composition of the silages due to deterioration result in increased pH, temperature and ammonia nitrogen (McDonald et al., 1991).

Alfalfa is a forage crop with great importance due to the worldwide use, high nutritional value and digestibility (Carvalho and Vilela, 1994). However, high concentration of organic acids, salts, proteins, and minerals result in a high buffering capacity (McDonald et al., 1991). Moreover, high buffering capacity and crude protein content, in combination with low WSC concentration indicate that the ensiling properties for the alfalfa are not ideal as suggested by Muck (2012).

In this context, the use of microbial inoculants as starters for alfalfa silage and microorganism that acting improving the aerobic stability of corn silage have been recommended (Muck and Kung, 1997; McAllister et al., 1998). The addition of inoculants containing beneficial homofermentative, heterofermentative or the combination of those LAB

in the ensiling process, aim to inhibit the growth of aerobic microorganisms, protease and deaminase activity of the plant and microorganisms resulting in improved dry matter recovery and aerobic stability of silages (Kung Jr. et al., 2003).

According to Muck (2013), the major international companies producing inoculants are based in Europe and North America. So, these products have been developed for cool-season grasses, whole-crop corn and alfalfa. The inoculants may or may not be effective when used on warm season grasses or tropical legumes, suggesting that environmental conditions can affect the physiology and metabolism of the inoculated strains and may influence their effects on the fermentation process.

The search for new LAB strains with desirable characteristics to improve fermentation of alfalfa silage and the aerobic stability of corn silage has been stimulated the development of studies looking for new microbial inoculants which result in good quality silages, with high nutritional value and lower losses due to deteriorating and pathogenic microorganisms.

Recently, silages of rehydrated grains have been used in Brazil. The process consists basically of hydration of the milled mature grain with 10-14% to reach the moisture necessary for ensiling, between 30 to 40% as fed (Gobetti et al., 2013). Despite the potential of rehydrated grain silages and the fact that silage-associated microorganisms may significantly affect both silage quality and ruminant health, little is known about the microbiota present in these silages.

Generally, the composition of microorganisms before and after ensiling has undergone great changes (Guan et al., 2018). Monitoring the changes of bacterial and fungal communities during ensiling process by amplicon sequencing of the 16S rRNA and internal transcribed spacer (ITS) genes are rapid tools which have allowed obtaining information about the presence of different microbial groups (Whiteley et al., 2012) enabling the thoroughly understanding of the fermentative process.

Based on that the experiments were carried out with the following objectives: 1) investigate the effects of novel LAB on alfalfa silage fermentation and in vitro dry matter digestibility; 2) evaluate the effects of wild Lactobacillus buchneri strains on microbial populations, fermentation profile, chemical composition, dry matter losses, and aerobic stability of corn silages; 3) explore the succession of bacterial and fungal populations involved in fermentation of rehydrated corn and sorghum grains and their silages after different days of fermentation and 4) evaluate the effect of ensiling on corn silage processing score, fermentation, and LCFA profile of whole-plant corn silage.

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CHAPTER 1 - NOVEL LACTIC ACID BACTERIA STRAINS AS INOCULANTS ON ALFALFA SILAGE FERMENTATION

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The experiment was performed using a completely randomized design (with three replicates) based on a 6 × 6 factorial assay with 6 inoculants (I): Control (CTRL), Commercial inoculant (CI), Lactobacillus pentosus 14.7SE (LPE), Lactobacillus plantarum 3.7E (LP), Pediococcus pentosaceus 14.15SE (PP), and Lactobacillus plantarum 3.7E + Pediococcus pentosaceus 14.15SE (LP+PP), and six fermentation periods (P): 1, 3, 7, 14, 28 and 56 days. Alfalfa was wilted for 6 h in the field, which increased the dry matter content to 368 g/kg as fed. The CP and yeast population decreased during the fermentation process. Silage inoculated with the PP strain had the lowest pH values beginning at 14 d of fermentation and the lowest acetic acid concentration on the last day of fermentation. New strains more efficiently regulated enterobacteria and mold populations at days 56 and 28, respectively. Silages inoculated with the PP strain had a higher coefficient of in vitro dry matter digestibility than LP silages. All of the tested novel strains resulted in positive effects on at least one chemical property of the silage during the fermentation process. However, the adding of P. pentosaceus can be indicated as the better for silage quality considering the tested treatments in the present study.

INTRODUCTION

Forage preservation via ensiling has become a global practice because it provides a consistent, reliable, and predictable feed supply for ruminant production systems. Unavoidable losses of highly digestible nutrients caused by plant respiration, plant microbial proteolytic activity, clostridial fermentation, microbial deamination, and decarboxylation of amino acids may negatively affect conservation efficiency, increase energy and nutrient losses, and cause an accumulation of anti-nutritional compounds in silage [1].

Alfalfa is a forage crop of great importance due to its worldwide use, high nutritional value and digestibility [2]. However, high concentrations of organic acids, salts, proteins, and minerals result in a high buffering capacity [3]. The high buffering capacity and CP, in combination with low water-soluble carbohydrates (WSC) concentrations, indicate that the ensiling properties of alfalfa are not ideal, as suggested by Muck [4].

Therefore, the use of microbial inoculants as starters for alfalfa silage is recommended [5]. Zielińska et al. [6] demonstrated that microbial inoculants altered many parameters of silages, but the strength of the effects on fermentation depended on the strain characteristics. One of the main challenges in the industry is the extent of variability in the effects of inoculant bacteria on the fermentation and preservation of silage, silage quality and animal performance, which were noted in several studies [5, 7]. The lack of inoculant effects on the process may be related to the ability of the inoculated bacterium to grow rapidly in the forage mass and effectively compete with the epiphytic flora as well as the presence of adequate substrate, and it may also be related to specificities between the forage, the microorganisms present in the inoculant and the weather conditions [8].

Muck [9] reported that the major international companies producing inoculants are based in Europe and North America. Therefore, these products have been developed for cool-season grasses, whole-crop corn and alfalfa. The inoculants may or may not be effective when used on warm season grasses or tropical legumes, which suggests that environmental conditions affect the physiology and metabolism of the inoculated strains and may influence their effects on the fermentation process.

Oliveira et al. [1] reported that Lactobacillus plantarum is the most commonly used silage inoculant. However, some lactic acid bacteria (LAB) species are also selected as silage inoculants because of their faster growth at high pH values (> 5) compared to L. plantarum. The authors suggested that more research was needed on the effects of infrequently used LAB as individual silage inoculants on silage fermentation because little is known about their related effects on silage quality.

Inoculants containing synergistic mixtures of LAB are used via the addition of microorganisms that act during different phases of fermentation. Some Pediococcus strains are more tolerant to high dry matter (DM) conditions than Lactobacillus spp. and exhibit a wider range of optimal temperatures and pH values for growth [10]. Silages treated with one or more bacteria often have a lower pH value and acetic acid, butyric acid, and ammonia nitrogen (NH_3-N) contents and also a higher lactic acid concentration and better DM recovery compared to untreated silages [5].

Based on that, in the search for new promising strains for silage inoculants, the purpose of the present study was to investigate the effects of L. pentosus 14.7SE, L. plantarum 3.7E, P. pentosaceus 14.15SE and a mixture of L. plantarum 3.7E and P. pentosaceus 14.15SE on the chemical composition, fermentative profiles and in vitro DM

digestibility of alfalfa silage under tropical conditions after 1, 3, 7, 14, 28 and 56 days of fermentation.

METHODS

Location and Climatic Conditions

The experiment was performed between June and August 2016 at the Department of Animal Science of the Federal University of Vicosa (Viçosa, MG, Brazil), located at 20°45' S latitude, 42°52' W longitude 648 m above sea level. The annual precipitation and average temperature in the year of the experiment were 1235.4 mm and 20.7°C, respectively.

Experimental Design

The experiment was performed using a completely randomized design (with three replicates) based on a 6 × 6 factorial assay (6 inoculants × 6 fermentation periods). The periods (P) were 1, 3, 7, 14, 28, and 56 days after fermentation. The following inoculants (I) were evaluated: 1- Control (CTRL); 2- Commercial inoculant (CI); 3- Lactobacillus pentosus 14.7SE (LPE); 4- Lactobacillus plantarum 3.7E (LP); 5- Pediococcus pentosaceus 14.15SE (PP); and 6- L. plantarum 3.7E + P. pentosaceus 14.15SE (LP+PP). The commercial inoculant Silobac (CHR Hansen´s®, Hørsholm, Denmark), which contains L. plantarum, P. pentosaceus, maltodextrin, sodium aluminosilicate and whey, was used to compare its effectiveness with the new strains.

Characterization of the Strains

The three wild strains of LAB used in this study belong to the microorganism bank of the Forage Laboratory of the UFV and were isolated from wilted and non-wilted alfalfa silages. The 16S rDNA sequences of the strains are deposited in the GenBank database with the following access numbers: L. pentosus 14.7SE - MH924298; L. plantarum 3.7E - MH924275; and P. pentosaceus 14.15SE - MH924301.

Growth tests at different temperatures (15 and 45°C), pH (3.5, 4.0, 4.5 and 8.5 at 37°C), salt concentrations (40 and 60 g/L of NaCl at 37°C), gas production and antimicrobial activities were performed in a previously study [11].

The efficiency in reducing pH was measured using a potentiometer after 24 h at 37ºC in alfalfa broth. Alfalfa broth was obtained from 100 g of herbage crushed in 400 ml of distilled water in an industrial blender for 1 min and was filtered and sterilized (121°C, 15

min). The strains were activated twice in MRS broth for 24 h, and one more time in tubes containing 3 ml of the alfalfa broth for 24 h. A sample (10%) of the inoculum was added to a tube containing 5 ml of the alfalfa broth (pH= 5.87). Samples after 24 h incubation were analyzed for metabolite production (lactic, acetic and propionic acids) using HPLC (Shimadzu Scientific Instruments, Columbia, MD) according to Siegfried et al. [12]. The characteristics of the strains are presented in Table S1.

The strains were selected based of their metabolite production, ability to induce a fast drop in pH, growing capacity in different conditions and broad-spectrum antimicrobial activity against pathogenic and harmful spoilage organisms, such as Listeria monocytogenes 19117, Listeria monocytogenes 7644, and Escherichia Coli K12.

Silage Production

The harvesting of alfalfa cv. Crioula (Medicago sativa cv. Crioula) was performed using a costal brush when the plants were at the early bud stage. Fresh alfalfa was wilted to a DM content of approximately 360 g/kg as fed and chopped into approximately 1.5-cm length particles.

Novel strains were cultured in MRS broth for 14 h, which was the average time that showed the maximum number of cells. Each inoculum was standardized using a spectrophotometer (630 nm) at an optical density of 0.05, in 20 ml of MRS broth, and the amount needed to reach the theoretical application rate of 10^5 colony forming units (cfu)/g of fresh weight were centrifuged at 1,000 g for 10 min. The supernatant was discarded.

Three replicated piles (each pile treated individually) containing approximately 10 kg of fresh alfalfa were prepared for each treatment (total of 18 piles). Inoculants were diluted in 15 ml of sterilized alfalfa broth plus 35 ml of water, which was sprayed uniformly on chopped forage. A total of 500 g of fresh alfalfa was packed into nylon-polyethylene bags (25 × 35 cm; Doug Care Equipment Inc., Springville, CA), and the air was evacuated from the bags using a vacuum sealer (Eco vacuum 1040, Orved, Italy). The same amount of alfalfa broth and water were applied to the CTRL silages. A total of 108 bags were prepared and stored in the laboratory at room temperature (range, 23-27°C). Three bags from each treatment were opened 1, 3, 7, 14, 28, and 56 d after fermentation.

Fermentative Profile and Microbial Populations

Twenty-five grams of the forage and silage samples from each mini-silo were homogenized in 225 ml of sterile Ringers solution (Oxoid, Basingstoke, UK) in an industrial

blender for 1 min. The aqueous extract was divided in two portions: one portion was used to measure the pH using a potentiometer and determine the concentrations of NH_3-N [13], WSC [14] and organic acids, as described previously.

Quantification of Microbial Populations

The second portion of the aqueous extracts was used to quantify the LAB, enterobacteria, yeast and mold populations. Serial dilutions were made in Ringers solution and plated using the plate technique in different culture media. Cultivation of the LAB population was performed on MRS agar (DifcoTM Lactobacilli MRS Agar®) at 37°C for 48 h. Culture of enterobacteria was performed on VRB agar (Violet Red Bile) at 37°C for 24 h, and the cultivation of mold and yeast was performed in Dextrose Potato Agar media containing a 1.5% tartaric acid solution (10% w/v) at 25°C for 96 h. The cfu was determined on plates containing 25 to 250 colonies.

Chemical Composition

Alfalfa samples before ensiling and their silages were dried in a forced-air oven at 55ºC for 72 h and milled in a Willey mill with a 1-mm sieve for determination of the DM (method 934.01) and CP (method 984.13), as described by the AOAC [15].

Acid detergent fiber (ADF) ([15], method 973.18) and neutral detergent fiber (NDF) using heat-stable α-amylase without sodium sulfite were only analyzed in the forage (day 0) and silage samples after 56 days of fermentation and corrected for residual ash [16]. Corrections of the NDF and ADF for nitrogen compounds were performed according to Licitra et al. [17].

In vitro Dry Matter Digestibility

In vitro DM digestibility (IVDMD) was performed on alfalfa silage samples from 56 d of fermentation. Dried 1-mm screen samples (0.5 g) were weighed in duplicates on F57 bags (Ankom Technology Corp.). Fermentation was performed in vitro using the DaisyII rotating jar in an incubator (Ankom Technology Corp.), according to methods described by Tilley and Terry [18] and adapted by Holden [19]. The analyses were replicated on two different occasions.

A composited inoculum was prepared with rumen fluid and rumen solids (pH= 6.09) collected from 3 cannulated lactating Holstein cows in mid-lactation that were fed a diet containing 70 g/kg of corn silage and 30 g/kg of concentrate mix (DM basis). Bags were removed from the jars after 48 h of fermentation, rinsed, and dried in a forced-air oven at 55ºC for 48 h. The coefficients of IVDMD was determined.

Statistical Analysis

The data were analyzed using the MIXED procedure of SAS® (v. 9.4 SAS Institute Inc., Cary, NC). The general model was given by Yijk=μ+Ii+Pj+(IP)ij+eijk, where Yijk = response variable; μ = overall mean; Ii = effect of inoculant i; Pj = effect of period j; (IP)ij = effect of the interaction between the level i of factor I and level j of factor P; and eijk = random residual term. The estimated means were compared using Tukey's test considering a significance level of 0.05.

RESULTS

The microbial populations and chemical composition characteristics of alfalfa forage prior the ensiling processes are shown in Table 1.

Table 1. Chemical composition (g/kg of DM, unless otherwise stated) and microbial populations (log cfu/g of fresh weight) of alfalfa forage before ensiling.

Item[1]	DM (g/kg)	CP	NDF	ADF	pH	WSC	LAB	Yeast	Molds	Ent
	368.1	175.4	403.1	270.7	6.54	23.9	6.54	5.19	5.16	6.37

[1]Item: DM = Dry matter; CP = Crude protein; NDF = Neutral detergent fiber; ADF = Acid detergent fiber; WSC = Water soluble carbohydrate; LAB = Lactic acid bacteria; ENT = Enterobacteria

The P-values and standard error of the mean of fermentation characteristics and microbial populations of alfalfa silages are shown in Table 2. There was an effect ($P<0.05$) of I and P on DM content and yeast population. CP content was affected only by P. Lower DM were observed in CTRL and LP silages than the other silages (360.5 vs. 365.5 g/kg as fed). However, this difference was biologically insignificant (Fig. 1). LP, PP, and LP + PP silages had lower yeast counts than CTRL (3.76 vs. 4.46 log cfu/g of fresh weight) (Fig. 1). The CP content and yeast population were reduced during the fermentation period (Fig. 2).

Table 2. Significance (P-values) of the tested and the standard error of the mean (SEM) for the fermentation profile variables of alfalfa silages treated with inoculants at different fermentation periods.

Item[1]	P-value			
	Inoculant	Period	Inoculant× Period	SEM
Dry matter	<.001	<.001	0.085	0.33
Crude protein	0.81	<.001	0.06	0.38
pH	<.001	<.001	<.001	0.06
WSC	<.001	<.001	<.001	0.04
NH_3-N	0.003	<.001	<.001	0.22
Lactic acid	<.001	<.001	<.001	0.13
Acetic acid	<.001	<.001	<.001	0.03
Propionic acid	<.001	<.001	<.001	0.014
Butyric acid	<.001	<.001	<.001	0.001
LAB[4]	<.001	<.001	<.001	0.16
Enterobacteria	<.001	<.001	<.001	0.21
Yeast	<.001	<.001	0.12	0.08
Molds	0.001	<.001	<.001	0.09

[1]Item: WSC = Water soluble carbohydrate; LAB = Lactic acid bacteria

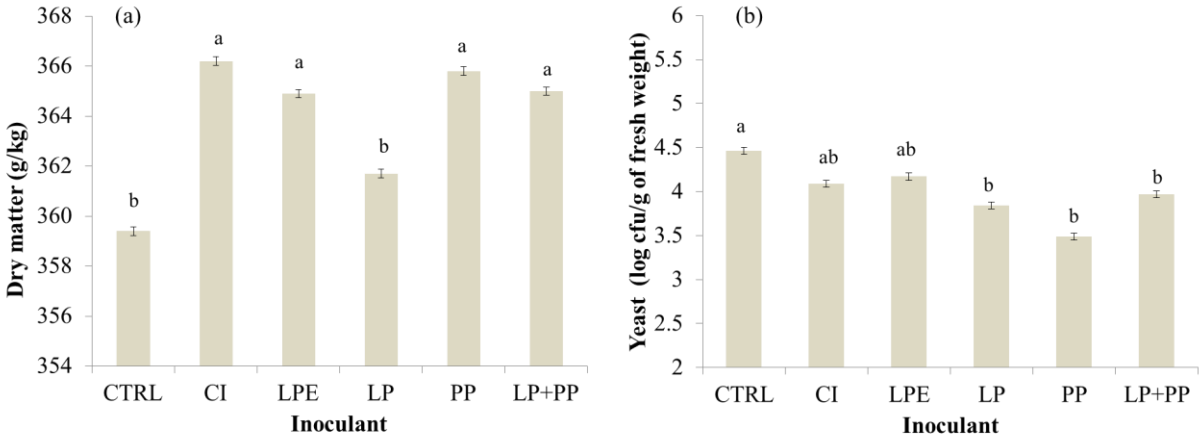

Figure 1 Effect of the microbial inoculants on the dry matter content (a) and yeast population (b) of alfalfa silages

[a-b]Means followed by different letters are significantly different according to Tukey's test ($P<0.05$).

CTRL = Control (without inoculant); CI = Commercial inoculant - Silobac; LPE = Lactobacillus pentosus; LP = Lactobacillus plantarum; PP = Pediococcus pentosaceus; LP+PP = Lactobacillus plantarum + Pediococcus pentosaceus.

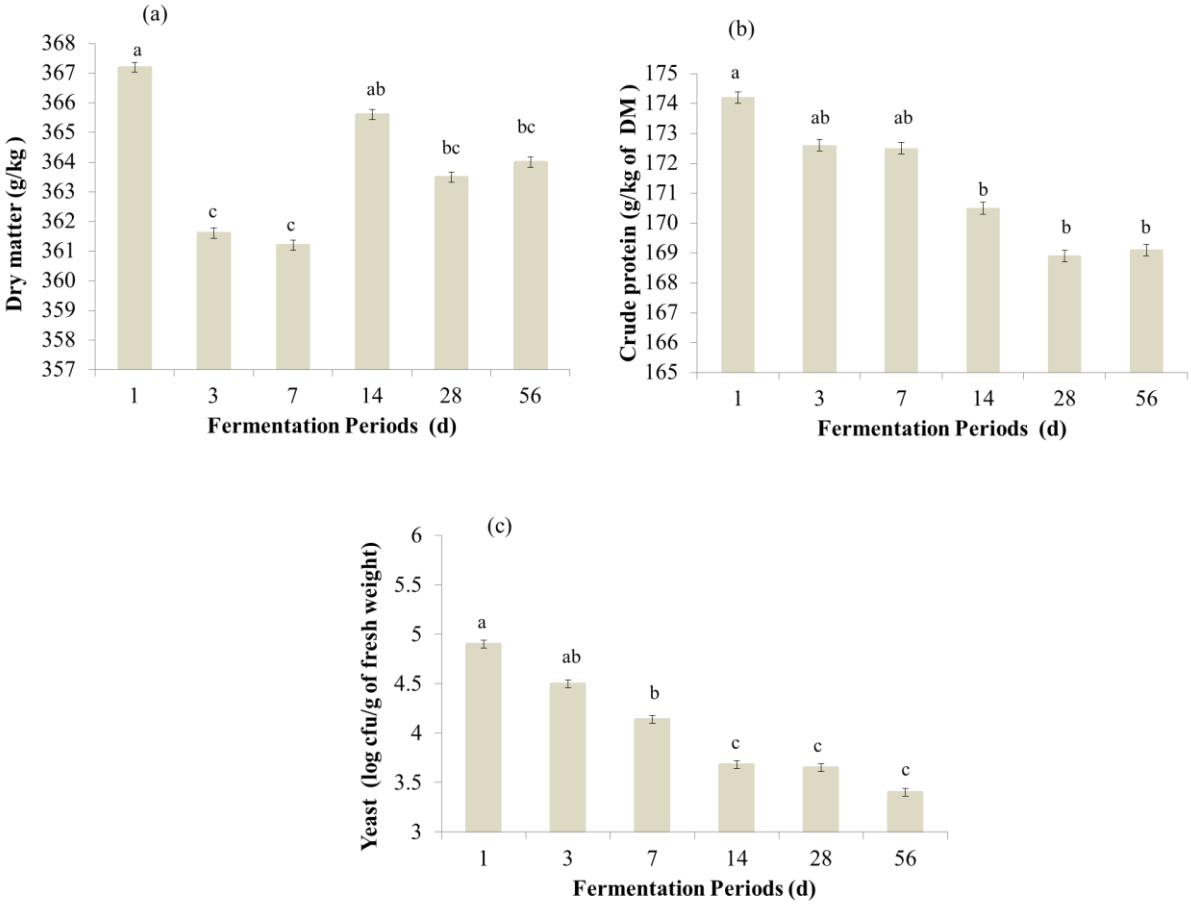

Figure 2. Effect of the fermentation period on the dry matter (a), crude protein (b) and yeast population (c) of alfalfa silages

[a-c]Means followed by different letters are significantly different according to Tukey's test ($P<0.05$).

CTRL = Control (without inoculant); CI = Commercial inoculant - Silobac; LPE = Lactobacillus pentosus; LP = Lactobacillus plantarum; PP = Pediococcus pentosaceus; LP+PP = Lactobacillus plantarum + Pediococcus pentosaceus.

The I×P interaction affected ($P<0.05$) the pH, WSC, NH_3-N, LAB, enterobacteria and mold populations (Table 2). The characteristics of alfalfa silages as a function of microbial inoculant within each fermentation period are shown in Table 3.

The LAB population was not affected by I only at 14 d of fermentation. The lowest counts were observed in CTRL silages in the first week of fermentation. Viable LAB counts increased from 6.54 log cfu/g in fresh forage to greater than 9 log cfu/g of fresh weight in silages. The population subsequently declined slowly after this peak. However, the LAB peak value for the inoculated silages occurred as early as 3 d after fermentation, and the peak was observed only after 28 d in non-inoculated silages. Lower counts of LAB was observed in PP silages at 28 d than other silages, except LP. However, PP silages had lower counts than LP at 56 d.

Table 3. Average fermentation profile, chemical composition and microbial populations of alfalfa silages as a function of the microbial inoculant within each fermentation period.

	Fermentation periods (days)					
Inoculant[1]	1	3	7	14	28	56
Lactic acid bacteria (log cfu/g of fresh weight)						
CTRL	6.89^{b}	8.42^{b}	9.05^{b}	9.20	9.42^{a}	8.71^{ab}
CI	7.47^{ab}	9.79^{a}	9.56^{a}	9.32	9.32^{a}	8.75^{ab}
LPE	7.66^{a}	9.94^{a}	9.66^{a}	9.38	9.20^{a}	8.55^{ab}
LP	7.81^{a}	9.83^{a}	9.52^{a}	9.45	9.13^{ab}	8.87^{a}
PP	7.42^{ab}	9.96^{a}	9.38^{a}	9.17	8.44^{b}	7.94^{b}
LP+PP	7.85^{a}	9.91^{a}	9.47^{a}	9.65	9.19^{a}	8.63^{ab}
Enterobacteria (log cfu/g of fresh weight)						
CTRL	5.89	7.29^{a}	6.84^{a}	5.07^{a}	3.98	4.48^{a}
CI	5.66	6.04^{ab}	5.04^{b}	3.48^{b}	3.13	2.79^{ab}
LPE	6.03	6.52^{ab}	4.80^{b}	3.08^{b}	3.30	2.45^{b}
LP	6.10	5.48^{b}	4.41^{b}	2.85^{b}	3.25	2.97^{b}
PP	6.73	6.06^{ab}	4.27^{b}	2.56^{b}	3.82	2.17^{b}
LP+PP	6.79	6.71^{ab}	5.00^{b}	2.84^{b}	3.15	2.52^{b}
Molds (log cfu/g of fresh weight)						
CTRL	5.06	4.60^{a}	4.23^{a}	4.45^{a}	3.84^{a}	2.68
CI	5.11	3.83^{b}	3.88^{ab}	3.68^{ab}	3.00^{ab}	2.50
LPE	5.25	4.03^{b}	3.59^{ab}	3.45^{ab}	2.49^{b}	2.18
LP	5.05	3.69^{b}	3.21^{ab}	3.56^{ab}	2.64^{b}	2.43
PP	5.08	3.88^{b}	2.97^{b}	3.23^{b}	2.43^{b}	2.25
LP+PP	5.00	3.82^{b}	2.99^{b}	3.26^{b}	2.32^{b}	2.47
Water soluble carbohydrates (g/kg of DM)						
CTRL	12.4^{ab}	16.0^{a}	9.8^{a}	7.5^{a}	3.0	3.4
CI	16.4^{a}	3.7^{b}	3.6^{b}	4.1^{b}	2.1	3.0
LPE	11.9^{ab}	2.6^{b}	5.0^{b}	3.5^{b}	2.6	3.6
LP	11.9^{ab}	4.6^{b}	3.0^{b}	3.3^{b}	2.6	3.6
PP	10.9^{b}	4.1^{b}	3.3^{b}	4.1^{b}	2.7	3.3
LP+PP	14.4^{ab}	4.5^{b}	3.3^{b}	3.5^{b}	2.9	3.2
pH						
CTRL	6.48	6.25^{a}	5.34^{a}	4.77^{ab}	4.60^{bc}	4.61^{bc}
CI	6.46	4.81^{bc}	4.58^{c}	4.65^{b}	4.74^{ab}	4.72^{b}
LPE	6.49	4.86^{bc}	4.78^{b}	4.79^{ab}	4.80^{a}	4.80^{ab}
LP	6.46	4.77^{c}	4.71^{bc}	4.76^{ab}	4.76^{a}	4.88^{a}
PP	6.52	4.88^{bc}	4.62^{c}	4.50^{c}	4.48^{c}	4.48^{c}
LP+PP	6.51	4.95^{b}	4.78^{b}	4.81^{a}	4.86^{a}	4.82^{ab}
NH_3-N (g/kg of total nitrogen)						
CTRL	20.2^{ab}	30.4^{ab}	46.0^{ab}	54.7^{a}	73.4^{ab}	80.9^{ab}
CI	10.6^{b}	24.3^{b}	59.9^{a}	48.1^{ab}	85.7^{a}	78.0^{ab}
LPE	26.3^{a}	25.7^{b}	58.9^{a}	54.7^{a}	81.8^{ab}	85.0^{a}
LP	14.0^{ab}	30.3^{ab}	40.0^{b}	54.6^{a}	71.2^{b}	84.6^{a}
PP	23.3^{ab}	41.7^{a}	46.3^{ab}	51.6^{ab}	59.9^{b}	69.4^{b}
LP+PP	27.6^{a}	32.9^{ab}	44.2^{b}	40.4^{b}	76.3^{ab}	83.4^{ab}

[a-c]Means within columns with different letters are significantly different according to Tukey's test ($P<0.05$).

[1]Inoculant: CTRL = Control (without inoculant); CI = Commercial inoculant - Silobac; LPE = Lactobacillus pentosus; LP = Lactobacillus plantarum; PP = Pediococcus pentosaceus; LP+PP = Lactobacillus plantarum + Pediococcus pentosaceus.

The I×P interaction did not affect the enterobacteria population at days 1 and 28. CTRL silages had the highest population 7 and 14 d after fermentation. The new strains controlled more efficiently the population at the end of the fermentation resulting in lower counts of these microorganisms. The mold population was not affected by interaction at 1 and 56 d. CTRL silages had the highest population of this microorganism at day 3 and higher counts than PP and LP+PP at 7 and 14 d of fermentation.

Non-inoculated silages had higher concentrations of residual WSC from 3 to 14 d, but no differences between silages was observed after 14 d. The pH was not affected by I only on the first day of fermentation. CTRL silages had the highest pH 3 and 7 d after ensiling. Inoculated silages had the highest pH decline rates in the first week of fermentation. PP resulted in the lowest pH values from 14 d of fermentation.

Inoculants affected the concentration of NH_3-N at all fermentation periods. Lower values were observed for LP and PP silages at 28 d compared to CI (65.5 vs. 85.7 g/kg TN). PP silages had lower concentrations than LP and LPE (69.4 vs. 84.8 g/kg TN) after 56 d of fermentation.

The I×P interaction also affected the production of lactic, acetic, propionic and butyric acids ($P<0.05$) (Table 2). The acid concentrations as a function of microbial inoculant within each fermentation period are shown in Table 4. An I×P effect was observed on the concentrations of lactic acid at 3, 7 and 28 d. CTRL silages had the lowest concentrations at 3 and 7 d. PP silages had the highest lactic acid values on day 28 of fermentation (40.6 vs. 34.9 g/kg DM).

Table 4. Average organic acids production of alfalfa silages as a function of the microbial inoculant within each fermentation period.

Inoculant[1]	Fermentation periods (days)					
	1	3	7	14	28	56
	Lactic acid (g/kg of DM)					
CTRL	7.7	6.3^{b}	18.6^{b}	26.5	34.4^{b}	33.2
CI	7.6	32.0^{a}	36.9^{a}	29.6	30.0^{b}	34.2
LPE	9.6	30.5^{a}	30.5^{a}	27.2	34.4^{b}	37.9
LP	7.7	27.8^{a}	35.3^{a}	34.3	32.9^{b}	39.9
PP	9.3	27.9^{a}	32.9^{a}	33.5	40.6^{a}	37.3
LP+PP	7.8	25.3^{a}	34.4^{a}	29.3	38.5^{b}	42.0
	Acetic acid (g/kg of DM)					
CTRL	3.7	5.0^{ab}	8.7^{a}	6.4^{b}	11.3^{a}	8.9^{ab}
CI	5.2	4.1^{ab}	7.0^{ab}	6.8^{b}	8.3^{ab}	8.2^{ab}
LPE	5.9	5.3^{ab}	7.2^{ab}	6.1^{b}	9.1^{ab}	10.4^{a}
LP	4.3	4.7^{ab}	6.3^{ab}	9.3^{a}	7.3^{b}	11.1^{a}
PP	5.5	4.0^{b}	5.1^{b}	6.4^{b}	6.5^{b}	6.4^{b}
LP+PP	4.5	6.8^{a}	6.5^{ab}	8.2^{b}	9.2^{ab}	10.7^{a}
	Propionic acid (g/kg of DM)					
CTRL	2.73	6.33^{a}	2.66^{a}	0.92	0.87	1.08
CI	2.25	5.76^{a}	0.98^{b}	1.06	0.96	1.05
LPE	2.52	3.08^{b}	2.79^{a}	0.96	0.94	1.06
LP	2.96	1.79^{c}	1.09^{b}	0.72	0.97	1.06
PP	2.69	0.93^{c}	1.12^{b}	0.98	1.04	1.14
LP+PP	2.62	1.02^{c}	0.87^{b}	0.90	0.90	1.13
	Butyric acid (g/kg of DM)					
CTRL	0.22	0.30	0.58^{a}	0.32	0.29	0.32
CI	0.23	0.31	0.48^{b}	0.30	0.31	0.23
LPE	0.24	0.42	0.33^{b}	0.25	0.31	0.45
LP	0.24	0.36	0.40^{b}	0.19	0.45	0.31
PP	0.22	0.40	0.27^{b}	0.23	0.45	0.43
LP+PP	0.26	0.31	0.33^{b}	0.27	0.30	0.46

[a-c]Means within columns with different letters are significantly different according to Tukey's test ($P<0.05$).

[1]Inoculant: CTRL = Control (without inoculant); CI = Commercial inoculant - Silobac; LPE = Lactobacillus pentosus; LP = Lactobacillus plantarum; PP = Pediococcus pentosaceus; LP+PP = Lactobacillus plantarum + Pediococcus pentosaceus.

The interaction did not affect the acetic acid concentration only at 1 d after fermentation. LP + PP and CTRL silages had higher concentrations than PP silages at days 3 and 7, respectively. The highest concentrations were observed in LP silages (9.3 g/kg DM) at day 14. Lower values were observed in LP and PP silages compared to CTRL (6.9 vs. 11.3 g/kg DM) at 28 d. PP silages had the lowest acetic acid production comparing to the new strains at 56 d.

Propionic acid was affected by the interaction at 3 and 7 d after fermentation. CTRL and CI silages had the highest concentration at day 3, and CTRL and LPE had the highest concentrations at day 7. There was a slight increase in butyric acid concentration in CTRL silages only 7 d after fermentation.

The chemical composition and IVDMD of alfalfa silages at 56 d of fermentation are presented in Table 5. The DM, CP, NDF and ADF of silages at 56 d of fermentation were unaffected (P>0.05) by I and averaged 364 g/kg as fed, 169.2, 388.0 and 259.0 g/kg DM, respectively. Silages inoculated with PP had higher coefficients of digestibility than LP silages (0.644 vs. 0.611).

Table 5. Average (with the respective standard error of the mean and ANOVA based P-value) chemical composition and coefficient of the in vitro dry matter digestibility of alfalfa silages treated with microbial inoculants at 56 d of fermentation (g/kg DM, unless otherwise stated).

Item[1]	Alfalfa silage day 56						SEM	P-value
	CTRL	CI	LPE	LP	PP	LP+PP		
Dry matter (g/kg)	358.0	369.0	366.9	362.9	364.4	363.0	0.11	0.09
Crude protein	171.7	171.5	168.8	167.9	167.2	168.1	1.33	0.31
NDF[3]	383.0	346.1	324.1	353.7	369.0	371.7	0.61	0.06
ADF[4]	265.1	255.8	258.2	253.6	256.5	268.0	0.28	0.72
IVDMD[5]	0.634[ab]	0.614[ab]	0.625[ab]	0.611[b]	0.644[a]	0.613[ab]	0.36	0.02

[a-b]Means within rows with different letters are significantly different according to Tukey's test (P<0.05).
[1]Item: NDF = Neutral detergent fiber; ADF = Acid detergent fiber; IVDMD = Coefficient of in vitro dry matter digestibility
CTRL = Control (without inoculant); CI = Commercial inoculant - Silobac; LPE = Lactobacillus pentosus; LP = Lactobacillus plantarum; PP = Pediococcus pentosaceus; LP+PP = Lactobacillus plantarum + Pediococcus pentosaceus.

DISCUSSION

Silage is a very complex fermentation matrix that exhibits variability in natural microbiota, chemical composition and nutrients, such as WSC and the nitrogenous components available for microbes [20]. The occurrence of desirable silage fermentations is guided by the amount and type of microorganisms present in the plant and the DM content, buffering capacity and WSC of the forage [3].

The average WSC of raw alfalfa was lower than the 40-60 g/kg DM recommended by Mahanna [21] as adequate for the occurrence of good fermentation of silage. However, studies on alfalfa silage also reported WSC between 10 and 40 g/kg DM [22, 23]. The LAB counts were higher than the minimum established by Muck [24] (5.0 log cfu/g fresh weight) as adequate for the occurrence of good fermentation of silage.

The faster increase in LAB counts observed in inoculated silages in early fermentation indicated that the LAB strains were competitive among the epiphytic communities. Microbial changes during this phase in successfully fermented silages are primarily the result of the disappearance of enterobacteria and the development of a dominant LAB population. The speed of this shift closely correlates with the rate of pH decline and lactic acid production [26]. The reduction in the LAB population after the peak in all assessed silages was expected because low pH and the lack of fermentable substrates result in bacterial death [27].

The reduction of pH is related to the conservation of the ensiled material. The fast initial acidification promotes a decrease in the enzyme-mediated proteolytic activity of the plant itself and controls the growth of enterobacteria and clostridia, which grow until an inhibitory concentration of non-dissociated acids and/or sufficiently low pH are reached [25].

In our study, the acidification induced by epiphytic bacteria fermentation in the CTRL silages reached the similar values of inoculated silages pH after 14 d. The highest pH values on days 3 and 7 in the CTRL silages reflected the low epiphytic LAB counts and its low efficiency in initiating fermentation and controlling undesirable microorganisms compared to the LAB strains, as suggested by Davies et al. [28]. The final pH values of all the silages were within the range of 4.48-4.88, which is considered adequate for legume silages, which usually stabilize when the pH drops to between 4.5 and 4.9 [29].

The changes in the WSC contents are related to the use of these carbohydrates by bacteria as substrates for growth, which results in the synthesis of primarily lactic acid [30]. As expected, the WSC concentrations of all silages were reduced during the fermentation. The highest residual WSC content in the CTRL silages in the first week of fermentation reflects the lower fermentation intensity in these silages, as evidenced by the lower LAB counts and lactic acid concentrations and higher pH values.

Zielińska et al. [6] found that some LAB strains developed more intensively in ensiled plants because of their role in the partial hydrolysis of starch, cellulose and xylans. This capacity may explain the lowest pH values of the PP silages with similar residual WSC concentrations at day 28 of fermentation, which was reflected by the highest conversion of substrate into lactic acid in these silages during the same period.

The reduction of CP content during the fermentation process was due to the plant and microbial proteolytic processes in the ensiled material, which change the nitrogenous compounds in silages and results in an increase in soluble N and NH_3-N [31], as observed in our study. According to Langston et al. [32] proteolysis results in the formation of peptides

and amino acids. The NH_3-N formation is a reflection of amino acid deamination, which characterizes the end of a putrefactive process. The NH_3-N concentrations were different between silages in our study, but no differences in CP concentrations were observed between inoculants, which suggests that the production of NH_3-N resulted from different intensities of the deamination of free amino acids in the material.

The higher NH_3-N concentrations of silages inoculated with LPE and LP strains compared to PP on the last day of fermentation were reflected by the higher pH and acetic acid concentrations in these silages, which indicated the growth of undesirable microorganism. Kung [10] and Oliveira et al. [1] reported that P. pentosaceus strain inoculation did not affect NH_3-N concentrations in silage, which may be related to its slower growth rate than other bacteria, but this effect was not observed in our study.

The main acids identified in the silages are acetic, butyric and lactic because these acids represent the highest concentrations of acids [33]. Kung et al. [31] demonstrated that lactic acid was generally found at the highest concentration in silages during the ensiling process and contributed the most to the decrease in pH during fermentation because it is approximately 10 to 12 times stronger than the other major acids. The concentrations of lactic acid in the silages were 20 to 40 g/kg DM, which are the concentrations commonly found in legume silages that were also reported by these authors.

The lower production of lactic acid in CTRL silages in the first week of fermentation reflects the lower LAB counts and their ability to dominate the fermentation, as discussed previously. Muck and Kung [5] found that silages treated with homofermentative bacteria resulted in lower silage pH compared to untreated silages because of the greater production of lactic acid, which may be more evident in legume than corn silage.

Although lower LAB counts were observed in the PP silages at day 28, higher lactic acid values were produced, which shows the efficiency of substrate utilization and the persistence of acidification of the strain. PP silages had lower pH than other inoculated silages at day 56 of fermentation with the same lactic acid concentration, which may be attributed to the lower production of NH_3-N and acetate in these silages and the reduced buffering effects of these compounds on the ensiled material [34].

The contents of acetic and butyric acids are primary negative indicators of the quality of the fermentation process and also correspond to silages that showed marked losses of dry matter and energy during fermentation. Lower concentrations of acetic acid in PP silages at the end of the fermentation period may result in higher DM recovery, and it indicates the

predominance of homolactic fermentation compared to the LP, LPE, and LP+PP silages. The highest concentrations of propionic acid at 3 and 7 d in CTRL silages may have resulted from secondary fermentations, especially because the concentrations of lactic acid were lower in this silage during this period.

Enterobacteria are generally the second most numerous bacterial group of the epiphytic microbiota active in the silo. Their population and rate of decline are used as indicators of silage quality because these microorganisms are main competitors with LAB for available sugars and result in gas losses and a reduction in the nutritional value of the silages [30].

The dominance of LAB, the faster drop in the pH induced by higher lactic acid production and the synergistic effects of the acids produced during the fermentation in our study resulted in the reduction of enterobacteria counts in all inoculated silages until 14 days of fermentation. The same reasons are attributed to the reducing of yeast population throughout the fermentation periods.

The studied variables did not affect the chemical composition of silages at 56 d of fermentation, which suggests that the attendant improvements in silage characteristics are often lacking even when the concentrations of supposedly explanatory metabolites increase in response to bacterial inoculation. This effect may occur because the explanatory metabolites only explain a fraction of the variability in the response to an inoculant [35]. The measurement of IVDMD is used to analyze the nutrient digestibility of feed ingredients [36, 15].The values of IVDMD in our study were similar to Nadeau et al. [37] and Rodrigues et al. [23], also in alfalfa silage.

In conclusion, all of the novel strains tested had a positive effect on at least one chemical property of the silage during the fermentation process. However, the addition of P. pentosaceus alone had a positive influence on all of the evaluated parameters and changed the characteristics of the silages; particularly, the strain enhanced the lactic acid content and decreased the pH, deteriorating microorganisms, and NH_3-N and acetic acid concentrations, which resulted in a better silage quality that surpassed the commercial inoculant. We suggest that this strain has potential for use as a silage inoculant, but it must be tested in different forages and in combination with other additives, such as heterofermentative bacteria or chemical additives. The results obtained at the laboratory scale must also be confirmed under more practical conditions.

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AUTHORS CONTRIBUTIONS

M.C.N.A performed the experiment, analyses and prepared the manuscript. O.G.P designed the experiment and contributed to the writing of the manuscript. V.P.S performed the chemical composition analyses. R.A.P performed the microbiology analyses. J.P.S.R. performed the in vitro analyses, and F.F.S. performed the statistical analyses. All the authors reviewed the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Characterization of lactic acid bacteria isolated from non-wilted and wilted alfalfa silages

Isolate ID		3.7 E	14.7 SE	14.15 SE
Identification		Lactobacillus plantarum	Lactobacillus pentosus	Pediococcus pentosaceus
Access number		MH924275	MH924298	MH924301
Control condition	MRS/37°C	+++	++++	+++
Temperature (°C)§	15	+	+	+
	45	-	+	+
pH§	3.5	+	+	+
	4	+	+	+
	4.5	+	+	+
	8.5	+	+	+
g/L NaCl§	40	+	+	+
	65	+	+	+
CO_2*		Ho	Ho	Ho
1†	Radius of inhibition‡	++	++	++
2†		++	++	++
3†		++	++	++
pH	After 24h	4.18	4.12	4.25
Metabolites (g/kg of DM)	Lactic acid	14.9	15.1	13.3
	Acetic acid	3.1	2.8	1.9
	Propionic acid	0	0.1	0.2

§: Growth measured by optical density (630 nm) - = Absence of growth; + = Presence of growth; *Ho: Homofermentative; He: Heterofermentative; †: Indicator microorganisms of the antimicrobial test. 1- Listeria monocytogenes 19117; 2- Listeria monocytogenes 7644; 3- Escherichia coli; ‡: (mm) : - (absence of inhibition halo), + (> 4 and ≤ 15), ++ (> 15 and ≤ 30), +++ (> 30).

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CHAPTER 2 – SHORT COMMUNICATION: EFFECT OF WILD STRAINS OF LACTOBACILLUS BUCHNERI ON THE FERMENTATION QUALITY AND AEROBIC STABILITY OF CORN SILAGE IN TROPICAL CONDITION

Manuscript formatted according to the Journal of the Science of Food and Agriculture requirements

Running title: Lactobacillus buchneri in corn silage

ABSTRACT

BACKGROUND: Corn silage is the most used silage worldly due to the easily corn cultivation and adaptability, high mass production, adequate fermentation and energy. In spite of the potentialities, the aerobic deterioration is its main limitation. Although there are ranges of inoculants based on L. buchneri available on the market, recent studies testing different strains as inoculant silage on a variety of forages in tropical regions are still inconsistent. In this context, it was evaluated the effects of 11 wild L. buchneri strains on fermentation profile and aerobic stability of corn silages after 90 days of fermentation.

RESULTS: A treatment effect was observed on pH, water-soluble carbohydrates, ammonia nitrogen (NH_3-N), lactic, acetic and propionic acids, ethanol and 1,2-propanediol concentrations. Non-inoculated silages had the lowest pH and 56.1, 56.4, and 56.9 silages had the highest values (3.65 vs. 3.84). The lowest NH_3-N concentrations were observed in 56.1 and 56.7 silages conversely the highest values were found in 56.8 and 56.21 (7.11 vs. 10.01 % total nitrogen). Inoculated silages with 56.1 strain had the highest acetic and propionic acids concentrations and higher ethanol production than commercial inoculant (CI), 56.7, 56.9, 56.22, 56.25, and 56.26 silages. The populations of enterobacteria and yeasts & molds, chemical composition and DM recovery after 90 d of fermentation were not affected by treatments. Silage treated with 56.1 strain had higher aerobic stability than non-inoculated silages (68.2 vs. 36.0 h).

CONCLUSION: Lactobacillus buchneri strain 56.1 has the potential to be used as microbial inoculant for corn silage.

KEY WORDS: acetic acid, dry matter recovery, microbial inoculant, pH, Zea mays

INTRODUCTION

Corn silage has become the predominant forage used in the production of ruminants diets worldwide because of minimized risks of production, elevated yield per area, and flexibility of harvesting corn for forage or grain (Allen et al., 2003). Moreover, uniquely in comparison with other forages, corn silage offers dairy nutritionists the opportunity to provide high energy along with physically effective neutral detergent fiber (NDF) concurrently (Ferraretto et al., 2018).

Due to the high temperature and relative humidity in tropical countries, the aerobic deterioration of silages nutrients is inevitable, causing significant losses and negative consequences in animal production system (Adesogan, 2010). Yeasts are the microorganisms responsible for the onset of aerobic deterioration in silages (Pahlow et al., 2003). After ensiling, the yeasts compete with the other microorganisms for fermentable substrates, and during the first weeks of fermentation, the population can reach 10^7 colony-forming unit (cfu)/g of fresh weight, with a gradual decrease during the subsequent storage stages (Jonsson & Pahlow, 1984). The survival of this microorganism during fermentation depends on the degree of anaerobiosis, the pH and the concentration of organic acids.

It should be noted that the concern with aerobic stability is not limited to dry matter (DM) losses, since the development of microorganisms, such as bacteria belonging to the genera Bacillus, Clostridium and Listeria and some filamentous fungi may influence the hygienic quality of silage by mycotoxins production (Lindgren et al., 2002). Thereby, in the mid-1990s, Muck (1996) suggested that the addition of Lactobacillus buchneri to silages might prove valuable in improving aerobic stability and nowadays it is the dominant species used in obligate heterofermentative lactic acid bacteria (LAB) silage additives (Muck et al., 2018).

Although there are range of inoculants based on L. buchneri available on the market, recent studies testing different strains as inoculant silage on a variety of forages in tropical regions are still inconsistent (Santos et al., 2015; Gomes et al., 2019; Rabelo et al., 2019). Base on that more research is needed on the use of new strains of L. buchneri, especially those isolated under tropical conditions with antimicrobial action that may result in significant effects on fermentation parameters and aerobic deterioration, and, consequently, improvements in silage quality.

In a previous study conducted in our lab, Silva et al. (2018) isolated 15 strains of L. buchneri from corn silage and four of them were evaluated as microbial inoculants in corn silage and four in sugarcane silage. The strains 56.1 and 56.4 were promising microbial inoculant in sugarcane fermentation, but the effects of these microorganisms were not evaluated in corn silage fermentation. In this context, we evaluated the effects of eleven wild L. buchneri including 56.1 and 56.4 strains on microbial populations, fermentation profile, chemical composition, DM loss, and aerobic stability of corn silages after 90 days of fermentation.

MATERIALS AND METHODS

The experiment was conducted between March and June 2016 at the Department of Animal Science of the Federal University of Vicosa (Viçosa, MG, Brazil), located at 20°45' South latitude, 42°51' West longitude and 648 m above sea level. The annual precipitation and average temperature the year of the experiment were 1235.4 mm and 20.7 °C, respectively.

The experiment was carried out under a completely randomized design with three replicates and 13 treatments. The evaluated treatments were the following: 1- non-inoculated (CRTL), 2- commercial L. buchneri strain (CI), and 11 wild strains of L. buchneri: 3- strain 56.1, 4- strain 56.2, 5- strain 56.4, 6- strain 56.7, 7- strain 56.8, 8- strain 56.9, 9- strain 56.21, 10- strain 56.22, 11- strain 56.25, 12- strain 56.26, 13- strain 56.27. The wild strains of L. buchneri isolated from corn silage, belonging to the culture collection of the Forage Laboratory of the UFV. The phenotypic characteristics of the strains are described in a study conducted by Silva et al. (2018). The commercial inoculant Lalsil® AS (Lallemand Animal Nutrition), which contains L. buchneri CNCM-I 4323 1.0 x 10^{11} cfu/g and sucrose, was used based on manufacture recommendation dosage to compare its effectiveness with new strains.

The wild strains were cultured in MRS broth for 14 h, and then the inoculum was standardized using a spectrophotometer (630 nm) at an optical density of 0.05, into 20 ml of MRS broth and cultured for 14 h. With this, the amount of inoculum needed to reach the theoretical application rate of 10^5 cfu/g of fresh forage was centrifuged at 1,000 g for 10 min and the supernatant was discarded.

Corn was harvested with an average DM of 27.36 % as fed, using a one-row pull-type forage harvester (JF-92 Z10) with a theoretical length chop of approximately 15 mm. Randomly, the chopped corn was divided in 39 piles of 10 kg and three replicated piles (each pile treated individually) were prepared for each treatment.

Inoculants were diluted in 50 ml of water and applied with the aid of a hand sprayer. The same water quantity was applied to the CTRL silages. Eight kilograms of the treated forage were ensiled in plastic buckets (experimental mini-silos) with 10 L capacity. The chopped forage were compacted in the buckets out by trampling and were stored in a temperature range, 23-27°C for 90 days.

Corn samples before ensiling and their silages were dried in a forced-air oven at 55 ºC for 72 h and milled in a Willey mill with a sieve of 1 mm. The DM (method 934.01), crude protein (CP; method 984.13) and acid detergent fiber (ADF; method 973.18) were determined as described by AOAC (1990). The NDF content was determined using heat-stable α-amylase without use of sodium sulfite and was corrected for residual ash (Mertens, 2002). Correction of the NDF and ADF for nitrogen compounds were performed according to Licitra et al. (1996).

Twenty-five grams of the forage and silage samples from each mini-silo were homogenized in 225 ml of sterile Ringers solution (Oxoid, Basingstoke, UK) for 1 min. The aqueous extract was divided in two portions, one of them was used to measure the pH using a potentiometer, determined the concentration of ammonia nitrogen (NH_3-N; Okuda et al., 1965), water-soluble carbohydrates (WSC; Nelson, 1944) and analysis of the organic acids by HPLC (Shimadzu Scientific Instruments, Columbia, MD) according to Siegfried et al. (1984).

The second portion of the aqueous extracts was used to quantify the LAB, enterobacteria, yeast and molds (yeast & molds) populations. Serial dilutions were made in Ringers solution and the plating by the plate technique was carried out in different culture media. Cultivation of the LAB population was performed on MRS agar (DifcoTM Lactobacilli MRS Agar) at 37 °C for 48 h; culture of enterobacteria was performed on VRB agar (Violet Red Bile) at 37 °C for 24 h; the cultivation of mold & yeasts was carried out in DPA media (Dextrose Potato Agar) containing 1.5% of tartaric acid solution (10% w./v.) at 25 °C for 96 h. The number of cfu was determined on plates containing between 25 and 250 colonies.

Apparent DM loss was calculated according to Jobim et al., (2007). After 90 days of fermentations 2 kg of silage from each silo were collected and placed in plastic buckets without a lid, the buckets were covered with 2 layers of cheesecloth to prevent drying and kept in a room at 21 ± 1°C for 7 days. Temperatures were measured every 15 min using data loggers (Impac, model MI-IN-D-2-L; São Paulo, Brazil), inserted into the silages mass at geometric center.

The aerobic stability was denoted by the time (h) before a 2 °C rise in silage temperature above ambient temperature (21 °C). It were also measured the maximum temperature (Tmax) reached by the mass and the time it took to reach those temperatures (HTmax). After 7 days of air exposure, 25 g of representative samples from each bucket were homogenized in 225 ml of sterile Ringers solution in industrial blender for 1 min and the pH, organic acids and the yeast & molds population were measured as described previously.

All microbial counts were converted into the logarithmic base (log10 cfu). The data were analyzed using the GLM procedure of SAS® (v. 9.4 SAS Institute Inc., Cary, NC). The general model was given by: $Yij=\mu+Ii+eij$, where Yij = response variable; μ = overall mean; Ii = effect of inoculant i and eij = random residual term. The estimated means were compared by Tukey's test considering a significance level of 0.05.

RESULTS AND DISCUSSION

The chemical composition of the corn forage agreed to hybrids produced in Brazil. The initial pH averaged 5.83 and the DM, CP, NDF and ADF contents averaged 27.36%, 5.45, 55.0 and 31.49% of DM, respectively. The WSC concentration (9.24% of DM) before ensiling was sufficient to ensure adequate ensiling (Haigh and Parker, 1985).

The adequate population of microorganisms in the silage dictates the sense of the fermentation and the proportions of the organic acids that will be produced, influencing the deterioration and the consumption of silages by the animals. The initial LAB population (7.4 log cfu/g fresh weight) was higher than the minimum established by Muck (1996) (5.0 log cfu/g fresh weight) as adequate for the occurrence of good fermentation of silage. Moreover, the initial populations of enterobacteria and yeast & molds averaged 7.25 and 6.46 log cfu/g fresh weight, respectively.

A treatment effect ($P < 0.05$) was observed on pH, WSC, NH_3-N, lactic, acetic and propionic acids, ethanol and 1,2-propanediol concentrations. The fermentation patterns of silages after 90 days of storage are shown in Table 1. The lowest pH was observed in CTRL silages, whereas, 56.1, 56.4, and 56.9 silages had the highest values (3.65 vs. 3.84). CTRL silages had higher residual WSC than CI, 56.2 and 56.7 silages and higher lactic acid concentration than CI and 56.4. Silages inoculated with 56.1 strain had the highest acetic and propionic acids concentrations and higher ethanol production than CI, 56.7, 56.9, 56.22, 56.25, and 56.26. Higher amounts of 1,2-propanediol was found in 56.25 silages than others

except CTRL and 56.1 while butyric acid was not affected (P = 0.99) by treatment and averaged 0.023% DM.

Table 1. Fermentation patterns (% of DM, unless otherwise stated) of corn silage treated with Lactobacillus buchneri strains after 90 days of fermentation (SEM, standard error of mean)

Treatment	Items								
	pH	WSC[†]	NH_3-N[‡]	LA[§]	AA[¶]	BA[¥]	PA[€]	ET[λ]	1,2-PD[£]
CTRL	3.65^{e}	1.08^{a}	8.04^{bcd}	5.91^{a}	1.24^{bc}	0.027	0.08^{d}	1.72^{ab}	0.30^{ab}
CI	3.70^{cde}	0.69^{b}	8.63^{abcd}	2.90^{b}	1.09^{c}	0.023	0.75^{d}	1.17^{b}	0.25^{bc}
56.1	3.84^{a}	0.81^{ab}	6.99^{d}	3.78^{ab}	2.53^{a}	0.023	3.02^{a}	2.62^{a}	0.27^{abc}
56.2	3.80^{abc}	0.54^{b}	7.49^{cd}	3.46^{ab}	1.47^{bc}	0.023	1.50^{bc}	1.49^{ab}	0.24^{bc}
56.4	3.85^{a}	0.79^{ab}	8.01^{bcd}	3.18^{b}	1.40^{bc}	0.027	1.65^{b}	1.62^{ab}	0.24^{bc}
56.7	3.76^{abcd}	0.70^{b}	7.23^{d}	4.30^{ab}	2.08^{ab}	0.023	1.50^{bc}	0.71^{b}	0.23^{c}
56.8	3.81^{ab}	0.71^{ab}	10.00^{a}	3.97^{ab}	1.54^{bc}	0.027	2.11^{b}	1.65^{ab}	0.23^{c}
56.9	3.83^{a}	0.82^{ab}	7.94^{bcd}	3.63^{ab}	1.44^{bc}	0.025	1.62^{b}	1.36^{b}	0.24^{bc}
56.21	3.71^{bcde}	0.78^{ab}	9.97^{a}	5.34^{ab}	1.33^{bc}	0.023	0.35^{d}	1.57^{ab}	0.24^{bc}
56.22	3.70^{cde}	0.83^{ab}	9.52^{ab}	3.81^{ab}	0.83^{c}	0.025	0.08^{d}	1.34^{b}	0.26^{bc}
56.25	3.71^{bcde}	0.77^{ab}	8.14^{bcd}	4.11^{ab}	0.66^{c}	0.023	0.12^{d}	0.80^{b}	0.33^{a}
56.26	3.67^{de}	0.88^{ab}	7.73^{cd}	3.50^{ab}	0.82^{c}	0.023	0.07^{d}	0.60^{b}	0.24^{bc}
56.27	3.68^{de}	0.87^{ab}	9.07^{abc}	5.06^{ab}	1.11^{c}	0.027	0.12^{d}	1.45^{ab}	0.22^{c}
SEM	0.01	0.03	0.18	0.39	0.13	0.0009	0.16	0.16	0.015
ANOVA P-value	<0.01	0.009	0.01	0.04	<0.01	0.99	<0.01	<0.01	0.0003

[a–e]Means within columns with different letters are statistically different under Tukey test (P < 0.05).
[†]Water-soluble carbohydrate; [‡]Ammonia nitrogen (% of total nitrogen); [§]Lactic acid; [¶]Acetic acid; [¥]Butyric acid; [€]Propionic acid, [λ]Ethanol, [£]1,2-Propanediol
CTRL:non-inoculated; CI: Commercial inoculant – Lalsil AS; 56.1-56.27 - Wild Lactobacillus buchneri strains

The lower pH in CTRL silages resulted from the higher lactic acid concentration found in these silages. Hence, untreated corn silage undergoes a homolactic fermentation resulting in relatively low pH values due to the high concentrations of lactate and low concentrations of acetic and propionic acids (Rooke and Hatfield, 2003).

The pH values in 56.1 silages reflected the lower lactic acid production or lactic acid consumption and it also may be attributed to buffering effects in the ensiled material caused by high concentrations of acetic and propionic acids and ethanol (Hashemzadeh-Cigari et al., 2013). Indeed, the moderate conversion of lactic acid to acetic acid, 1,2-propanediol and ethanol by some L. buchneri strains often results in silages 0.1 to 0.2 pH units higher than untreated silage (Oude Elferink et al., 2001). However, all pH values were within the range of 3.6 and 4.0 which according to Kung et al. (2018) reflects adequate fermentation for restricting the growth of undesirable microorganisms.

High residual WSC concentration as observed in CTRL silages are desirable and suggests that plant sugars were less extensively fermented by the epiphytic bacteria compared with those in CI, 56.2 and 56.7 silages, reflecting a more efficient fermentation in the silo and indicate greater availability of energy-yielding substrates for ruminal microbes (Arriola et al., 2011). However, high residual WSC concentrations together with high lactic acid content may also induce spoilage microorganism growth (Weinberg et al., 1993), reducing the aerobic stability of silages as observed in our study.

During the fermentation process, the initial proteolysis is mediated primarily by plant enzymes, while subsequent amino acid degradations occur by the action of microorganisms (Heron et al., 1986). The NH_3-N formed in this process alters the fermentation course, inhibiting a rapid drop in the pH of the ensiled mass, and affect silage consumption, reducing the efficiency in the use of nitrogen for protein synthesis by rumen microorganisms (McKersie, 1985). The lowest NH_3-N concentrations were observed in 56.1 and 56.7 silages conversely the highest amounts were found in 56.8 and 56.21 silages (7.11 vs. 10.0 % of total nitrogen).

The population of LAB, enterobacteria, yeasts & molds are present in Figure 1. It was observed a treatment effect ($P = 0.003$) only on LAB population. Silages inoculated with CI and 56.25 strains had higher counts than 56.4 and 56.22 (7.05 vs. 5.68 log cfu/g of fresh weight). The populations of enterobacteria and yeast & molds averaged 0.43 and 5.40 log cfu/g of fresh weight, respectively.

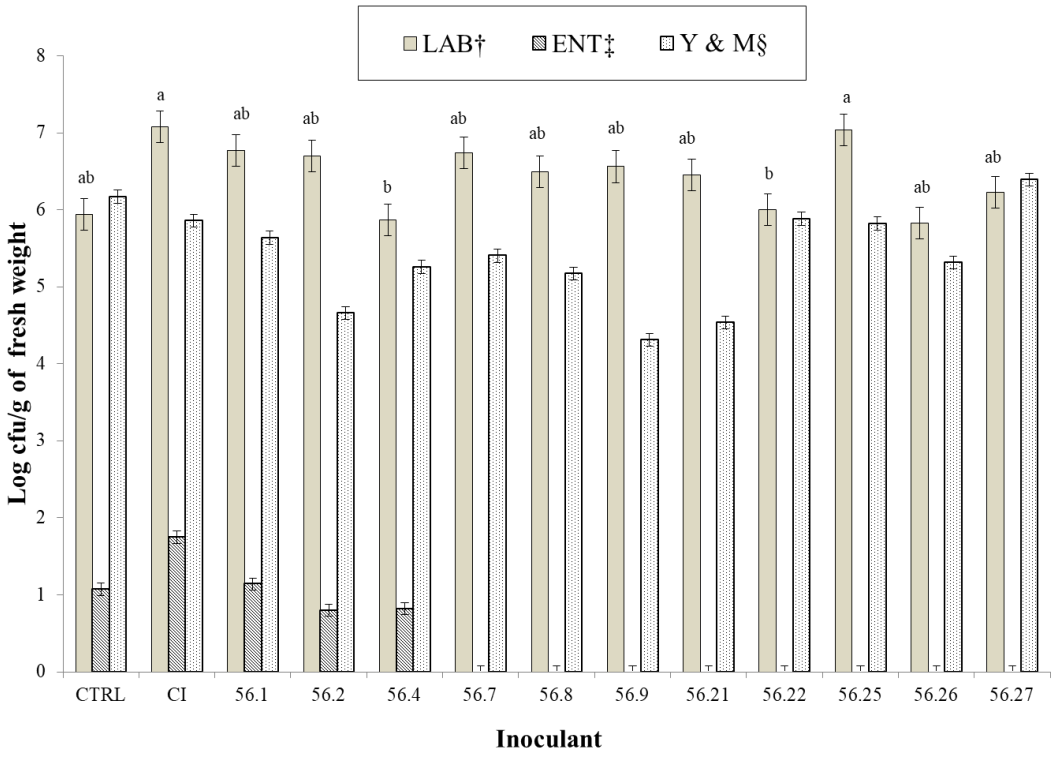

Figure 1. Microbial populations of corn silage treated with Lactobacillus buchneri strains after 90 days of fermentation

[a–b]Means within columns with different letters are statistically different under Tukey test ($P < 0.05$)

[†]lactic acid bacteria - ANOVA P-value = 0.003; [‡]enterobacteria - ANOVA P-value = 0.43; [§]yeast and molds - ANOVA P-value = 0.48

CTRL: non-inoculated; CI: Commercial inoculant – Lalsil AS; 56.1-56.27 – Wild Lactobacillus buchneri strains

The growth of enterobacteria in silage is undesirable because they compete with the LAB for the available sugars, and also degrade protein (Oude Elferink et al., 1999). In our study, the low counts of this microorganism were expected due to the rapid and sufficient drop in silage pH during the ensiling process (McDonald et al. 1991).

Enumeration of yeasts & molds in silages may be useful because, high numbers of yeasts in silages are usually associated with high concentrations of ethanol, and their numbers are often inversely related to the aerobic stability of silages. Controversially, in our study, the higher aerobic stability ($P = 0.009$) observed for 56.1 silage compared with non-inoculated (68.2 vs. 36.0 h, Table3) silage was accompanied by higher ethanol concentration and no changes on yeasts & molds counts. Silva et al. (2018) also observed the absence of inoculant effect on yeast & molds counts after fermentation, and it was attributed to the high initial population of these microorganisms in raw material.

We must be careful when interpreting the yeasts and molds populations in silages for various reasons. First, analytical laboratories enumerate the total number of yeasts but do not differentiate between those that are lactate assimilators and those that are not. Second, yeast may be able to grow on selective agar during enumeration, but this does not necessarily reflect their metabolic capabilities in silage. Thus, silages with a moderate amount of yeasts can still be relatively aerobically stable. Third, especially in corn silages, other microorganism such as Acetobacter bacteria can initiate aerobic spoilage because they are able to oxidize lactate and acetate to carbon dioxide and water (Spoelstra et al., 1988), and thus silages with low yeast numbers can be aerobically unstable. In this context, the increased aerobic stability observed in 56.1 silages may be related to inhibiting effect on specific yeasts species and other deteriorating microorganisms silages due to the accumulation of compounds with antifungal capacity (e.g., acetic and propionic acids) and the reduced concentrations of lactic acid (Wilkinson and Davies, 2013).

The chemical composition and dry matter recovery of silages after 90 days of fermentation are shown in Table 2. The DM, CP, NDF, ADF and DM recovery were not affected ($P > 0.05$) by the treatments and averaged 26.8 %, 5.54, 50.54 and 32.99 % of DM and 97.09 %, respectively.

Table 2. Chemical composition (% of DM, unless otherwise stated) and dry matter recovery of corn silage treated with Lactobacillus buchneri strains after 90 days of fermentation (SEM, standard error of mean)

Treatment	Items				
	DM[†] (%)	CP[‡]	NDF[§]	ADF[¶]	DM rec (%)[¥]
CTRL	25.71	5.47	54.91	32.99	91.92
CI	26.02	5.84	50.59	33.35	95.96
56.1	26.07	5.69	51.58	35.40	92.76
56.2	26.80	5.67	52.00	33.28	95.53
56.4	26.27	5.75	49.67	33.04	95.03
56.7	26.72	5.51	50.54	29.74	88.77
56.8	26.82	5.47	51.10	31.91	97.10
56.9	26.51	5.67	48.82	34.13	97.09
56.21	26.90	5.15	51.01	34.93	98.87
56.22	27.28	5.39	49.79	29.51	99.72
56.25	27.09	5.69	48.65	28.01	97.87
56.26	27.12	5.54	49.78	28.32	99.16
56.27	27.61	5.51	49.17	28.22	97.63
SEM	0.23	0.06	0.54	0.60	4.32
ANOVA P-value	0.97	0.71	0.79	0.07	0.20

[†]Dry matter; [‡]Crude protein; [§]Neutral detergent fiber; [¶]Acid detergent fiber; [¥] Dry matter recovery

CTRL: non-inoculated; CI: Commercial inoculant – Lalsil AS; 56.1-56.27 – Wild Lactobacillus buchneri strains.

The fermentation pattern of silages after 7 days of air exposure are presented in Table 3. The DM, yeast & molds population, Tmax, HTmax and pH were not affected ($P > 0.05$) by treatments and averaged 26.62 %, 9.00 log cfu/g fresh weight, 40.73 and 69.17 h and 6.20, respectively.

Table 3. Fermentation pattern and aerobic stability of corn silage treated with Lactobacillus buchneri strains after 7 days of air exposure at 21°C (SEM, standard error of mean)

Treatment	Items					
	DM[†]	pH	Y & M[‡]	AE[§]	Tmax[¶]	HTmax[¥]
CTRL	26.19	6.36	9.16	36.00[b]	41.76	50.17
CI	25.26	6.61	9.11	53.62[ab]	41.83	67.92
56.1	27.05	6.16	8.54	68.25[a]	38.83	86.04
56.2	26.49	6.15	8.46	63.41[ab]	40.73	88.14
56.4	26.62	6.08	8.67	59.62[ab]	39.75	84.75
56.7	26.51	6.24	8.76	48.00[ab]	40.83	69.17
56.8	26.69	6.20	9.15	60.58[ab]	39.33	87.17
56.9	26.95	6.00	8.90	56.00[ab]	39.76	80.42
56.21	26.93	6.31	9.09	38.41[ab]	41.00	55.00
56.22	27.41	6.07	9.00	48.25[ab]	39.80	62.67
56.25	26.51	6.26	9.19	56.75[ab]	39.83	77.25
56.26	26.62	6.25	8.94	39.50[ab]	41.6	57.38
56.27	27.13	6.13	9.10	37.25[ab]	40.76	59.75
SEM	0.72	0.16	0.23	3.80	1.50	3.94
ANOVA P-value	0.98	0.19	0.34	0.009	0.87	0.09

[a–b]Means within columns with different letters are statistically different under Tukey test ($P < 0.05$).
[†]Dry matter (%); [‡] Yeast & molds (log cfu/g of fresh weight); [§]Aerobic stability (h); [¶]Maximum temperature (°C); [¥] Time to reach the maximum temperature (h)
CTRL: non-inoculated; CI: Commercial inoculant – Lalsil AS; 56.1-56.27 – Wild Lactobacillus buchneri strains.

The organic acids, ethanol and 1,2-propanediol concentrations of corn silage after 7 days of air exposure are present in Table 4. The production of lactic, acetic and butyric acids and ethanol were not affected ($P > 0.05$) by treatments and averaged 0.22, 0.28, 0.02 and 1.39 % DM, respectively. Higher concentration of propionic acid was observed in 56.1 and 56.4 silages than others except 56.8. Silages inoculated with 56.27 strain had the lowest 1,2-propanediol concentration (0.24 % DM). After air exposure, all silages spoiled and had high yeast & molds population. The increased pH observed in the silages can be due to the reduction of organic acids concentrations by volatilization or utilization as substrate by yeasts, molds or bacteria under aerobic conditions (Carvalho et al., 2014; Oude Elferink et al., 2001).

Table 4. Organic acids, ethanol and 1,2-propanediol concentrations (% of DM) of corn silage treated with Lactobacillus buchneri strains after 7 days of air exposure at 21°C (SEM, standard error of mean)

Treatment	Items					
	LA[†]	AA[‡]	BA[§]	PA[¶]	Ethanol	1,2-Propanediol
CTRL	0.18	0.15	0.027	0.07^{bc}	1.25	0.26^{a}
CI	0.23	0.33	0.023	0.08^{bc}	0.92	0.27^{a}
56.1	0.45	0.33	0.025	0.16^{a}	1.32	0.30^{a}
56.2	0.29	0.19	0.023	0.07^{bc}	1.63	0.25^{a}
56.4	0.23	0.78	0.030	0.17^{a}	1.68	0.35^{a}
56.7	0.14	0.22	0.015	0.08^{bc}	1.83	0.25^{a}
56.8	0.29	0.25	0.027	0.11^{ab}	0.69	0.26^{a}
56.9	0.26	0.24	0.020	0.07^{bc}	1.48	0.28^{a}
56.21	0.25	0.29	0.023	0.07^{bc}	0.52	0.24^{a}
56.22	0.17	0.26	0.027	0.07^{bc}	1.59	0.28^{a}
56.25	0.10	0.22	0.027	0.06^{bc}	2.08	0.32^{a}
56.26	0.17	0.23	0.023	0.06^{bc}	2.26	0.27^{a}
56.27	0.04	0.09	0.020	0.03^{c}	0.81	0.12^{b}
SEM	0.03	0.05	0.001	0.008	0.15	0.018
ANOVA P-value	0.09	0.60	0.85	< 0.01	0.27	< 0.01

[a–b] Means within columns with different letters are statistically different under Tukey test ($P < 0.05$).

[†]Lactic acid; [‡]Acetic acid; [§]Butyric acid; [¶]Propionic acid

CTRL: non-inoculated; CI: Commercial inoculant – Lalsil AS; 56.1-56.27 – Wild Lactobacillus buchneri strains

In conclusion, the inoculation of corn with the L. buchneri strain 56.1 resulted in silage with high concentrations of propionic and acetic acids, low NH_3-N production, and also higher aerobic stability than non-inoculated silages. The L. buchneri 56.1 has the potential to be used as microbial inoculant in corn silage. Future studies are needed to confirm the results in large-scale silos.

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CHAPTER 3 - BACTERIOME OF REHYDRATED CORN AND SORGHUM GRAIN SILAGES TREATED WITH MICROBIAL INOCULANTS IN DIFFERENT FERMENTATION PERIODS

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Due to the co-evolved intricate relationships, and mutual influence between changes in the microbiome and silage fermentation quality, we explored the succession of bacterial populations, and evaluated the impacts caused by microbial inoculants on epiphytic bacterial community of rehydrated corn and sorghum grains and their silages by next-generation sequencing after 0, 3, 7, 21, 90 and 360 days of fermentation. The inoculants were composed of 1- non-inoculated (CTRL); 2- Lactobacillus plantarum + Propionibacterium acidipropionici (Inoc1) and 3- Lactobacillus buchneri (Inoc2). Analyses of the relative abundance of bacterial communities revealed the presence of 9 and 6 phyla, for corn and sorghum grains samples, respectively. Proteobacteria was predominantly in both grains at the beginning of the fermentation and Firmicutes phylum throughout the fermentation periods. Bacilli class was found predominantly in both grains. Inoculant containing Lactobacillus plantarum and Propionibacterium acidipropionici was more efficient in promoting a sharply growth of Lactobacillus ssp. and maintaining greater stability of the bacterial community during longer periods of storage in both grains silages. Species of Lactobacillus and Weissella are the main bacteria involved in the fermentation of rehydrated corn and sorghum grain silages.

INTRODUCTION

Corn and sorghum grains have been used in concentrates offered to ruminants with the objective of providing energy, mainly from its starch content (Oliveira et al., 2015). Grain endosperm is the morphological structure that contains the highest amount of starch and it determines the economic and nutritional value of the grain, since the structure and composition of the starch and its physical interaction with the grain protein can alter its digestibility (Rooney & Pflugfelder, 1986).

The endosperm effect on digestibility can be manipulated by grain processing (Taylor and Allen 2005). Rehydrated grain silages is a promising technique to improve the nutritive value of grains (da Silva et al., 2018) and consist of the hydration of the milled mature grain

with 10-14% of moisture to achieve 30 e 40% of fresh weight, which favors its fermentation and storage as silage (Pereira et al., 2013). Among grains, after this process, sorghum grain is the one which has the highest gains in digestibility followed by corn and other cereals (Theurer, 1986).

During the ensiling process increasing in digestibility is due to partially degradation of hydrophobic starch-protein matrix surrounding starch granules by proteolysis (Philippeau and Michalet-Doreau, 1997), promoting greater solubilization of prolamins and greater number (and surface area) of individual starch granules for potential attack by rumen bacteria (Hoffman et al., 2011).

Forage often naturally contains many detrimental types of bacteria, due to this, adding a microbial inoculant to the ensiled material in order to dominate the fermentation results in higher quality silage (Kung, 2001) and greater aerobic stability (McAllister et al., 1998). According to Si et al. (2018) silage and its microbiota have co-evolved intricate relationships, and mutual influence exists between changes in the microbiome and silage fermentation parameters such as the positive correlation of Lactobacillus plantarum and lactic acid content.

Generally, the composition of microorganisms before and after ensiling has undergone great changes (Guan et al., 2018). Monitoring these changes during ensiling would be helpful for thoroughly understanding and improving the ensiling process. Despite the potential of rehydrated grain silages and the fact that silage-associated microorganisms may significantly affect both silage quality and ruminant health, there are few studies on evaluation of microorganism population and its dynamics over the fermentative process of rehydrated corn and sorghum grains, particularly using next generation sequencing for bacteriome identification.

Recently, molecular methods have been used to evaluate the quality, activity and dynamics of the complex community of microorganisms involved in forage preservation (Gallagher et al., 2018; Xu et al., 2018; Guan et al., 2018) presenting as an advantage the avoiding of underestimating the microbial diversity of silages (Ercolini, 2004).

In this context, we explored the succession of bacterial populations, identified microorganisms that are dominantly involved in ensiling and evaluated the impacts caused by microbial inoculants on epiphytic bacterial community of rehydrated corn and sorghum grain and their silages after 0, 3, 7, 21, 90 and 360 d of fermentation.

MATERIALS AND METHODS

Location and climatic conditions

The experiment was conducted between January 2016 and January 2017 at the Department of Animal Science of the Federal University of Vicosa (Viçosa, MG, Brazil), located at 20°45' S latitude, 42°52' W longitude 648 m above sea level. The annual precipitation and average temperature the year of the experiment were 1235.4 mm and 20.7 °C, respectively.

Ensiling and sampling

The samples used in this experiment were obtained from a previous study conducted by Pimentel (2017) (unpublished data) which evaluated the effect of inoculant and period of fermentation on rehydrated corn and sorghum grain silages. The characterization of rehydrated corn and sorghum grains before fermentation and their silages obtained in this previous study are shown in Table 1 and Table 2, respectively.

Briefly, the experiment was carried out under a completely randomized design (with three replicates) based on a 2 × 3 × 6 factorial assay, with two grains (corn -**CG** and sorghum-**SG**), three inoculants and six fermentation periods (0, 3, 7, 21, 90 and 360 days). The evaluated treatments were: Corn control (**CG-CTRL**); Corn Inoculant 1- (**CG-Inoc1**); Corn Inoculant 2 - (**CG-Inoc2**); Sorghum control (**SG-CTRL**); Sorghum Inoculant 1- (**SG-Inoc1**); Sorghum Inoculant 2 - (**SG-Inoc2**). The inoculants were composed of **CTRL** –non-inoculated; **Inoc1 -** Lactobacillus plantarum and Propionibacterium acidipropionici (Lalsil® Milho, Lallemand Animal Nutrition) and **Inoc2 -** Lactobacillus buchneri (Lalsil® AS, Lallemand Animal Nutrition).

The CG and SG were grossly disintegrated in a mill retrofitted with 3 mm mesh sieves. Prior to fermentation, the milled CG and SG were rehydrated with water to moisture content at 30%. After, inoculants were dissolved in distilled water at the dosage recommended by the manufacturer, were sprayed on 500 g of rehydrated grains and mixed uniformly by hand before packing into plastic film bags (25.4 cm × 35.56 cm) and vacuumed with a vacuum sealer (Eco vacuum 1040, Orved, Italy). The same amount of water was applied to CTRL silages.

The bags were stored in the laboratory at room temperature (range, 23-27°C) and 18 bags were opened on 0, 3, 7, 21, 90 and 360 days after fermentation. It was prepared representative composite samples of each treatment in each fermentation period totalizing six samples per fermentation period and 36 total samples.

The data were analyzed using the MIXED procedure of SAS® (v. 9.4 SAS Institute Inc., Cary, NC). The estimated means were compared by Tukey's test considering a significance level of 0.05.

Table 1. Average chemical composition (% DM, unless otherwise stated) pH and microbial populations (log cfu/g of fresh weight) of rehydrated corn and sorghum grains before fermentation.

Item	Corn grain			Sorghum grain			SEM
	CTRL	Inoc1	Inoc2	CTRL	Inoc1	Inoc2	
DM (% as fed)	67.49	67.98	68.86	69.01	68.65	68.40	0.18
WSC[2]	1.97	2.12	2.05	1.12	1.09	1.10	0.10
pH	5.88	5.77	5.91	6.35	6.39	6.40	0.05
LAB[3]	5.46	5.43	5.11	5.04	5.32	4.84	0.08
Enterobacteria	4.76	4.92	4.94	5.51	5.54	5.29	0.08
Fungi	5.75	6.42	5.76	4.29	4.65	4.59	0.18

[1]Dry matter; [2]Water soluble carbohydrate; [3]Lactic acid bacteria. **CTRL:** Non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

Table 2. Average (with respective standard error of the mean and ANOVA based P-value) fermentation profile (% DM) and microbial population (log cfu/g of fresh weight) of rehydrated corn and sorghum grain silages throughout the fermentation period.

Inoculant	Days	Grain	pH	WSC[1]	Fermentation products					Microbial counts		
					LA[2]	AA[3]	BA[4]	PA[5]	NH_3-N	LAB[6]	ENT[7]	Fungi
	3	**CG**	4.77	3.56	0.25	0.05	0.06	0.001	2.86	8.9	6.65	5.64
	7	**CG**	4.32	3.33	0.35	0.06	0.08	0.001	3.19	8.71	5.28	5.37
CTRL	21	**CG**	4.10	2.33	0.27	0.06	0.04	0.001	4.02	8.22	3.94	5.01
	90	**CG**	3.87	2.05	0.76	0.11	0.12	0.002	4.63	6.74	2.61	2.91
	360	**CG**	3.81	2.72	1.16	0.21	0.03	0.254	5.13	3.86	0	1.97
	3	**CG**	4.03	2.62	0.66	0.05	0.05	0.001	3.15	9.58	4.48	5.72
	7	**CG**	3.9	2.88	0.90	0.05	0.05	0.001	3.42	8.38	3.81	5.51
Inoc1	21	**CG**	3.94	3.07	0.74	0.06	0.04	0.002	3.85	5.53	2.76	5.12
	90	**CG**	3.93	2.50	0.85	0.05	0.07	0.005	4.30	4.91	0.92	4.72
	360	**CG**	3.93	2.95	1.07	0.11	0.03	0.231	3.89	4.36	0	3.62
	3	**CG**	4.77	2.9	0.23	0.04	0.06	0	3.26	9.37	7.11	5.55
	7	**CG**	4.14	2.4	0.67	0.13	0.15	0	4.21	9.70	3.24	4.79
Inoc2	21	**CG**	4.00	0.75	0.37	0.13	0.09	0.001	3.48	9.50	0	4.16
	90	**CG**	3.98	2.33	0.95	0.27	0.19	0.001	5.62	4.72	1.03	2.03
	360	**CG**	3.98	1.86	1.10	0.24	0.03	0.455	6.55	3.95	0	1.97
	3	**SG**	5.22	0.63	0.27	0.03	0.05	0.001	2.35	9	8.08	4.73
	7	**SG**	4.73	0.68	0.35	0.04	0.07	0	2.59	9.18	6.19	5.97
CTRL	21	**SG**	4.46	0.56	0.33	0.06	0.06	0.001	2.95	8.51	3.11	5.65
	90	**SG**	4.44	0.75	0.42	0.08	0.12	0.001	3.65	8.55	1.76	4.09
	360	**SG**	4.15	0.78	1.01	0.10	0.09	0.244	4.63	6.38	0	1.39
	3	**SG**	4.1	0.37	0.60	0.04	0.02	0.002	1.76	9.8	4.51	3.69
	7	**SG**	4.03	0.48	0.77	0.05	0.03	0.002	2.12	9.08	3.92	3.88
Inoc1	21	**SG**	4.01	0.44	0.33	0.05	0.01	0.006	2.07	7.10	2.62	4.46
	90	**SG**	4.00	0.68	0.76	0.04	0.04	0.003	2.59	6.40	0	3.46
	360	**SG**	3.92	0.69	0.87	0.30	0.08	0.472	3.01	3.87	0	2.79
	3	**SG**	4.79	0.39	0.29	0.03	0.06	0	2.26	9.53	6.91	4.41
	7	**SG**	4.52	0.49	0.40	0.07	0.09	0.001	2.86	9.46	3.99	4.19
Inoc2	21	**SG**	4.58	0.43	0.24	0.12	0.08	0.001	3.00	9.54	2.93	4.06
	90	**SG**	4.46	0.47	0.30	0.39	0.15	0.004	3.64	8.22	0	3.36
	360	**SG**	4.36	0.56	0.43	0.60	0.05	0.716	5.56	5.48	0	3.73

SEM		0.09	0.36	0.14	0.08	0.03	0.10	0.39	0.51	0.61	0.85
P-value	I	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.03	< 0.01	< 0.01	< 0.01	0.05
	G	< 0.01	< 0.01	< 0.01	0.03	0.15	0.12	< 0.01	< 0.01	0.25	0.11
	D	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
	I × G	< 0.01	< 0.01	< 0.01	< 0.01	0.14	0.49	< 0.01	0.36	0.10	< 0.01
	I × D	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
	G × D	< 0.01	0.012	< 0.01	< 0.01	< 0.01	0.02	0.05	< 0.01	< 0.01	0.02
	I × D × G	< 0.01	< 0.01	< 0.01	< 0.01	0.73	0.59	0.24	< 0.01	< 0.01	0.21

[1]Water soluble carbohydrate; [2]Lactic acid; [3]Acetic acid; [4]Butyric acid; [5]Propionic acid; [6]Lactic acid bacteria; [7]Enterobacteria. **CG:** Corn grain; **SG:** Sorghum grain. **CTRL:** non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri. I: Inoculant effect; G: Grain effect; D: Day of fermentation effect; I × G: Interaction effect inoculant × grain; I × D: Interaction effect inoculant × day; G × D: Interaction effect grain × day; I × D × G: Interaction effect inoculant × day × grain.

DNA extraction, 16S rRNA amplicon library preparation and sequencing

The 36 samples were crushed in liquid nitrogen and the total DNA was extracted by using the NucleoSpin® Soil DNA extraction kit (Macherey-Nagel, Düren, Germany), as per the manufacturer's recommendation. The DNA was quantified using Nanodrop spectrophotometer (Thermo Scientific®) and checked for quality on an agarose gel (1.4%).

Briefly, PCR amplicon libraries targeting the 16S rRNA encoding gene present in metagenomic DNA were produced using a barcoded primer set adapted for the Illumina HiSeq2000 and MiSeq (Caporaso et al., 2012). DNA sequence data was then generated using Illumina paired-end sequencing at the Environmental Sample Preparation and Sequencing Facility (ESPSF) at Argonne National Laboratory. Specifically, the V4 region of the 16S rRNA gene (515F-806R) was PCR amplified with region-specific primers that include sequencer adapter sequences used in the Illumina flowcell (Caporaso et al. 2011, 2012).

The reaction was supplemented with a custom PNA blocker designed to prevent the amplification of contaminating host sequences (mitochondrial or plastid). These blockers clamp onto host sequences during the PCR process and prevent their amplification (Lundberg et al., 2013). The reverse amplification primer also contained a twelve base barcode sequence that supports pooling of up to 2,167 different samples in each lane (Caporaso et al. 2011, 2012). Each 25 µL PCR reaction contained 8.5 µL of MO BIO PCR Water (Certified DNA-Free), 12.5 µL of QuantaBio's AccuStart II ToughMix (2x concentration, 1x final), 1 µL Golay barcode tagged Reverse Primer (5 µM concentration, 200 pM final), 1 µL Forward Primer (5 µM concentration, 200 pM final), 1 µL PNA blocker (mitochondrial or plastid, 25 µM concentration, 1 µM final) (Lundberg et al., 2013), and 1 µL of template DNA. The conditions for PCR were as follows (Lundberg et al., 2013): 95 °C for 45 s to denature the DNA, with 35 cycles at 95 °C for 15 s, 78 °C for 10 s (for PNA annealing), 50 °C for 30 s (for Primer annealing), and 72 °C for 30 s, with a cooldown to 4 °C once the cycling was completed. Amplicons were then quantified using PicoGreen (Invitrogen) and a plate reader (Infinite® 200 PRO, Tecan). Once quantified, volumes of each of the products were pooled into a single tube so that each amplicon was represented in equimolar amounts. This pool was then cleaned up using AMPure XP Beads (Beckman Coulter), and then quantified using a fluorometer (Qubit, Invitrogen).

After quantification, the molarity of the pool was determined and diluted down to 2 nM, denatured, and then diluted to a final concentration of 6.75 pM with a 10% PhiX spike

for sequencing on the Illumina MiSeq. Amplicons were sequenced on a 251bp x 12bp x 251bp MiSeq run using customized sequencing primers and procedures (Caporaso et al., 2012).

Bioinformatics analyzes

Raw sequencing reads obtained from 16S rRNA amplicon sequencing were subjected to different quality filtering steps. Sequences that showed bases with a maximum expected error of 0.5 of probability were removed and the remaining sequences were grouped into OTUs using the program Usearch v.11 (Edgar et al., 2013) with a threshold of 97% of similarity. Chimeras were also removed by the Uparse algorithm. The taxonomic annotation was performed using the BLAST (Basic Local Alignment Search Tool) method of QIIME v.1.9.1 (Caporaso et al., 2010) using the SILVA databases. Contaminant sequences such as chloroplasts and mitochondria were removed through the result of taxonomic annotation.

Alpha diversity metrics (Chao1 richness, evenness and Simpson diversity) and beta diversity metrics (using weighted UniFrac distance) were calculated with the WGCNA package, stat packages, and the ggplot2 package in R software (Version 2.15.3).

RESULTS

It was generated a total of 1,581,953 high-quality reads. 783,146 of the reads with an average of 21,754 per sample were originated from the CG samples and 798,807 reads with an average of 22,189 reads per sample were originated from SG in different days of fermentation. The number of sequences was standardized relative to the minimum number of 12,297 sequences obtained from a single sample.

The bacterial communities in CG and SG samples are presented in Table S1 and Table S2, respectively. A total of 257 and 119 OTUs were detected in CG and SG samples, respectively. Rarefaction curves at 97 % identity OTUs are shown in Figure S1. Sequencing depth was sufficient to fully describe the diversity of the bacterial populations in silages as rarefaction curves reached a plateau for sequences.

The β-diversity analysis (Figure 1) was performed to compare the microbial community compositions between the different inoculants and ensiling periods. The initial bacterial communities (day 0) were more similar for SG than CG, irrespective of treatment. However, over the course of ensiling, a clear changing in the distribution and structure of the bacterial communities were observed.

Two different groups were formed by CG samples. As early as 3 d of fermentation the community diverged forming one group which remained similar until 90 d. After 360 d, the communities in the samples converged again getting closer to the initial population.

The populations of SG-CTRL and SG-Inoc2 silages were similar during the intermediate periods. After 360 d, the SG population converged again but it did not approach to the initial community as observed in CG silages.

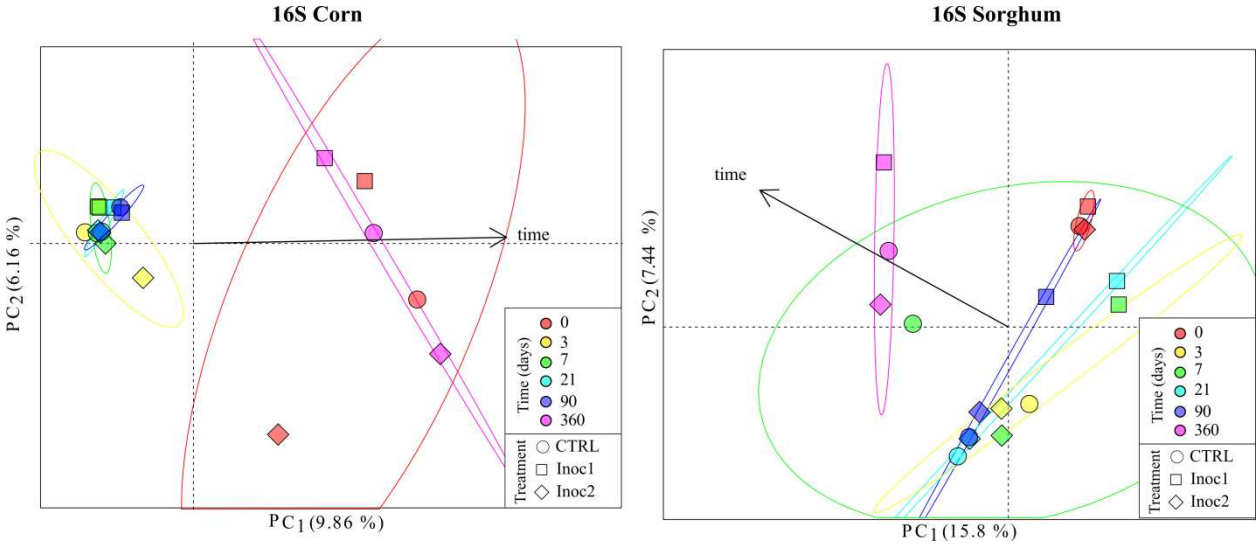

Figure 1. Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance of bacterial communities according to sampling time (0, 3, 7, 21, 90 and 360 d) and silage type for rehydrated corn and sorghum grain silages. **CTRL:** non-inoculated; **Inoc1:** Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2:** Lactobacillus buchneri.

Diversity analysis

The Simpson diversity index of rehydrate corn and sorghum grain silages are shown in Figure 2. The initial diversity of CG had similar values between treatments. There was a reduction in the diversity during the fermentation period, mainly for CG-Inoc1 silages, due to the lower evenness in these silages, since the Chao 1 richness reduced in the 3 d and remained similar between the treatments until the end of the fermentation period. The diversity of the CG at 360 d increased approaching the initial values. This response was also observed previously in the PCoA analysis.

Samples of SG had lower initial diversity index than CG and increased from 3 d. The diversity response of the silages in the different treatments were similar except for SG-Inoc1 that had lower initial numbers and reduced the values after 90 d due to the reduction of evenness.

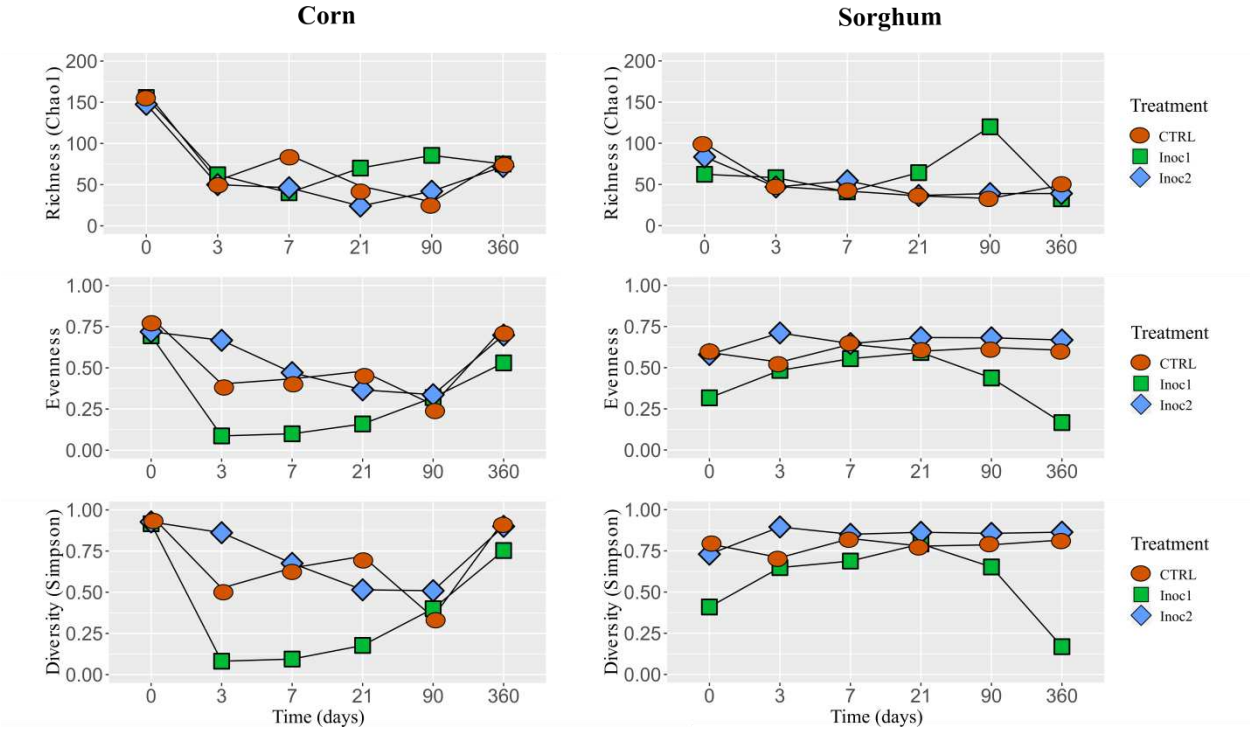

Figure 2. Chao 1 Richness, evenness and Simpson diversity of rehydrated corn and sorghum grain silages throughout the fermentation period (0, 3, 7, 21, 90 and 360 d). **CTRL**: non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

Taxonomic composition - Phylum

Analyses of the relative abundance of bacterial communities revealed the presence of 9 and 6 phyla, for CG and SG, respectively (Figure 3). The phyla Actinobacteria, Bacteroides, Deinococcus-Thermus, Firmicutes and Proteobacteria were found in both grains, only Cyanobacteria was found in SG and Acidobacterias, Chloroflexi, Planctomycetes and Verrucomicrobia were found in CG samples.

The predominance (> 80%) of Proteobacteria was observed in both grains at the beginning of the fermentation (day 0) except in CG-CTRL and CG-Inoc1, which had 51% of Proteobacteria and 61% of Actinobacteria, respectively. In all CG silages Firmicutes phylum dominated (> 84%) the fermentation from 3 to 90 d after ensiling. As observed previously at 360 d there was a tendency in the bacterial community to return its initial diversity with the replacement of Firmicutes by Proteobacteria and Actinobacteria. However, CS-Inoc1 had smaller and CS-Inoc2 greater replacement of the Firmicutes phylum in the last period evaluated.

The bacterial community changes in SG silages were less pronounced in SG-CTRL and SG-Inoc2. As early as 3 d of fermentation it was observed 85% of Firmicutes on SG-Inoc1 which unlike CG silages, the predominance of this phylum was extended up to 360 d.

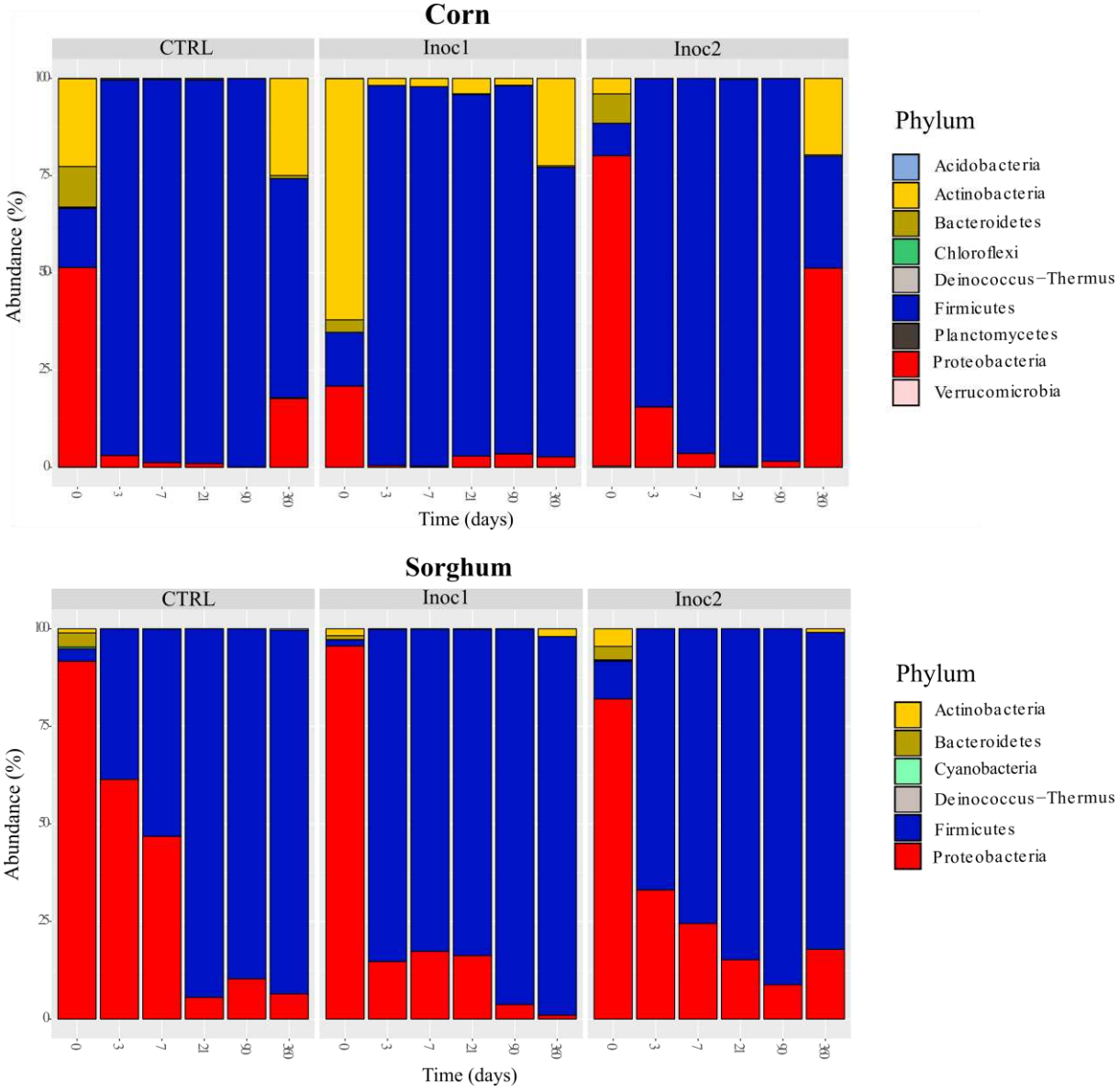

Figure 3. Phyla taxonomic profiles of bacterial communities of rehydrated corn and sorghum grain silages after 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL**: non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

Taxonomic composition - Class

As observed for phyla, the number of classes found in CG samples was higher than that of SG (16 vs. 9) (Figure 4). The classes Actinobacteria, Alphaproteobacteria, Bacilli, Bacteroidia, Clostridia, Deinococci, Erysipelotrichia, and Gammaproteobacteria, were commonly found in both grains. Oxyphotobacteria was present only in SG and Acidobacteria,

Deltaproteobacteria, Negativicutes, Planctomycetacia, Rubrobacteria, Thermoleophilia, TK10, Verrucomicrobiae, were found only in CG. The Bacilli and Gammaproteobacteria classes were the main representatives of the Firmicutes and Proteobacteria phyla, respectively. During the fermentation period, changes observed in these two main phyla, were mainly result of increased or reduced numbers of those two classes.

Both inoculants favored Bacilli growth in CG and SG, however, as observed in CG, bacteria present in Inoc1 were more effective in promoting changes in the bacterial population of SG as early as the 3rd day of fermentation.

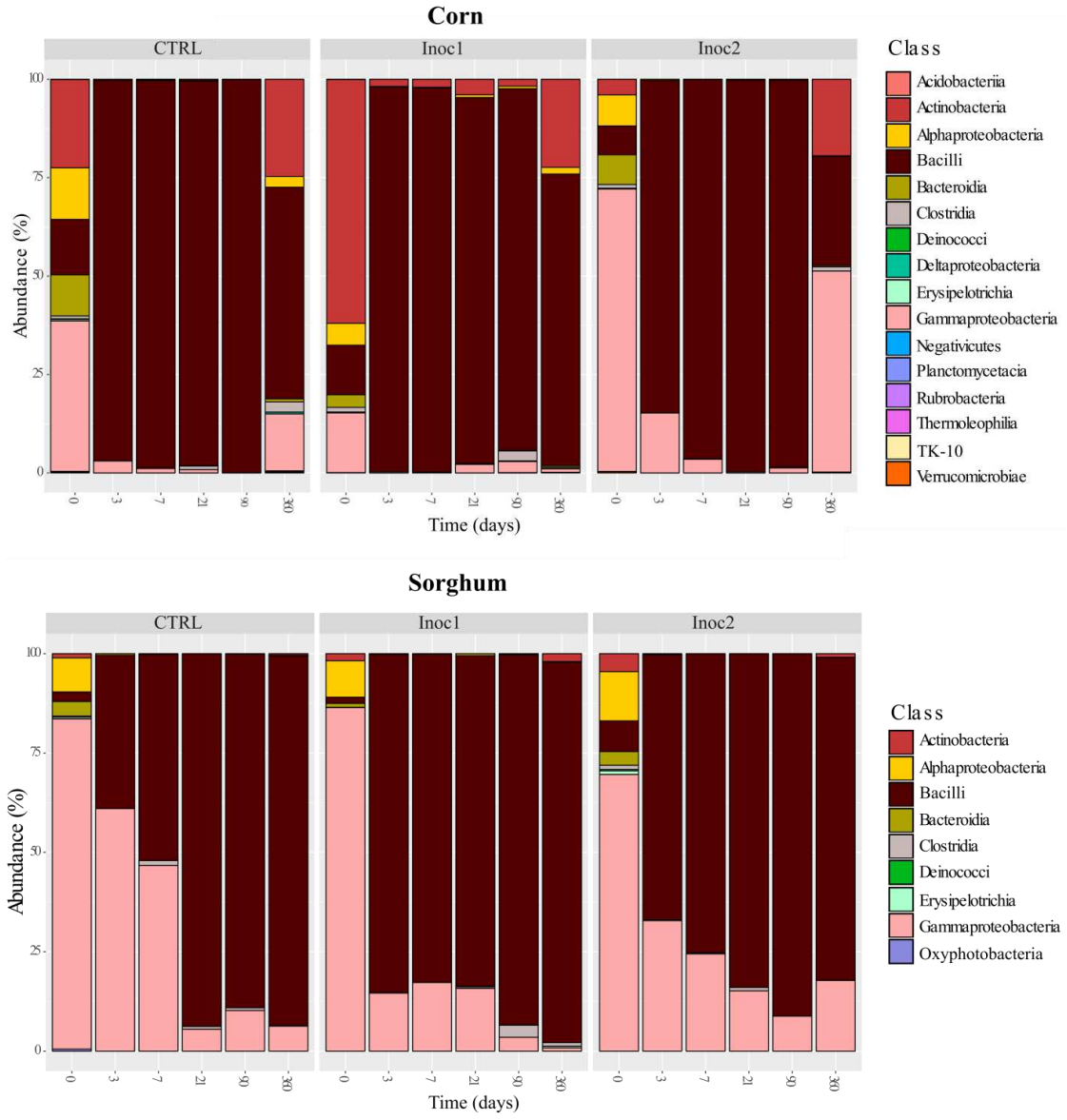

Figure 4. Classes taxonomic profiles of bacterial communities of rehydrated corn and sorghum grain silages after 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL:** non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2:** Lactobacillus buchneri.

Taxonomic composition - Genus

The dynamics of the main genera in CG are present in Figure 5. Although the Bacilli class was found predominantly in both grains in the intermediate periods of fermentation the succession of the genera representing this class were distinguished among the silages.

The initial genera compositions of the CG were uneven among the treatments. In the CS-CTRL the fermentation from the 3rd day was predominantly by Weissela with gradual replacement by the Lactobacillus genus, which represented 93% of the genera at 90 d. Bacteria present in Inoc1 induced the predominance (> 80%) of Lactobacillus from 3 to 90 d of fermentation.

Although Lactobacillus represented ≅ 90% of the bacteria in CS-Inoc2 at 21 and 90 d, the inoculant was not efficient to prevent the development of Weissella and other genera on 3 d and 7 d as Inoc1. It was observed an increased evenness in silages of all treatments at 360 d, resulting in the substitution of Lactobacillus. CS-Inoc1 silages were the ones with the highest Lactobacillus counts (50%), evidencing the greater efficiency of Inoc1 in maintaining the equilibrium of the bacterial population in the final period evaluated. CS-Inoc2 silages were the ones which mostly tended to return to the initial population profile with the relative abundance of 32% of Acinetobacter and 37% of others genera.

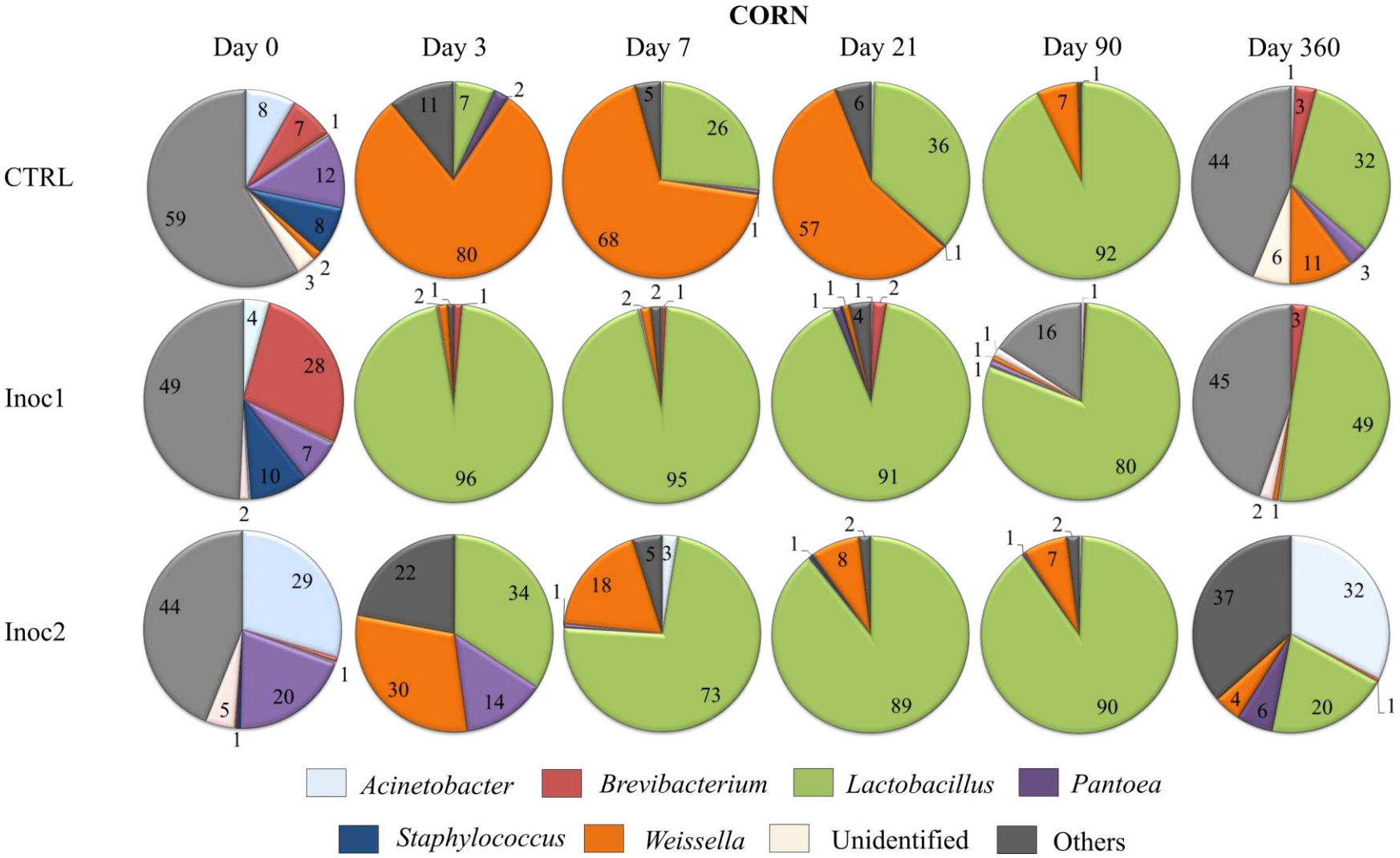

Figure 5. Main genera dynamics (%) of bacterial communities of rehydrated corn grain silages after 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL:** non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2:** Lactobacillus buchneri.

The dynamics of the genera profile in SG are showed in Figure 6. The changes of the bacterial community in inoculated SG silages were more complex than CG silages, indicating the more complicated interactions between the bacterial flora. Differently from CG, which the initial populations were heterogeneous, the SG had lower initial richness and evenness which was a reflection of the predominance (51-80%) of the Pantoea genus, mainly in SS-Inoc1 silages (80%). Throughout the fermentation period, gradual but not total genus substitution occurred by different proportions of Weissella, Lactobacillus and bacteria belonging to Enterobacteriaceae family.

The bacterial replacement was less pronounced in SG silages during the fermentation period than CG. The presence of high percentage (> 85%) of Lactobacillus as observed in CG-Inoc1 in the initial stages of fermentation occurred only in SG-Inoc1 silages from of 90 d. The SG-Inoc2 had similar bacterial succession as SG-CTRL from 90 d with the presence of different genera in the bacterial community.

As observed in CG at 360 d, there were changes in bacterial taxonomic composition, mainly the replacement of Lactobacillus by Weissella in SG-CTRL silages and Weissella and Kosakonia in SG-Inoc2. However, the changes in SG were mainly at the family level, while in the CG substitutions were also observed at the phylum rank. SG-Inoc1 presented the greatest stability of the bacterial composition at 360 d, with 93% represented by Lactobacillus.

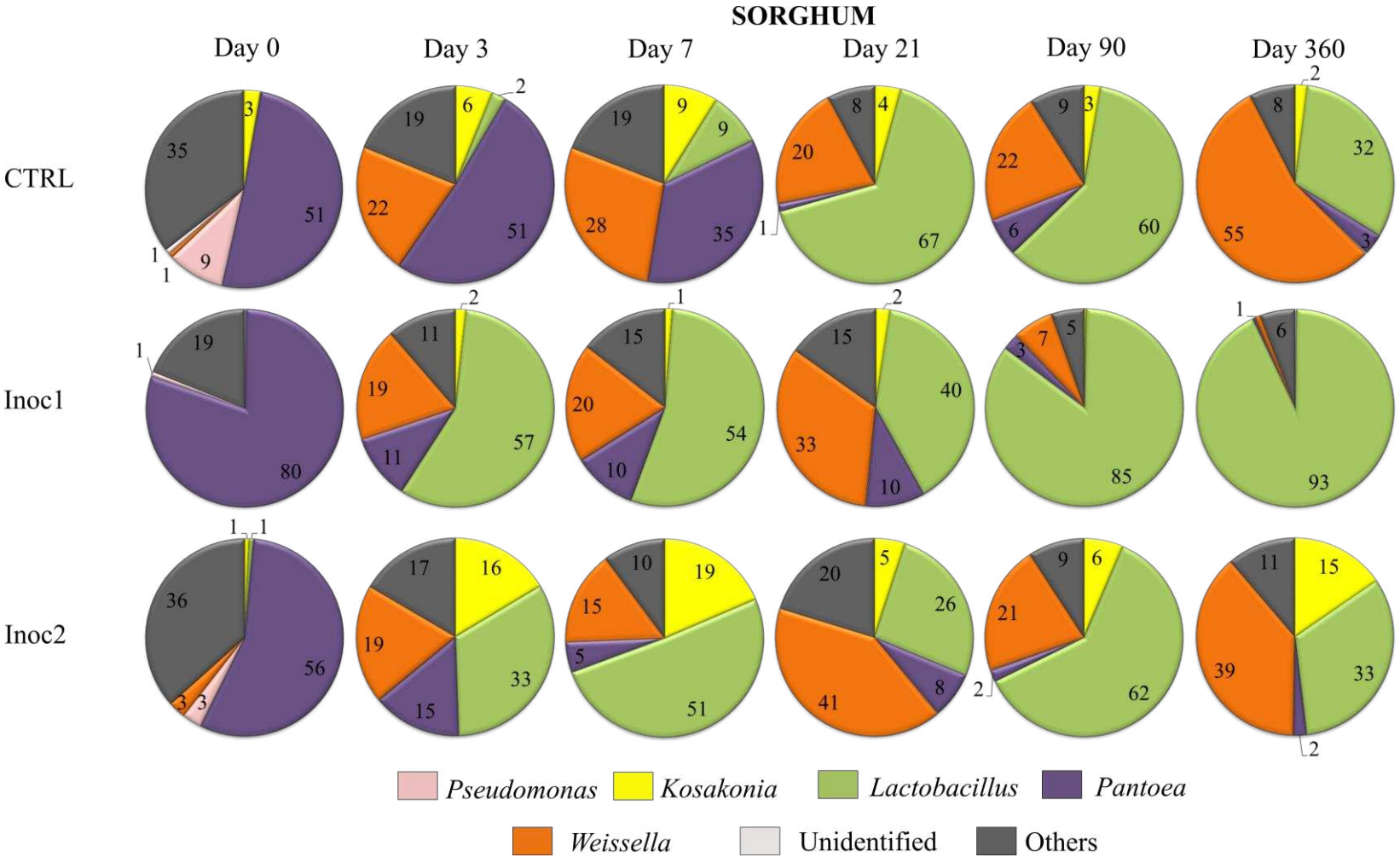

Figure 6. Main genera dynamics (%) of bacterial communities of rehydrated sorghum grain silages after 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL:** non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2:** Lactobacillus buchneri

DISCUSSION

L. plantarum has been reported to be the most commonly used silage inoculant (Oliveira et al., 2017). This specie produces lactic acid that acts to rapidly reduce pH and improve fermentation (Santos et al., 2013). To minimize aerobic degradation, others bacteria, such as propionic and heterolactic bacteria, can be used for controlling microorganisms that promote aerobic deterioration (Zhang et al., 2010).

Lin et al. (1992) reported that epiphytic lactic acid bacteria (**LAB**) play a major role in silage fermentation and its numbers have become a significant factor in predicting the adequacy of silage fermentation and determining whether to apply silage bacterial inoculants and its effectiveness. Variations in the epiphytic populations in both materials in this study can be justified by environmental factors such as rainfall and humidity (Guan et al., 2018) and chemical composition including moisture and water soluble carbohydrate (**WSC**) (McEniry et al., 2010).

The similar initial bacterial communities of SG, reflected the minimal immediate impact of the treatments to the microbial community at the onset of the time-course, as well as the absence of significant differences in exogenous microbial contaminants that could alter the microbial make up (Gallagher et al., 2018).

The predominance of Proteobacteria in the raw material was also reported by Romero et al. (2018) and McGarvey et al. (2013) in samples of corn and alfalfa, respectively. Moreover, the presence of Actinobacteria in the initial composition of CG-Inoc1 was much higher (61%) than observed by the first authors in corn samples (4.5%).

After fermentation, the high abundance for Firmicutes and low Proteobacteria in both grains silages was also observed in wilted whole-crop oats ensiled for 217 d (Romero et al., 2017) and alfalfa silage inoculated with different microbial inoculants (Si et al., 2018). Hence, environmental conditions developed during ensiling contributed to the growth of Firmicutes phylum (Liu et al., 2019), ensuring the conservation of small grain silages (Dunière et al., 2017).

In grass silage most of the reads assigned to the phylum Firmicutes were related to the order Lactobacillales comprising the three most prevalent genera Lactobacillus, Lactococcus and Weissella (Eikmeyer et al., 2013). In our study, in addition, the Acinetobacter genus also had influential quantities in CG-Inoc2 silage at 360 d.

Acinetobacter ssp. are aerobic, non-fermenting bacteria which can be found in different environments (Kämpfer and Glaeser, 2012). Some species can survive in an

anaerobic environment in the presence of acetate as a substrate (Fuhs and Chen, 1975), requiring energy from carbohydrate degradation (Satoh et al., 1996). The increased abundance of Acinetobacter in CG-Inoc2 silage at 360 d may have resulted from the increased acetate concentration produced by inoculated L. buchneri strain as observed at 90 d of fermentation (Table 2), and this may partly explain the small, though important, DM losses sometimes observed in silages that had been treated with this heterofermentative bacteria at ensiling (Filya, 2003).

The reduction of bacterial diversity in Inoc1 silages from 3 d in CG and 90 d in SG silages can be explained by the dominance of Lactobacillus when inoculant containing L. plantarum was applied. Low bacterial diversity as a result of the high abundance of Lactobacillus was also reported by Ogunade et al. (2017) in corn silage. Hence, the more the abundance of a dominant bacterium, the less diverse the microbial community (Polley et al., 2007; Allen et al., 2009).

According to McDonald et al. (1991) in general after anaerobic fermentation, the complex microbial communities of the raw materials are gradually replaced by LAB, and Lactobacillus can become a dominant genus in successful silages. Indeed, analyzing the Tables 1 and 2, LAB culture-dependent counts increased and enterobacteria and fungi reduced gradually throughout the fermentation period in all treatments. The increased Lactobacillus population in silages could be due to the ability of usage WSC (McDonald et al, 1991), starch or cellulose to increase in abundance because the expression of amylases and both 1–4 and 1–6 glucosidases in amylolytic LAB isolated from sorghum has been previously reported by Velikova et al. (2016).

Muck (2008), reported that the current strains of L. buchneri are rather slow compared with other species. So other LAB may do the primary work of fermentation and after active fermentation is done, the L. buchneri strains slowly convert lactic to acetic acid. This means that their effect on aerobic stability may take a while to be observed, typically 45 to 60 d after fermentation.

In our study although the Lactobacillus genus represented 73 and 51%, in CG-Inoc2 and SG-Inoc2 silages, respectively, at 7 d of fermentation, data from Table 2 suggest that the production of acetic acid by L. buchneri occurred after 90 d, mainly in SG-Inoc2 silages. So we can speculate that other species of the Lactobacillus genus worked as starter cultures in the initial fermentation of these silages as suggested by Muck (2008).

Although there was P. acidipropionici in the composition of Inoc1, interestingly no representatives of this genus were found in silages of both grains. This propionic bacteria belongs to the class Actinobacteria, which were present mainly in the initial population of CG-Inoc1 and was represented by the genera Brevibacterium, Streptomyces, Arthrobacter and others.

According to Filya et al. (2004) the combination of P. acidipropionici and L. plantarum do not look promising in protecting wheat, sorghum and maize silages upon aerobic exposure. In general, Propionibacterium have been effective in situations where the decline in pH is slow and (or) when the final pH of silage has been relatively high (> 4.2 to 4.5) (Rahman et al., 2017) what was observed in our study, mainly in SG silages. Thereby, the absence of this genus in all samples was unexpected especially in recent inoculated samples (day 0).

Bacteria assigned to Weissella genus are strictly heterofermentative, producing a mixture of lactate and acetate as the major end products of sugar metabolism (Fusco et al., 2015; Graf et al., 2016). Some species have been isolated from a wide range of sources such as soil, fresh vegetables, meat, fish, fermented silage and foods (Björkroth et al., 2002; Sirirat et al., 2008; Valerio et al., 2009).

In agreement to Muck (2013), who suggested that the biggest deviation in microbial community appearance to be in corn silage in warm climates where Weissella and Leuconostoc species contributed to early stages of fermentation, in our study the fermentation in CG-CTRL silages until 21 d of fermentation was also predominantly by Weissella spp. In addition, it was influential the presence of this genus on SG silages throughout the fermentation periods.

The study of Pang et al. (2011) indicates that perhaps several Weissella spp. could improve silage quality. Furthermore, Ndagano et al. (2011) reported the production of acetate as well as other antifungal compounds, such as 3-hydroxy fatty acids and phenyllactate, by Weissella paramesenteroides isolated from fermented Manihot esculenta. However, Cai et al. (1998) concluded that heterofermentative strains of W. paramesenteroides did not improve silage quality and may cause some fermentation loss, due to the greater growth of aerobic bacteria and clostridia, higher pH, butyric acid and ammonia nitrogen contents and lower lactate concentration in alfalfa and Italian ryegrass silages. The effect of Weissella genus in silage fermentation still contradictory and unclear.

Spoiled silages were sometimes characterized by high levels of Enterobacteriaceae family (Kraut-Cohen et al. 2016). Several species are also closely linked to human and animal disease such as Salmonella and Escherichia coli. The influential presence of Enterobacteriaceae in SG silages, was mainly represented by the genus Pantoea followed by smaller proportions of Kosakonia.

The Pantoea genus is a highly diverse group whose members are found in aquatic and terrestrial environments, and in association with plants, insects, humans and animals (Walterson and Stavrinides, 2015). McGarvey et al. (2013) also reported that Pantoea was one of the most commonly found genera in the bacterial community of alfalfa. The presence of this genus throughout the fermentation period in SG silages contradicts the finding by Si et al. (2018) in which the genus disappeared by 30 d in alfalfa silage. The authors also reported that this bacteria was negative correlated with acetic acid, NH_3-N concentration, and positive correlated with WSC content. Jacxsens et al. (2003) reported that Pantoea agglomerans are able to ferment sugars to acids under anaerobic condition and it can also use lactic acid causing nutrient loss.

Addition of Inoc1in both grains resulted in silages dominated by the Lactobacillus genus with much less diversity than CTRL and Inoc2. SG-Inoc2 silages could sustain more Pantoea, Kosakonia and others Enterobacteriaceae that can potentially include pathogenic species of bacteria. Thus, adding Inoc1 may have also contributed to the safety of SG silages.

Large quantity of others undesirable microorganisms may develop in silage when the pH is insufficiently reduced or when oxygen is available. Some species of Bacillus, Clostridium, Listeria, Mycobacterium, Yersinia and Salmonella are known to associate with disease of human or animal (Dunière et al., 2013). Species of Pseudomonas and Klebsiella could produce biogenic amines, which often linked to a decrease in the protein content and nutritional value of the silage (Silla Santos, 1996). Among those genera, in our study, only Bacillus, Clostridium and Pseudomonas were detected in both grains and Mycobacterium in CG samples (data not shown). However, the presences of OTUs belonging to those genera were relatively low in all silages, reflecting the trace amounts of butyric acid present in the silages (Table 2).

An increased richness and diversity with significant decrease in Firmicutes and increase of Proteobacteria in the bacterial community were also reported by Liu et al. (2019) in barley silages after prolonged aerobic exposure time. Due to this, the reason for the changes of the bacterial community at 360 d in our study is unclear, because the experimental

conditions of anaerobiosis were constant throughout the period. In addition, studies analyzing the bacterial community are limited to shorter fermentation periods, making it difficult to correlate the observed results with previous studies.

Contrary to that reported by Dunière et al. (2013) in which due to the acidification, the silage can be stored for a long time. Changes in the final community of silages in both grains after 360 d breaks the premise that bacterial community stabilizes after a given fermentation period, suggesting that even with a constant pH, changes in the microbial profile of grain silages may result in undesired changes in the fermentation profile of silages stored for long periods as observed in our study such as higher concentrations of NH_3-N and propionic acid and lower LAB counts.

CONCLUSIONS

The effects of the inoculants on bacterial succession were different among the grains. The inoculant containing Lactobacillus plantarum and Propionibacterium acidipropionici was more efficient in promoting a sharply growth of Lactobacillus and maintaining greater stability of the bacterial community during longer periods of storage in silages of both grains. Species of Lactobacillus and Weissella are the main bacteria involved in the fermentation of rehydrated silages of corn and sorghum grains. The bacterial communities of rehydrated corn and sorghum grain silages do not remain stabilized after 360 days of storage.

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SUPPORTING INFORMATION

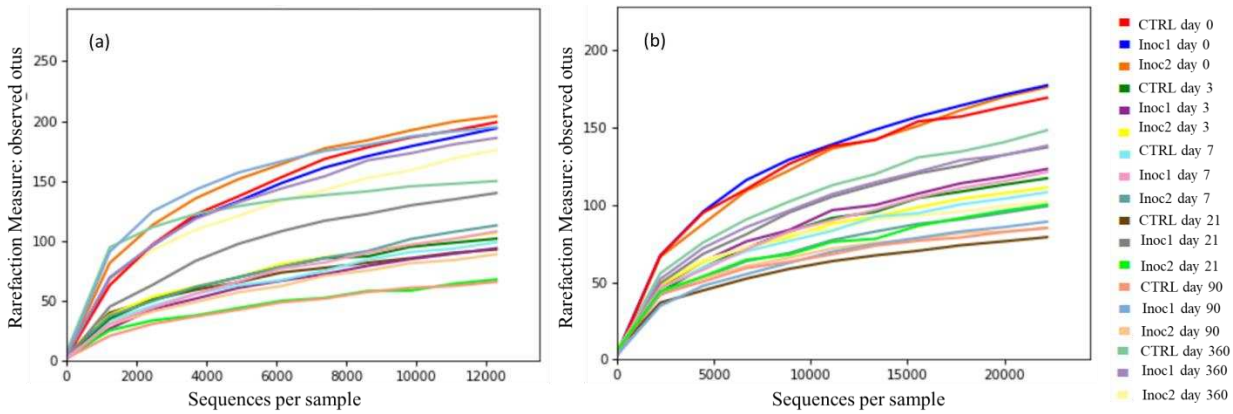

Figure S1. Rarefaction curves showing the sampling effort and number of bacterial OTUs observed in rehydrated corn (a) and sorghum (b) grain silages after 0, 3, 7, 21, 90 and 360 d of fermentation. **CTRL:** non-inoculated**; Inoc1:** Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

Table S1. Bacterial abundance in rehydrated corn grain and theirs silages throughout 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL**: non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

Corn grain	CTRL						Inoc1						Inoc2					
Day	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Acetobacterales, Acetobacteraceae, Craurococcus	3	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1
Acetobacterales, Acetobacteraceae, Roseomonas	7	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Actinomycetales, Actinomycetaceae, Actinomyces	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacillales, Bacillaceae, Bacillus	15	0	0	0	0	42	7	1	1	2	31	23	3	763	932	980	964	214
Bacillales, Bacillaceae, Geobacillus	0	0	0	0	0	4	0	0	0	0	0	0	6	0	0	0	0	0
Bacillales, Bacillaceae, Oceanobacillus	0	0	0	0	0	31	0	0	0	0	3	156	18	0	0	0	0	0
Bacillales, Bacillaceae, Ureibacillus	0	0	0	0	0	0	0	0	0	0	2	2	14	0	0	0	0	0
Bacillales, Bacillaceae, Virgibacillus	0	0	0	0	0	3	0	0	0	0	0	37	0	0	0	0	0	1
Bacillales, Family XII, Exiguobacterium	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Bacillales, Paenibacillaceae, Brevibacillus	1	0	0	0	0	0	0	0	0	0	1	0	0	3	0	1	3	2
Bacillales, Paenibacillaceae, Cohnella	1	0	0	0	0	0	0	0	0	0	1	1	2	0	1	0	0	0
Bacillales, Paenibacillaceae, Paenibacillus	9	1	0	1	1	10	8	0	1	1	24	0	10	1	0	0	0	2
Bacillales, Paenibacillaceae, Saccharibacillus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacillales, Planococcaceae, Domibacillus	4	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
Bacillales, Planococcaceae, Lysinibacillus	4	0	0	0	0	21	8	0	0	2	76	76	213	181	9	4	5	78
Bacillales, Planococcaceae, Rummeliibacillus	0	0	0	0	0	2	0	0	0	0	1	6	0	0	0	0	0	0
Bacillales, Planococcaceae, Solibacillus	3	0	0	0	0	2	1	0	0	0	4	2	1	0	0	0	0	0
Bacillales, Staphylococcaceae, Staphylococcus	100	1	1	0	0	2	121	0	2	4	1	1	9	0	0	0	0	0
Bacillales, Thermoactinomycetaceae, Shimazuella	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteroidales, Dysgonomonadaceae, uncultured	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Bdellovibrionales, Bdellovibrionaceae, Bdellovibrio	0	0	0	0	0	0	2	0	0	0	0	0	0	96	247	289	286	35

Corn grain	CTRL						Inoc1						Inoc2					
Day	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Betaproteobacteriales, Burkholderiaceae, Burkholderia-Caballeronia-Paraburkholderia	21	0	0	0	0	0	6	0	0	1	1	0	8	0	0	0	0	2
Betaproteobacteriales, Burkholderiaceae, Achromobacter	6	0	0	0	0	0	6	0	0	0	0	0	8	0	0	7	0	0
Betaproteobacteriales, Burkholderiaceae, Ambiguous taxa	8	0	0	0	0	0	0	0	0	0	0	0	4	11	5	2	3	7
Betaproteobacteriales, Burkholderiaceae, Comamonas	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Betaproteobacteriales, Burkholderiaceae, Massilia	25	0	0	0	0	0	6	0	0	0	0	0	13	8	1	0	0	4
Betaproteobacteriales, Burkholderiaceae, NA	3	0	0	0	0	0	0	0	0	0	0	0	42	0	0	0	1	4
Betaproteobacteriales, Burkholderiaceae, Verticia	2	0	0	0	0	0	2	0	0	0	0	0	5	0	0	0	0	0
Betaproteobacteriales, Neisseriaceae, uncultured	1	0	0	0	0	37	0	0	0	0	0	0	0	0	0	0	0	0
Caulobacterales, Caulobacteraceae, Brevundimonas	61	0	1	0	0	0	16	1	0	0	0	2	10	0	1	0	3	63
Caulobacterales, Caulobacteraceae, Caulobacter	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Caulobacterales, Caulobacteraceae, Phenylobacterium	2	0	0	0	0	0	1	0	0	1	0	0	8	0	0	0	1	1
Chitinophagales, Chitinophagaceae, Filimonas	23	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0
Chitinophagales, Chitinophagaceae, Niabella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chitinophagales, Chitinophagaceae, Taibaiella	2	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Chitinophagales, Chitinophagaceae, Terrimonas	1	0	0	0	0	0	5	0	0	0	0	0	5	0	0	0	0	0
Chitinophagales, Chitinophagaceae, uncultured	3	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0
Chitinophagales, Saprospiraceae, Phaeodactylibacter	0	0	0	0	0	2	0	0	0	0	0	0	2	0	0	0	0	0
Chloroflexi, TK10, uncultured bacterium	2	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	1	0
Chthoniobacterales, Chthoniobacteraceae, Candidatus Udaeobacter	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
Clostridiales, Christensenellaceae, Christensenellaceae R-7 group	1	0	0	0	0	3	0	0	0	0	0	0	2	0	0	0	0	0
Clostridiales, Clostridiaceae 1, Clostridium sensu stricto 1	0	0	0	0	0	4	1	0	0	0	9	4	2	0	0	0	0	1

Corn grain	CTRL						Inoc1						Inoc2					
Day	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Clostridiales, Clostridiaceae 1, Clostridium sensu stricto 12	0	0	0	12	0	5	0	0	0	0	0	0	18	0	0	0	0	0
Clostridiales, Clostridiaceae 1, Clostridium sensu stricto 13	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Clostridiales, Clostridiaceae 1, Clostridium sensu stricto 3	0	0	0	0	0	0	7	0	0	0	1	0	0	0	0	0	0	0
Clostridiales, Clostridiaceae 1, Clostridium sensu stricto 8	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0
Clostridiales, Clostridiaceae 1, Clostridium sensu stricto 9	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Clostridiales, Clostridiaceae 2, Alkaliphilus	1	0	0	0	0	4	0	0	0	0	2	0	0	0	0	0	0	0
Clostridiales, Family XI, Tissierella	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0
Clostridiales, Lachnospiraceae, Anaerocolumna	3	0	0	0	0	2	1	0	0	0	4	0	0	84	51	16	25	19
Clostridiales, Lachnospiraceae, NA	1	0	0	0	0	0	6	0	0	0	10	0	0	0	0	0	0	0
Clostridiales, Peptostreptococcaceae, Romboutsia	2	0	0	0	0	2	0	0	0	0	3	0	0	0	0	0	0	0
Clostridiales, Peptostreptococcaceae, Sporacetigenium	0	0	0	0	0	0	0	0	0	0	3	0	1	0	0	0	0	0
Corynebacteriales, Corynebacteriaceae, Corynebacterium 1	45	0	0	0	0	132	18	0	0	0	3	79	4	0	0	0	0	0
Corynebacteriales, Corynebacteriaceae, Lawsonella	6	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Corynebacteriales, Mycobacteriaceae, Mycobacterium	11	1	0	0	0	0	21	1	2	2	0	0	0	0	0	0	0	0
Corynebacteriales, Nocardiaceae, Gordonia	3	0	0	0	0	2	17	1	1	0	0	0	0	0	0	0	0	3
Cytophagales, Spirosomaceae, Dyadobacter	2	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
Cytophagales, Spirosomaceae, Larkinella	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Deinococcales, Deinococcaceae, Deinococcus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diplorickettsiales, Diplorickettsiaceae, uncultured	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacteriales, Enterobacteriaceae, Cronobacter	10	4	0	1	0	0	8	0	0	0	0	0	1	0	0	0	0	1
Enterobacteriales, Enterobacteriaceae, Izhakiella	2	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Enterobacteriales, Enterobacteriaceae, Kosakonia	11	0	0	0	0	72	2	1	0	4	3	1	1	0	0	0	0	0

Corn grain	CTRL						Inoc1						Inoc2					
Day	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Enterobacteriales, Enterobacteriaceae, NA	6	0	0	1	0	0	1	0	0	0	0	4	4	0	0	0	0	0
Enterobacteriales, Enterobacteriaceae, Pantoea	152	33	10	4	0	41	92	2	1	17	13	3	0	0	0	0	0	1
Enterobacteriales, Enterobacteriaceae, Sodalis	6	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Erysipelotrichales, Erysipelotrichaceae, Erysipelatoclostridium	2	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0
Erysipelotrichales, Erysipelotrichaceae, Turicibacter	3	0	0	0	0	0	1	0	0	0	2	0	0	0	0	0	0	0
Flavobacteriales, Flavobacteriaceae, Flavobacterium	5	0	0	0	0	0	3	0	0	0	0	0	1	0	0	0	0	0
Flavobacteriales, Flavobacteriaceae, NS3a marine group	0	0	0	0	0	5	0	0	0	0	0	0	4	0	0	0	0	0
Flavobacteriales, Weeksellaceae, Chryseobacterium	69	0	1	0	0	0	7	0	0	0	0	0	4	0	2	0	0	1
Flavobacteriales, Weeksellaceae, Elizabethkingia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	82
Flavobacteriales, Weeksellaceae, Empedobacter	0	0	0	0	0	0	1	0	0	0	1	0	4	0	0	0	0	0
Frankiales, Geodermatophilaceae, Geodermatophilus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Lactobacillales, Enterococcaceae, Enterococcus	2	36	10	31	2	14	3	0	0	0	0	5	0	0	0	0	0	0
Lactobacillales, Lactobacillaceae, Lactobacillus	5	86	346	475	1223	424	4	1265	1260	1203	1042	644	27	0	0	2	0	162
Lactobacillales, Lactobacillaceae, Pediococcus	0	43	17	23	1	7	2	2	0	0	0	1	1	0	0	0	0	0
Lactobacillales, Leuconostocaceae, Leuconostoc	0	9	2	2	0	5	0	0	0	0	0	0	13	0	0	0	0	130
Lactobacillales, Leuconostocaceae, Weissella	20	1047	900	756	90	137	3	21	23	13	10	12	9	0	0	0	0	0
Lactobacillales, Streptococcaceae, Lactococcus	8	46	22	3	2	2	3	0	0	0	0	3	115	1	0	0	1	12
Lactobacillales, Streptococcaceae, Streptococcus	1	0	0	0	0	0	0	0	0	0	1	0	6	3	0	0	2	0
Micrococcales, Brevibacteriaceae, Brevibacterium	91	2	2	3	0	45	356	17	9	29	4	32	0	0	0	3	0	0
Micrococcales, Cellulomonadaceae, Cellulomonas	0	0	0	0	0	0	0	0	0	0	0	0	1	12	2	0	1	4
Micrococcales, Dermabacteraceae, Brachybacterium	15	0	0	0	0	1	86	1	0	0	0	5	0	0	0	0	0	0

Corn grain	CTRL						Inoc1						Inoc2					
Day	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Micrococcales, Microbacteriaceae, Curtobacterium	2	0	0	0	1	0	2	0	0	0	0	0	0	0	0	0	0	12
Micrococcales, Microbacteriaceae, NA	2	0	0	0	0	0	5	0	0	0	0	1	1	0	0	0	0	0
Micrococcales, Micrococcaceae, Arthrobacter	3	0	0	0	0	138	6	0	0	0	0	168	0	1	0	0	0	0
Micrococcales, Micrococcaceae, Glutamicibacter	0	0	0	0	0	0	1	0	0	0	0	0	10	0	0	0	0	0
Micrococcales, Micrococcaceae, Kocuria	21	1	0	0	0	3	91	1	2	3	1	2	3	0	0	0	0	0
Micrococcales, NA, NA	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Micrococcales, NA, NA	0	0	0	0	0	0	1	0	0	0	0	5	1	0	0	0	0	0
Micrococcales, Promicromonosporaceae, Cellulosimicrobium	3	0	0	0	0	0	2	0	0	0	0	0	0	61	3	3	7	0
Micromonosporales, Micromonosporaceae, NA	0	0	0	0	0	0	0	0	0	0	2	1	1	0	0	0	0	0
Micromonosporales; Micromonosporaceae; Actinocatenispora	0	0	0	0	0	0	3	0	0	0	0	0	2	0	0	0	0	0
Myxococcales, Polyangiaceae, NA	0	0	0	0	0	6	0	0	0	0	0	0	10	0	0	0	0	1
NA	4	0	1	0	0	0	4	0	0	0	0	0	0	1	0	0	0	0
Nitrosococcales, Methylophagaceae, Methylophaga	0	0	0	0	0	5	0	0	0	0	0	0	41	0	0	0	2	0
Pasteurellales, Pasteurellaceae, uncultured	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Planctomycetales, Gimesiaceae, uncultured	0	0	0	0	0	3	0	0	0	0	0	0	2	0	0	0	0	0
Pseudomonadales, Moraxellaceae, Acinetobacter	102	2	3	3	0	9	54	1	1	4	7	2	3	0	1	0	0	3
Pseudomonadales, Moraxellaceae, Alkanindiges	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonadales, Pseudomonadaceae, Pseudomonas	16	0	1	0	1	20	3	0	0	1	5	2	5	0	0	0	0	0
Pseudonocardiales, Pseudonocardiaceae, Actinophytocola	1	0	0	0	0	0	15	0	1	0	2	0	0	0	0	0	0	0
Pseudonocardiales, Pseudonocardiaceae, NA	3	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Pseudonocardiales, Pseudonocardiaceae, Pseudonocardia	2	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Rhizobiales, Beijerinckiaceae, Bosea	2	0	0	0	0	0	1	0	0	0	0	0	0	3	0	0	0	0
Rhizobiales, Beijerinckiaceae, Camelimonas	0	0	0	0	0	0	2	0	0	2	0	0	1	0	0	0	0	0

Corn grain	CTRL						Inoc1						Inoc2					
Day	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Rhizobiales, Beijerinckiaceae, Chelatococcus	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Rhizobiales, Beijerinckiaceae, Methylobacterium	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
Rhizobiales, Devosiaceae, Devosia	3	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Rhizobiales, Kaistiaceae, Kaistia	21	0	0	0	0	0	3	0	0	1	0	0	2	0	0	0	0	7
Rhizobiales, Rhizobiaceae, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	10	0	0	0	0	0	8	0	1	2	0	0	15	64	22	7	4	9
Rhizobiales, Rhizobiaceae, Aureimonas	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Rhizobiales, Rhizobiaceae, NA	0	0	0	0	0	0	1	0	0	0	0	0	208	0	17	0	1	0
Rhizobiales, Rhizobiaceae, Ochrobactrum	21	0	0	0	0	0	30	1	1	2	1	0	24	0	0	0	0	0
Rhizobiales, Rhizobiaceae, uncultured	0	0	0	0	0	12	0	0	0	0	0	17	0	0	0	0	0	0
Rhizobiales, Xanthobacteraceae, NA	2	0	0	0	0	0	0	0	0	0	2	0	22	0	1	0	0	7
Rhodobacterales, Rhodobacteraceae, NA	0	0	0	0	0	22	0	0	0	0	4	1	188	2	17	0	5	425
Rhodobacterales, Rhodobacteraceae, Paracoccus	4	0	0	0	0	0	0	0	0	0	0	2	7	0	0	0	0	0
Rickettsiales, Anaplasmataceae, Wolbachia	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rickettsiales, Rickettsiaceae, Rickettsia	0	0	0	0	0	0	1	0	0	0	1	0	2	0	0	0	0	0
Rubrobacterales, Rubrobacteriaceae, Rubrobacter	1	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0
Selenomonadales, Veillonellaceae, Anaerospora	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
Solibacterales; Solibacteraceae (Subgroup 3); Bryobacter	1	0	0	0	0	0	1	0	0	0	0	0	9	0	0	0	0	4
Solirubrobacterales, Solirubrobacteraceae, Conexibacter	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Solirubrobacterales, Solirubrobacteraceae, Patulibacter	1	0	0	0	0	0	1	0	1	0	0	0	8	0	0	0	0	0
Sphingobacteriales, Sphingobacteriaceae, Arcticibacter	2	0	0	0	0	0	0	0	0	0	0	0	0	8	3	2	1	0
Sphingobacteriales, Sphingobacteriaceae, Mucilaginibacter	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Sphingobacteriales, Sphingobacteriaceae, Olivibacter	0	0	0	0	0	0	6	0	0	0	0	0	2	0	0	0	0	0
Sphingobacteriales, Sphingobacteriaceae, Pedobacter	0	0	0	0	0	0	2	0	0	0	0	0	7	0	0	0	0	0

Corn grain		CTRL						Inoc1						Inoc2					
Day		0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Sphingobacteriales, Solitalea	Sphingobacteriaceae,	0	0	0	0	0	3	0	0	0	0	0	5	5	0	0	0	0	0
Sphingobacteriales, Sphingobacterium	Sphingobacteriaceae,	18	1	0	1	0	0	13	0	0	2	0	0	16	0	1	0	0	1
Sphingomonadales, Altererythrobacter	Sphingomonadaceae,	1	0	0	0	0	0	1	0	0	0	0	0	0	4	4	3	2	0
Sphingomonadales, Novosphingobium	Sphingomonadaceae,	5	0	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Sphingomonadales, Sphingobium	Sphingomonadaceae,	7	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0
Sphingomonadales, Sphingomonas	Sphingomonadaceae,	7	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Streptomycetales, Streptomyces	Streptomycetaceae,	63	0	0	1	0	3	161	3	10	15	10	0	9	0	0	0	0	0
Streptosporangiales, Nocardiopsis	Nocardiopsaceae,	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0
Verrucomicrobiales, Luteolibacter	Rubritaleaceae,	0	0	0	0	0	0	0	0	0	0	0	0	5	7	1	0	2	7
Verrucomicrobiales, Prosthecobacter	Verrucomicrobiaceae,	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrionales, Vibrionaceae, Vibrio		0	0	0	0	0	6	0	0	0	0	2	0	3	1	0	0	0	0
Xanthomonadales, Dokdonella	Rhodanobacteraceae,	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Xanthomonadales, Rhodanobacteraceae, Dyella		54	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Xanthomonadales, Luteimonas	Xanthomonadaceae,	4	0	0	0	0	0	1	0	0	1	0	0	14	0	0	0	0	0
Xanthomonadales, Pseudoxanthomonas	Xanthomonadaceae,	5	0	0	0	0	0	3	0	0	0	0	0	2	0	0	0	0	0
Xanthomonadales, Stenotrophomonas	Xanthomonadaceae,	21	1	1	2	0	0	9	0	0	1	6	0	14	0	0	0	0	0

Table S2. Bacterial abundance in rehydrated sorghum grain and theirs silages throughout 0, 3, 7, 21, 90 and 360 days of fermentation. CTRL: non-inoculated; Inoc1: Lactobacillus plantarum and Propionibacterium acidipropionici and Inoc2: Lactobacillus buchneri.

Sorghum grain	CTRL						Inoc1						Inoc2					
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Acetobacterales, Acetobacteraceae, Roseomonas	1	0	0	0	0	0	3	0	0	1	0	0	1	0	0	0	0	0
Bacillales, Bacillaceae, Bacillus	2	0	0	0	0	1	0	3	0	0	0	20	7	0	0	0	0	0
Bacillales, Paenibacillaceae, Paenibacillus	4	0	0	0	0	0	1	0	0	0	0	0	7	0	0	0	0	0
Bacillales, Paenibacillaceae, Saccharibacillus	3	0	0	0	0	0	2	0	0	0	0	0	3	0	0	0	0	0
Bacillales, Planococcaceae, Domibacillus	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0
Bacillales, Planococcaceae, Lysinibacillus	0	0	0	0	0	2	4	0	0	0	0	4	6	0	0	0	0	0
Bacillales, Planococcaceae, Solibacillus	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0
Bacillales, Staphylococcaceae, Staphylococcus	7	0	1	0	0	7	2	0	0	0	0	0	6	1	2	1	0	6
Betaproteobacteriales, Burkholderiaceae, Ambiguous taxa	3	0	0	0	0	0	3	0	0	0	0	0	1	0	0	0	0	0
Betaproteobacteriales, Burkholderiaceae, Comamonas	2	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0
Betaproteobacteriales, Burkholderiaceae, Massilia	26	0	0	0	0	0	15	0	0	1	0	0	28	0	1	0	0	0
Betaproteobacteriales, Burkholderiaceae, NA	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Caulobacterales, Caulobacteraceae, Brevundimonas	0	0	0	0	0	1	1	0	0	2	0	0	3	0	0	0	0	0
Chitinophagales, Chitinophagaceae, Filimonas	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
Clostridiales, Clostridiaceae 1, Clostridium sensu stricto 1	1	0	0	0	0	0	1	0	0	0	0	9	10	0	1	0	0	0
Clostridiales, Clostridiaceae 1, Clostridium sensu stricto 12	0	0	16	9	9	3	0	0	0	5	40	0	0	2	2	11	0	1

Sorghum grain	CTRL						Inoc1						Inoc2					
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Clostridiales, Peptostreptococcaceae, Ambiguous_taxa	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Clostridiales, Peptostreptococcaceae, Romboutsia	1	0	0	0	0	0	0	0	0	0	0	3	3	0	0	0	0	0
Corynebacteriales, Corynebacteriaceae, Corynebacterium 1	2	0	1	0	0	4	3	0	0	0	0	14	23	0	0	0	0	9
Corynebacteriales, Nocardiaceae, Rhodococcus	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
Deinococcales, Deinococcaceae, Deinococcus	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0
Enterobacteriales, Enterobacteriaceae, Cronobacter	16	41	35	1	18	7	5	25	37	43	6	0	1	24	9	29	4	3
Enterobacteriales, Enterobacteriaceae, Kosakonia	33	80	117	55	34	26	1	22	16	31	4	2	9	216	244	66	84	202
Enterobacteriales, Enterobacteriaceae, Pantoea	627	677	461	17	83	46	1038	142	138	127	35	5	680	192	66	101	25	28
Erysipelotrichales, Erysipelotrichaceae, Turicibacter	5	0	0	0	0	0	0	0	0	0	0	5	11	0	1	0	0	0
Flavobacteriales, Weeksellaceae, Apibacter	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0
Flavobacteriales, Weeksellaceae, Chryseobacterium	18	0	0	0	0	0	7	1	0	2	0	0	18	0	0	0	0	0
Flavobacteriales, Weeksellaceae, Elizabethkingia	6	0	0	0	0	0	3	1	0	0	0	0	15	0	0	0	0	0
Frankiales, Geodermatophilaceae, Geodermatophilus	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Kineosporiales, Kineosporiaceae, Quadrisphaera	6	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0
Lactobacillales, Aerococcaceae, Aerococcus	0	0	0	0	0	0	1	0	0	0	0	0	4	0	0	0	0	0
Lactobacillales, Carnobacteriaceae, Desemzia	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0

Sorghum grain	CTRL						Inoc1						Inoc2					
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Lactobacillales, Carnobacteriaceae, uncultured	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Lactobacillales, Enterococcaceae, Enterococcus	1	58	72	36	38	33	1	13	11	27	6	0	4	66	42	109	39	53
Lactobacillales, Lactobacillaceae, Lactobacillus	2	31	117	877	796	419	1	756	718	521	1120	1217	9	436	669	346	810	429
Lactobacillales, Lactobacillaceae, Pediococcus	1	24	29	38	30	5	0	21	20	17	1	0	0	59	48	52	33	33
Lactobacillales, Leuconostocaceae, Leuconostoc	0	19	13	9	8	22	0	43	42	70	12	3	0	31	11	6	34	30
Lactobacillales, Leuconostocaceae, Weissella	9	283	373	267	288	725	1	246	257	437	88	11	35	256	203	537	279	504
Lactobacillales, Streptococcaceae, Lactococcus	1	95	80	11	16	12	2	38	41	24	2	0	9	31	15	56	9	9
Micrococcales, Dermatophilaceae, NA	1	0	0	0	0	0	2	0	0	0	0	0	1	0	0	0	0	0
Micrococcales, Microbacteriaceae, Curtobacterium	1	0	0	0	0	0	14	0	0	0	0	0	9	0	0	0	0	0
Micrococcales, Microbacteriaceae, NA	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Micrococcales, Micrococcaceae, Arthrobacter	0	0	0	0	0	1	0	0	0	0	0	12	0	0	0	0	0	3
Micrococcales, Micrococcaceae, Glutamicibacter	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Micrococcales, Micrococcaceae, Kocuria	1	0	0	0	0	0	3	0	0	0	0	0	9	0	0	0	0	0
Nostocales, Chroococcidiopsaceae, Chroococcidiopsis PCC 7203	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nostocales, Phormidiaceae, NA	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonadales, Moraxellaceae, Acinetobacter	159	5	4	0	0	1	25	2	5	4	0	3	73	1	1	3	2	0
Pseudomonadales, Pseudomonadaceae, Pseudomonas	116	1	0	0	0	3	9	0	0	1	1	1	39	0	0	1	1	0
Rhizobiales, Beijerinckiaceae, Methylobacterium	3	0	0	0	0	0	1	0	0	0	0	1	7	0	0	0	0	0

Sorghum grain	CTRL						Inoc1						Inoc2					
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Rhizobiales, Rhizobiaceae, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	4	1	0	0	0	0	1	0	0	0	0	0	4	0	0	0	0	1
Rhizobiales, Rhizobiaceae, Aureimonas	13	0	1	0	0	0	7	1	1	0	1	0	18	0	0	0	0	0
Rhizobiales, Rhizobiaceae, Ochrobactrum	4	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0
Rhodobacterales, Rhodobacteraceae, Paracoccus	1	0	0	0	0	1	2	0	0	0	0	1	22	0	0	0	0	0
Rhodobacterales, Rhodobacteraceae, Rubellimicrobium	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Sphingobacteriales, Sphingobacteriaceae, Sphingobacterium	21	0	0	0	0	0	0	0	0	0	1	0	8	0	0	0	0	0
Sphingomonadales, Sphingomonadaceae, Novosphingobium	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0
Sphingomonadales, Sphingomonadaceae, Sphingomonas	80	3	0	0	1	0	102	0	0	3	2	0	91	2	0	0	0	0
Xanthomonadales, Xanthomonadaceae, Stenotrophomonas	44	2	0	0	0	0	23	1	32	0	0	0	14	0	1	0	0	0
Xanthomonadales, Xanthomonadaceae, Xanthomonas	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0

CHAPTER 4 - MYCOBIOME OF REHYDRATED CORN AND SORGHUM GRAIN SILAGES TREATED WITH MICROBIAL INOCULANTS IN DIFFERENT FERMENTATION PERIODS

Manuscript formatted according to the Fungal Diversity requirements

ACKNOWLEDGMENTS

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ABSTRACT

Fungal growth during ensiling and after aerobic exposure leads to loss of nutrients and reductions in the palatability and feed value of silage. Therefore, understanding the microbial community involved in ensiling can avoids losses with the aerobic deterioration, guaranteeing the adequate hygienic conditions of the ensiled material. Faced with this, we explored the diversity, community succession and evaluated the impacts caused by Lactobacillus plantarum + Propionibacterium acidipropionici and Lactobacillus buchneri inoculants on epiphytic fungi community of rehydrated corn and sorghum grains and their silages by ITS rRNA Illumina Miseq sequencing after 0, 3, 7, 21, 90 and 360 days of fermentation. Ascomycota, Basidiomycota and Mucoromycota phyla were found in both grains. Saccharomycetes and Eurotiomycetes classes were predominantly found in corn grain, whereas the Dothideomycetes together with Saccharomycetes were the main classes in sorghum grain fungal fermentation. Aspergillus ssp. represented 51-89% of the initial population of corn grain samples while Alternaria and unidentified Pleosporales were present in the initial community of sorghum grain samples. Aspergillus spp. molds were predominant in rehydrated corn grain fermentation while the yeast Wickerhamomyces anomalus was the major fungal in rehydrated sorghum grain silages. The addition of inoculant did not have an effect on fungal population of rehydrated sorghum grain silages.

Keywords acetic acid, ITS rRNA, fungi, silage

INTRODUCTION

The production of corn and sorghum grains has been increasing exponentially in Brazil and generating a series of problems related to transport and storage logistics. As much of the grain production is destined to animal feed, one of the alternatives to minimize losses is the use of the ensiling process. In addition to reducing costs with taxes, transportation, and storage, as well as reducing losses from insect and rodent attacks, under appropriate management conditions, silage can also reduce or eliminate the development of molds, which is the main problem of grains storage (Reis et al. 2001).

The rehydrated grain silage consists basically of hydration of the milled mature grain with 10-14% to reach the moisture necessary for silage, between 30 to 40% of fresh weight (Gobetti et al. 2013). During the ensiling process occurs hydration of the protein matrix, loss of organization and rupture of endosperm cells, promoting partial rupture of the protein matrix of the grains, reducing the negative effect of the hard texture of the endosperm on the starch digestibility of mature stage grains (Sullins and Rooney 1971).

Undesirable microorganisms may develop in silage when the pH is insufficiently reduced or when oxygen is available by secreting extracellular enzymes, which break down complex organic polymers into monomers, which then can be used for its growth (May et al. 2001). Fungal growth during ensiling and after aerobic exposure leads to loss of nutrients and reductions in the palatability and feed value of silage (O'Brien et al. 2007).

After air exposure, yeasts are generally the starters of aerobic deterioration, consuming sugars and acids, raising silage temperature and pH (Pahlow et al. 2003). After, Bacilli and other aerobic bacteria are able to grow in an increased pH material, increasing temperature further. Finally, molds complete the silage deterioration (Borreani et al. 2018). The aerobic deterioration of silage caused by filamentous fungi results in losses of nutrients and energy, as well as the risk of contamination by mycotoxins (Lindgren et al. 2002).

Understanding the microbial community involved in ensiling is important to ensure effective conservation of silage (Peng et al. 2018). Using next generation sequencing to investigate the fungal succession of silage could facilitate the development of additives that could act synergistically with defined populations to improve the quality and aerobic stability of silages (Dunieri et al. 2017).

Recent studies have evaluated fungal microbiome in corn, sweet sorghum and small grain silages (Dunieri et al. 2017, Romero et al. 2018, Gallagher et al. 2018). However, to the

best of our knowledge, literature evaluating the fungal population of rehydrated corn and sorghum grains and their silages is scarce, particularly using the next-generation sequencing as mycobiome identification technique.

Based on that, expecting a rapidly changing silage environment and different effects of microbial inoculants on fungi communities of silages. We explored the diversity, community structure and evaluated the impacts caused by microbial inoculants on epiphytic fungi community of rehydrated corn and sorghum grain and their silages after 0, 3, 7, 21, 90 and 360 d of fermentation.

MATERIALS AND METHODS

Location and climatic conditions

The experiment was conducted between January 2016 and January 2017 at the Department of Animal Science of the Federal University of Vicosa (Viçosa, MG, Brazil), located at 20°45' S latitude, 42°52' W longitude 648 m above sea level. The annual precipitation and average temperature the year of the experiment were 1235.4 mm and 20.7 °C, respectively.

Ensiling and sampling

The samples used in this experiment were obtained from a previous study conducted by Pimentel (2017) (unpublished data) which evaluated the effect of inoculant and period of fermentation on rehydrated corn and sorghum grain silages.

Briefly, the experiment was carried out under completely randomized design (with three replicates) based on a 2 × 3 × 6 factorial assay, with two grains (corn -**CG** and sorghum-**SG**), three inoculants and six fermentation periods (0, 3, 7, 21, 90 and 360 days). The evaluated treatments were: Corn control (**CG-CTRL**); Corn Inoculant 1- (**CG-Inoc1**); Corn Inoculant 2 - (**CG-Inoc2**); Sorghum control (**SG-CTRL**); Sorghum Inoculant 1- (**SG-Inoc1**); Sorghum Inoculant 2 - (**SG-Inoc2**). The inoculants were composed of **CTRL** – non-inocuated; **Inoc1**- Lactobacillus plantarum and Propionibacterium acidipropionici (Lalsil® Milho, Lallemand Animal Nutrition) and **Inoc2**- Lactobacillus buchneri (Lalsil® AS, Lallemand Animal Nutrition).

The CG and SG were grossly disintegrated in a mill retrofitted with 3 mm mesh sieves. Prior to fermentation, the milled CG and SG were rehydrated with water to moisture content at 30%. After, inoculants were dissolved in distilled water at the dosage recommended by the manufacturer, were sprayed on 500 g of rehydrated grains and mixed uniformly by

hand before packing into plastic film bags (25.4 cm × 35.56 cm) and vacuumed with a vacuum sealer (Eco vacuum 1040, Orved, Italy). The same amount of water was applied to CTRL silages.

The bags were stored in the laboratory at room temperature (range, 23-27°C) and 18 bags were opened on 0, 3, 7, 21, 90 and 360 days after fermentation. It was prepared representative composite samples of each treatment in each fermentation period totaling six samples per fermentation period and 36 total samples.

DNA extraction and sequencing

The 36 samples were crushed in liquid nitrogen and the total DNA was extracted by using the NucleoSpin® Soil DNA extraction kit (Macherey-Nagel, Düren, Germany), as per the manufacturer's recommendation. The DNA was quantified using Nanodrop spectrophotometer (Thermo Scientific®) and checked for quality on an agarose gel.

Genomic DNA was amplified using a custom barcoded ITS primer set, adapted for the Illumina HiSeq2000 and MiSeq. These primers were designed by Kabir Peay's lab at Stanford University (Plos one; Smith, Peay 2014). The reverse amplification primer also contained a twelve base barcode sequence that supports pooling of up to 2,167 different samples in each lane (Caporaso 2010, Caporaso 2012). Each 25 µl PCR reaction contained 12 µl of MoBio PCR Water (Certified DNA-Free), 10 µl of 5 Prime HotMasterMix (1x), 1 µl of Forward Primer (5 µM concentration, 200 pM final), 1 µl Golay Barcode Tagged Reverse Primer (5 µM concentration, 200 pM final), and 1 µl of template DNA. The conditions for PCR were follows: 94 °C for 3 min to denature the DNA, with 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s; with a final extension of 10 min at 72 °C to ensure complete amplification. Amplicons were quantified using PicoGreen (Invitrogen) and a plate reader. Once quantified, different volumes of each of the products were pooled into a single tube so that each amplicon was represented equally. This pool was then cleaned up using the UltraClean® PCR Clean-Up Kit (MoBIO), and then quantified using the Qubit (Invitrogen). After quantification, the molarity of the pool was determined and diluted down to 2 nM, denatured, and then diluted to a final concentration of 6.75 pM with a 10 % PhiX spike for sequencing on the Illumina MiSeq.

Bioinformatics analyzes

Sequences that showed bases with a maximum expected error of 0.5 of probability were removed and the remaining sequences were grouped into OTUs using the program Usearch v.11 (Edgar et al. 2013) with a threshold of 97% of similarity. Chimeras were also removed by the Uparse algorithm. The ITSx v.1.0.11 program (Bengtsson-Palme et al. 2013) was used to remove non-fungal ITS1 sequences. The taxonomic annotation was performed using the BLAST (Basic Local Alignment Search Tool) method of QIIME v.1.9.1 (Caporaso et al. 2010) using the UNITE databases. Contaminant sequences such as chloroplasts and mitochondria were removed through the result of taxonomic annotation. Alpha diversity metrics (Chao1 richness, evenness and Simpson diversity) and beta diversity metrics (using weighted UniFrac distance) were calculated with the WGCNA package, stat packages, and the ggplot2 package in R software (Version 2.15.3).

RESULTS

It was generated a total of 2,569,416 high-quality reads. CG samples originated 1,587,182 of the reads with an average of 44,088 per sample and 982,334 reads with an average of 27,284 reads per sample were originated from SG in different days of fermentation. The quality of the sequences present in the SG-Inoc2 at 360 d was not good enough to identify the fungal community. The number of sequences was standardized relative to the minimum number of 2,863 sequences obtained from a single sample

The fungal communities in CG and SG samples are presented in supplementary Table S1 and Table S2, respectively. A total of 71 and 109 OTUs were detected in CG and SG samples, respectively. Rarefaction curves at 97 % identity OTUs are shown in Supplementary Figure 1. Sequencing depth was sufficient to fully describe the diversity of the fungal populations in silages as rarefaction curves reached a clear plateau for sequences.

The principal coordinate analysis based on weighted Unifrac distance of fungal communities in CG and SG are shown in Figure 1. There were changes in the mycobiome community throughout the fermentation period in both grains. CG samples were more grouped than SG except for CG-Inoc1. SG samples formed 3 groups according to fermentation day. Independent of the treatment samples were grouped in initial population (0 d), intermediaries and last day of fermentation (360 d), contrary to corn community that final population grouped closer to initial groups.

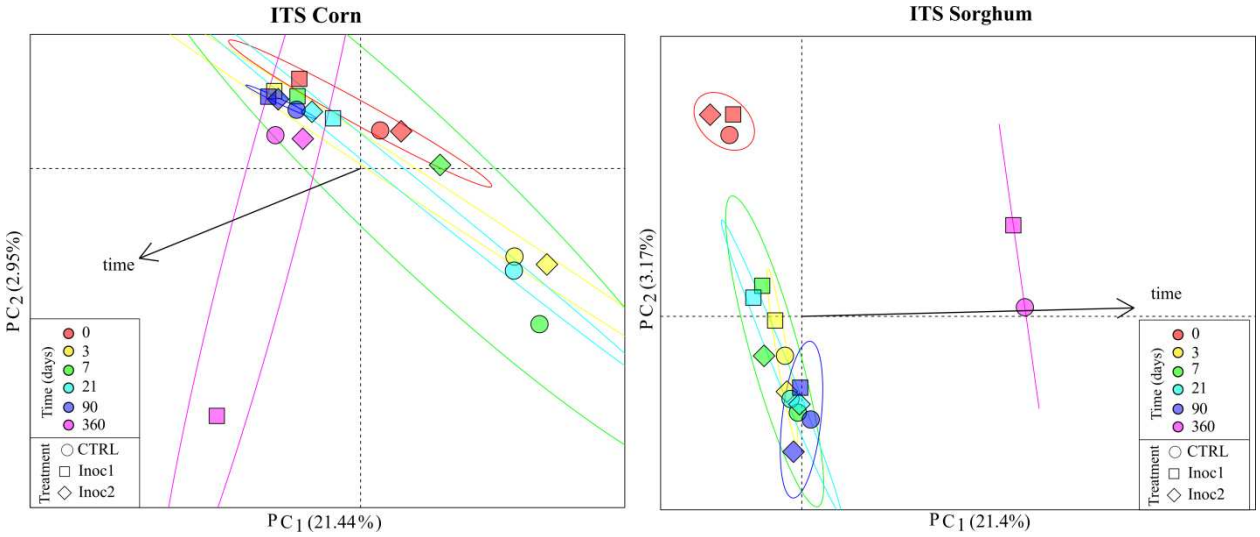

Fig. 1 Principal Coordinate Analysis (PCoA) based on Bray-Curtis distance of fungal communities according to sampling time (0, 3, 7, 21, 90 and 360 d) and silage of rehydrated corn and sorghum grains. **CTRL:** non-inoculated; **Inoc1:** Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2:** Lactobacillus buchneri.

Diversity analysis

The Simpson diversity index of corn and sorghum grain silages are shown in Figure 2. There was fluctuation of mycobiome diversity throughout the fermentation period in all treatments. CG-Inoc1 silages had lower diversity in the beginning of the fermentation than others CG treatments. At 90 d, there was drop in the diversity of all treatments and then increased again after 360 d mainly due to variation of evenness. In general, SG diversity was higher than CG samples. As observed in CG at 90 d, there was a reduction in diversity in all SG treatments, especially in SG-Inoc2 silages, and increased again after 360 d of fermentation.

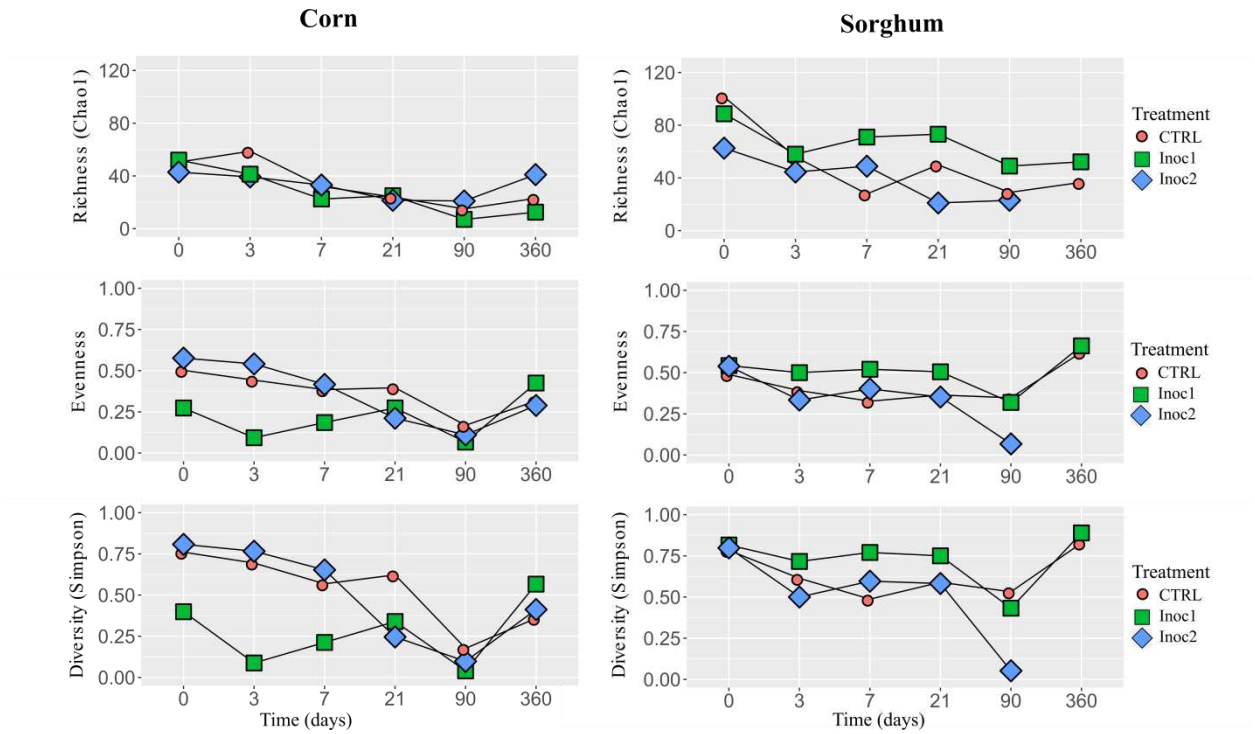

Fig. 2 Fungal Chao 1 Richness, evenness, Simpson diversity of rehydrated corn and sorghum grain silages throughout the fermentation period (0, 3, 7, 21, 90 and 360 d). **CTRL:** non-inoculated; **Inoc1:** Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2:** Lactobacillus buchneri.

Taxonomic composition – Phylum

The Phyla Ascomycota, Basidiomycota and Mucoromycota were found in both grains (Figure 3). The predominance of Ascomycota occurred in all samples with few variations in the presence of the other phyla.

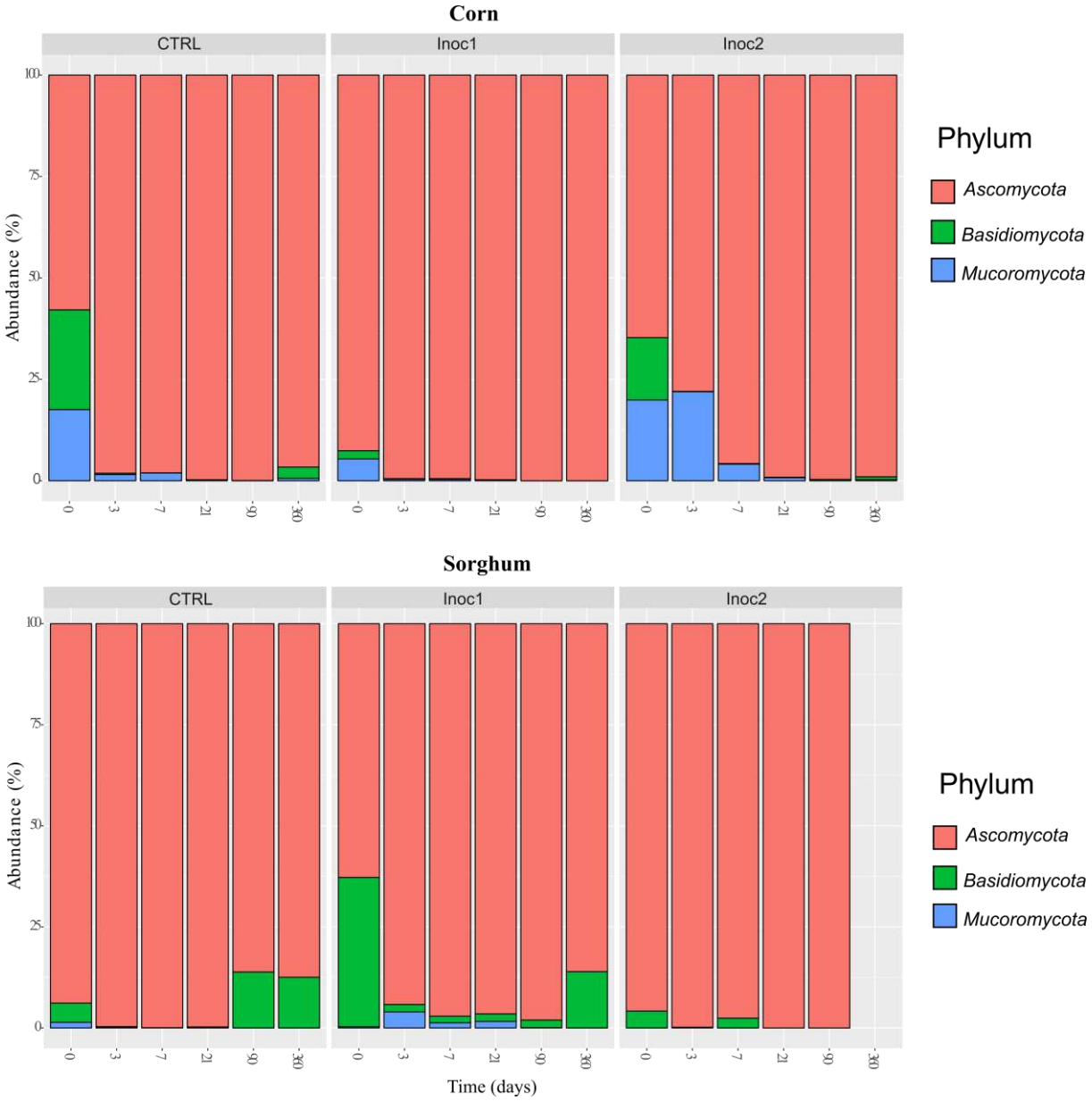

Fig. 3 Phyla taxonomic profiles of fungal communities of rehydrated corn and sorghum grain silages after 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL:** non-inoculated; **Inoc1:** Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2:** Lactobacillus buchneri.

Taxonomic composition - Class

Unidentified fungi and other eleven classes were found in both grains (Figure 4). The classes Agaricomycetes, Dothideomycetes, Eurotiomycetes, Microbotryomycetes, Mucoromycetes, Saccharomycetes, Sordariomycetes, Tremellomycetes and Wallemiomycetes were found in both grains, Orbiliomycetes and Pezizomycetes were found only in CG samples and Agaricostilbomycetes and Cystobasidiomycetes in SG samples. Saccharomycetes and

Eurotiomycetes classes were predominantly found in CG, whereas the Dothideomycetes together with Saccharomycetes were the main classes in SG fungal fermentation.

In CG-CTRL and CG-Inoc2 silages, about half of the initial populations were microorganisms belonging to the Eurotiomycetes class with smaller proportions of Sordariomycetes and Tremellomycetes. On day three of fermentation there was a large increase of Saccharomycetes in both silages with gradual replacement by Eurotiomycetes which its predominance (>80 %) was extended up to 360 d.

Dothideomycetes accounted for 56-92% of the initial population of SG. Tremellomycetes were also in significant amounts prior to fermentation in SG-Inoc1 silages. Sharply the initial populations were replaced by Saccharomycetes with dominance extended up to 90 d in all silages. At 360 d influential amounts of Eurotiomycetes replaced the Saccharomycetes microorganism in SG-CTRL and SG-Inoc1 silages.

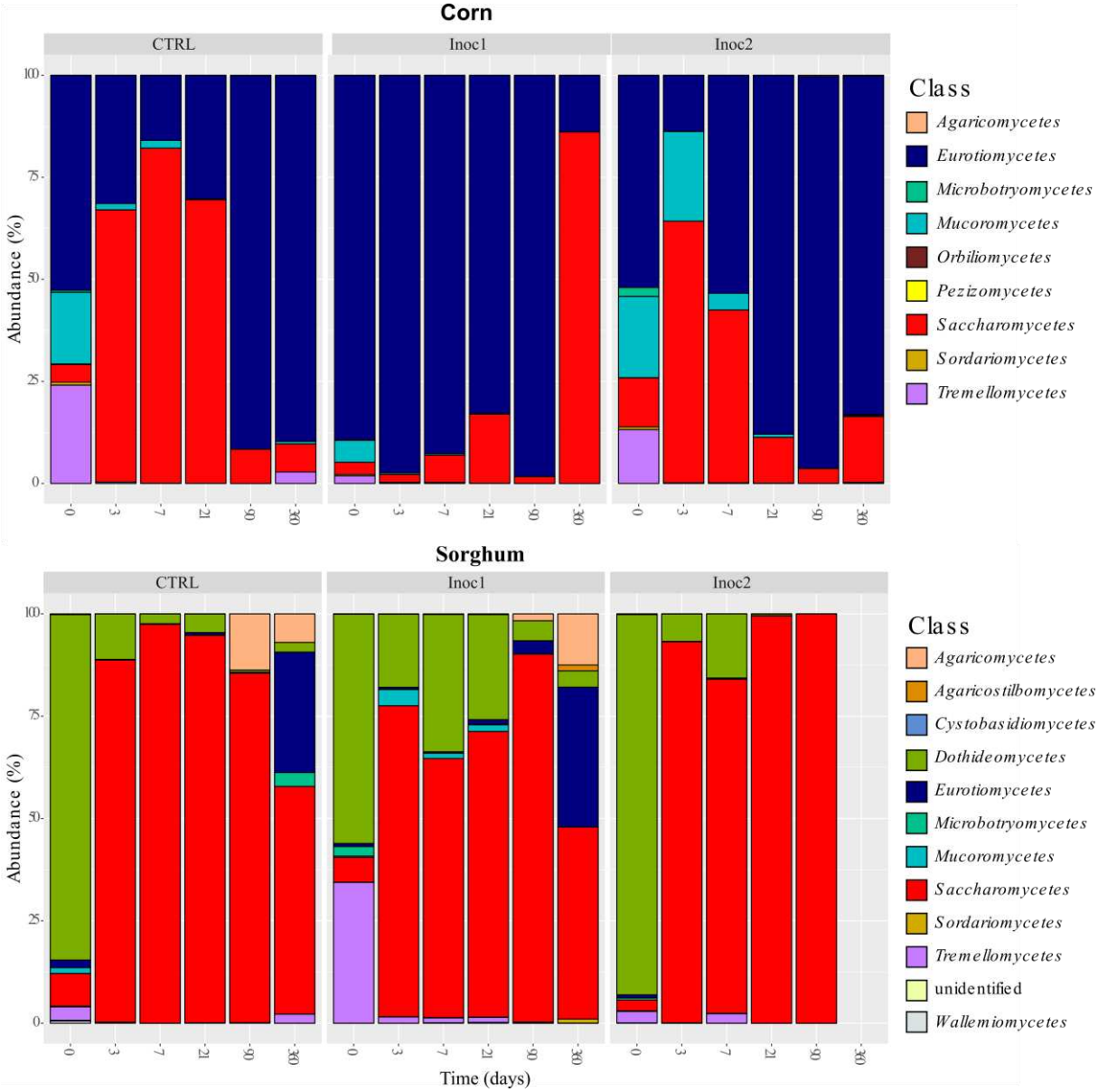

Fig. 4 Class taxonomic profiles of fungal communities of rehydrated corn and sorghum grain silages after 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL**: non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

Taxonomic composition - Genus

The main genera dynamics of corn grain silages are shown in Figure 5. Aspergillus ssp. represented 51-89% of the initial population of CG samples. Its predominance (89%) in CG-Inoc1 silages at 0 d resulted in the lowest initial diversity in this silages and it was also responsible for the sharp reduction in diversity in all CG silages at 90 d.

In CG-CTRL silages as early as 3 d there was a growth of Wickerhamomyces (60%) yeasts that dominated the fermentation until 21 d. Aspergillus returned to dominate from the

90 d until the end of the fermentation. Similar response was observed in CG-Inoc2, however at 7 d Aspergillus has already represented 54% and 88% of the genera at 21 d.

Bacteria present in Inoc1 resulted in fermentation with the predominance of Aspergillus up to 90 d. However, at 360 d, 86% of the genera were represented exclusively by unidentified yeasts belonging to the order Saccharomycetales.

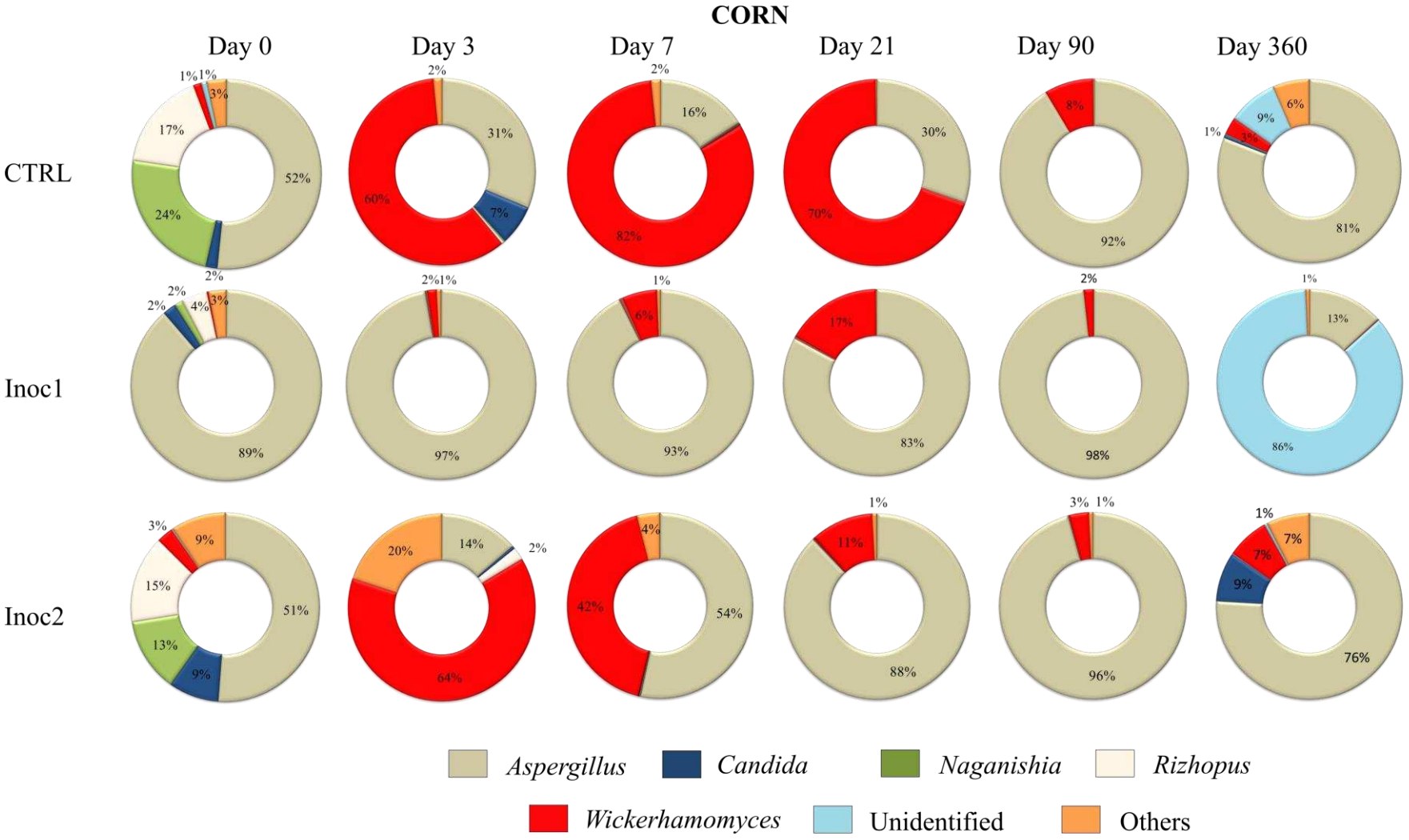

Fig. 5 Main genera dynamics (%) of fungal communities of rehydrated corn grain silages after 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL**: non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

The main genera dynamics of sorghum grain silages are shown in Figure 6. Greater participation of different genera were observed in initial samples of SG than CG. Alternaria spp. accounted for 30-60 % of the initial population of the silages. Moreover larger quantities of unidentified fungi bellonging to Pleosporales order were also observed mainly in SG-Inoc 2 samples (60 %).

Wickerhamomyces anomalus were predominant in the population of all silages at intermediate periods of fermentation with small participations of other genera. As previously observed, the diversity of SG-CTRL and SG-Inoc 1 silages at 360 d increased due to increased evenness values which reflected the greater participation of other genera such as Monascus, Candida, Aspergillus during the silage fermentation.

Some OTUs in SG samples were classified at species level such as: Amylostereum chailletii, Aspergillus flavus, Exserohilum turcicum, Hyphoderma setigerum, Kwoniella heveanensis, Kwoniella mangrovensis, Monascus purpureus, Mucor circinelloides, Nigrospora oryzae, Phialemoniopsis curvata, Resinicium saccharicola, Rhizopus arrhizus, Rhodotorula diobovata, Rhodotorula mucilaginosa, Schizophyllum commune, Setophoma sacchari, Sterigmatomyces halophilus, Strelitziana eucalypti, Wallemia ichthyophaga, Wickerhamomyces anomalus, Xeromyces bisporus and Zygoascus hellenicus.

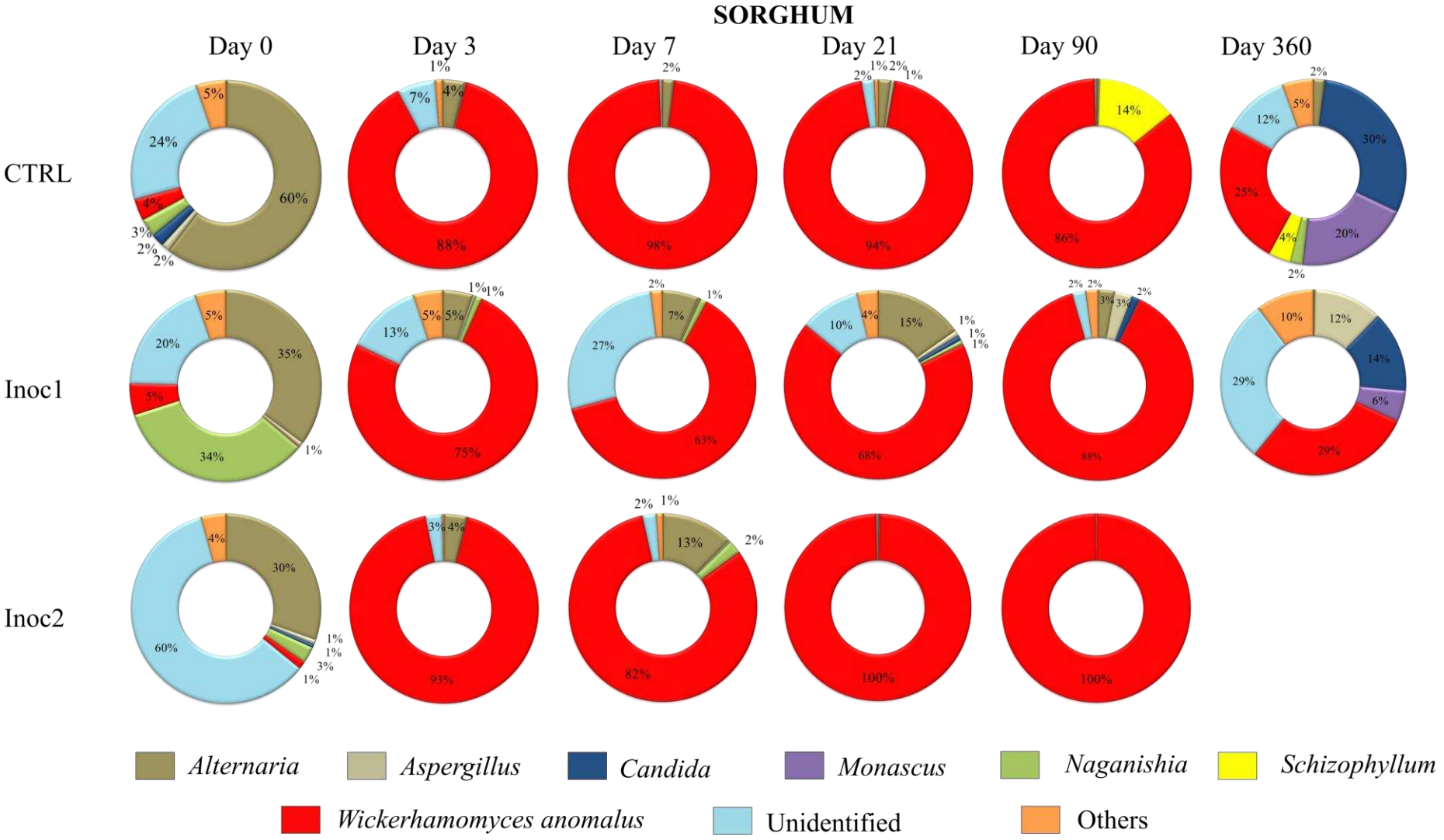

Fig. 6 Main genera dynamics (%) of fungal communities of rehydrated sorghum grain silages after 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL**: non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

DISCUSSION

Studies showed that climatic conditions affect all stages of silage production and utilization, especially in the hot and humid areas because microbial proliferation is strongly influenced by temperature (Bernardes et al. 2018). These climatic factors not only affect forage crop growth and disease incidence, but also influence the silage fermentation and aerobic stability (Kim and Adesogan 2006).

High amounts of silage have been lost and the cost of production may suffer negative consequences due to aerobic deterioration. Elimination of fermentation losses is not possible, but the use of silage additives may help minimize it (Borreani et al 2018). Propionic bacteria and heterofermentative bacteria producing acetate have been studied to reduce the deterioration of silages after exposure to air (Arriola et al. 2011; Da Silva et al. 2018). However, inoculation of bacteria can influence in different ways the fermentation characteristics and silage nutritional value according to the epiphytic bacteria present in the raw material (Si et al. 2018) and each individual strain from different silage materials (Liu et al. 2019).

As noted the epiphytic mycobiome profile among grains before fermentation was different resulting in different effects of the microbial inoculant on fungal population of the ensiled materials. Although there was a difference in the fungal profile between the grains, samples within the same grain were not altered before fermentation, evidencing the minimal immediate impact of the treatments to the microbial community at the onset of the time-course, as well as the absence of significant differences in exogenous microbial contaminants that could alter the microbial make up (Gallagher et al. 2018).

The predominance of Ascomycota phylum followed by lower quantities of Basidiomycota and Zygomycota phyla was also observed in wilted oat, corn and their silages (May et al. 2001, Romero et al. 2017, 2018). However, in our study Mucoromycota was found in the place of Zygomycota phylum. Moreover, Ascomycota and Basidiomycota were also the main phyla in Purple prairie clover (Dalea purpurea Vent.) (Peng et al. 2018).

Ascomycota group is of particular relevance to humans as sources of medicinally compounds and food making products, but also as pathogens of humans and plants. According to Romero et al. (2018) aerobic stability increased at high relative abundance of unidentified Ascomycota species in corn silage, suggesting that this microorganism may have limited the growth of other microbes responsible of aerobic spoilage.

Although the predominance of Ascomycota phyla was observed in all samples, the mycobiome profiles were different among grains. The predominance of unidentified species of Aspergillus in CG sample, Alternaria and other unidentified fungal belonging to the Pleosparales order in SG samples before fermentation, evidence the presence of high abundance of molds in pre-ensiled grains even without visible symptoms of fungal contamination. Pleosporales molds are usually associated with leaf spot of the plant. Thereby, the presence of this genus was expected since many fungi inhabit the plant phyllosphere as it matures in the field and may still be present before absolute anaerobiosis is achieved (May et al. 2001).

Most of fungal microorganisms are strict aerobes; however a few filamentous fungi and several yeasts are capable of fermentative growth. In all mixed microbial populations of silages, certain species are better adapted than others to reduced O_2 tensions, lower pH, and higher concentrations of carbon dioxide and organic acids (Pelhate 1977), such as some species of Aspergillus, Penicillium, Fusarium and Monascus (Gallo et al. 2015).

According to Gulbis et al. (2016) the most frequently species isolated from corn silage are fungal belonging to the genera Alternaria, Fusarium and Penicilliumm. In the present study, Penicillium and Fusarium spp. were presented in low abundance during the silage fermentation. Throughout the fermentation period, in general the CG fermentation was dominated by Aspergillus molds whereas the Wickerhamomyces anomalus yeasts were predominant in SG samples. Thus, the persistence and predominance of Aspergillus during the fermentative period in CG silages, evidences the better tolerance of this genus to ensiling condition than Alternaria and other Pleosparal molds that were present in SG before fermentation.

Indeed, Aspergillus spp. are worldwide distributed mold and it is one of the most predominant species found in different silages in Brazil (Keller et al. 2012). It requires high temperature and low water activity for growth and it can also survive under microaerophilic conditions and acidic environment (Pereyra et al. 2008). Moreover, mycotoxins produced by Aspergillus species is one of the four major toxins found in corn silage (Mansfield 2005).

The predominance of Aspergillus genus in CG-Inoc1 silages since the beginning of fermentation suggests the inoculant inhibited the growth of some yeasts specially Wickerhamomyces species. However, the increased abundance of unidentified yeasts belonging to Saccharomycetales order was unexpected and unclear at 360 d.

Several factors are known to affect the yeast flora composition in silage such as the amount of air ingress during silage storage and the type of crop that is ensiled (Pahlow et al. 2003). As the anaerobic conditions were guaranteed and kept constant throughout the fermentation period, it suggests that the different yeast community profile among grains may be affected by crop type and other factors (Santos et al. 2017).

As discussed previously, the presence of some yeast in silage is associated with anaerobic fermentative loss and aerobic deterioration after aerobic exposure of silage during animal feeding or damage in the sealing of the silo (Pahlow et al. 2003). In addition to losses in the quality of silage, yeasts may also be opportunistic pathogens (Kurtzman et al. 2011a) and reduce the in vitro aNDF digestion (Santos et al. 2014).

Yeasts such Wickerhamomyces species found in this study belong to the order of Saccharomycetales and are frequently associated with spoilage or processing of food and grain products. Saccharomycetales spp. was also predominant in the fungal core microbiome of small grain silages (Duniere et al. 2017).

W. anomalus, formerly known as Pichia anomala, Hansenula anomala or Candida pelliculosa was assigned to the genus Wickerhamomyces (Kurtzman, 2011b). It is a biotechnologically relevant yeast specie present in a very diverse habitat (Padilla et al. 2018). The predominance of this specie during the fermentation of SG silages is related to the ability of this specie to tolerate extreme environmental conditions like oxidative, salt, and osmotic stress, as well as pH and temperature shocks (Walker, 2011). However, due to these characteristics, this microorganism can be a spoilage in several products such as silage and dairy products (Kitamoto et al. 1999, Passoth et al. 2006).

According to Druvefors et al. (2005) W. anomalus has promising features as alternatives to chemical fungicides in storage of cereal grains by production of metabolites derived from glycolysis with antifungal action, rather than to competition for nutrients or activity of cell wall lytic enzymes. In addition, ethanol and ethyl acetate were also considered responsible for the antifungal activity of this specie. In this context, the effects of W. anomalus in silage fermentation still need to be elucidated.

CONCLUSION

The mycobiome diversity were different among corn and sorghum grains. Aspergillus spp. were predominant in rehydrated corn grain fermentation while Wickerhamomyces anomalus was the major fungal species in rehydrated sorghum grain silages. The addition of inoculant did not have an effect on fungal population of rehydrated sorghum grain silages, while the mix of Lactobacillus plantarum and Propionibacterium acidipropionici controled the growth of Wickerhamomyces yeast until 90 d of fermentation in rehydrated corn grain silages.

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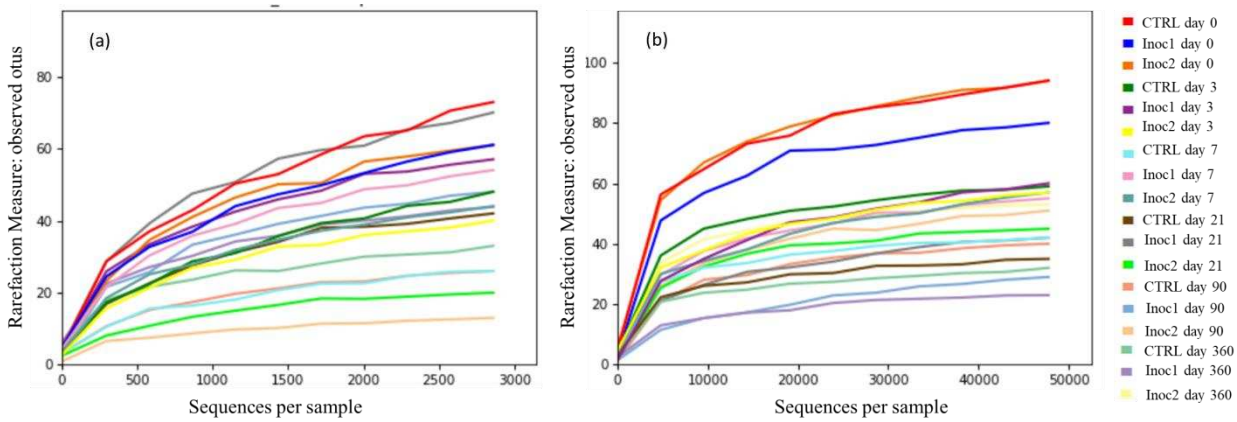
SUPPLEMENTARY INFORMATION



Supplementary Figure 3. Rarefaction curves showing the sampling effort and number of fungal OTUs observed in rehydrated corn (a) and sorghum (b) grain silages after 0, 3, 7, 21, 90 and 360 d of fermentation. **CTRL:** non-inoculated**; Inoc1:** Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

Table S1. Fungal abundance in rehydrated corn grain silages throughout 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL**: non-inoculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

Corn grain	CTRL						Inoc1						Inoc2					
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Ascomycota, Classe	0	0	0	0	0	25	0	0	0	0	0	0	0	0	0	0	0	1
Ascomycota, unidentified	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Basidiomycota, NA	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	3
Cantharellales, unidentified	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Chaetothyriales, Chaetothyriales Incertae sedis, Sarcinomyces	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chaetothyriales, Herpotrichiellaceae, Exophiala	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Corticiales, Vuilleminiaceae, Vuilleminia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Cystofilobasidiales, NA	0	0	0	0	0	48	0	0	0	0	0	0	0	0	0	0	1	4
Diaporthales, Diaporthaceae, NA	3	0	1	0	0	0	1	3	0	0	0	0	2	1	0	0	0	0
Eurotiales, Aspergillaceae, Aspergillus	1470	897	456	866	2616	2300	2534	2784	2653	2372	2814	377	1458	392	1527	2516	2739	2171
Eurotiales, Aspergillaceae, Monascus	0	0	0	0	0	166	0	0	0	0	0	20	0	0	0	0	0	193
Eurotiales, Aspergillaceae, Penicillium	14	0	0	0	0	0	8	1	0	0	0	0	2	0	0	0	0	2

Corn grain	CTRL						Inoc1						Inoc2					
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Eurotiales, Aspergillaceae, Xeromyces	7	2	0	1	1	0	0	0	0	0	1	0	19	1	1	1	6	1
Eurotiomycetes, NA	8	0	0	0	0	62	5	1	0	0	0	0	0	0	0	0	1	0
Filobasidiales, Filobasidiaceae, Naganishia	677	6	1	1	0	3	42	3	3	0	0	0	360	1	4	0	0	0
Filobasidiales, NA	0	0	0	0	0	28	0	0	0	0	0	0	0	0	0	0	0	4
Hymenochaetales, Hymenochaetales Incertae sedis, Resinicium	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Hypocreales, Nectriaceae, Fusarium	15	1	0	0	0	0	8	1	0	0	0	0	14	3	1	0	0	0
Mucorales, Mucoraceae, Mucor	16	30	44	5	0	17	29	11	10	1	0	0	138	565	111	16	0	5
Mucorales, Rhizopodaceae, Rhizopus	483	14	11	1	0	0	124	0	0	4	0	0	428	63	6	6	0	3
Orbiliales, unidentified,	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pezizales, Ascodesmidaceae, Cephaliophora	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Pleosporales, NA	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Pleosporales, Pleosporaceae, Alternaria	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pleosporales, unidentified	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Polyporales, Fomitopsidaceae, Dacryobolus	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

Corn grain	CTRL						Inoc1						Inoc2					
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Polyporales, Hyphodermataceae, Hyphoderma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0
Polyporales, NA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Polyporales, unidentified	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Polyporales, Xenasmataceae, Phlebiella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2
Russulales, Peniophoraceae, Peniophora	0	1	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0
Saccharomycetales, Debaryomycetaceae, Debaryomyces	17	1	0	0	0	0	10	0	0	0	0	0	11	1	0	0	0	1
Saccharomycetales, NA	12	4	1	1	0	88	5	2	4	1	0	2461	5	0	1	4	0	0
Saccharomycetales, Phaffomycetaceae, Cyberlindnera	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Saccharomycetales, Phaffomycetaceae, Wickerhamomyces	39	1703	2345	1985	240	89	7	45	175	475	48	4	81	1819	1206	311	98	211
Saccharomycetales, Saccharomycetales Incertae sedis, Candida	56	199	3	3	0	16	64	8	13	8	0	1	245	13	2	6	5	246
Saccharomycetales, Trichomonascaceae, Blastobotrys	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	3
Saccharomycetales, Trichomonascaceae, Zygoascus	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
Sordariomycetes, unidentified	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Sporidiobolales, Sporidiobolaceae, Rhodotorula	14	0	0	0	0	0	5	0	0	0	0	0	63	1	0	0	0	5

Corn grain	CTRL						Inoc1						Inoc2					
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	360
Trechisporales, Hydnodontaceae, Trechispora	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Tremellales, Cryptococcaceae, Kwoniella	8	3	0	0	0	0	11	1	2	2	0	0	15	0	0	3	0	0
Wallemiales, Wallemiaceae, Wallemia	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Xylariales, Xylariales Incertae sedis, Phialemoniopsis	2	0	0	0	0	0	1	0	0	0	0	0	3	0	0	0	1	0

Table S2. Fungal abundance in rehydrated sorghum grain silages throughout 0, 3, 7, 21, 90 and 360 days of fermentation. **CTRL**: non-inculated; **Inoc1**: Lactobacillus plantarum and Propionibacterium acidipropionici and **Inoc2**: Lactobacillus buchneri.

Sorghum grains	CTRL						Inoc1						Inoc2				
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90
Eurotiales, Aspergillaceae, Aspergillus	12	2	2	15	5	1	5	11	3	22	85	332	6	1	7	2	0
Eurotiales, Aspergillaceae, Aspergillus, Aspergillus flavus	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Hypocreales, Nectriaceae, Fusarium	1	0	0	0	0	1	0	0	0	0	0	28	0	0	0	0	0
Pleosporales, Phaeosphaeriaceae, Setophoma, Setophoma sacchari	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
Pleosporales, Pleosporaceae, Bipolaris	0	0	0	0	0	0	2	0	0	0	0	0	2	0	0	0	0
Sporidiobolales, Sporidiobolaceae, Rhodotorula	0	0	0	0	0	0	6	1	1	1	0	0	0	0	0	0	0
Agaricales, Schizophyllaceae, Schizophyllum, Schizophyllum commune	1	0	0	0	392	108	0	0	0	0	1	0	0	0	0	0	0
Agaricales, Strophariaceae, Psilocybe	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Agaricomycetes, NA	0	0	0	0	0	0	0	0	0	0	0	183	0	0	0	0	0
Agaricomycetes, unidentified	0	0	0	0	0	0	0	0	0	0	5	110	0	0	0	0	0

Sorghum grains	CTRL						Inoc1						Inoc2				
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90
Agaricostilbales, Agaricostilbaceae, Sterigmatomyces, Sterigmatomyces halophilus	0	0	0	0	0	0	0	0	0	0	0	40	0	0	0	0	0
Ascomycota, NA	2	0	0	4	0	0	2	5	0	1	0	0	4	6	0	0	0
Ascomycota, unidentified	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Auriculariales, Exidiaceae, Heterochaete, NA	0	0	0	0	0	0	0	0	0	1	30	0	0	0	0	0	0
Auriculariales, NA	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Basidiomycota, NA	9	1	0	1	0	0	4	4	2	3	0	0	8	0	2	0	0
Cantharellales, Ceratobasidiaceae, Ceratobasidium	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0
Capnodiales, Mycosphaerellaceae, NA	2	0	0	1	0	0	0	0	0	0	0	0	2	0	0	0	0
Capnodiales, NA	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0
Chaetothyriales, Chaetothyriales Incertae sedis, Sarcinomyces	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chaetothyriales, Chaetothyriales Incertae sedis, Strelitziana, Strelitziana eucalypti	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Corticiales, NA	0	0	0	0	0	85	0	0	0	0	0	0	0	0	0	0	0
Cystobasidiomycetes Incertae sedis, Symmetrosporaceae, Symmetrospora	1	0	0	1	0	0	1	0	2	0	0	0	2	1	0	0	0

Sorghum grains	CTRL						Inoc1						Inoc2				
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90
Cystofilobasidiales, NA	0	0	0	0	1	0	0	0	0	11	6	0	0	0	0	0	0
Diaporthales, unidentified	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
Dothideomycetes, Pleosporales, unidentified	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0
Dothideomycetes, unidentified	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0
Eurotiales, Aspergillaceae, Aspergillus	0	0	0	0	0	1	0	0	0	0	0	3	0	0	0	0	0
Eurotiales, Aspergillaceae, Aspergillus, Aspergillus flavus	3	0	0	1	2	1	5	0	2	2	1	5	3	0	0	0	0
Eurotiales, Aspergillaceae, Monascus, Monascus purpureus	0	0	0	0	0	547	0	0	0	0	1	154	0	0	0	0	0
Eurotiales, Aspergillaceae, Penicillium	0	0	0	0	0	56	0	0	0	0	0	15	0	0	0	0	0
Eurotiales, Aspergillaceae, Xeromyces, Xeromyces bisporus	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Eurotiomycetes, NA	0	0	0	0	0	210	0	0	0	1	3	456	0	0	0	0	0
Filobasidiales, Filobasidiaceae, Naganishia	35	1	0	1	1	61	451	28	30	18	0	1	33	3	62	0	0
Hymenochaetales, Hymenochaetales Incertae sedis, Resinicium	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0

Sorghum grains	CTRL						Inoc1						Inoc2				
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90
Hymenochaetales, Hymenochaetales Incertae sedis, Resinicium, Resinicium saccharicola	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
Hypocreales, Clavicipitaceae, Claviceps	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
Hypocreales, Nectriaceae, Fusarium	1	0	0	0	0	0	0	1	1	0	0	0	2	0	0	0	0
Mucorales, Mucoraceae, Mucor, Mucor circinelloides	15	2	2	0	0	0	3	97	33	38	2	0	0	1	0	0	0
Mucorales, Rhizopodaceae, Rhizopus, Rhizopus arrhizus	1	0	0	1	0	0	1	0	0	2	0	0	0	0	0	0	0
Pleosporales, Massarinaceae, Saccharicola	0	8	0	0	0	0	0	0	0	0	0	0	0	0	16	0	0
Pleosporales, Massarinaceae, Stagonospora	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0
Pleosporales, Periconiaceae, Periconia	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pleosporales, Phaeosphaeriaceae, NA	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Pleosporales, Pleosporaceae, Alternaria	687	88	53	59	7	54	467	125	163	375	77	8	362	106	331	5	1
Pleosporales, Pleosporaceae, Bipolaris	10	7	1	4	0	0	6	2	3	17	3	0	12	2	5	0	0

Sorghum grains	CTRL						Inoc1						Inoc2				
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90
Pleosporales, Pleosporaceae, Exserohilum	1	0	1	0	0	0	0	1	0	0	0	0	4	0	1	0	0
Pleosporales, Pleosporaceae, Exserohilum, Exserohilum turcicum	13	7	1	10	3	0	16	8	1	15	1	0	19	4	5	0	0
Pleosporales, Pleosporaceae, NA	4	0	0	3	0	0	2	0	1	2	0	0	0	1	0	0	0
Pleosporales, Sporormiaceae, Preussia	0	0	0	0	0	0	1	0	0	0	0	53	3	0	0	0	0
Pleosporales, unidentified	231	147	10	49	5	12	246	299	693	219	42	48	692	71	59	7	0
Polyporales, Hyphodermataceae, Hyphoderma, Hyphoderma setigerum	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
Russulales, Peniophoraceae, Peniophora	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Russulales, Stereaceae, Amylostereum, Amylostereum chailletii	0	0	0	0	0	0	0	0	0	0	0	46	0	0	0	0	0
Saccharomycetales, Debaryomycetaceae, Debaryomyces,	1	1	0	0	0	0	3	2	2	0	0	103	0	0	0	0	0
Saccharomycetales, NA	22	3	0	2	1	15	3	6	3	15	2	3	7	0	2	0	1
Saccharomycetales, Phaffomycetaceae, Wickerhamomyces, Wickerhamomyces anomalus	42	2038	2684	2627	2438	695	71	1841	1615	1676	2475	819	16	2561	2173	2843	2860

Sorghum grains	CTRL						Inoc1						Inoc2				
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90
Saccharomycetales, Saccharomycetales Incertae sedis, Candida	25	2	0	5	0	832	3	1	4	23	39	398	8	2	1	1	0
Saccharomycetales, Trichomonascaceae, Blastobotrys	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Saccharomycetales, Trichomonascaceae, Zygoascus, Zygoascus hellenicus	0	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
Sporidiobolales, Sporidiobolaceae, Rhodotorula	0	0	0	1	0	0	12	0	2	3	0	0	2	0	0	0	0
Sporidiobolales, Sporidiobolaceae, Rhodotorula, Rhodotorula diobovata	0	0	0	0	0	0	3	1	0	0	0	0	3	0	0	0	0
Sporidiobolales, Sporidiobolaceae, Rhodotorula, Rhodotorula mucilaginosa	0	0	0	0	0	94	10	0	0	0	0	0	1	0	1	0	0
Trechisporales, unidentified	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0
Tremellales, Cryptococcaceae, Kwoniella, Kwoniella heveanensis	1	0	0	0	0	0	4	5	0	0	0	0	0	0	0	0	0
Tremellales, Cryptococcaceae, Kwoniella, Kwoniella mangrovensis	1	2	0	0	0	0	0	4	0	1	0	0	0	0	0	0	0

Sorghum grains	CTRL						Inoc1						Inoc2					
Days	0	3	7	21	90	360	0	3	7	21	90	360	0	3	7	21	90	
Trichosphaeriales, Trichosphaeriaceae, Nigrospora, Nigrospora oryzae	0	0	0	0	0	0	1	0	0	1	2	0	0	0	1	0	0	
Wallemiales, Wallemiaceae, Wallemia, Wallemia ichthyophaga	6	2	0	2	2	0	0	0	3	5	0	0	1	0	0	0	0	
Xylariales, Xylariales Incertae sedis, Phialemoniopsis, Phialemoniopsis curvata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	

CHAPTER 5 - CASE STUDY: EFFECT OF ENSILING ON CORN SILAGE PROCESSING SCORE, FERMENTATION AND LONG-CHAIN FATTY ACIDS PROFILE IN WHOLE-PLANT CORN SILAGE

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Running Head: Ensiling and corn silage processing score

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ABSTRACT

Fat and starch composition vary in whole-plant corn silage with maturity and grain content. In addition, it is well known that prolonged storage time ensiling improves

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fermentation and starch digestibility of whole-plant corn silage. However, studies evaluating the effects of ensiling on corn silage processing score (**CSPS**) and long-chain fatty acid (**LCFA**) profile are needed. Therefore, we aimed to evaluate the effect of ensiling on the fermentation profile, CSPS and long-chain fatty acids (LCFA) profile of whole-plant corn. Eleven corn hybrids were obtained at harvest. Each of the 11 samples was homogenized manually and allocated into 4 samples of approximately 600 g each. Each of the 4 samples was randomly assigned to 1 of 2 treatments (0 or 120 d of ensiling) and vacuum-sealed in nylon-polyethylene standard barrier vacuum pouches. Representative samples from 0 and 120 d were analysed for nutrient, fermentation profile, CSPS, and LCFA profile. Concentration of dry matter was unaffected ($P > 0.10$) by ensiling and averaged 36.2% as fed. Crude fat was 0.42%-units greater ($P = 0.005$) while pH levels were lower ($P = 0.001$) after 120 d compared with 0 d of ensiling. The effects on pH is likely attributed to 7.7%-, 1.0%- and 1.2%-units greater ($P < 0.02$) lactic, acetic and isobutyric acids concentrations, respectively, for 120 d compared with 0 d. Conversely, water soluble carbohydrate concentrations were reduced ($P = 0.001$) by 9.9%-units for 120 d in comparison with 0 d. Concentrations of ammonia-N increased ($P = 0.001$) with ensiling, as expected. Starch concentrations and CSPS was unaffected ($P > 0.10$) by ensiling and averaged 31.2% of DM and 28.8%, respectively. No effects of ensiling were observed on LCFA profile of major FA including C16:0, C18:0, C18:1, C18:2, and C18:3 FA ($P > 0.10$). Ensiling improved fermentation profile. Further research is warranted to elucidate under which conditions ensiling time enhances the CSPS.

Key words: corn silage processing score, ensiling, fermentation profile**,** long-chain fatty acids

INTRODUCTION

Whole-plant corn silage is the main forage source fed to dairy cows in the United States and thus, one of the primary sources of nutrients for milk production. According to Mir (2004) fat and starch composition of whole-plant corn silage vary with maturity and grain content; although most of the energy supplied to ruminants comes from the starch and fiber fractions of silage, its fat content also contributes as an energy source. It is well-established that ensiling and prolonged storage improve fermentation profile and starch digestibility of whole-plant corn silage (Der Bedrosian et al., 2012; Ferraretto et al., 2015a). However, to the best of our knowledge, literature evaluating the effect of ensiling on long-chain fatty acids (**LCFA**) profile and corn silage processing score (**CSPS**) in whole-plant corn silage is scarce;

but preliminary data suggest that CSPS is improved with ensiling (Ferraretto et al., 2015b). Perhaps proteolysis associated with disruption of the starch-protein matrix during ensiling (Hoffman et al., 2011) may dissociate starch granules and thereby reduce mean particle size of kernels.

Even though whole-plant corn silage contains relatively low levels of total fatty acids (**FA**), the presence of 70% of unsaturated FAs (**UFA**) in total FA (Mir, 2004), makes it the major source of UFA in ruminant diets. Fat and FA metabolism and digestion in ruminants are of considerable interest to scientists and the agricultural industry because of the increased use of dietary fat supplements and the specific and compelling effects of FA, from dietary and rumen origin, on ruminant metabolism and human health (Lock et al. 2006). An extensive metabolism of lipid occurs in the rumen and this has a major impact on the profile of FA available for absorption and tissue utilization. Therefore, better understanding of potential changes in the LCFA profile of whole-plant corn silage would aid nutritionists to better formulate and manipulate the FA profile of ruminant diets and thereby improve FA utilization.

Therefore, the objective of this study was to evaluate the effect of ensiling on CSPS, fermentation, and LCFA profile of whole-plant corn silage. We hypothesized that CSPS would increase and the profile of LCFA would change due to the fermentation process.

MATERIALS AND METHODS

Eleven corn hybrids (used as experimental units) were grown on an irrigated field at the Plant Science Research and Education Center (Citra, FL) on plots (1.8 x 6.1 m) under equal fertilizer application, weed control, and tillage management. Whole-plant corn was harvested at targeted DM concentration of 32% using an adapted one-row pull-type forage harvester (New Holland model 707), set with a chop length of approximately 16 mm. Each of the 11 samples was homogenized manually, divided and allocated into 4 samples of approximately 600 g each using a quartering technique. Each of the 4 samples was randomly assigned to 1 of 2 treatments (0 or 120 d of ensiling) and vacuum-sealed in nylon-polyethylene standard barrier vacuum pouches (3.5-mil thickness, 25.4 x 35.6 cm; Doug Care Equipment Inc., Springville, CA) using an external clamp vacuum machine (Bestvac; distributed by Doug Care Equipment Inc., Springville, CA). Mini-silos were stored at room

temperature (approximately 20ºC) in the dark until targeted ensiling period was reached. All samples were frozen for at least 7 d to ensure protocol similarity among all samples.

Mini-silos were thawed overnight in the refrigerator (approximately 4^{o}C), and 4 representative sub-samples from each mini-silo were collected. The first sub-sample was dried in a forced-air oven at 60°C for 48 h to determine DM concentration, and subsequently ground to pass a 1-mm screen using a Wiley mill (Thomas Scientific, Philadelphia, PA) for starch and water-soluble carbohydrates (**WSC**). Starch was analysed following the colorimetric method of Hall (2015), and the anthrone reaction assay was used to quantify WSC (Ministry of Agricultural, Fisheries, and Food, 1986).

A second sub-sample containing 20 g of undried and unground sample was diluted 10-fold (mass basis) in double distilled water, blended for 30 s in a high-speed stomacher (Lab-Blender 400, Tekmar Company, Cincinnati, OH), and filtered through a filter funnel with a 2-mm filter screen. The extract was collected, and pH was immediately measured in duplicate using a pH meter (Orion 710+; Thermo Fisher Scientific Inc., Waltham, MA). Two 20-mL aliquots of extract were separated, stabilized by adding 0.2 mL of 50% sulfuric acid, and centrifuged (7,000 × g) for 15 min at 4°C. The supernatant liquid was frozen (−20°C) for subsequent analysis for organic acid and ammonia-N concentrations. Organic acid concentrations were determined as described by Muck and Dickerson (1988) using high-performance liquid chromatography (**HPLC**; Merck Hitachi Elite La-Chrome; Hitachi L2400, Tokyo, Japan). Briefly, 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) and a flow rate of 0.7 mL/min at 46°C. Ammonia-N was analyzed with a Technicon Auto Analyzer (RFA-300, Alpkem Corporation, Clackamas, OR) adapted from the Noel and Hambleton (1976) method for colorimetric ammonia quantification.

The third sub-sample was dried in a forced-air oven at 60°C for 48 h, but not ground, and analyzed for CSPS (% of starch passing through a 4.75 mm sieve; Ferreira and Mertens 2005). Last, the fourth sub-sample was freeze-dried (FreeZone 6 Liter Console Freeze Dry System with Purge Valve – model 7753022; Labconco, Kansas City, MO) for 36 h, ground to pass a 1-mm screen using a Wiley mill (Thomas Scientific, Philadelphia, PA) and sent to the Agricultural Experimental Station Chemical Laboratories at the University of Missouri, Columbia for total crude fat and LCFA profile analysis. Total crude fat was determined

according to AOCS (2012, official Method Ca 5b-71). Preparation of methyl ester of FA were performed according to AOCS (2012; method Ce 2-66), and LCFA profile analysis was performed according to AOAC International (2012; method 996.06).

Data were analyzed using Proc Glimmix of SAS with the fixed effect of ensiling. Duplicates for each hybrid within an ensiling time were averaged and used for statistical analysis; hybrids were used as experimental units and not as a treatment. Means were determined using the least squares means statement. Statistical significance and trends were declared at $P \leq 0.05$ and $P > 0.05$ to $P \leq 0.10$, respectively.

RESULTS AND DISCUSSION

Effect of ensiling on fermentation profile and CSPS of whole-plant corn forage is in Table 1. Concentrations of DM and starch were unaffected ($P > 0.10$) by ensiling and averaged 36.2% of as fed and 31.2% of DM, respectively. Previous studies have reported a minor increase of 2%-units, on average, in DM concentration over an extended ensiling period (Der Bedrosian et al., 2012; Ferraretto et al., 2015a). Likewise, the literature is consistent with minimal or no changes in starch concentration due to ensiling (Der Bedrosian et al., 2012; Ferraretto et al., 2015a). Contrary to our hypothesis, CSPS was unaffected ($P > 0.10$) by ensiling and averaged 28.8% of starch passing through the 4.75 mm sieve. Lack of an effect on CSPS is in disagreement with our previous findings that observed 10 and 7% greater CSPS on ensiled samples after 30 or 120 d of fermentation, respectively, compared with unfermented samples (Ferraretto et al., 2015b). Interestingly, however, the magnitude of the increase was greater for the study with lower CSPS despite its shorter fermentation period (30 vs. 120 d). Based on our previous findings, we hypothesized that ensiling would enhance CSPS to a greater extent in poorly (< 50% of starch passing through 4.75 mm sieve) compared with adequate or optimally (> 50% or 70% of starch passing through 4.75 mm sieve, respectively) processed silage. Nevertheless, in the present study the whole-plant corn forage was very poorly processed but CSPS was not improved and further research is warranted to elucidate the threshold at which CSPS would improve throughout the fermentation process. Measurements of pH were lower ($P = 0.001$) for 120 d compared with 0 d of ensiling. This is likely related to the 7.7%-, 1.0%- and 1.2%-units greater ($P < 0.02$) lactic, acetic and isobutyric acids concentrations, respectively, for 120 d compared with 0 d. Propionate and butyrate were not detected. Conversely, WSC concentrations were reduced (P

= 0.001) by 9.9%-units for 120 d in comparison with 0 d. Concentrations of ammonia-N increased (P = 0.001) with ensiling, as expected. Ensiling effects on pH, organic acids and ammonia-N were as expected and indicates that the fermentation process was adequate in the current study.

Table 1. Effect of ensiling on nutrient composition, corn silage processing score (CSPS) and fermentation profile in whole-plant corn silage

Item	Ensiling time, d		SEM	P-value
	0	120		
Nutrient				
DM, % as fed	36.6	35.6	0.65	0.29
Starch, % of DM	31.4	31.1	1.56	0.89
Water soluble carbohydrates, % of DM	11.0	1.1	0.5	0.001
CSPS, % starch passing through a 4.75 mm sieve	28.8	28.8	1.37	0.97
Crude fat, % of DM	2.27	2.69	0.09	0.01
Fermentation profile				
pH	5.74	4.00	0.02	0.001
Lactic acid, % of DM	0.03	7.74	0.52	0.001
Acetic acid, % of DM	0.00	1.01	0.07	0.001
Isobutyric acid, % of DM	0.43	1.59	0.30	0.01
Total acids, % of DM	0.47	10.3	0.75	0.001
Ammonia-N, % of DM	0.11	0.57	0.02	0.001

Crude fat was 0.42%-units greater (P = 0.01) for 120 d than 0 d and the response was consistent with findings from previous studies. For example, the meta-analytic review by Glasser et al. (2013) estimated a slight increase in ether extract content (0.26% units) with ensiling on various crops. Similarly, Elgersma et al. (2003) reported greater concentrations of crude fat in perennial ryegrass after fermentation (2.7 vs. 3.9 % DM before and after, respectively) and suggested that during the ensiling process certain fractions, most likely cell wall components, are hydrolyzed. The hydrolysis of cell wall components may release ether-soluble components resulting in greater crude fat concentrations. We speculate similar response on cell wall hydrolysis during fermentation in our study and greater fat concentrations may be attributed to the release of hexane-soluble components. The effect of ensiling on FA profile is in Table 2. According to Kalač and Samková (2010), extensive lipolysis of membrane lipids occurs during ensiling; the extent of this process may be affected by maturity, species and cultivar. During the fermentation, the microbial population may increase the concentration of FAs that are of microbial origin, such as odd- and branched-chain FA (Vlaeminck et al., 2006). Moreover, isomerization and hydrogenation by lactic acid

bacteria may also be observed (Ogawa et al., 2005). Contrary to our hypothesis, the majority of FA were unaffected by ensiling ($P > 0.10$) in our study. Previous studies have observed equivocal responses on FA content and composition of forages in response to ensiling. Alves et al. (2011) reported that ensiling did not affect total FA content of whole-plant corn silage; however, FA composition was affected, mostly by decreasing the proportions of C18:2n-6 and C18:3n-3. Elgersma et al. (2003) concluded that ensiling lowered 33% on average the total FA content with marked difference in the contents of free FA and esterified FA between unfermented and fermented grasses. Furthermore, ensiling also affected the FA composition of the total fat, increasing the proportions of C16:0 and C18:2 and reducing the proportions of C18:1 and C18:3 FA (Elgersma et al., 2003).

Table 2. Effect of ensiling on fatty acids profile in whole-plant corn silage

FA, % of total	Ensiling time, d		SEM	P-value
	0	120		
14:0 (Myristic acid)	0.30	0.31	0.03	0.81
15:0 (Pentadecylic acid)	0.46	0.45	0.04	0.94
16:0 (Palmitic acid)	18.11	17.93	0.44	0.77
16:1n-9 (Palmitoleic acid)	0.17	0.21	0.02	0.09
17:0 (Margaric acid)	0.53	0.45	0.03	0.07
18:0 (Stearic acid)	2.72	2.74	0.08	0.84
18:1n-9 (Oleic acid)	23.31	23.18	1.01	0.93
18:2n-6 (Linoleic acid)	44.09	45.45	1.15	0.41
18:3n-3 (Linolenic acid)	7.68	6.81	0.78	0.44
20:0 (Arachidic acid)	0.94	0.96	0.03	0.05
20:1n-9 (Gondoic acid)	0.37	0.35	0.01	0.07
22:0 (Behenic acid)	0.57	0.55	0.03	0.59
24:0 (Lignoceric acid)	0.76	0.72	0.04	0.49
SFA[1]	24.38	24.00	0.60	0.66
UFA[2]	75.62	76.00	0.61	0.65
MUFA[3]	23.85	23.74	1.01	0.93
PUFA[4]	51.77	52.26	0.79	0.64

[1] SFA- saturated fatty acid; [2] UFA- unsaturated fatty acid; [3] MUFA- monounsaturated fatty acids; [4] PUFA-polyunsaturated fatty acid.

In our study, the concentration of palmitoleic (C16:1n-9) and arachidic (C20:0) acids tended ($P < 0.10$) to increase with ensiling. Conversely, margaric (C17:0) and gondoic (C20:1n-9) acids tended ($P < 0.10$) to be reduced after 120 d compared with 0 d. Overall, FA in fresh forage were predominantly UFA (75.62% of total FA) where 51.8% was polyunsaturated fatty acid (**PUFA**) and 23.9% was monounsaturated fatty acid (**MUFA**). Burr et al. (1932) proposed that UFA are incorporated into cell membranes and functions as precursors for other UFA that are key for metabolic regulation and cell membrane function and are essential to life for all mammals. Among all measured LCFA, linoleic acid (C18:2n-6) was observed in the greatest proportion followed by oleic (C18:1n-9) and palmitic (16:0) acid, averaging 44.8, 23.2, 18.0% of total FA, respectively. These results are in agreement with Mir (2004), who reported that whole-plant corn silage contains greater concentrations of linolenic (C18:3n-3), oleic and linoleic acids. Interestingly, however, concentration of palmitic acid was greater than linolenic acid in our study.

IMPLICATIONS

Ensiling did not affect CSPS. Further research is warranted across a wide range of CSPS values to elucidate under which conditions ensiling and ensiling time enhances this parameter. Fatty acids in whole-plant corn silage were not or minimally affected by ensiling. These findings highlight that UFA content derived from whole-plant corn silage at harvesting would be adequate to formulate diets.

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GENERAL CONCLUSIONS

The addition of P. pentosaceus alone had a positive influence on all evaluated parameters in alfalfa silage, resulting in better silage quality. The inoculation of corn with L. buchneri 56.1 resulted in silage with high concentrations of propionic and acetic acids and low NH_3-N production, and also had higher aerobic stability than non-inoculated silages.

The inoculant containing Lactobacillus plantarum and Propionibacterium acidipropionici was more efficient in promoting a sharply growth of Lactobacillus and maintaining greater stability of the bacterial community during longer periods of storage in silages of both grains and controled the growth of Wickerhamomyces yeast until 90 d of fermentation in rehydrated corn grain silages. Species of Lactobacillus e Weissella were the main bacteria involved in the fermentation of rehydrated silages of corn and sorghum grains and Aspergillus and Wickerhamomyces anomalus were the predominant fungal genera present in rehydrate corn and sorghum grain silages, respectively. The bacterial and fungal communities of rehydrated corn and sorghum grain silages do not remain stabilized after a long storage period.

Ensiling did not or minimally affect fatty acids in whole-plant corn silage and did not affect corn silage processing score.