LEANDRO DIEGO DA SILVA

EVALUATION OF SILAGE CROP AND DIETARY CRUDE PROTEIN LEVELS FOR BEEF CATTLE AND SELECTION OF *Lactobacillus buchneri* FROM CORN SILAGE FOR USE AS INOCULANT

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: July 29, 2016

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DEDICATION

To my parents Geraldo da Silva and Ivanete da Silva; my stepmother Maria da Conceição Gonçalves da Silva; my brothers Ivan Manoel da Silva and Ivo Leandro da Silva, and my beloved fiancee Michele Oliveira Santos. I am grateful to have you in my life.

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I thank God for giving me health, love and peace.

To the Department of Animal Science of the *Universidade Federal de Viçosa* (UFV), *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES), *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* (FAPEMIG), and *Instituto Nacional de Ciência e Tecnologia de Ciência Animal* (INCT-CA) for all financial support.

BIOGRAPHY

Leandro Diego da Silva, son of Geraldo da Silva and Ivanete da Silva, was born in Itu, São Paulo, Brazil, on May 14, 1986. He started the undergrad in Animal Science at *Universidade Federal de Viçosa* in 2006, and obtained a Bachelor of Science degree in Animal Science in 2010.

In August 2010, he started the Master's program, with major in forage conservation at the same University, submitting to the dissertation defense on August 31, 2012.

In September 2012, he started the Doctorate program, continuing work on forage conservation, defending his thesis to obtain the *Doctor Scientiae* degree in Animal Science on July 29, 2016.

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RESUMO

SILVA, Leandro Diego da, D.Sc., Universidade Federal de Viçosa, julho de 2016. **Avaliação de tipos de silagem e níveis de proteína bruta na dieta de bovinos de corte e seleção de *Lactobacillus buchneri* da silagem de milho para uso como inoculante.** Orientador: Odilon Gomes Pereira. Coorientadores: Sebastião de Campos Valadares Filho e Karina Guimarães Ribeiro.

Capítulo 1 - Foram conduzidos dois experimentos para avaliar os efeitos de tipo silagem e níveis de proteína bruta (PB) na dieta de bovinos Nelore. As dietas experimentais foram constituídas por silagem de *Stylosanthes* (SSt) ou silagem de milho (SM) com dois níveis de PB, 110 e 130 g PB/kg de dieta na matéria seca (MS). A relação volumoso: concentrado foi de 50:50 com base na MS. No Experimento 1, foram utilizados quatro bovinos com média de peso corporal inicial de 450 ± 37,9 kg, canulados no rúmen e abomaso. Os animais foram distribuídos em um quadrado latino 4 × 4, em esquema fatorial 2 × 2, com quatro períodos de 16 dias. Neste experimento, foram coletadas amostras de líquido ruminal, digesta abomasal, fezes, urina e sangue. Além disso, foram estimados os parâmetros cinéticos de degradação da MS, PB e fibra em detergente neutro (FDN). No Experimento 2, 40 bovinos com média de peso corporal inicial de 374 ± 16,5 kg, distribuídos em um delineamento inteiramente casualizado, em esquema fatorial 2 × 2, com dez repetições. O experimento teve duração de 99 dias, dividido em um período inicial de 15 dias de adaptação e um período de 84 dias de coleta de dados. No Experimento 2, foram avaliados o consumo, a digestibilidade dos nutrientes e o desempenho dos animais. As frações potencialmente degradáveis da MS, PB e FDN da SSt foram inferiores em relação a SM (P<0,004). As dietas contendo SSt apresentaram menor digestibilidade ruminal da matéria orgânica (MO) e menor digestibilidade total da MO, PB e FDN em comparação com as dietas contendo SM (P<0,050). As dietas contendo SSt apresentaram valores mais elevados de pH ruminal em comparação com dietas contendo SM (P=0,010). A concentração

ruminal de N-NH_3 foi mais elevada para o nível de 130 g PB/kg de dieta, em comparação com 110 g PB/ kg de dieta (P<0,001). Os níveis de PB das dietas afetaram o nitrogênio ureico no sangue (P<0,001) e na urina (P=0,017), e em ambas as dietas contendo SSt e SM foram superiores para o nível de 130 g de PB/kg de dieta. As dietas contendo SSt apresentaram maior consumo de MS, MO, PB, FDN e fibra em detergente neutro indigestível (FDNi) em comparação dietas contendo SM (P<0,008). No entanto, a quantidade de nutrientes digeridos (kg/dia) foi semelhante entre os tratamentos (P>0,050). O tipo de silagem e nível de PB na dieta não apresentaram efeito sobre o desempenho produtivo (P>0,050), porém as dietas contendo SSt tenderam a diminuir a eficiência alimentar (P=0,085). Contudo, a SSt e o nível de 110 g PB/kg de dieta podem ser utilizados para alimentação na terminação de bovinos confinados. **Capítulo 2** - Foram isoladas cento e cinquenta e uma cepas de bactérias do acido lático (BAL) da silagem de planta inteira de milho. As identificações foram baseadas na análise da sequência de rDNA 16S. As espécies predominantes foram *Lactobacillus plantarum* 53,0%, *Pediococcus pentosaceus* 11,9%, *Lactobacillus buchneri* 9,9%, *Lactobacillus pentosus* 5,3%, *Weissella cibaria* 4,6%, e *Lactobacillus brevis* 4,0%. As estirpes de *Lactobacillus buchneri* foram predominantes no dia 56 de ensilagem. Oito estirpes de *L. buchneri* foram pré-selecionadas de um total de 15 estirpes com base na taxa de crescimento e na produção de metabólitos. As estirpes selecionadas foram avaliadas na fermentação e estabilidade aeróbia de silagens de milho e cana-de-açúcar. Não houve melhoria no teor de MS, população de mofo e leveduras, perdas de MS e estabilidade aeróbia da silagem de milho (P>0,050) inoculadas com as estirpes 56.22, 56.27, 56.28, e 56.29. Enquanto as estirpes 56.1, 56.4 e a comercial 40788, quando inoculadas em silagem de cana-de-açúcar, apresentaram os maiores teores de MS e menores de perda de MS em comparação com a silagem contole (P<0,050). A estirpe comercial

apresentou a menor população de mofos e leveduras após 90 dias de ensilagem (*P* = 0,024). No entanto, a estabilidade aeróbia não foi afetada. Após a exposição ao ar, todas as silagens deterioram e apresentaram elevada população de mofos e leveduras. A pré-seleção de estirpes de *L. buchneri* com base na produção de ácido acético apresentou os melhores resultados no processo de ensilagem. Assim, as estirpes 56.1 e 56.4 são promissoras para utilização como inoculante para silagem de cana-de-açúcar.

ABSTRACT

SILVA, Leandro Diego da, D.Sc., Universidade Federal de Viçosa, July, 2016. **Evaluation of silage crop and dietary crude protein levels for beef cattle and selection of *Lactobacillus buchneri* from corn silage for use as inoculant.** Adviser: Odilon Gomes Pereira. Co-advisers: Sebastião de Campos Valadares Filho and Karina Guimarães Ribeiro.

Chapter 1 - Two trials were conducted to evaluate the effects of silage crop and levels of dietary crude protein (CP) in Nellore beef cattle. The experimental diets consisted of *Stylosanthes* (StS) or corn silage (CS) with two levels of dietary CP, 110 and 130 g CP/kg diet on dry matter (DM) basis. The forage to concentrate ratio was 50:50 on a DM basis. In Experiment 1, four bulls were used, with an average initial body weight (BW) of 450 ± 37.9 kg, surgically cannulated in the rumen and abomasum. The bulls were assigned to a 4 × 4 Latin square with a 2 × 2 factorial design of treatments, and four 16-day periods. In this experiment, we collected ruminal fluid, abomasal digesta, feces, urine, and blood. In addition, the kinetic parameters of DM, CP and neutral detergent fiber (NDF) degradation were estimated. In Experiment 2, 40 bulls with an average initial BW of 374 ± 16.5 kg were distributed into a 2 × 2 factorial in a randomized design with ten replicates. The trial lasted 99 days and was divided into a 15-day period for adaptation and an 84-day period for data collection. The intake and digestibility of nutrients, as well as animal performance were evaluated in Experiment 2. The potentially degradable fractions of DM, CP, and NDF of StS were lower than CS (P<0.004). The diets containing StS showed lower ruminal digestibility of organic matter (OM) and lower total digestibility of OM, CP, and NDF than diets containing CS (P<0.050). The StS-based diets showed higher values for ruminal pH in comparison with CS-based diets (P=0.010). The ruminal NH_3-N concentration was higher for the level of 130 g CP/kg diet in comparison with 110 g CP/kg diet (P<0.001). The dietary CP level affected urea nitrogen in blood (P<0.001) and urine (P=0.017), which in the

diets containing StS and CS were higher at the level of 130 g CP/kg diet. The diets containing StS showed higher intake of DM, OM, CP, NDF, and indigestible neutral detergent fiber (iNDF) than diets containing CS (P<0.008). However, the amount of digested nutrients (kg/d) was similar between treatments (P>0.050). The silage crop and dietary CP levels had no effect on productive performance (P>0.050), but the StS-based diet tended to decrease feed efficiency (P=0.085). Thus, StS, as well as the level of 110 g CP/kg diet, can be used to feed finishing beef cattle. **Chapter 2** - One hundred and fifty-one LAB strains were isolated from whole-plant corn silage in tropical weather. Their identifications were based on sequence analysis of 16S rDNA. The predominant species were *Lactobacillus plantarum* 53.0%, *Pediococcus pentosaceus* 11.9%, *Lactobacillus buchneri* 9.9%, *Lactobacillus pentosus* 5.3%, *Weissella cibaria* 4.6%, and *Lactobacillus brevis* 4.0%. *Lactobacillus buchneri* strains were predominant at day 56 of ensiling. Eight *L. buchneri* strains from a total of 15 were preselected, based on growth rate and metabolites production. The strains selected were evaluated on fermentation and aerobic stability of corn and sugarcane silages, four strains in each crop. There was no improvement of the inoculation with the strains 56.22, 56.27, 56.28, and 56.29 on the DM content, yeast and molds population, DM losses, and aerobic stability of corn silage (P > 0.050). Inoculated silages had lower concentration of acetic acid and higher ethanol in comparison with the control silage (P < 0.050). While the strains 56.1, 56.4, and 40788 showed highest DM content, and lowest DM losses when applied in sugarcane silage. At day 90 of ensiling, the commercial strain showed lowest population of yeasts and molds in sugarcane silage. However, the aerobic stability was not affected. After air exposure, all silages deteriorate and had high population of yeast and molds. Preselection of *L. buchneri* strains based on acetic acid production showed

the best results on silage fermentation. Thus, the strains 56.1 and 56.4 are promising for use as an inoculant in sugarcane silages.

GENERAL INTRODUCTION

Grazing is the most common and economical way to feed cattle, however it is cannot be done over the entire year, due the climatic conditions that limit the grasses growth. The availability of pastures in livestock systems depends on seasons, because the factors that affect plant growth (e.g. temperature, luminosity, and rainfall) are different for each season, which leads to periods with high forage production and periods of its shortage. In the winter, for example, there is no forage production enough to feed the animals (Doonan et al., 2004).

The choice of suitable forage conservation process to provide constantly feed, essentially depends of the climatic conditions at harvest. In hot areas with dry seasons, probably the haymaking is the best choice for forage preservation, because it is a simple technology, where the fresh crop is dehydrated after cutting and the material is stable and preserved after reach an adequate moisture content. However, in tropical regions with hot and humid climates, it is difficult to produce high quality hay, due to high humidity and frequent rainfall at the optimum stage of maturity for crop with better nutritional value. In this context, ensiling is an important method of forage preservation because it is not too dependent on weather as the haymaking. In addition, in many parts of world the silage is the major source of energy in the total mixed rations of ruminants (Chiba et al., 2005; Adesogan, 2009).

The knowledge about silage fermentation provides technology improvement to produce high quality silages. In addition, crops that were once considered inappropriate to ensiling, mainly legumes are routinely ensiled in many farms nowadays. Theoretically, all forage crops can be conserved as silage, if the ensiling techniques as the finely chopped, well-packed in the silo and complete sealed through of plastic sheet are done carefully to promote adequate anaerobic conditions. However, the crop

intrinsic characteristics will direct the fermentation pathway and affect the final silage quality.

Inoculants are used with the purpose of increasing the initial population of LAB ensuring efficient fermentation to produce lactic and acetic acids that accelerate the pH reduction (Pahlow et al., 2003). Microbial inoculants include one or more of these bacteria: *Lactobacillus plantarum*, *L. acidophilus*, *L. salivarius*, *L. brevis*, *L. buchneri*, *Pediococcus acidilactici*, *P. pentacaceus*, *Enterococcus faecium*, and *Streptococcus bovis*. Some combinations are used in accordance with the LAB capacity and potential of synergistic actions. However, *Lactobacillus plantarum* is the most common species used (Kung et al., 2003). According to Muck (2010) the inoculant should be added at a rate that is at least 10% of the epiphytic population to fermentation improvement. For commercial inoculants recommendation ranges from 1×10^5 to 1×10^6 colony-forming units (cfu)/g of fresh forage.

However, during the feed-out phase, when opening the silo, the presence of oxygen allows the development of molds, yeasts, and aerobic bacteria that consume the silage nutrients. The length of time that silage remains cool and does not spoil after it is exposed to air is called of aerobic stability. There are chemical and biological additives that are used to improve the aerobic stability by inhibit aerobic spoilage, mainly yeasts and acetic acid bacteria, because these microorganisms are responsible to initiate the aerobic deterioration. Generally, the chemical additives are more expensive and difficult to handle than are biological, and successful treatment depends on application rate (Kung et al., 2003). Biological additives based on heterofermentative LAB, such as *Lactobacillus buchneri*, which anaerobically degrade lactic acid to acetic acid and 1,2-propanediol causing a yeast inhibition (Oude-Elferink, 2000).

Recently, the interest in using legume crops for silage making in livestock systems has increased because it is an important source of protein in animal production, especially *Stylosanthes* because it has adequate characteristics for ensiling and provides satisfactory results regarding animal performance (Heinritz et al., 2012; Souza et al., 2014; Bureenok et al., 2016). However, more studies are needed to evaluate *Stylosanthes* cv. Campo Grande and its interaction with dietary crude protein (CP), and how it affects animal performance. In addition, the quantity and quality of dietary CP are one of the main factors associated with animal performance under tropical conditions, due to the positive relationship between dietary CP and forage intake (Leng, 1990; Detmann et al., 2014). However, the improvement in nutritional status with protein inclusion, mostly with soybean meal in the diet, can increase the production cost, which can reduce the profitability (Valadares Filho et al., 2006).

Based on that the experiments were carried out with the following objectives: 1) Evaluate the effects of the silage crop (*Stylosanthes* spp. and corn) and two levels of dietary crude protein (CP), i.e. 110 and 130 g CP/kg DM, on the intake, ruminal and total digestibility of nutrients, ruminal fermentation, efficiency of nitrogen usage, and performance in beef cattle. 2) Isolate and identify the *L. buchneri* that colonize the tropical corn silage during the fermentation process, and evaluate its effect on silage fermentation.

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CHAPTER 1

Effects of silage crop and dietary crude protein levels on digestibility, ruminal fermentation, nitrogen use efficiency, and performance of finishing beef cattle

Paper published at the Animal Feed Science and Technology

ABSTRACT

Two trials were conducted to evaluate the effects of silage crop and levels of dietary crude protein (CP) in Nellore beef cattle. The experimental diets consisted of *Stylosanthes* (StS) or corn silage (CS) with two levels of dietary CP, 110 and 130 g CP/kg diet on dry matter (DM) basis. The forage to concentrate ratio was 50:50 on a DM basis. In Experiment 1, four bulls were used, with an average initial body weight (BW) of 450 ± 37.9 kg, surgically cannulated in the rumen and abomasum. The bulls were assigned to a 4 × 4 Latin square with a 2 × 2 factorial design of treatments, and four 16-day periods. In this experiment, we collected ruminal fluid, abomasal digesta, feces, urine, and blood. In addition, the kinetic parameters of DM, CP and neutral detergent fiber (NDF) degradation were estimated. In Experiment 2, 40 bulls with an average initial BW of 374 ± 16.5 kg were distributed into a 2 × 2 factorial in a randomized design with ten replicates. The trial lasted 99 days and was divided into a 15-day period for adaptation and an 84-day period for data collection. The intake and digestibility of nutrients, as well as animal performance were evaluated in Experiment 2. The potentially degradable fractions of DM, CP, and NDF of StS were lower than CS (P<0.004). The diets containing StS showed lower ruminal digestibility of organic matter (OM) and lower total digestibility of OM, CP, and NDF than diets containing CS (P<0.050). The StS-based diets showed higher values for ruminal pH in comparison with CS-based diets (P=0.010). The ruminal NH_3-N concentration was higher for the level of 130 g CP/kg diet in comparison with 110 g CP/kg diet (P<0.001). The dietary CP level affected urea nitrogen in blood (P<0.001) and urine (P=0.017), which in the diets containing StS and CS were higher at the level of 130 g CP/kg diet. The diets containing StS showed higher intake of DM, OM, CP, NDF, and indigestible neutral detergent fiber (iNDF) than diets containing CS (P<0.008). However, the amount of

digested nutrients (kg/d) was similar between treatments (*P*>0.050). The silage crop and dietary CP levels had no effect on productive performance (*P*>0.050), but the StS-based diet tended to decrease feed efficiency (*P*=0.085). Thus, StS, as well as the level of 110 g CP/kg diet, can be used to feed finishing beef cattle.

Keywords: feedlot, corn silage, legume silage, *Stylosanthes*

Abbreviations: StS, *Stylosanthes* silage; CS, corn silage; DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; iNDF, indigestible neutral detergent fiber; BW, body weight; N, nitrogen; NH_3−N, ammonia nitrogen

INTRODUCTION

In tropical regions, silage making is a suitable source of high quality forage during the dry season, because of the seasonal variation in forage production through the year. In addition, silage is the main method of forage conservation used in tropical regions because it is difficult to produce a good-quality hay under high relative humidity and frequent rainfall during the harvest period. Furthermore, corn and sorghum silage are the main crops used in feedlot cattle (Adesogan, 2009; Millen et al., 2009).

Recently, the interest in using legume crops for silage making in livestock systems has increased because it is an important source of protein in animal production, especially *Stylosanthes* because it has adequate characteristics for ensiling, and provides satisfactory results regarding animal performance (Heinritz et al., 2012; Souza et al., 2014; Bureenok et al., 2016).

The genus *Stylosanthes* is native from America and most of the species occur naturally in South America. *Stylosanthes* cv. Campo Grande is a mix of two species, *Stylosanthes macrocephala* and *S. capitata*, and has the potential to be adopted in tropical regions because it does not require nitrogen fertilization, and it can be utilized to recover degraded pastures, and to improve animal performance (Valle et al., 2001; Paciullo et al., 2003). In addition, this legume can adapt well to infertile soils, including sandy soils, with an annual dry matter (DM) yield of 8-15 t/ha (Fernandes et al., 2005; Moreira et al., 2015).

However, more studies are needed to evaluate *Stylosanthes* cv. Campo Grande and its interaction with dietary crude protein (CP), and how it affects animal performance. In addition, the quantity and quality of dietary CP are one of the main factors associated with animal performance under tropical conditions, due to the positive relationship between dietary CP and forage intake (Leng, 1990; Detmann et al. 2014). However, the

improvement in nutritional status with protein inclusion, mostly with soybean meal in the diet, can increase the production cost, which can reduce the profitability (Valadares Filho et al., 2006).

Based on that, it was hypothesized that StS can replace corn silage (CS) in diets with a forage to concentrate ratio of 50:50 for feedlot cattle in Brazil. The objective of this study was to evaluate the effects of the silage crop (*Stylosanthes* spp. and corn) and two levels of dietary crude protein (CP), i.e. 110 and 130 g CP/kg DM, on the intake, ruminal and total digestibility of nutrients, ruminal fermentation, efficiency of nitrogen usage, and performance in beef cattle.

MATERIALS AND METHODS

Experimental area and climatic conditions

The experiments were conducted and crops grown at the Experimentation, Research and Extension Center of Triângulo Mineiro ("Central de Experimentacão, Pesquisa e Extensão do Triângulo Mineiro, CEPET") of the Federal University of Vicosa ("Universidade Federal de Viçosa, UFV"), MG, Brazil. This research station is located at an average altitude of 620.2 m, 18.41°S latitude and 49.34°W longitude. The climate is classified by Köppen standards as Aw, i.e., hot and humid, with a rainy season in the summer and a dry season in the winter, and an annual average precipitation of 1,500 mm.

Ensiling of Stylosanthes and corn

The *Stylosanthes* cv. Campo Grande crop was seeded in November 2012 in a 6-ha area by broadcasting seeds at an application rate of 3 kg/ha. At sowing, 100 kg/ha of simple superphosphate [phosphorus pentoxide (P_2O_5) 180 g/kg, calcium (Ca) 160 g/kg, and sulfur (S) 80 g/kg] was applied. Harvesting was performed at flowering,

approximately 150 days after sowing. Harvested material was chopped into a theoretical particle length of 2 cm using a JF-92 Z10 forage harvester (JF Agricultural Machinery, SP, Brazil), and ensiled in three pile silos (35 tons each, approximately) which were opened after 120 days of storage.

Corn hybrid (SHS 4070; Santa Helena, MG, Brazil) was seeded in December 2012 in a 5-ha area using an SHM 11/13 seeder (Semeato, RS, Brazil) at an application rate of 60,000 seeds/ha (80-cm row spacing). At sowing, 300 kg/ha of fertilizer, 8-28-16 (N-P-K), was applied, and a top-dressing of 300 kg/ha of ammonium sulfate was applied 30 days later. Corn plants were harvested approximately 120 days after sowing, when kernels reached the hard dough stage, and was stored in three pile silos (45 ton each, approximately) which were opened after 150 days of storage.

Experimental diets

The experimental diets consisted of StS or CS with two levels of dietary CP, i.e. 110 and 130 g CP/kg DM. The forage to concentrate ratio was 50:50 on a DM basis. The diets were formulated to meet the requirements for a daily gain of 1.00 and 1.50 kg per day according to BR-Corte, the Brazilian system of nutrient requirements for Nellore and crossbred cattle (Valadares Filho et al., 2010). Three concentrates were prepared with 110, 150 and 190 g CP/kg DM. The CS-based diets were formulated with the concentrates containing 150 and 190 g CP/kg DM, and the StS-based diets were with the concentrates containing 110 and 150 g CP/kg DM, to reach the dietary CP levels of 110 and 130 g CP/kg diet, respectively. The chemical composition of the feedstuffs is shown in Table 1. The ingredient proportion and the chemical composition of the diets and concentrates are shown in Table 2.

Experimental design, animal management, and data collection

Two experiments were conducted simultaneously, from August to November of

2013. The management and care of animals was performed in accordance with the guidelines and recommendations of the Committee of Ethics on Animal Studies at the UFV. All animals were treated with ivermectin (Bayer, SP, Brazil) administered by a subcutaneous injection at the rate of 1 mL/50 kg body weight (BW) for the control of parasites. All animals were housed in individual 10-m^2 pens with feeders and water troughs and were fed twice daily at 0800h and 1600h for *ad libitum* intake, allowing for a maximum of 100 g orts/kg diet.

In the first experiment (EXP 1), four Nellore bulls with an average initial BW of 450 ± 37.9 kg were previously surgically cannulated in the rumen and abomasum. The animals were assigned to a 4 × 4 Latin square with balanced single-square design without an extra period (Lucas, 1957), with a 2 × 2 (silages × levels of dietary CP) factorial design of the treatments. The treatments were StS or CS with two levels of dietary CP, i.e. 110 and 130 g CP/kg DM. Each experimental period lasted for 16 d: 12 d for adaptation to the diet and 4 d for data collection.

Daily samples of feed and orts were collected on day 12 to 16 of the experimental periods, placed in labeled plastic bags, and stored in a freezer at -15°C.

To calculate the flow of abomasal nutrients and rumen digestibility coefficient, a double marker system was employed in which cobalt-ethylenediamine tetra-acetic acid (Co-EDTA) was used as the liquid phase and a small particle marker, and indigestible neutral-detergent fiber (iNDF) was used as particulate phase marker. Co-EDTA (6 g/day) was infused in the rumen of each animal starting three days before abomasal digesta sampling (Mariz et al., 2013). The flow of DM and the reconstitution factor were calculated from the concentrations of the markers at different stages of abomasal digesta as described by France and Siddons (1986). Total tract digestibility of nutrients was determined based on feed to feces ratio of iNDF as an internal marker (Schneider

and Flatt, 1975; Huhtanen et al., 1994)

Ruminal fluid, abomasal digesta, feces, and urine were collected every 9 hours during the 13th to the 15th day of each experimental period. The time points of sample collections were: 0000h, 0900h, 1800h, 0300h, 1200h, 2100h, 0600h and 1500h.

At each collection, 500 mL of abomasal digesta was sampled via the abomasal cannula and stored at -15°C. At the end of the experimental period, the samples for the evaluation of ruminal flow were thawed at room temperature and a composite sample was made for each animal, resulting in a 4 L of sample of abomasal digesta. These samples were filtered through a 100-μm nylon filter (Sefar Nitex 100/44, Sefar, Thal, Switzerland), thus obtaining two phases: the phase retained on the filter was called the particulate phase and the filtered portion corresponded to the liquid phase and small particles. Each phase was packed and identified in plastic trays and were dried at 55°C for 72 h in a forced air oven.

Rumen fluid samples were analyzed for pH, ammonia nitrogen (NH_3-N), and volatile fatty acids (VFA). To determine ruminal pH, 50 mL of ruminal fluid was collected, and the pH was immediately measured after collection using a digital pH meter (Tecnal, SP, Brazil). Following the pH measurement, 1 mL of 1:1 H_2SO_4 diluted with distilled water was added to the sample and it was stored in a freezer at -15°C for subsequent analysis of ruminal NH_3-N and VFA.

Fecal grab samples were collected directly from the rectum, stored in plastic bags and refrigerated. At the end of each experimental period, a fresh sample of approximately 350 g per animal was dried at 55°C for 72 h in a forced air oven.

Urine samples were obtained by massaging the external genitalia of the bulls. At the end of each experimental period, 50 mL urine was obtained per animal. The urine was filtered, and 10 mL aliquots were removed and immediately diluted in 40 mL of

0.036 N H_2SO_4 to prevent bacterial destruction of the purine derivatives (PD) and uric acid precipitation. These samples were stored at -15°C for later analysis of urea, creatinine, allantoin, and uric acid. An undiluted urine sample was also stored at -15°C to determine the yield of total nitrogen compounds.

Blood collection was performed (on the 16th day of each experimental period) by puncturing the jugular vein 4 h after the morning feeding, using vacuum tubes containing a coagulation accelerator and separator gel (BD Vacutainer SST II Advance, SP, Brazil). Immediately, the samples were centrifuged and blood serum was stored at -15°C for subsequent determination of urea nitrogen.

In Experiment 2, (EXP 2), 40 Nellore bulls with an average initial BW of 374 ± 16.5 kg were distributed into a 2 × 2 factorial arrangement in a randomized design. The trial lasted 99 days and was divided into a 15-d adaptation period and an 84-d data collection period. Following adaptation (d 15) to the facilities, handling, and diets, and at end (d 99) of the experiment, the bulls were weighed after fasting for 16 h. Four animals were slaughtered at the end of the adaptation period to estimate the initial dressing of all bulls at the beginning of the experiment. They represented the mean of the total group.

During the trial, samples of feeds and orts were collected daily, and a composite sample was made that was packed in labeled plastic bags and stored in a freezer at -15°C. The composite samples were obtained by collecting weekly 30 g of dry sample from each animal. Fecal grab samples (approximately 350 g) were collected between days 42 and 44 at collection intervals of 28 h to determine the total tract digestibility of nutrients, as described for EXP 1.

All bulls from EXP 2 were slaughtered at the end of the experiment to determine the dressing, which was calculated as the proportion between the carcass weight and the

final body weight after fasting. Thus, the carcass average daily gain (CADG) was calculated using the following equation: CADG (kg/day) = {[FBW × (DRSf/1000)] - [IBW × (DRSi/1000)]}/n; where FBW is the final body weight after fasting (kg), IBW is the initial body weight after fasting (kg), DRSf is dressing (g/kg) weight after slaughter at the end of the experiment, DRSi is the dressing weight after slaughter at the end of the adaptation period (beginning of the experimental period), and "n" is the number of evaluated days.

In situ ruminal degradability

Rumen-cannulated bulls from EXP 1 were used to incubate feeds for the determination of *in situ* estimates of digestibility. The bulls were fed StS and concentrate (a diet with 130 g CP/kg DM). The StS and CS samples were dried for 72 hours in the forced air oven at 55°C and ground to a particle size of 2 mm. Samples (5 g) were placed in 10 × 20 cm nylon bags with 50 µm pores, each silage in duplicate, and animal that represented a replicate. The bags were fixed to a steel chain with a weight at the end, allowing sample immersion in the rumen. The incubation times were 0, 2, 4, 8, 16, 24, 48, 72, 96, 120 and 144 hours. A sequential incubation and simultaneous removal scheme was used. Following incubation, the bags were rinsed manually with tap water, after this were placed in a forced air oven at 55°C for 72 h. The concentrations of DM (ID 934.01) and CP (ID 984.13) were measured in the incubation residues, according to AOAC (1990), and NDF was measured according to Mertens (2002).

The kinetic parameters of DM and CP degradation were estimated using the first-order asymptotic model proposed by Ørskov and McDonald (1979), described by the equation: $Yt = a + b \times (1 - e^{-kd \times t})$, where Yt = fraction degraded over time t (g/kg), a = soluble fraction (g/kg), b = potentially degradable fraction (g/kg), kd = degradation

rate (h^{-1}), and t = time, the independent variable (h). NDF degradability was estimated using the exponential decay model, adjusted for the lag time, proposed by Mertens and Loften (1980) described by the equation: $R = B \times e^{-k(t-L)} + U$, where R is residue remaining at time t, B is the digestible fraction, k is the digestion rate constant, L is the discrete lag time, and U is the indigestible fraction.

Laboratory analysis

For the pH and organic acid analysis of silages, water extracts were prepared by adding 25 g of fresh silage to 225 mL of sterile Ringers solution (Oxoid, Basingstoke, UK) in a blender and homogenized at medium setting for 1 min; the pH was measured using a pH meter (Tecnal, SP, Brazil). The water extract was filtered through Whatman 54 filter paper (Whatman, Florham, NJ) and 10 mL was acidified with 1:1 H_2SO_4 diluted with distilled water, and a sample was frozen prior to the analysis of NH_3-N. One milliliter of the acidified extract was mixed with 0.2 mL of 250 g/kg meta-phosphoric acid and centrifuged at 10,000 g × 15 min, for subsequently analyses of lactic acid, acetic acid, propionic acid, and butyric acid by high-performance liquid chromatography (HPLC; SPD-10 AVP, Shimadzu, OR, USA).

Rumen fluid samples were treated with calcium hydroxide [$Ca(OH)_2$] and cupric sulfate ($CuSO_4$) to determine the concentrations of VFA by HPLC (Siegfried et al. 1984). The HPLC (SPD-10 AVP, Shimadzu) apparatus was equipped with a refractive index detector, and used an Aminex HPX-87H column (BIO-RAD, CA, USA) with the mobile phase containing 0.005 M sulfuric acid and a flow rate of 0.6 mL/min at 50ºC. The concentration of NH_3-N was determined using a colorimetric method according to Chaney and Marbach (1962).

The samples of feed, orts, feces, and abomasal digesta were dried at 55°C for 72 h in a forced air oven, ground in a Wiley mill (Wiley mill, Arthur H. Thomas, PA,

USA) with a 1-mm screen, and stored in plastic containers for the determination of DM (method 934.01; AOAC, 1990). Organic matter was determined by ash (OM; method 924.05; AOAC, 1990). Crude protein was calculated by determining total N using the micro-Kjeldhal technique (CP; method 920.87; AOAC, 1990) and using a fixed conversion factor (6.25). The ether extract (EE) was determined gravimetrically after extraction with petroleum ether in a Soxhlet apparatus (method 920.85; AOAC, 1990). The following parameters were also measured: NDF (Mertens, 2002), ADF (method 973.18; AOAC, 1990), and sulfuric acid lignin (Robertson and Van Soest, 1981). The NDF and ADF contents were corrected for ash according to Mertens (2002) and protein was determined according to Licitra et al. (1996). The protein fractionation for feedstuffs was determined according to the method described by Licitra et al. (1996). The samples used for the determination of iNDF were milled to 2 mm. For this, 1.0 g of sample in F57 bags (Ankom®, NY, USA) was previously weighed. The bags were incubated with samples for 288 hours in the rumen of bulls (Huhtanen et al., 1994). Once removed from the rumen, the *in situ* bags were rinsed manually with tap water and dried at 55°C for 48 hours. Then, NDF analysis was performed as described previously.

Non-fibrous carbohydrates (NFC) were calculated as follows according to Detmann and Valadares Filho (2010): NFC (g/kg) = 1000 - [(CP - urea derived CP + urea) + NDF + EE + ash], where CP = crude protein, NDF = neutral detergent fiber and EE = ether extract; hemicellulose = NDF – ADF and cellulose = ADF - lignin residue after treatment with sulfuric acid.

The cobalt analysis was performed by atomic absorption spectroscopy, according to the method described by Williams et al. (1962).

Urine and blood samples were analyzed for urea by the enzymatic-colorimetric method (urea CE; Labtest Diagnóstica, MG, Brazil). Uric acid in the urine was also

determined (uric acid; Labtest Diagnóstica). For the analysis of urinary creatinine, the alkaline picrate method (creatinine; Labtest Diagnóstica) was used. Allantoin was analyzed by a colorimetric method according to Fujihara et al. (1987) as described by Chen and Gomes (1992).

The total daily urine volume was estimated as the ratio between the calculated daily urinary creatinine excretion and urine creatinine concentration. Daily creatinine excretion was calculated using the equation for estimation of urinary creatinine excretion (UCE): UCE (g/day) = 0.0345 × $SBW^{0.9491}$, where SBW = shrunk body weight, which was obtained by Silva et al. (2012) for Nellore cattle. Total urinary nitrogen was determined by the micro-Kjeldhal technique. The daily urinary excretion of nitrogenous compounds was calculated by multiplying the concentration of nitrogen in the samples with the estimated urine volume. Purine derivative (PD; allantoin and uric acid) excretion was calculated by multiplying the urine volume, which was estimated within 24 h, by the PD concentration of the urine samples. Absorbed purines (Y, mmol/day) were calculated from the PD excretion (X, mmol/day) using the equation $Y = [X - (0.30 \times BW^{0.75})]/0.80$, in which 0.80 is the recovery of absorbed purines as PD and $0.30 \times BW^{0.75}$ is the endogenous contribution to purine excretion (Barbosa et al., 2011). Microbial nitrogen synthesis (Nmic) was calculated as: Nmic (g N/day) = 70 × absorbed PD/(0.93 × 1000 × 0.137) where 70 is the N content of purines (mg N/mmol), 0.93 represents the true digestibility of purines, and 0.137 is the average N-purines: N-total ratio in the bacteria that were isolated from the rumen (Barbosa et al., 2011).

Statistical analysis

Marquardt regression was used to fit the models for *in situ* degradability using the NLIN procedure of SAS, version 9.1 (SAS System Inc., Cary, NC, USA). All other data were analyzed using the MIXED procedure of SAS. Homogeneity of variances

between treatments was assumed, and the results were subjected to analysis of variance and means were compared by the F-test. All statistical procedures were conducted using 0.050 as the critical probability level for type I error.

Data from ruminal and total digestibility, ruminal fermentation, and nitrogen efficiency (EXP 1) were analyzed in a 4 × 4 Latin square with a 2 × 2 factorial design of treatments. The following statistical model was used:

$Y_{ijkl} = \mu + \alpha_i + \beta_j + a_k + p_l + (\alpha\beta)_{ij} + \varepsilon_{ijkl}$,

where Y_{ijkl} = dependent variable, μ = overall mean, α_i = fixed effect of the i^{th} silage crop, β_j = fixed effect of the j^{th} level of CP, a_k = random effect of the k^{th} animal, p_l = random effect of the l^{th} period, $(\alpha\beta)_{ij}$ = interaction between silage crop and level of CP, and ε_{ijkl} = random error assuming a normal independent distribution (NID) (0; $\sigma^2\varepsilon$).

Nutrient intake, total digestibility and animal performance from EXP 2 were subjected to analyses of variance in a 2 × 2 factorial arrangement with complete randomized design with ten replicates. The initial BW of bulls was considered to be a covariate, according to the following model:

$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma(X_k - \overline{X}) + \varepsilon_{ijk}$,

where Y_{ijkl} = dependent variable, μ = overall mean, α_i = fixed effect of the i^{th} silage crop, β_j = fixed effect of the j^{th} level of CP, $(\alpha\beta)_{ij}$ = interaction between silage crop and level of CP, γ = regression coefficient or functional relationship with the covariate, X_k = observed value of the covariate applied to the k^{th} experimental unit, $\overline{X}$ = mean value for covariate, and ε_{ijkl} = random error assuming a normal independent distribution (NID) (0; $\sigma^2\varepsilon$).

RESULTS

In situ degradation kinetics (Exp 1)

The soluble (a) and the potentially degradable (b) fractions of DM in StS were lower than in CS (P=0.002; Table 3). However, the DM degradation rate in StS was higher than in CS (P=0.001). StS contained a larger soluble fraction of CP than CS (P=0.031), but the potentially degradable fraction was lower than CS (P=0.001). The rate of CP degradation of StS was higher than CS (P=0.011). The NDF degradable fraction in StS was lower than in CS (P=0.004). The indigestible fraction, the lag time, and the NDF degradation rate were higher in StS than in CS (P<0.027; Table 3).

Ruminal and total digestibility, and ruminal fermentation (EXP 1)

There were no S × CP interactions for ruminal digestibility of the measured nutritional components (P>0.050). The diets containing StS had lower ruminal digestibility of DM, OM and EE than diets containing CS (P<0.044). The ruminal digestibility of CP, NDF and NFC was not affected by S (P>0.050). There was no effect of dietary CP levels on ruminal digestibility (P>0.050). An effect of dietary CP levels was observed only for the total digestibility of CP, which was higher for the diet with 130 g CP/kg DM (P=0.012). The StS-based diets showed lower total digestibility of DM, OM, CP, and NDF than CS-based diets (P<0.005). The digestibility of NFC was not affected by the treatments (P>0.050; Table 4).

Ruminal fermentation was not affected by the interaction S × CP (P>0.050; Table 5). The ruminal pH was affected only by S (P=0.010). The StS-based diets showed higher values for ruminal pH in comparison to CS-based diets. In both silage-based diets, the concentration of ruminal NH_3-N was higher with the level of 130 g CP/kg DM (P<0.001). Rumen VFA concentration was not affected by treatments (P>0.050). The StS-based diets showed higher molar proportions of acetate and lower propionate in comparison to CS-based diets (P<0.001).

Nitrogen use efficiency (EXP 1)

Nitrogen use efficiency was not affected by the interaction S × CP (P>0.050; Table 6). There was effect of S on urinary nitrogen, which was lower for StS-based diets than CS-based diets (P=0.026). The dietary CP levels affected the blood urea nitrogen and urinary urea nitrogen, which in both diets containing StS or CS were higher with 130 g CP/kg DM (P<0.018). The microbial protein supply was not affected by the treatments (P>0.050).

Nutrient intake, total digestibility and animal performance (EXP 2)

There was no effect of the interaction S × CP on the variables of EXP 2 (P>0.050; Table 7). The StS-based diets showed higher intake of DM, OM, CP, NDF, and iNDF than CS-based diets (P<0.008). However, the intake of NFC and total digestible nutrients (TDN) were not affected by the treatments (P>0.050). The intake of CP was lower for the diets containing 110 g CP/kg DM than diets containing 130 g CP/kg DM (P<0.001). When expressed as g/kg of BW, animals fed StS-based diets had higher intake of DM and NDF than those fed CS-based diets (P<0.001; Table 7).

Animals fed StS-based diets had lower total digestibility of DM, OM, CP, and NDF than those fed CS-based diets (P<0.001; Table 7). However, the amounts digested in kg/day were similar among treatments (P>0.050). There was no effect of dietary CP levels on the total digestibility for all nutrients (P>0.050). S and dietary CP had no effect on productive performance parameters (P>0.050). The average daily gain for all treatments was 1.31 kg/day (Table 8).

DISCUSSION

StS had a naturally adequate end fermentation pattern when harvested at flowering, in accordance with others studies on the same cultivar (Souza et al., 2014; Da Silva et al., 2015). In the present study, CS was harvested with high moisture, which

drives the fermentation process to more production of acetic acid, and does not represent typical CS produced under tropical conditions.

In situ degradation kinetics (EXP 1)

The intrinsic characteristics of legumes result in faster degradation rates, even though they have a greater indigestible fraction compared with grasses. The higher DM degradation rate for StS compared with CS can be partially explained by the quantity and arrangement of mesophyll cells. In leaves of legumes the mesophyll cells are loosely arranged that allows for a high degree of colonization by rumen microorganisms. In contrast, the leaves of C4 grasses, like CS, are mostly composed of bundle sheath cells, which are slowly or only partially degraded (Wilson, 1993). However, the greater lignin content of StS compared with CS is one of the major factors that limits degradability (Buxton and O'Kiely, 2003).

Ruminal and total digestibility, and ruminal fermentation (EXP 1)

The *in vivo* digestibility of DM, CP, and NDF followed the same pattern of *in situ* degradation. Although legume leaves have an arrangement of the mesophyll cells that leads to a faster degradation rate, it is not enough to overcome the strong interaction between lignin and hemicellulose in the cell wall that results in lower total digestibility of nutrients. Moreover, the lignin content and consequently the high iNDF content of StS compared with CS are the main reasons for the lower total digestibility of OM, CP, and NDF observed with the StS-based diets. Furthermore, Huhtanen and Khalili (1991) showed a negative relationship between NDF digestibility and the total amount of NDF in the rumen.

In the present study, the StS-based diets showed the ruminal digestibility of CP similar to CS-based diets, despite having a lower total apparent digestibility of CP. This can be explained by the proteolysis that occurs during the fermentation process of StS,

which increases the proportion of non-protein nitrogen, thus increasing rumen nitrogen availability. Souza et al. (2014), in a study that varied the proportions of StS replacing CS for growing beef cattle, observed an increase in the ruminal digestibility of CP by increasing the proportion of StS in the diet, which did not influence the total digestibility. However, the CP indigestible fraction in StS is higher than that in CS, which results in lower total digestibility of CP. In the present study, the highest dietary CP level increased the total digestibility of CP in both StS and CS-based diets, probably due to the addition of a CP source that was high digestible (soybean meal and urea). In addition to the dilution of endogenous nitrogen losses (Swanson, 1977), due to the intake of DM, CP and NDF were not affected (EXP 1, data not shown).

When the dietary content of EE is low, ruminal digestibility of EE may result in a negative coefficient (Mariz et al., 2013). This pattern can occur due to the synthesis of fatty acids by rumen microbial fermentation and microbial *de-novo* lipogenesis (Vlaeminck et al., 2006).

The high ruminal pH of StS-based diets could be attributed to a buffering effect in the rumen due to the high buffering capacity observed in legume silage (Heinritz et al., 2011). However, the range of the ruminal pH in all experimental diets was similar and did not compromise rumen fermentation (Van Soest, 1994; Hoover, 1996).

The concentration of ruminal NH_3-N was not affected by the silage crop, even though the StS showed higher concentrations of NH_3-N and soluble protein than CS. This may have occurred due to equilibrium between the release of NH_3-N in the rumen by microorganism fermentation, absorption via the rumen epithelium, and the liquid passage rate. The effect of dietary CP levels on ruminal NH_3-N was expected due the concentration of ruminal NH_3-N, which showed exponential growth with the dietary CP content (Detmann et al., 2014). An interesting result was observed for dietary CP levels,

because in spite of the 20 g CP/kg DM difference, the 110 g CP/kg diet resulted in a concentration of ruminal NH_3-N that could be considered below the minimum recommended for fiber degradation under tropical conditions (Detmann et al., 2009). However, in our study, the dietary level of 130 g CP/kg DM did not increase NDF digestibility, but showed a trend toward increasing the NDF digestibility of diets containing StS. In addition, Detmann et al. (2009) indicate that a decrease in the assimilation of NH_3-N by rumen microorganisms occurs at dietary CP levels higher than 109 g CP/kg DM.

The same level of rumen available energy could probably explain the similarity of rumen VFA concentration among treatments. However, StS-based diets increased the proportion of acetate and decreased propionate in comparison to CS-based diets, because the pattern of rumen VFA production depends on the diet composition (Bergman, 1990). In this context, diets with large amounts of non-fiber carbohydrates results in fermentation to high propionate proportion, whereas diets containing high fiber content leads to high acetate production, which explains the results observed in this study for CS and StS, respectively (Vlaeminck et al., 2006).

Nitrogen use efficiency (EXP 1)

The StS-based diets had a greater quantity of metabolizable protein than the CS-based diets; based on that the amounts of N-intake and N-feces were similar and the excretion of N-urine was lower for the diets containing StS. However, there seemed to be an equilibrium, and the nitrogen balance was not influenced by treatments. If the availability of ruminal NH_3-N exceeds its utilization by microorganisms, an increase in urinary urea occurs (Russell et al., 1992). However, to achieve a continuous nitrogen supply to the gastrointestinal tract for microbial growth, the animal is able to regulate the amount of urinary nitrogen and nitrogen that is recycled to the rumen (Van Soest,

1994; Detmann et al., 2014). The urinary urea-N followed the same pattern as urea-N in blood, confirming the assertion by Harmeyer and Martens (1980) that the amount of urinary urea-N is mainly influenced by its concentration in the blood.

In our study, reducing the level from 130 to 110 g CP/kg diet did not decrease nitrogen use efficiency. In addition, Galles et al. (2011) studying the reduction of dietary CP from 135 to 116 g CP/kg diet in cattle, observed a reduction in excreted nitrogen, particularly N-urinary, without affecting retention, and concluded that eliminating the dietary N-content that exceeds the requirements of finishing beef cattle provides an opportunity to reduce overall nitrogen losses to the atmosphere.

The microbial nitrogen supply obtained from the experimental diets can be considered adequate. The similarity in microbial nitrogen supply among the diets, also observed by Da Silva et al. (2015) with sheep, indicates that StS can replace CS in diets without compromising the growth of ruminal microorganisms.

Nutrient intake, total digestibility and performance (EXP 2)

Animals fed a diet containing StS increased their intake of nutrients to meet their nutritional requirements, especially for energy, because of the low digestibility of StS. This may explain why the StS diets had higher OM intake than CS, but because of the low digestibility, this resulted in similar amounts of digested nutrients between the diets (Table 7). Based on that, we can affirm that there was no limitation in intake because of a possible rumen filling effect due to the high content of iNDF observed in StS. Although StS has high levels of lignin, in diets with 500 g concentrate/kg DM, the ruminal filling effect was not observed. In addition, legume particles are generally more susceptible to digestion and have a lower ruminal retention time in comparison with grasses (Waghorn et al., 1989).

It is likely that the lack of an effect on the intake of NFC and TDN were due to the low concentration of these fractions in StS, which were consistently lower than in CS, as observed in previous studies (Souza et al., 2014; Da Silva et al., 2015).

The dietary CP levels affected only the CP intake and did not affect the intake of other nutrients, which was expected. According to Detmann et al. (2014) the improvement in animal performance, in relation to diets with nitrogen deficiency, occurs when the increase in dietary CP content positively affects the nutrient intake. In our study, the lack of an effect on TDN intake and performance supports the idea that a diet containing 110 g CP/kg DM is enough to meet the CP requirement for similar weight gain with a diet containing 130 g CP/kg DM. In addition, the classic recommendations for the protein requirements of finishing beef cattle are between 120-140 g CP/kg DM (Galyean, 1996; Bailey and Duff, 2005). However, some studies conducted in Brazil with finishing feedlot cattle have shown that the productive performance is not reduced with the use of 110 g CP/kg diet (Cavalcante et al., 2005; Amaral et al., 2014). Obeid et al. (2006) used CS and 400 g concentrate/kg DM with four dietary CP levels and concluded that a level of 90 g CP/kg diet resulted in lower ADG (1.20 kg/day), while the levels 110, 130, and 150 g CP/kg diet were similar (1.40 kg/day).

In the present study, the absence of an effect on animal performance was probably due to the similar TDN intake between treatments. However, StS-based diets trended to decrease feed efficiency due the low total digestibility and high intake of nutrients in comparison with CS-based diets. Importantly, the use of StS in a 110 g CP/kg diet caused a reduction in the amount of soybean meal in the diet and would probably reduce the cost of feed with concentrate.

CONCLUSIONS

StS and a level of 110 g CP/kg diet can be used to feed finishing feedlot beef cattle, with satisfactory productive performance without compromising rumen fermentation and nitrogen use efficiency. Although the StS-based diet tended to decrease feed efficiency, StS is a suitable source of forage in tropical regions. Its production costs need to be evaluated in further studies.

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Table 1
Chemical composition of the feedstuffs

	Stylosanthes silage	Corn silage	Ground corn	Wheat meal	Soybean meal
Chemical composition, g/kg of dry matter					
Dry matter, g/kg	304	276	909	911	925
Organic matter	879	956	986	949	932
Crude protein	117	75.8	82.7	184	509
Neutral detergent fiber	577	538	93.1	351	123
Acid detergent fiber	342	271	23.6	100	77.0
iNDF[a]	346	193	6.47	73.7	5.08
Lignin	93.8	38.8	3.97	32.2	3.22
Ether extract	11.6	16.7	41.8	28.1	17.3
Non fiber carbohydrates	172	325	768	385	283
Cellulose	224	230	19.3	73.3	75.9
Hemicellulose	234	267	69.4	251	46.1
pH	4.22	3.76			
Protein fractionation[b], g/kg of total nitrogen					
Fraction A	341	218	100	119	42.7
Ammonia	111	46.4			
Fraction B_1	235	258	234	294	150
Fraction B_2	179	358	585	475	763
Fraction B_3	142	126	70.1	95.3	31.1
Fraction C	100	37.6	10.2	14.0	12.4
Organic acids, g/kg of dry matter					
Lactic acid	45.6	86.1			
Acetic acid	43.6	73.4			
Propionic acid	4.76	5.76			
Butyric acid	0.372	0.352			

Values are means of five samples.
[a] Indigestible neutral detergent fiber.
[b] A: nonprotein nitrogen, B_1, B_2 and B_3: true protein with fast, variable and slow degradation, respectively, C: indigestible.

Table 2
Ingredients proportion and the chemical composition (g/kg of dry matter)

Items	Treatments[a]			
	CS-110	CS-130	StS-110	StS-130
Ingredients proportion				
Stylosanthes silage	-	-	500	500
Corn silage	500	500	-	-
Ground corn	421	421	421	421
Wheat meal	30.0	-	60.0	30.0
Soybean meal/Urea[b]	38.0	68.0	8.00	38.0
Mineral Mix[c]	11.0	11.0	11.0	11.0
Chemical composition of the diets				
Dry matter, g/kg	583	584	596	597
Organic matter	951	946	917	912
Crude protein	112	132	111	131
Neutral detergent fiber	323	315	349	342
Acid detergent fiber	151	150	187	186
iNDF[d]	101	99.5	180	178
Ether extract	27.3	26.9	25.2	24.8
Non fiber carbohydrates	488	471	431	413
Total digestible nutrients	687	674	616	636
Chemical composition of the concentrates				
Dry matter, g/kg	890	891	889	890
Organic matter	945	935	956	945
Crude protein	145	185	105	145
Neutral detergent fiber	107	92.2	122	107
Acid detergent fiber	30.8	26.6	33.0	30.8
iNDF[d]	10.1	6.01	14.3	10.1
Ether extract	38.0	37.1	38.8	38.0
Non fiber carbohydrates	687	678	696	687

[a] CS-110: diet containing corn silage and 110 g CP/kg (CP), CS-130: corn silage and 130 g CP/kg, StS-110: *Stylosanthes* silage and 110 g CP/kg, StS-130: *Stylosanthes* silage and 130 g CP/kg.
[b] Soybean meal 830 g/kg with urea 170 g/kg and the proportion was 9 parts of urea and 1 part of ammonium sulphate.
[c] Calcium 150 ng/kg, cobalt 671 mg/kg, copper 979 mg/kg, phosphorus 90.0 g/kg, iodine 685 g/kg, manganese 944 mg/kg, magnesium 11.0 g/kg, sulfur 160 g/kg, fluoride 710 mg/kg, selenium 23.3 mg/kg, sodium 160 g/kg and zinc 3.60 g/kg.
[d] Indigestible neutral detergent fiber.

Table 3
Effect of silage crop on *in situ* degradation kinetics

Parameters[a]	Silage crop		SEM[b]	*P*-value
	Stylosanthes	Corn		
Dry matter				
a, g/kg	119	167	1.41	0.002
b, g/kg	336	565	6.64	0.002
k, g/kg h^{-1}	0.356	0.129	0.065	0.001
Crude protein				
a, g/kg	351	312	1.67	0.031
b, g/kg	221	434	10.4	0.001
k, g/kg h^{-1}	0.349	0.294	0.067	0.011
Neutral detergent fiber				
B, g/kg	402	708	8.87	0.004
U, g/kg	548	268	8.10	0.005
L, h	2.91	1.07	0.55	0.027
k, g/kg h^{-1}	0.284	0.102	0.054	0.021

[a] a: soluble fraction, b: potentially degradable fraction, k: degradation rate, B: digestible fraction, L: lag time, U: indigestible fraction.
[b] SEM: standard error of means.

Table 4
Effects of silage crop and dietary crude protein levels (110 and 130 g CP/kg DM) on ruminal and total apparent digestibility of nutrients in Nellore bulls (EXP 1)

Items[a]	*Stylosanthes* silage		Corn silage		SEM[b]	*P*-value[c]		
	110	130	110	130		S	CP	S × CP
Ruminal[d], g/kg DM								
DM	384	398	520	490	20.2	<0.001	0.610	0.206
OM	481	519	570	557	21.5	0.044	0.619	0.348
CP	366	498	461	529	40.7	0.351	0.158	0.621
EE	17.5	-188	77.4	262	74.9	0.017	0.898	0.052
NDF	408	488	526	514	21.8	0.080	0.357	0.229
NFC	590	613	705	651	42.5	0.147	0.753	0.431
Total[e], g/kg DM								
DM	603	650	695	705	13.7	0.001	0.071	0.225
OM	646	678	721	727	13.5	0.005	0.302	0.481
CP	593	693	721	750	18.4	0.002	0.012	0.116
EE	630	692	697	696	19.3	0.175	0.237	0.225
NDF	456	521	606	597	18.7	0.001	0.194	0.098
NFC	808	798	804	820	11.8	0.655	0.865	0.538

[a] DM: dry matter, OM: organic matter, CP: crude protein, EE: ether extract, NDF: neutral detergent fiber, NFC: non-fiber carbohydrates.
[b] SEM: standard error of means.
[c] Probability of a silage (S), crude protein (CP), and interaction S with CP (S × CP) effects.
[d] Ruminal apparent digestibility (g/kg): (nutrient intake – nutrient abomasal flow)/nutrient intake) × 1000.
[e] Total apparent digestibility (g/kg): (nutrient intake – nutrient excretion)/nutrient intake) × 1000.

Table 5
Effects of silage crop and dietary crude protein levels (110 and 130 g CP/kg DM) on ruminal fermentation in Nellore bulls (EXP 1)

Items[a]	*Stylosanthes* silage		Corn silage		SEM[b]	*P*-value[c]		
	110	130	110	130		S	CP	S × CP
pH	6.64	6.71	6.49	6.51	0.039	0.010	0.486	0.686
NH_3−N	6.09	10.3	6.50	10.6	0.439	0.662	<0.001	0.942
VFA	65.0	72.6	58.0	61.9	2.90	0.124	0.293	0.727
Volatile fatty acids, molar proportions								
Acetate	0.688	0.704	0.660	0.653	0.006	0.001	0.552	0.162
Propionate	0.158	0.156	0.198	0.205	0.007	<0.001	0.718	0.496
Butyrate	0.154	0.140	0.142	0.142	0.005	0.604	0.454	0.415
Ace/Pro	4.39	4.54	3.36	3.22	0.178	<0.001	0.993	0.317

[a] NH_3−N: Ammonia nitrogen (mg/dL), VFA: Total volatile fatty acids (mM), Ace/Pro: acetate to propionate ratio.
[b] SEM: standard error of means.
[c] Probability of a silage (S), crude protein (CP), and interaction S with CP (S × CP) effects.

Table 6
Effects of silage crop and dietary crude protein levels (110 and 130 g CP/kg DM) on efficiency of nitrogen usage in Nellore bulls (EXP 1)

Items[a]	*Stylosanthes* silage		Corn silage		SEM[b]	*P*-value[c]		
	110	130	110	130		S	CP	S × CP
Nitrogen balance, g								
N-intake	140	146	160	172	11.1	0.159	0.527	0.832
N-feces	59.8	50.7	48.6	47.7	4.56	0.331	0.487	0.565
N-urine	65.3	82.5	88.5	102	9.13	0.026	0.077	0.831
NB	15.1	13.3	22.4	21.8	3.61	0.086	0.760	0.880
UUN	65.3	82.5	88.5	102	5.97	0.434	0.018	0.876
Blood serum, mg/dL								
BUN	9.55	15.3	9.79	14.9	0.782	0.928	0.002	0.663
Microbial protein supply								
Nmic	70.7	58.6	90.2	75.8	5.35	0.067	0.172	0.905
Emic	95.0	87.2	89.8	84.3	4.35	0.648	0.474	0.899

[a] N: nitrogen, NB: nitrogen balance, BUN: blood urea nitrogen, UUN: urinary urea nitrogen, Nmic: microbial nitrogen synthesis (g N/d), Emic: microbial synthesis efficiency (g CPmic/kg TDN).
[b] SEM: standard error of means.
[c] Probability of a silage (S), crude protein (CP), and interaction S with CP (S × CP) effects.

Table 7
Effects of silage crop and dietary crude protein levels (110 and 130 g CP/kg DM) on nutrient intake and total apparent digestibility of finishing Nellore bulls (EXP 2).

Items[a]	*Stylosanthes* silage		Corn silage		SEM[b]	*P*-value[c]		
	110	130	110	130		S	CP	S × CP
Intake, kg/day								
DM	10.36	10.33	9.14	9.55	0.144	<0.001	0.416	0.345
OM	9.61	9.50	8.79	9.11	0.121	0.008	0.622	0.333
CP	1.18	1.36	1.02	1.24	0.024	<0.001	<0.001	0.521
NDF	3.28	3.34	2.92	2.99	0.051	<0.001	0.439	0.904
iNDF	1.54	1.59	0.88	0.93	0.056	<0.001	0.175	0.949
NFC	4.88	4.59	4.66	4.71	0.060	0.665	0.306	0.140
TDN	6.02	6.03	6.10	6.24	0.078	0.341	0.619	0.699
Intake, g/kg body weigth								
DM	24.2	23.9	21.4	22.3	0.286	<0.001	0.576	0.235
NDF	7.66	7.72	6.85	6.98	0.106	<0.001	0.606	0.852
Total digestibility[d], g/kg								
DM	609	610	667	654	7.38	<0.001	0.657	0.565
OM	625	629	690	677	7.71	<0.001	0.729	0.499
CP	660	663	751	755	10.7	<0.001	0.809	0.990
NDF	481	484	598	554	10.1	<0.001	0.126	0.079
Quantity digested[e], kg/day								
DM	6.17	6.46	6.28	6.01	0.081	0.256	0.985	0.067
OM	6.02	6.28	6.04	5.86	0.077	0.180	0.786	0.150
CP	0.821	0.896	0.837	0.825	0.017	0.396	0.331	0.183
NDF	1.67	1.72	1.58	1.63	0.030	0.155	0.371	0.955

[a] DM: dry matter, OM: organic matter, CP: crude protein, NDF: neutral detergent fiber, iNDF: indigestible neutral detergent fiber, NFC: non-fiber carbohydrates, TDN: total digestible nutrients.
[b] SEM: standard error of means.
[c] Probability of a silage (S), crude protein (CP), and interaction S with CP (S × CP) effects.
[d] Total apparent digestibility = (nutrient intake – nutrient excretion)/nutrient intake) × 1000.
[e] Quantity digested = (nutrient intake × nutrient digestibility)/1000.

Table 8
Effects of silage crop and dietary crude protein levels (110 and 130 g CP/kg DM) on the productive performance of finishing Nellore bulls (EXP 2)

Items[a]	*Stylosanthes* silage		Corn silage		SEM[b]	*P*-value[c]		
	110	130	110	130		S	CP	S × CP
FE	123	135	138	141	3.24	0.086	0.213	0.463
STF	2.20	2.20	2.40	2.30	0.071	0.317	0.738	0.739
ADG	1.29	1.40	1.23	1.31	0.034	0.303	0.175	0.802
CADG	0.777	0.824	0.762	0.786	0.029	0.640	0.535	0.841
Dressing	542	539	543	540	2.90	0.886	0.634	0.998

[a] FE: feed efficiency (g gain/kg DM intake), SFT: subcutaneous fat thickness - slaughterhouse JBS® (mm), ADG: average daily gain (kg/day), CADG: carcass average daily gain (kg/day), Dressing (g/kg).
[b] SEM: standard error of means.
[c] Probability of a silage (S), crude protein (CP), and interaction S with CP (S × CP) effects.

CHAPTER 2

Effect of applying *Lactobacillus buchneri* isolated from tropical corn silage on fermentation and aerobic stability of corn and sugarcane silages

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ABSTRACT

One hundred and fifty-one LAB strains were isolated from whole-plant corn silage in tropical weather. Their identifications were based on sequence analysis of 16S rDNA. The predominant species were *Lactobacillus plantarum* 53.0%, *Pediococcus pentosaceus* 11.9%, *Lactobacillus buchneri* 9.9%, *Lactobacillus pentosus* 5.3%, *Weissella cibaria* 4.6%, and *Lactobacillus brevis* 4.0%. *Lactobacillus buchneri* strains were predominant at day 56 of ensiling. Eight *L. buchneri* strains from a total of 15 were preselected, based on growth rate and metabolites production. The strains selected were evaluated on fermentation and aerobic stability of corn and sugarcane silages, four strains in each crop. There was no improvement of the inoculation with the strains 56.22, 56.27, 56.28, and 56.29 on the DM content, yeast and molds population, DM losses, and aerobic stability of corn silage ($P > 0.050$). Inoculated silages had lower concentration of acetic acid and higher ethanol in comparison with the control silage ($P < 0.050$). While the strains 56.1, 56.4, and 40788 showed highest DM content, and lowest DM losses when applied in sugarcane silage. At day 90 of ensiling, the commercial strain showed lowest population of yeasts and molds in sugarcane silage. However, the aerobic stability was not affected. After air exposure, all silages deteriorate and had high population of yeast and molds. Preselection of *L. buchneri* strains based on acetic acid production showed the best results on silage fermentation. Thus, the strains 56.1 and 56.4 are promising for use as an inoculant in sugarcane silages.

INTRODUCTION

Climate in tropical countries generally is warm and wet, which is stimulatory to crop's growth. In addition, ensiling is the main method to forage preservation, once there are many difficulties for hay production, which would be another option, due to high humidity and frequent rainfall (Bernardes and Rêgo, 2014). However, the weather also stimulates microbial growth and increases fermentation losses by aerobic spoilage of silage (Ashbell et al., 2002). Furthermore, crops containing high concentration of starch or sugars, such as corn and sugarcane respectively, probably will have more yeasts due to the large amount of available substrate.

Although the whole-plant corn is the most widely used crop as silage in the word, studies about microbial diversity of this silage in tropical climate are rare. In addition, corn has adequate characteristics for good fermentation in a silo, but severe losses can occurs when the silo is opened (Santos et al., 2015). Also, sugarcane has high sugar content and low buffering capacity, which favor lactic acid production and fast pH drop, but normally had high yeast population that leads to intense alcoholic fermentation and excessive dry matter loss during ensilage (Pedroso et al., 2011). Thus, these recent studies have shown that the application of *L. buchneri* can increase the aerobic stability of corn silage and reduce losses of sugarcane silage.

The *Lactobacillus buchneri*, an obligate heterolactic acid bacterium, improves aerobic stability by increasing acetic acid concentration and decreasing molds and yeasts of silages (Driehuis et al., 1999). In addition, the microbiological additive can be more economical than chemical preservatives, mainly depending on moisture, application rate, silo size, climate and packing density (Kung, 2010). Furthermore, a novel microbial additive adapted to our condition could be used to improve silage fermentation more efficiently (Santos et al., 2015).

Based on that, it was hypothesized that a strain of *L. buchneri* isolated from corn silage can improve the fermentation pattern of corn and sugarcane silages in tropical conditions. The objective of this study were to isolate and identify the *L.buchneri* that colonize tropical corn silage during fermentation process and screening of strains for use as silage inoculant in corn and sugarcane to determine its effect on silage fermentation and aerobic stability.

MATERIALS AND METHODS

Experimental area and climatic conditions

The experiments were conducted and crops grown at the Department of Animal Science of the Federal University of Vicosa (Universidade Federal de Viçosa - UFV, Viçosa, Minas Gerais, Brazil) between January and March 2014. Vicosa is located at 20°45’ South latitude, 42°51’West longitude and 657 meters above sea level with a mean annual rainfall of 1341 mm. The climate is classified by Köppen standards as Cwb, i.e., winters are dry, and summers can be rainy.

Tropical corn silage characterization and LAB identification

Corn (*Zea mays*) plants were harvested when kernels reached the one-third milk-line stage. Harvested material was chopped at 2.5 cm length by using a JF-92 Z10 forage harvester (JF Agricultural Machinery, SP, Brazil). Approximately 500 g of chopped material was immediately conditioned in nylon-polyethylene bags (25 × 35 cm; Doug Care Equipment Inc., Springville, CA) to produce mini-silos, and the air was evacuated from the bags using a vacuum sealer (Eco vacuum 1040, Orved, Italy). The mini-silos were stored at room temperature and opened after 0, 1, 3, 7, 14, 28, and 56 d of ensiling. Three replicates were prepared for each sampling date. In all opening days, the fresh samples were destined for dry matter (DM) determination of (at 105^{o}C for 18

h), and the water extract were prepared for chemical analysis and identification of lactic-acid bacteria (LAB).

The water extracts from the silage and fresh forage samples were prepared by homogenizing 25 g of sample in 225 mL of sterile Ringer's solution (Oxoid, Hampshire, England) in an industrial blender for 1 min, after this was divided into two portions.

One portion was subjected to serial dilutions ranging from 10^{-1} to 10^{-10} for microbial analysis (Table 1). Pour plates were prepared with MRS (Difco, São Paulo, Brazil) agar for LAB, and Potato Dextrose Agar (PDA; Difco, Sao Paulo, Brazil) containing 1.5% of tartaric acid solution (10% w. / v.) for yeasts and molds (Y&M). The MRS plates were incubated at 37°C for 48 h in the anaerobic jars (Permution®, Curitiba, PR, Brazil). The PDA plates were incubated aerobically at 25°C for 5 d. All colonies were counted on plates with 25 to 250 well-isolated colony-forming units.

For the identification of LAB species were isolates at random a number of colonies corresponding to the square root of the total contained on the MRS agar plates (Holt et al., 1994). The isolates were further purified by streaking individual colonies onto MRS agar containing bromocresol purple and $CaCO_3$ as indicators. All LAB were detected by a yellowish colony and a clear zone caused by the dissolution of $CaCO_3$. Pre-selected cells grown in 5 mL of MRS broth at 37°C for 18h were used for 16S rDNA gene sequence. First, the DNA was extracted using a commercial kit (Wizard® Genomic DNA Purification kit, Promega, Madison, WI, USA) with the following modifications. The samples were centrifuged (Mikro 200 R, Sigma-Aldrich, São Paulo, Brazil) at 10,000 g × 5 min and washed with saline solution 0.85%. The cells were resuspended with 480 μL of EDTA (50 mM) and immediately added to 50 μL of lysozyme at 50 $mg.mL^{-1}$. The concentration of extracted DNA was evaluated in a Nanodrop spectrophotometer (Thermo Scientific 2000, Waltham, MA, USA) and

stocked at – 20°C. The 16S rDNA gene sequence coding region was amplified by PCR in a PCR thermal cycler (Eppendorff®, Hamburg, Germany). The sequences of the PCR products were determined directly with a sequencing kit using the prokaryotic 16S ribosomal DNA universal primers P027F (GAGAGTTTGATCCTGGCTCAG) and 1492R (TACGG(C/T)TACCTTGTTACGACTT) (Heuer et al., 1997). The PCR reaction was performed in microfuge tubes containing 50 µL of the reaction mixture: DNA (approximately 60 ng); Reaction buffer 10X (Tris-HCl 0.1 mol l^{-1}, pH 8.0, KCl 0.5 mol l^{-1}); $MgCl_2$ (1.5 mmol l^{-1}, pH8.0); dNTP mix (Promega, Madison WI USA); GoTaq® DNA Polymerase (Promega); Primer P027F (0.6 µmol l^{-1}), 1492R (0.6 µmol l^{-1}) and autoclaved milli-Q water. The reaction conditions used were: 94°C × 5 min; 30 cycles (denaturation: 94°C × 30 seconds; 60°C × 30 seconds); polymerization: 72°C × 2 min; final extension: 72°C × 5 min. The PCR reaction mixture was checked by run on agarose gel (1.4%) electrophoresis with TRIS borate – EDTA buffer (Thermo Scientific). The gel was stained with 0.5 mg mL^{-1} ethidium bromide and bands were visualized on UV light. The PCR product was sent to the Macrogen© (Seoul, Korea) for purification and sequencing. Sequence similarity searches were performed using the DNA database of GenBank, and the Basic Local Alignment Search Tool for nucleotide (http://www.ncbi.nlm.nih.gov/BLAST). The 16S rRNA gene sequences that show similarity greater than 97% were considered as belonging to the same Operational Taxonomic Unit (Altschul et al., 1990).

In another water-extract portion, the pH was measured using a potentiometer. After this, the water extract was filtered through Whatman 54 filter paper (Whatman, Florham, NJ), and 10 mL was acidified with 1:1 H_2SO_4 diluted with distilled water for the chemical analysis (Table 1). The water-soluble carbohydrates (WSC) were quantified by a colorimetric procedure (Nelson, 1944). One milliliter of the extract

acidified was centrifuged at 10,000 g × 15 min and subsequently analyzed for lactic acid, acetic acid, propionic acid, butyric acid, and ethanol by high-performance liquid chromatography (HPLC; SPD-10 AVP, Shimadzu, OR, USA). The HPLC apparatus was equipped with a refractive index detector, and used an Aminex HPX-87H column (BIO-RAD, CA, USA) with the mobile phase containing 0.005 M sulfuric acid, and a flow rate of 0.6 mL/min for organic acids and of 1.0 mL/min for ethanol, at 50ºC.

Characterization and preselection of *L. buchneri* strains

Among 151 LAB isolated, 15 were identified as *L. buchneri* (Table 2). Physiological and biochemical tests were utilized for *L. buchneri* characterization and preselection (Table 3 and Fig. 1). First, all isolated *L. buchneri* strains were cultivated in MRS broth for 16 h at 37°C. After this period, the inoculum was standardized using a spectrophotometer (630 nm) at an optical density of 0.05, into 10 ml of MRS broth, which was incubated at 37°C with two replicates. The rate growth and medium pH in MRS broth were evaluated each 3 h. Growth rate was monitored by cell counting in microdrops; specific growth rate (h^{-1}) was determined as the angular coefficient of the exponential growth line as a function of time ($LnX = \mu t + LnX_0$). Gas production in MRS broth with a Durham tube were determined at 37°C for 48 hours. Growth at different temperatures was observed in MRS broth after incubation at 15, 37 and 45°C for 24 hours. Growth at pH, 3.5, 4.0, 4.5 and 8.5 was observed in MRS broth after incubation at 37°C for 24 hours. Salt tolerance was tested in MRS broth containing 4.0 and 6.0% NaCl at 37°C for 24 hours. Also, were evaluate the growth in corn silage broth and fresh sugar cane broth, after 12 hours. The forage broth was crushed out from 100g of forage mixed with 400 ml distilled water in a squeezer, then filtered and sterilized (121°C, 15 min). Samplings for metabolite production (lactic acid, acetic acid, and ethanol) analysis by HPLC were taken after 3, 6, 9, 12 and 24 h of growth in MRS

broth at 37°C (Fig. 1). Based on the characteristics of the *L. buchneri* strains (Table 3 and Fig 1), eight strains were chosen for evaluation as silage inoculant, and the criteria will be presented in the results session.

Evaluation of *L. buchneri* strains on silage fermentation

Two experiments were conducted, one with corn silage and other with sugarcane silage, and the plants characteristics before ensiling are shown in Table 4. Eight isolated *L. buchneri* strains were selected and evaluated, four in corn silage and four in sugarcane silage. The corn (*Zea mays*) plants with kernels at hard dough stage of maturity, and the sugar cane (*Saccharum* spp.) plants with approximately 16 months old were harvested. The whole plants were harvested and chopped with a cut length of 2.5 cm. The isolated strains 56.22, 56.27, 56.28, and 56.29 were evaluated in corn silage. The strains 56.1, 56.4, 56.9, 56.26 and the commercial inoculant "Lalsil *Cana*" (*L. buchneri* NCIMB 40788; Lallemand, Goiás, Brazil) were evaluated in sugarcane silage. For all the treatments, the theoretical application rate was 1.0×10^6 cfu/g of fresh weight through 1% of cooled distilled water. The corn and sugarcane silages without inoculants were used as control and applied just 1% of cooled distilled water.

Eight kg of chopped forage were mixed with the inoculants. Approximately seven kg of treated material were conditioned in plastic buckets (mini-silos), 25 cm diameter and 25 cm height, sealed with tight lids. The chopped forage was compacted to a density of 571±16.7 kg/m^3 of fresh forage for corn silage and 573±19.8 kg/m^3 for sugarcane silage. Four mini-silos replicates were prepared. The mini-silos were stored at room temperature (25 ± 2°C) and opened after 90 d of storage.

After mini-silo opening, apparent DM recovery was determined and samples of approximately 2 kg were removed from each mini-silo and returns to the buckets to assess the aerobic stability. Temperatures were measured each 30 min using data

loggers (Escort mini; Impac, São Paulo, Brazil) inserted into the silage mass at geometric center using a ruler. The aerobic stability was defined as the number of hours the silage remained stable before rising more than 2°C above the ambient temperature (Kung et al., 2003). For fermentation characteristics, the forage samples were prepared as previously described.

Statistical analyses

Data from microbial counts were transformed into the logarithmic base (log_{10} cfu). The variance analysis and multiple comparisons of data were performed by the PROC MIXED procedures of SAS (SAS Institute Inc., Cary, NC, US) and the means were separated by Tukey's test ($P \leq 0.05$).

RESULTS

Isolation and identification of LAB from tropical corn silage

Corn silage fermentation and identification of isolated LAB are shown in Table 1. The DM average was 29.9%. The pattern of fermentation was typical from whole-plant corn silage (Table 1). One hundred and fifty-one strains of LAB were identified based on sequence analyses of their 16S rDNA.

In the first three days of ensling the LAB species *Pediococcus pentosaceus, Lactobacillus plantarum* and *Weissella* were the most identified. The *L. plantarum* was the predominant specie until the 28th day of storage. At day 56, the predominant species were *L. buchneri* followed by *Streptococcus salivarius* and *L. casei*. The predominant species of tropical corn silage, considering isolates in MRS agar for all the days of ensiling were *Lactobacillus plantarum* 53.0%, *Pediococcus pentosaceus* 11.9%, *L. buchneri* 9.9%, *L. pentosus* 5.3%, *Weissella cibaria* 4.6%, and *L. brevis* 4.0%. Fifteen strains of *L. buchneri* were found only at day 56 of ensiling (Table 1). The similarity of

16S rDNA sequence compared to the access code in GenBank for the isolated *L. buchneri* strains are show in the Table 2.

Preselection of *L. buchneri* strains based on phenotypic characteristics and metabolite production

The general pattern of phenotypic characteristics were that the medium with lower values of pH and temperature decreased the growth. The *L. buchneri* with the same access code in GenBank showed differences on phenotypic characteristics. They showed a good growth rate decreasing the pH in MRS broth, besides the high number of colonies in broth of corn silage and sugarcane (Table 3). All the *L. buchneri* strains produce lactic acid, acetic acid, and ethanol in different amounts (Fig 1).

We selected for inoculation in corn silage the strains 56.22 (showed good growth at high temperature, and good growth rate), 56.27 (good growth at acid pH, and good growth rate), 56.28 (highest production of lactic acid, and lower final pH), and 56.29 (good production of acetic acid). While for sugarcane silage were selected the strains 56.1 (good production of acetic acid), 56.4 (good production of acetic acid, and good growth at high temperature), 56.9 (lower ethanol production, and good growth at high temperature), and 56.26 (lower ethanol production, and good growth rate).

Effects of *L. buchneri* strains on fermentation characteristics and aerobic stability of corn silage

The *L. buchneri* strains did not affect the DM content, yeast and molds population, DM losses, WSC, lactic acid, propionic acid, butyric acid, and ethanol of corn silage after 90 d of ensiling ($P > 0.050$). The pH, LAB population, and acetic acid were affected by inoculants ($P < 0.050$). The 56.27, 56.28, and 56.29 strains showed lower pH values than the control silage. The LAB number was greater for the silages containing the strains 56.22 and 56.27 in comparison with the control silage ($P =$

0.003). The *L. buchneri* strains 56.22, 56.28, and 56.29 decreased the concentration of acetic acid in comparison with the control silage ($P = 0.041$; Table 5).

After seven days of air exposure, there was observed no improvement of inoculation with the *L.buchneri* strains in the aerobic stability of silage when compared to the control ($P = 0.289$). The overall mean for aerobic stability of corn silage was of 32.7 h. The treated and untreated silages showed high pH, high number of yeasts and molds, and low concentrations of WSC and organic acids (Table 5).

Effects of *L. buchneri* strains on fermentation characteristics and aerobic stability of sugarcane silage

The treatment with *L. buchneri* strains affected the DM content, LAB population, yeasts and molds population, DM losses, and concentrations of WSC, lactic acid and ethanol of sugarcane silage after 90 d of ensiling ($P < 0.050$; Table 6). The silage pH and the concentrations of acetic, propionic, and butyric acids were not affected by the treatments ($P > 0.050$). The silages inoculated with the isolated strains 56.1 and 56.4, and the commercial strain 40788 showed the highest DM content ($P = 0.004$). The higher LAB number than the control silage were observed to the isolated strains 56.4 and 56.9 ($P = 0.015$). At 90 d of ensiling, the inoculated silages with the isolated strains 56.1 and 56.9, and the commercial strain 40788 showed lower yeasts and molds population than the control silage ($P = 0.024$). The lower DM losses in comparison with the control silage were observed for the silages inoculated with the isolated strains 56.1 and 56.4, and the commercial strain 40788 ($P < 0.001$). The highest WSC concentration were observed for the silages inoculated with the isolated strains 56.1 and 56.4 ($P < 0.001$). The isolated strains 56.1 and 56.4, and the commercial strain 40788 showed lower lactic acid concentration than the control silage ($P = 0.023$). Regarding the ethanol concentration, the inoculated silages with the strains 56.1, 56.4,

and 40788 showed lower values than the inoculated silages with the strains 56.9 and 56.26, while the control silage showed intermediate values ($P = 0.016$).

There was no effect of the inoculation with the strains of *L. buchneri* on the aerobic stability, maximum temperature reached, DM content, pH, WSC, lactic acid, butyric acid, and ethanol concentrations of sugarcane silage after seven days of air exposure ($P > 0.050$; Table 6). The overall mean for aerobic stability was of 58.2 h ($P = 0.524$). The inoculated silage with the strain 56.9 showed higher population of yeasts and molds than the control silage; the others strains showed intermediates values ($P = 0.024$). The strains 56.1, 56.4, and 40788 showed lower acetic acid and propionic acid concentrations than the control silage after seven days of air exposure ($P = 0.001$).

DISCUSSION

Isolation and identification of LAB from tropical corn silage

Whole plant corn had good characteristics to production of high quality silage. Results of our study demonstrated that there a quickly drop in pH with the consumption of WSC and production of organic acids, besides the epiphytic LAB succession during the pre-ensiling and ensiling periods of corn.

The isolation of LAB using MRS medium under anaerobic conditions, followed the analysis of 16S rDNA fragment, allows the identification of different species in fresh forage and silage (Ávila et al., 2014; Ni et al., 2015). In our study, the growth of *L. plantarum*, *P. pentosaceus*, and *W. cibaria* were replaced by *L. buchneri*, *S. salivarius*, *L. casei,* and *L. brevis* during the silage fermentation. Probably, the substrates availability and the pH could induce the shift of LAB species. Our observations that the *L. buchneri* accounted for a larger proportion at day 56 of ensiling can be due the ability of this species to use lactic acid as energy source (Oude-Elferink et al., 2001).

Several studies have investigated the diversity of epiphytic LAB during the corn ensiling process. In general, *L. plantarum* and *P. pentosaceus* were two predominant LAB species at the beginning of corn silage fermentation. While *L. buchneri* and *L. brevis* were widely detected few days after ensiling (Lin et al., 1992; Brusetti et al., 2006; Stevenson et al., 2006; Parvin et al., 2010; Zhou et al., 2016). It is generally recognized that for a high quality silage, the fermentation should starts with homofermentative LAB species. These species quickly grow and decreasing the pH. However, when the pH becomes acid and substrate limited, heterofermentative LAB species can replace them and eventually predominate (McDonald et al., 1991).

Preselection of *L. buchneri* strains

Among fifteen *L. buchneri* identified in our study, we found seven different GenBank access codes (Table 2). In order to evaluate the different access codes in silage fermentation, a preselection of the *L. buchneri* strains was carried out in an attempt to identify the best strains. Because that the strains of same species might show differences in the metabolism and, consequently, on the ability to survive in the silage environment (Santos et al., 2015). In addition, the different strains of the same species generally result in distinct patterns of silage fermentation (Saarisalo et al., 2007; Ávila et al., 2014; Carvalho et al., 2014).

The effects of microbial inoculants on silage fermentation are mainly due the ability of quickly dominate the fermentation and produce metabolites, besides the changes in pH. In addition, an inoculant to be effective, the crop and the LAB selected must be compatible. Thus, these characteristics can be employed as a selection criterion of new inoculants (Saarisalo et al., 2007; Ávila et al., 2014, Ni et al., 2015). In our study, the preselection of *L. buchneri* strains for starter cultures in silages were based

mainly on growth rate and metabolites production to improve the fermentation and/or aerobic stability.

Effects of *L. buchneri* strains on corn silage

The DM and WSC contents of fresh chopped corn (35.5% and 9.41% respectively; Table 4) at ensiling were considered adequate and all silages showed good fermentation pattern and low DM losses. The average values of pH obtained in the present study for all silages range in the pH interval considered adequate for the corn silage (Kung Jr. and Shaver, 2001). However, the inoculated silages presented a lower value of pH in comparison to control silage. It was no expected, because of some *L. buchneri* strains can degrade lactic acid into acetic acid raising the pH (Driehuis et al., 1999; Oude-Elferink et al., 2001; Kung Jr. et al, 2003). Although, in our study, for all *L. buchneri* strains, the lactic acid was the major product of the fermentation, besides of acetic acid and ethanol, when evaluated their metabolites production.

Although none improvement of inoculant was observed on silage fermentation, there is an evidence that the strains survival due the greater LAB count of the inoculated silages. At day 90 of ensiling, the greater LAB population of inoculated silages than the control silage probably was due the fact that in acid conditions some LAB may lose viability, and just specialized LAB, such as *L. buchneri*, can remain active (Oude-Elferink et al., 2000). Reduction of LAB population during the fermentation process also has been reported by other authors; it is related to resistance of LAB to acidic conditions (Li and Nishino, 2011; Assis et al., 2014).

In our study, the populations of yeasts and molds in the fresh forage were greater than the previous studies (Filya, 2003, Assis et al., 2014; Zhou et al., 2016). This difference in diversity could be attributed to factors as corn hybrid and environmental conditions. However, this high population can be a reason because the treatments had

no effect on population of yeasts and molds. Probably because of these microorganisms can survive under anaerobic conditions and a wide range of pH (McDonald et al., 1991). However, the ethanol contents of silages in this study are in the interval acceptable (Kung Jr. and Shaver, 2001; Li and Nishino, 2011).

After air exposure, all silages deteriorate and had high population of yeast and molds. The high DM content and low concentration of acetic, propionic and butyric acids, and ethanol of silages can be due the volatilization. In addition, yeasts and molds, and aerobic bacteria can metabolize WSC and lactic acid under aerobic conditions. Furthermore, because of the reduction in the concentrations of lactic, acetic, propionic and butyric acids the pH increased (McDonald et al., 1991; Oude Elferink et al., 2001; Carvalho et al., 2014).

For corn silage, the preselection of *L. buchneri* strains based on growth at high temperature and acid pH, and production of lactic and acetic acids had no improvement on the fermentation and aerobic stability of corn silage. It can be due the greater population of yeasts and molds in the fresh forage and/ or absence of metabolites production by the *L. buchneri* strains in sufficient amounts, as acetic acid that can inhibit these microorganisms (Kung Jr et al., 2003).

Effects of *L. buchneri* strains on sugarcane silage

The main problem of sugarcane silage are due the high WSC content and the proliferation of yeast. Natural fermentation of sugarcane silage often results in high DM losses and ethanol concentration (Kung and Stanley, 1982).

In our study, the higher DM content and lower DM losses of silages inoculated with 56.1, 56.4, and commercial 40788 strains of *L. buchneri* are mainly due to higher content of residual WSC. In these inoculated silages, the acetic acid concentration had no significant differences, but was numerically higher, resulting in decreased

consumption of WSC by yeasts, although the inoculant effect on the population of yeasts and molds were variable. This variation probably is due the inhibitory effect of ethanol on yeasts population of sugarcane silage (Ávila et al., 2014; Carvalho et al., 2014).

Inoculants containing heterofermentative LAB that produces high concentrations of acetic acid are more suitable for yeast control because of the inhibitory effect of this acid (Kung et al., 2003). Increase in the concentration of acetic acid as a final product of the fermentation is because that some *L. buchneri* strains do not have the acetaldehyde dehydrogenase enzyme, responsible for the reduction of the acetaldehyde to ethanol, and thus synthesize mainly acetic acid (McDonald et al., 1991; Axelsson, 2004). The fungistatic effect of acetic acid is due to lipophilicity. This acid, in acid pH, can permeate the cell membrane; inside the cell, in neutral pH, the disassociation of acetic acid releasing protons, which decreases the intracellular pH and can lead the microorganisms to death (Danner et al., 2003).

At day 90 of ensiling, the strains 56.1, 56.9, and 40788 significantly decrease the population of yeasts and molds in comparison with control silage. Although the strains that resulted in the best fermentation patterns were 56.1, 56.4, and 40788. These inoculated silages had better-quality fermentation than the control silage, because of the preservation of WSC and decreased ethanol concentrations. Indeed, those isolated strains were selected according to the highest acetic acid production, as noted in the study of Ávila et al. (2014).

Usually in sugarcane silage occurs concurrently with the consumption of WSC the increases of ethanol concentration. Ethanol production in silage is normally associated with fermentation of WSC and organic acids by yeast. However, some heterofermentative LAB such as *L. buchneri* can converts sugars into ethanol (Liu et al.,

2008). It could explain the increased ethanol concentration observed in our study for the inoculated silages with the strains 56.9 and 56.26.

The population of yeasts and molds in the fresh sugarcane was higher than in other published studies (Ávila et al., 2014). According to McDonald et al. (1991), the silages containing population of yeast and molds larger than five-log cfu g^{-1} are more susceptible to aerobic deterioration. In tropical regions, the aerobic deterioration during feeding-out is common. In addition, the high concentration of lactic acid and residual WSC of good quality silage are substrate for yeast, molds, and aerobic bacteria (McDonald et al., 1991).

In general, the *L. buchneri* can enhance the fermentation of sugarcane silage resulting in low DM losses and aerobic stability increased (Ávila et al., 2009; Pedroso et al., 2008; Roth et al., 2010; Ávila et al., 2014). However, the improvement on aerobic stability can be due other antimicrobial substance, besides the acetic acid. For example, some *L. buchneri* strains can produce bacteriocin that may be responsible to enhance the aerobic stability (Yildirim, 2001; Kleinschmit and Kung Jr, 2006).

In our study, after air exposure, all silages deteriorate and had high population of yeasts and molds. Beyond the effects described for the corn silage experiment, the sugarcane silages that showed a higher concentration of ethanol resulted in high concentration of acetic acid and low pH. This pattern can be due the effects of aerobic acetic bacteria; belong to family *Pseudomonodaceae*, mainly *Acetobacter* and *Gluconobacter* genera, this bacteria can converts WSC and/or ethanol into acetic acid, aerobically (Rizzon, 2006).

CONCLUSIONS

The appearance of *L. buchneri* occurs naturally in the 56th d of ensiling corn in tropical conditions. The isolated strains when applied in corn silage showed no beneficial effect. However, the preselection of *L. buchneri* strains based on acetic acid production showed the best results for sugarcane silage. Thus, the strains 56.1 and 56.4 are promising for use as an inoculant in sugarcane silages.

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Table - 1. Fermentation characteristics and epiphytic lactic acid bacteria succession of tropical corn silage.

Items	Days of ensiling						
	0	**1**	**3**	**7**	**14**	**28**	**56**
Chemical composition (% of DM[1])							
Dry matter (% of FM[2])	30.4	29.7	28.7	29.4	29.5	30.3	31.0
pH	6.05	3.79	3.80	3.69	3.60	3.66	3.7
WSC[3]	13.7	10.2	5.78	5.25	4.24	3.25	1.13
Lactic acid	-	1.35	2.01	2.75	3.08	4.10	3.50
Acetic acid	-	1.46	1.98	2.03	2.27	2.13	2.01
Propionic acid	-	0.119	0.129	0.152	0.149	0.170	0.113
Butyric acid	-	0.013	0.016	0.019	0.017	0.017	0.011
Microorganisms (log_{10} cfu/g of FM)							
Lactic acid bacteria	5.07	8.33	8.13	7.41	6.86	5.88	4.45
Yeasts and molds	6.63	6.23	5.12	3.75	4.33	5.29	5.23
Enterobacteria	6.78	5.94	3.89	2.13	ND	ND	ND
Epiphytic lactic acid bacteria succession (%)							
Lactobacillus plantarum	39	42	72	85	81	67	-
Lactobacillus buchneri	-	-	-	-	-	-	60
Lactobacillus pentosus	6	-	8	5	19	4	-
Lactobacillus brevis	-	-	4	-	-	17	4
Lactobacillus casei	-	-	-	5	-	-	12
Lactobacillus fermentum	-	-	-	-	-	4	-
Lactobacillus sp	-	-	-	-	-	4	-
Pediococcus pentosaceus	17	46	16	-	-	-	-
Weissella sp	5	-	-	-	-	-	-
Weissella confusa	11	-	-	-	-	-	-
Weissella cibaria	22	12	-	-	-	-	-
Streptococcus salivarius	-	-	-	-	-	-	16
Uncultured	-	-	-	5	-	4	8

[1]Dry matter.
[2]Fresh matter.
[3]Water-soluble carbohydrates.
ND, not detected (< 2 log_{10} cfu g^{-1} FM).

Table - 2. *Lactobacillus buchneri* identification

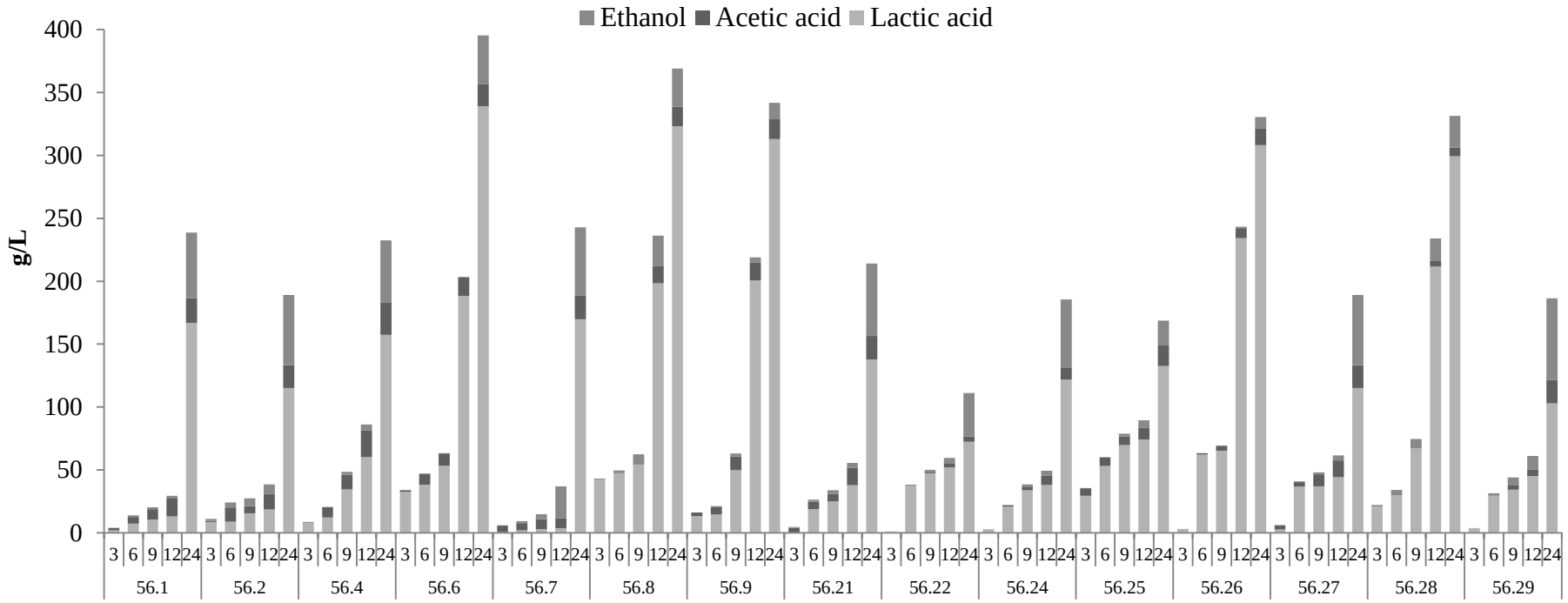
Identification	Species	Similarity	GenBank access code
56.1	*Lactobacillus buchneri* FQ027	98%	KF418820.1
56.2	*Lactobacillus buchneri* mze12	97%	KP062948.1
56.4	*Lactobacillus buchneri* JNLAB-4	99%	KC336485.1
56.6	*Lactobacillus buchneri* mze12	99%	KP062948.1
56.7	*Lactobacillus buchneri* L4APL6.2	99%	KM005146.1
56.8	*Lactobacillus buchneri* JNLAB-4	99%	KC336485.1
56.9	*Lactobacillus buchneri* mze12	99%	KP062948.1
56.21	*Lactobacillus buchneri* mze12	98%	KP062948.1
56.22	*Lactobacillus buchneri* L4APL6.2	99%	KM005146.1
56.24	*Lactobacillus buchneri* L4APL6.2	98%	KM005146.1
56.25	*Lactobacillus buchneri* JNLAB-4	98%	KC336485.1
56.26	*Lactobacillus buchneri* JNLAB-4	99%	KC336485.1
56.27	*Lactobacillus buchneri* NRRL B-30929	98%	CP002652.1
56.28	*Lactobacillus buchneri* FD2	99%	JN188387.1
56.29	*Lactobacillus buchneri* MF161	98%	KJ994455.1

Identification based on ~1500 base pair gene that codes for a portion of the 16S rDNA.

Table - 3. Phenotypic characteristics of *L. buchneri* strains isolated from tropical corn silage after 24 hours.

Item	***Lactobacillus buchneri* strains**														
	56.1	**56.2**	**56.4**	**56.6**	**56.7**	**56.8**	**56.9**	**56.21**	**56.22**	**56.24**	**56.25**	**56.26**	**56.27**	**56.28**	**56.29**
Growth[a] at pH:															
3.5	+	+	+	++	+	++	++	+	+	+	+	++	++	+	+
4.0	++++	++++	++++	++++	++++	++++	++++	++++	+++	++++	++++	+++	+++	+++	++++
4.5	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	+++	+++	++++
8.5	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Growth at temperature (°C):															
15	+	+	+	+++	+	++++	+++	+	+	+	+	+++	++	+++	+
37	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
45	++	+++	+++	++++	+++	++++	+++	+++	+++	++	++	+++	+++	+++	++
Growth in NaCl (%):															
4.0	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++
6.0	+	+++	++	+++	+	+++	+++	+++	++++	+++	+	+++	++	+++	+
CO_2[b]	++	+++	+++	+	+++	+	+	+++	+++	+++	+++	+	+++	+	+++
pH[c]	4.45	4.38	4.45	3.86	4.42	4.44	4.46	4.44	4.53	4.44	4.45	3.91	4.45	3.86	4.43
μ[d]	0.136	0.097	0.167	0.161	0.182	0.239	0.197	0.193	0.202	0.158	0.162	0.314	0.205	0.194	0.203
Cells count at broth[e] (log cfu/mL):															
Corn silage	7.76	7.90	7.71	7.74	6.48	7.08	7.57	7.45	6.96	6.87	7.38	7.23	6.65	6.52	6.74
Sugarcane	8.02	8.08	8.04	8.16	7.43	7.45	7.57	7.60	7.34	7.43	7.40	8.53	7.67	8.08	7.58
MRS	8.72	8.85	8.72	9.32	9.30	9.52	9.83	9.38	9.60	9.45	9.49	9.38	9.32	9.34	9.32

[a]Growth measured by optical density (630nm) : <0.3 = +; >0.3 to <0.6 = ++; >0.6 to <0.9 = +++; >0.9 = ++++.
[b]CO_2 = carbon dioxide gas in MRS broth with Durham tubes: <1/3 of tube = +; >1/3 to <2/3 = ++; >2/3 = +++.
[c]pH in MRS broth (initial pH 6)
[d]μ = specific growth rate (h^{-1}).
[e]Forage broth = 100g of forage mixed with 400 ml of distilled water; MRS = de Man, Rogosa, and Sharpe broth.



End products as time function (3, 6, 9, 12 and 24h) for each *L. buchneri* strains

Fig 1. Lactic acid, acetic acid and ethanol production as a time function by *L. buchneri* strains in MRS broth.

Table - 4. Plant characterization before ensiling.

Item	Corn	Sugarcane
Dry matter (% of FM[1])	35.5	31.9
Water-soluble carbohydrates (% of DM[2])	9.41	38.5
pH	5.76	5.54
Lactic acid bacteria (log cfu/g of FM)	6.87	6.29
Yeasts and molds (log cfu/g of FM)	5.86	6.63

[1]Fresh matter.
[2]Dry matter.

Table - 5. Fermentation pattern and aerobic stability (% of DM, unless stated otherwise) of corn silage treated with isolated *Lactobacillus buchneri* strains after 90 d of ensiling and after 7 d of air exposure at 23°C.

Item	Control	*L. buchneri* strains				SEM[1]	*P*-value
		56.22	56.27	56.28	56.29		
After 90 d of ensiling							
DM[2]	34.9	35.4	35.1	34.4	35.1	0.158	0.315
pH	3.77^{a}	3.74^{ab}	3.72^{b}	3.73^{b}	3.72^{b}	0.005	0.015
LAB[4]	4.35^{b}	6.19^{a}	6.66^{a}	5.45^{ab}	5.28^{ab}	0.216	0.003
Y&M[5]	5.39	5.80	5.65	5.03	5.12	0.181	0.633
DM losses	3.07	3.01	3.32	3.32	3.26	0.054	0.213
WSC[6]	1.51	1.39	1.65	1.27	1.30	0.066	0.348
Lactic acid	3.88	3.45	4.09	3.97	4.01	0.116	0.446
Acetic acid	1.22^{a}	0.981^{b}	1.10^{ab}	0.992^{b}	0.964^{b}	0.031	0.041
Propionic acid	0.055	0.057	0.049	0.044	0.069	0.003	0.129
Butyric acid	0.658	0.390	0.396	0.297	0.296	0.047	0.115
Ethanol	1.14	1.57	1.98	1.82	1.60	0.119	0.282
After 7 d of air exposure at 23°C							
Stability (h)	31.9	32.5	32.9	28.9	37.4	1.24	0.289
Max. temp. (°C)	39.0	38.9	38.5	38.8	37.5	0.250	0.328
DM[2]	33.8	34.5	34.8	33.8	34.6	0.236	0.590
pH	6.11	6.18	6.24	6.30	6.22	0.029	0.395
Y&M[5]	7.93^{c}	8.36^{bc}	8.77^{ab}	8.99^{a}	8.96^{a}	0.098	0.001
WSC[6]	0.291	0.402	0.500	0.394	0.647	0.045	0.129
Lactic acid	0.709	0.452	0.540	0.568	0.466	0.033	0.117
Acetic acid	0.191	0.226	0.240	0.190	0.196	0.013	0.691
Propionic acid	0.033	0.038	0.046	0.026	0.028	0.003	0.195
Butyric acid	0.156	0.216	0.158	0.221	0.357	0.027	0.109
Ethanol	0.235^{b}	0.582^{ab}	0.359^{b}	1.58^{a}	1.48^{a}	0.173	0.014

[1]Standard error of mean.
[2]Dry matter content (% of FM[3]).
[3]Fresh matter.
[4]Enumeration of lactic acid bacteria (log cfu/g of FM[3]).
[5]Enumeration of yeasts and molds (log cfu/g of FM[3]).
[6]Water-soluble carbohydrates (% of DM).
[a–c]Means in rows with unlike superscripts differ ($P < 0.05$).

Table - 6. Fermentation pattern and aerobic stability (% of DM, unless stated otherwise) of sugarcane silage treated with isolated *Lactobacillus buchneri* strains after 90 d of ensiling and after 7 d of air exposure at 23°C.

Item	Control	*L. buchneri* strains					SEM[1]	*P*-value
		56.1	56.4	56.9	56.26	40788		
After 90 d of ensiling								
DM[2]	24.7^{bc}	29.0^{a}	29.0^{a}	26.8^{ab}	23.6^{c}	28.6^{a}	0.571	0.004
pH	3.39	3.42	3.44	3.48	3.47	3.50	0.014	0.192
LAB[4]	6.35^{c}	6.72^{bc}	7.46^{ab}	7.77^{a}	6.60^{c}	6.69^{bc}	0.146	0.015
Y&M[5]	5.56^{a}	4.79^{bc}	4.93^{abc}	4.66^{bc}	5.19^{ab}	4.42^{c}	0.110	0.024
DM losses	26.0^{ab}	13.7^{c}	14.6^{c}	20.9^{bc}	30.7^{a}	15.1^{c}	1.60	<0.001
WSC[6]	2.22^{c}	7.36^{a}	7.01^{a}	4.93^{b}	2.66^{c}	3.50^{bc}	0.478	<0.001
Lactic acid	5.98^{a}	3.65^{b}	3.80^{b}	4.59^{ab}	5.79^{a}	3.89^{b}	0.282	0.023
Acetic acid	3.30	3.80	3.71	3.55	2.57	3.60	0.231	0.716
Propionic acid	0.366	0.333	0.361	0.316	0.273	0.344	0.014	0.447
Butyric acid	0.335	0.401	0.419	0.382	0.350	0.295	0.021	0.628
Ethanol	21.6^{ab}	14.1^{b}	15.4^{b}	31.8^{a}	38.5^{a}	15.2^{b}	2.11	0.016
After 7 d of air exposure at 23°C								
Stability (h)	56.0	54.5	60.5	50.6	67.2	60.6	2.51	0.524
Max. temp. (°C)	39.5	38.0	43.0	41.5	38.0	37.4	0.780	0.221
DM[2]	26.2	27.2	27.2	28.0	25.1	28.2	0.490	0.526
pH	3.49	4.13	4.36	3.59	3.63	4.91	0.170	0.078
Y&M[5]	7.28^{b}	9.10^{ab}	9.41^{a}	8.78^{ab}	7.90^{ab}	8.72^{ab}	0.230	0.024
WSC[6]	3.40	4.13	2.47	5.52	2.77	4.50	0.429	0.327
Lactic acid	1.95	1.06	0.811	1.43	1.29	1.24	0.116	0.079
Acetic acid	14.4^{a}	2.36^{bc}	0.646^{c}	9.34^{ab}	13.1^{a}	2.97^{bcd}	1.29	<0.001
Propionic acid	1.52^{a}	0.701^{bcd}	0.410^{d}	1.00^{abcd}	1.41^{ab}	0.597^{cd}	0.106	0.001
Butyric acid	0.251	0.141	0.188	0.355	0.204	0.259	0.026	0.251
Ethanol	4.60	4.52	7.94	3.11	7.57	3.10	1.34	0.868

[1]Standard error of mean.
[2]Dry matter content (% of FM[3]).
[3]Fresh matter.
[4]Enumeration of lactic acid bacteria (log cfu/g of FM[3]).
[5]Enumeration of yeasts and molds (log cfu/g of FM[3]).
[6]Water-soluble carbohydrates (% of DM).
[a–d]Means in rows with unlike superscripts differ ($P < 0.05$).

APPENDIX

Epiphytic lactic acid bacteria (LAB) isolated from tropical corn silage and identified based on the access code of GenBank.

Identification	LAB species	Similarity	Access code
SM0.1	*Lactobacillus plantarum* strain S-CO1(2)	99%	KP056258.1
SM0.3	*Lactobacillus plantarum* strain LCN 56	99%	JX003604.1
SM0.4	*Weissella confusa* strain HTBS-BGB-001	97%	KM505155.1
SM0.5	*Weissella cibaria* strain PON100420	97%	KM668164.1
SM0.6	*Pediococcus pentosaceus* strain BMG 74	97%	EU080993.1
SM0.7	*Weissella cibaria*	100%	KM668164.1
SM0.8	*Weissella cibaria* SCWL	97%	KM922568.1
SM0.9	*Weissella sp.* KLDS 7.0701	98%	EU600924.1
SM0.10	*Weissella cibaria* 860308	99%	AB469386.1
SM0.11	*Lactobacillus plantarum*	98%	AB572045.1
SM0.12	*Pediococcus pentosaceus* strain E3-76	98%	KP189233.1
SM0.13	*Weissella confusa* 3199O2	98%	KF598896.1
SM0.14	*Lactobacillus plantarum* strain S-CO1(2)	99%	KP056258.1
SM0.15	*Lactobacillus plantarum* strain B21	99%	CP010528.1
SM0.16	*Pediococcus pentosaceus* strain NBRC 106014	98%	JX141317.1
SM0.17	*Lactobacillus pentosus* strain LT32	97%	JQ288726.1
SM0.18	*Lactobacillus plantarum* strain LAG1	99%	KJ806295.1
SM0.20	*Lactobacillus plantarum* strain TL4	98%	JQ937330.1
SM1.1	*Pediococcus pentosaceus* strain NBRC 106014	97%	JX141317.1
SM1.2	*Weissella cibaria* strain PON100420	99%	KM668164.1
SM1.3	*Weissella cibaria* strain PON100420	99%	KM668164.1
SM1.5	*Lactobacillus plantarum* strain B21	98%	CP010528.1
SM1.6	*Pediococcus pentosaceus* strain E24-168	96%	KP189228.1
SM1.7	*Pediococcus pentosaceus* strain E3-76	99%	KP189233.1
SM1.8	*Weissella cibaria* strain PON100420	100%	KM668164.1
SM1.9	*Pediococcus pentosaceus* strain NBRC 106014	99%	JX141317.1
SM1.10	*Lactobacillus plantarum* strain B21	99%	CP010528.1
SM1.11	*Lactobacillus plantarum* strain B21	99%	CP010528.1
SM1.12	*Pediococcus pentosaceus* strain E3-76	98%	KP189233.1
SM1.14	*Pediococcus pentosaceus* strain V3-103	97%	KP189226.1
SM1.15	*Lactobacillus plantarum* strain TW11-4	97%	KJ026590.1
SM1.16	*Pediococcus pentosaceus* strain E3-76	99%	KP189233.1
SM1.17	*Pediococcus pentosaceus* strain E3-76	99%	KP189233.1
SM1.18	*Lactobacillus plantarum* strain C11(5)	99%	KJ420401.1
SM1.19	*Pediococcus pentosaceus* strain E24-168	96%	KP189228.1
SM1.20	*Lactobacillus plantarum* strain gp41	98%	KM495872.1

SM1.22	*Lactobacillus plantarum*	96%	JN587506.1
SM1.24	*Pediococcus pentosaceus*	98%	AB921219.1
SM1.26	*Lactobacillus plantarum* S-CO1(2)	99%	KP056258.1
SM1.28	*Pediococcus pentosaceus* E24-168	94%	KP189228.1
SM1.29	*Lactobacillus plantarum* strain gp41	98%	KM495872.1
SM1.30	*Lactobacillus plantarum* strain L601(LBF2)F02	98%	KM269713.1
SM3.1	*Lactobacillus pentosus* LT32	98%	JQ288726.1
SM3.2	*Lactobacillus brevis* LU2	98%	JQ236621.1
SM3.3	*Lactobacillus plantarum* MF266	99%	KJ994400.1
SM3.4	*Lactobacillus plantarum* MF176	99%	KJ994448.1
SM3.5	*Lactobacillus plantarum* B21	97%	CP010528.1
SM3.6	*Lactobacillus plantarum* UFSBC 317	97%	JQ580980.1
SM3.7	*Pediococcus pentosaceus* E3-68	96%	KP189232.1
SM3.8	*Lactobacillus plantarum* MF266	99%	KJ994400.1
SM3.9	*Lactobacillus plantarum* S4	99%	KJ160209.1
SM3.10	*Lactobacillus plantarum* B21	100%	CP010528.1
SM3.11	*Lactobacillus plantarum* SM34	99%	KJ690749.1
SM3.14	*Lactobacillus plantarum* TW11-4	98%	KJ026590.1
SM3.15	*Lactobacillus plantarum* MF176	99%	KJ994448.1
SM3.16	*Lactobacillus plantarum*	99%	AB889712.1
SM3.17	*Pediococcus pentosaceus* DSPV 029SA	99%	JQ322223.1
SM3.18	*Lactobacillus plantarum* TW11-4	98%	KJ026590.1
SM3.19	*Pediococcus pentosaceus* SBR7	99%	KF013201.1
SM3.20	*Lactobacillus plantarum* subsp. *plantarum* TW14-1	98%	KJ026598.1
SM3.21	*Lactobacillus plantarum* MF266	98%	KJ994400.1
SM3.22	*Pediococcus pentosaceus*	97%	KF013201.1
SM3.23	*Lactobacillus plantarum* B21	98%	CP010528.1
SM3.25	*Lactobacillus plantarum* subsp. *plantarum* TW14-1	97%	KJ026598.1
SM3.26	*Lactobacillus plantarum* gp102	97%	KM495892.1
SM3.27	*Lactobacillus pentosus* LT32	97%	JQ288726.1
SM3.29	*Lactobacillus plantarum* strain gp102	97%	KM495892.1
SM7.1	*Lactobacillus pentosus* strain LT32	97%	JQ288726.1
SM7.3	*Lactobacillus plantarum* strain TW11-4	97%	KJ026590.1
SM7.4	*Lactobacillus casei* strain MO11	98%	KJ739522.1
SM7.7	Uncultured *Bacillus sp.* clone 99	97%	KM819173.1
SM7.8	*Lactobacillus plantarum*	99%	AB889712.1
SM7.10	*Lactobacillus plantarum* gp102	99%	KM495892.1
SM7.11	*Lactobacillus plantarum* subsp. *plantarum* P-8	100%	CP005942.2
SM7.14	*Lactobacillus plantarum* JJ 29	99%	JN573605.2
SM7.15	*Lactobacillus plantarum* B21	100%	CP010528.1

SM7.19	*Lactobacillus plantarum* B21	99%	CP010528.1
SM7.20	*Lactobacillus plantarum* B21	99%	CP010528.1
SM7.21	*Lactobacillus plantarum* LAG1	100%	KJ806295.1
SM7.22	*Lactobacillus plantarum* SM30	99%	KJ690745.1
SM7.25	*Lactobacillus plantarum* S-CO1(2)	99%	KP056258.1
SM7.26	*Lactobacillus plantarum* B21	98%	CP010528.1
SM7.27	*Lactobacillus plantarum* subsp. *plantarum* P-8	99%	CP005942.2
SM7.28	*Lactobacillus plantarum* MF176	99%	KJ994448.1
SM7.29	*Lactobacillus plantarum* gp41	99%	KM495872.1
SM7.30	*Lactobacillus plantarum* subsp. *plantarum* P-8	99%	CP005942.2
SM14.1	*Lactobacillus brevis*	99%	KM495920.1
SM14.2	*Lactobacillus plantarum* LAG1	99%	KJ806295.1
SM14.3	*Lactobacillus pentosus* LT32	98%	JQ288726.1
SM14.5	*Lactobacillus plantarum* B21	98%	CP010528.1
SM14.12	*Lactobacillus pentosus* LT32	96%	JQ288726.1
SM14.13	*Lactobacillus pentosus* LT32	96%	JQ288726.1
SM14.14	*Lactobacillus plantarum* S4	99%	KJ160209.1
SM14.18	*Lactobacillus plantarum* B21	99%	CP010528.1
SM14.19	*Lactobacillus plantarum* B21	99%	CP010528.1
SM14.20	*Lactobacillus plantarum* B21	99%	CP010528.1
SM14.22	*Lactobacillus plantarum* BIM B-538	97%	JF965386.1
SM14.24	*Lactobacillus plantarum* S4	99%	KJ160209.1
SM14.25	*Lactobacillus plantarum* B21	99%	CP010528.1
SM14.26	*Lactobacillus plantarum* MF176	100%	KJ994448.1
SM14.28	*Lactobacillus plantarum* S-CO1(2)	99%	KP056258.1
SM14.29	*Lactobacillus plantarum* LAG1	99%	KJ806295.1
SM14.30	*Lactobacillus plantarum* CT2	98%	KJ160193.1
SM28.1	*Lactobacillus plantarum* MF266	99%	KJ994400.1
SM28.2	*Lactobacillus sp.* 3.8.18	97%	JX826520.1
SM28.4	*Lactobacillus plantarum* LBRH013	98%	HM101322.1
SM28.5	*Lactobacillus fermentum* 3-7-2	99%	KJ690913.1
SM28.6	*Lactobacillus plantarum* C	100%	KJ160192.1
SM28.7	*Lactobacillus plantarum* B21	99%	CP010528.1
SM28.8	*Lactobacillus plantarum* B21	100%	CP010528.1
SM28.9	*Lactobacillus plantarum* A1	99%	KJ160190.1
SM28.10	*Lactobacillus plantarum* S4	99%	KJ160209.1
SM28.11	*Lactobacillus brevis* E6-120	98%	KP221637.1
SM28.12	*Lactobacillus plantarum* gp41	99%	KM495872.1
SM28.13	*Lactobacillus brevis* E6-120	99%	KP221637.1
SM28.16	*Lactobacillus brevis* gp71	99%	KM495920.1
SM28.18	Uncultured *Bacillus sp.* Clone 99	99%	KM819173.1

SM28.19	*Lactobacillus brevis* LMG 11992	97%	EU194344.1
SM28.20	*Lactobacillus plantarum* subsp. *plantarum* P-8	100%	CP005942.2
SM28.21	*Lactobacillus plantarum* subsp. *plantarum* P-8	100%	CP005942.2
SM28.22	*Lactobacillus pentosus* LT32	97%	JQ288726.1
SM28.24	*Lactobacillus plantarum* MF176	99%	KJ994448.1
SM28.25	*Lactobacillus plantarum* subsp. *plantarum* P-8	99%	CP005942.2
SM28.26	*Lactobacillus plantarum* subsp. *plantarum* P-8	99%	CP005942.2
SM28.28	*Lactobacillus plantarum* subsp. *plantarum* P-8	99%	CP005942.2
SM28.29	*Lactobacillus plantarum* subsp. *Plantarum* P-8	99%	CP005942.2
SM28.30	*Lactobacillus plantarum* MF266	98%	KJ994400.1
SM56.1	*Lactobacillus buchneri* FQ027	98%	KF418820.1
SM56.2	*Lactobacillus buchneri* mze12	97%	KP062948.1
SM56.3	*Lactobacillus casei* str. Zhang	99%	CP001084.2
SM56.4	*Lactobacillus buchneri* JNLAB-4	99%	KC336485.1
SM56.5	*Lactobacillus casei* 7A04A1	99%	KJ764639.1
SM56.6	*Lactobacillus buchneri* mze12	99%	KP062948.1
SM56.7	*Lactobacillus buchneri* L4APL6.2	99%	KM005146.1
SM56.8	*Lactobacillus buchneri* JNLAB-4	99%	KC336485.1
SM56.9	*Lactobacillus buchneri* mze12	99%	KP062948.1
SM56.10	*Lactobacillus casei* 7A04A1	97%	KJ764639.1
SM56.11	*Streptococcus salivarius* GD32	99%	KF928788.1
SM56.12	*Streptococcus salivarius* GD32	99%	KF928788.1
SM56.13	*Streptococcus salivarius* GD32	99%	KF928788.1
SM56.14	*Streptococcus salivarius* GD32	99%	KF928788.1
SM56.15	Uncultured *bacterium* clone ncd2800b06c1	97%	KF101483.1
SM56.16	*Lactobacillus brevis* s MF398	97%	KJ994371.1
SM56.17	Uncultured *bacterium* clone ncd654b10c1	99%	HM288104.1
SM56.21	*Lactobacillus buchneri* mze12	98%	KP062948.1
SM56.22	*Lactobacillus buchneri* L4APL6.2	99%	KM005146.1
SM56.24	*Lactobacillus buchneri* L4APL6.2	98%	KM005146.1
SM56.25	*Lactobacillus buchneri* JNLAB-4	98%	KC336485.1
SM56.26	*Lactobacillus buchneri* JNLAB-4	99%	KC336485.1
SM56.27	*Lactobacillus buchneri* NRRL B-30929	98%	CP002652.1
SM56.28	*Lactobacillus buchneri* FD2	99%	JN188387.1
SM56.29	*Lactobacillus buchneri* MF161	98%	KJ994455.1