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

Effect of ensiling temperature on microbial inoculants and performance of dairy cows fed corn grain silage with sodium benzoate

Viviane Carnaval Gritti

Thesis presented to obtain the degree of Doctor in Science. Area: Animal Science and Pastures

Piracicaba
2021

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Animal Scientist

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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

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To my family

I DEDICATE

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

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CONTENTS

RESUMO

Efeito da temperatura de ensilagem em inoculantes microbianos e desempenho de vacas leiteiras alimentadas com silagem de grãos de milho com benzoato de sódio

Aditivos são recomendados para silagens à base de milho em condições tropicais, como silagem de milho de plantas inteiras, grãos de milho com alta umidade e grãos de milho rehidratados, para evitar o perdas no armazenamento do silo e maior estabilidade aeróbica. Benzoato de sódio é um sal de um ácido orgânico e tem propriedades antifúngicas e é usado como aditivo químico para silagens. O mecanismo do benzoato de sódio para evitar a presença de microrganismos indesejáveis nas silagens está relacionado com a forma não dissocida de ácido benzoico, que pode passar pela membrana celular e liberar o hidrogênio no citoplasma microbiano. Mas, a influência do benzoato de sódio no desempenho de animais ainda é pouco explorada. Outro tipo de aditivo adequado para silagens à base de milho são os inoculantes contendo bactérias ácido lácticas (LAB), que podem ser divididas em dois grandes grupos, bactérias homo-fermentativas e bactérias hetero-fermentativas. Temperaturas mais elevadas normalmente levam a alteração de populações microbianas homoláticas para heteroláticas, mas a maioria dos conhecimentos microbiológicos de silagem se concentra em condições ideais de fermentação, em vez de condições sob extremos ambientais. O objetivo do primeiro estudo foi avaliar o desempenho, o comportamento alimentar e os parâmetros ruminais das vacas leiteiras Holandesas alimentadas com dietas com HMC ou RCG, como a principal fonte de amido, adicionada ou não com 0,2% de matéria fresca de benzoato de sódio (BEN). Além disso, foram avaliadas a composição química e física, o perfil fermentativo e a digestibilidade de HMC ou RCG com ou sem benzoato de sódio. Como principais resultados em silagens HMC e RCG, benzoato de sódio reduziu a concentração de ácido butírico e proteína solúvel. Além disso, o benzoato de sódio aumentou a digestibilidade total do amido e a produção de leite, e também interferindo no comportamento alimentar das vacas. No entanto, o RCG não afetou o desempenho das vacas quando comparado ao HMC. Mesmo o HMC e o RCG diferindo no perfil de fermentação, a umidade dos grãos na colheita não afetou o desempenho dos animais, sendo o RCG uma alternativa eficiente ao HMC. Benzoato de sódio reduziu a solubilidade da proteína, produtos de fermentação, desenvolvimento de microrganismos deterioradores e melhorou a digestibilidade do amidoem (%), afetando o comportamento de mastigação da vaca leiteira e aumentando a produção de leite em 0,8 kg/dia. Os objetivos do segundo ensaio foram estudar os padrões de fermentação entre as silagens inoculadas com LAB expostas a altos e baixos níveis de estresse térmico e temperaturas de ensilagem correspondentes. Os inoculantes comerciais foram aquecidos a 30 ºC (LHS) e 40ºC (HHS) por 24h e incubados a 30 ºC e 45ºC para testar o crescimento. Alguns inoculantes diminuíram o crescimento quando incubadas a 45ºC e o estresse térmico não aumentou a adaptação de alguns inoculantes, não teve diferença para inoculante 1 e aumentou o crescimento de 11 inoculantes para altas temperaturas. A alta temperatura da ensilagem diminuiu a formação de produtos finais de fermentação e aumentou o pH. HHS antes ensilagem foi eficiente na queda do pH para alguns inoculantes. No geral, a exposição prévia ao estresse térmico de culturas inoculantes de silagem produziu efeitos variados sobre o desempenho de inoculantes na cultura e no silo. Em particular, a exposição prévia ao alto estresse térmico resultou em menores valores de pH de silagem e perfis variados de fermentação quando comparados ao LHS ou controles não inoculados.

Palavras-chave: Aditivos químicos, Inoculantes microbianos, Produção de leite, Produtos de fermentação

ABSTRACT

Effect of ensiling temperature on microbial inoculants and performance of dairy cows fed corn grain silage with sodium benzoate

Silage additives are recommended for corn-based silages in tropical conditions, such as whole-plant corn silage, high moisture corn, and rehydrated corn grain, to prevent losses at storage and enhanced aerobic stability. Sodium benzoate is a salt of an organic acid and has antifungal properties and it is used as a chemical additive for silages. The mechanism of sodium benzoate to prevent the presence of undesirable microorganisms in silages is related to the undissociated form of benzoic acid, which may pass across the cell membrane and release the hydrogen in the cytoplasm. But, the influence of sodium benzoate on animal performance still unknown. Another type of additives suitable for corn-based silages is the inoculants containing lactic acid bacteria (LAB), which can be divided into two major groups, homo-fermentative bacteria and hetero-fermentative bacteria. Higher ensiling temperatures typically lead to a shift from homolactic to heterolactic microbial populations, but the majority of silage microbiological knowledge focuses on optimal fermentation conditions, rather than optimal outcomes under environmental extremes. The objective of the first study was to evaluate the performance, feeding behavior, and ruminal parameters of Holstein lactating dairy cows fed diets with HMC or RCG, as the main source of starch, added or not 0.2% fresh matter of sodium benzoate (BEN). Also, to evaluate the chemical and physical composition, the fermentative profile, and the digestibility of HMC or RCG with or without sodium benzoate. As main results in both HMC and RCG silages, sodium benzoate reduced butyric acid concentration, reduced soluble protein. Moreover, sodium benzoate increased total-tract starch digestibility and milk production, and changed the cows eating behavior. However, RCG did not affect the performance of cows when compared to HMC. Even HMC and RCG differing in fermentation profile, grain moisture at harvesting did not affect dairy cow's performance, being RCG an efficient alternative of HMC. Even HMC and RCG differing in fermentation profile, grain moisture at harvesting did not affect dairy cow's performance, being RCG an efficient alternative of HMC. Sodium benzoate reduced protein solubility, fermentation end-products, development of spoilage microorganisms and improve starch digestibility, affecting dairy cow’s chewing behavior and increasing milk yield by 0.8 kg/day. The objectives of the second trial were to study the patterns of fermentation between silages inoculated with LAB exposed to high- and low- levels of heat stress and corresponding ensiling temperatures. Commercial inoculants were heat-stressed at 30 ºC and 40ºC for 24h incubated at 30 ºC and 45ºC to test growth. Some inoculants decreased the growth when incubated at 45ºC and heat-stress did not increase the adaptation of some inoculants, had no difference for inoculant 1 and increased the growth for inoculant 11 to high temperatures. The high temperature of ensiling decreased the formation of end products and increased the pH. HHS before ensiling was efficient in dropping the pH for some inoculants. Overall, prior exposure to heat stress of silage inoculant cultures produced varied effects on the performance of inoculants in culture and in the silo. In particular, prior exposure to high heat stress resulted in lower silage pH values and varied fermentation profiles when compared to LHS or uninoculated controls

Keywords: Chemical additives, Inoculants, Milk yield, Fermentation end products

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1. INTRODUCTION

Silage production in tropical regions can be challenging in many aspects, one of those is the high temperature. High temperatures above 35 ºC can lead to the development of spoilage microorganisms, because of the insufficient production of weak acids in the silage to prevent them to grow (Bernardes et al., 2018). Moreover, clostridia showed an optimum growing temperature at 37 ºC (McDonald et al., 1991) and can grow even in temperatures above 45 ºC. In this way, silage additives are recommended for corn-based silages in tropical conditions, such as whole-plant corn silage (WPCS), high moisture corn (HMC), and rehydrated corn grain (RCG), because they are considered high-cost associated feedstuffs for animals, mainly dependent of the grain proportion.

Sodium benzoate is a chemical additive for silages, especially in warm climates, because of its effect against yeasts and other microorganisms, which are more active in an environment with high temperatures (Bernardes et al., 2015). It is a salt of an organic acid and has antifungal properties. The mechanism of sodium benzoate to prevent the presence of undesirable microorganisms in silages is related to the undissociated form of benzoic acid, which may pass across the cell membrane and release the hydrogen in the cytoplasm, causing the reduction or stopping of the microbial cell growth (Lambert and Stratford, 1999).

Another type of additives suitable for corn-based silages is the inoculants containing lactic acid bacteria (LAB), which can be divided into two major groups, homofermentative bacteria and heterofermentative bacteria. The inoculation of homofermentative LAB (*Lactobacillus plantarum; Pediococcus spp.; Enterococcus faecium*) in silages aims to the conversion of water-soluble sugars into lactic acid, strictly. On the other hand, heterofermentative bacteria (*Lactobacillus buchneri*), besides lactic acid, can produce acetic acid and other minor compounds (Muck et al., 2018). Lactic acid bacteria found in silages, on average, have the optimum growth temperature around 30 ºC, but environmental conditions in the field can reach higher temperatures, leading to a shift in population between homo and heterofermentative, and harming the effectiveness of silage inoculation (Wilkinson and Muck, 2018).

Besides fermentation, the most critical phase for any of the corn-based silages is the feed-out, when silages get in contact with oxygen, and yeasts can start the aerobic deterioration (Ranjit and Kung, 2000). As a result, there is an increase in silage pH, which can stimulate the growth of harmful microorganisms (e. g. clostridia). Therefore, it is important to use additives

as a tool to decline yeast and clostridia population in silages, avoiding the risks of transferring mycotoxins or pathogens from silage to milk (Ogunade et al., 2016).

Therefore, two studies were performed to investigate the effects of additives in WPCS, HMC, and RCG. The first study evaluated the effects of HMC and RCG, with or without sodium benzoate during ensiling, in Holstein cows' diets, attaining the animal performance and the fermentative profile of silages. HMC and RCG presented different original microorganism population previously (Carvalho-Estrada et al., 2020), which could affect the fermentative profile and the availability of nutrients for dairy cows. The second study had as objective evaluate the fermentative profile of various bacterial inoculants, previously acclimated in high temperatures and then applied to WPCS, which was also stored in an environment set at high temperatures. The premise was that the acclimatization could benefit the LAB to survive in high temperature of store conditions.

The purpose of this thesis was to evaluate strategies to enhance fermentation, minimize aerobic deterioration, and prevent the growth of undesirable microorganisms in corn-based silages, by applying sodium benzoate to high moisture corn and rehydrated corn grain, and evaluating the performance of Holstein cows. Also, evaluate the fermentative profile of whole-plant corn silages inoculated with a range of different LAB adapted to high temperatures.

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2. PERFORMANCE OF DAIRY COWS FED HIGH MOISTURE CORN OR REHYDRATED CORN GRAIN SILAGES WITH THE ADDITION OF SODIUM BENZOATE

ABSTRACT

High moisture corn silages (HMC) and rehydrated corn grain silages (RCG) are considered feedstuffs with high nutritional value for dairy cows and the use of additives, such as sodium benzoate, are indicated to avoid losses during silage fermentation and feed-out phase. The objective of this study was to evaluate the performance, feeding behavior, and ruminal parameters of Holstein lactating dairy cows fed diets with HMC or RCG, as the main source of starch, with or without 0.2% fresh matter of sodium benzoate (BEN). Also, the objective was to evaluate the chemical and physical composition, the fermentative profile, and the digestibility of HMC or RCG added or not sodium benzoate. Silages were ensiled in two-hundred liters drums, with 6 replicates, and the treatments were: HMC without the addition of sodium benzoate (HMC CON); HMC with sodium benzoate (HMC BEN); RCG without sodium benzoate (RCG CON); RCG with sodium benzoate (RCG BEN). The experimental design for the performance trial was a 4 × 4 Latin square, with a 2 × 2 factorial arrangement of treatments, where 24 Holstein cows were used, half primiparous and half multiparous, fed diets with the treatments as the main starch source. As main results in both HMC and RCG silages, sodium benzoate tended (P = 0.08) to reduce soluble protein in 6%, when compared to control treatment. Moreover, sodium benzoate increased total-tract starch digestibility (P = 0.05), by 0.5% lead to an increase (P = 0.05) of 0.8 kg/d in milk production, and changed the cows eating behavior, by increasing (P = 0.01) one more meal per day. However, RCG did not affect the performance of cows when compared to HMC. Therefore, RCG can be considered as an efficient alternative to HMC, and sodium benzoate demonstrated to be a suitable additive to these silages, by reducing protein solubility and other fermentation end-products, improving starch digestibility, and altering dairy cow's behavior, resulting in more milk yield per day.

Keywords: chemical additives, fermentation, milk yield, soluble protein

2.1. INTRODUCTION

Corn grains ensiling is a common processing method used in dairy cow's diets both in North (Ferraretto et al., 2013) and South America (Daniel et al., 2019). The main advantage of ensiling corn grains is the increase in ruminal and total-tract starch digestibility (Firkins et al., 2001). The increase in starch availability of grain silages during the fermentation process is mainly

due to bacteria (Junges et al., 2017) and it increases over time, where the greater effect occurs in the first 60 days, due to the decrease in the prolamin content (Fernandes et al., 2020).

The rehydration of corn grain for silage gained popularity mainly as a strategy in economic, agronomic, and labor aspects. High moisture corn (HMC) is harvested earlier and then rehydrated corn grain (RCG) is prepared occasionally based on dry corn grain availability which can be bought when prices are lower. Furthermore, RCG is easier than HMC due to the larger harvesting window, optimal moisture content, machine availability, and high labor demands (Daniel et al., 2019).

Although there are common characteristics between HMC and rehydrated corn grain (RCG) silages, the microbial population can differ from one to another. According to Carvalho-Estrada et al. (2020), the greater exposition of the corn grain, before rehydration (RCG), to environmental conditions, mainly lower moisture and less water activity (aw), in comparison to HMC can lead to a lower wild (epiphytic) population of *Lactobacilli* which open up the opportunity for increase undesirable bacterial population composed of greater counts of clostridia and enterobacteria.

One of the strategies to avoid the growth of undesirable microorganisms and consequently losses in corn-based silages in a warm climate is the use of chemical additives (Bernardes et al., 2018). Sodium benzoate is a salt of an organic acid and known to have antifungal properties, which the undissociated form of benzoic acid may pass across the cell membrane and release the hydrogen in the microbial cytoplasm, causing the reduction or stopping of the microbial cell growth (Lambert and Stratford, 1999). However, in grain silages where prolamin degradation is an important step to release the starch matrix, sodium benzoate at a certain dosage might have an antimicrobial activity that would impair the proteolysis during fermentation (Da Silva et al., 2015), and lead to lower starch availability. As it was reported by Junges et al. (2017) proteolysis of prolamins in silages is mainly dependent on microbial proteolytic activity.

High moisture corn and rehydrated corn grain silages are considered feedstuffs with high nutritional value for dairy cows and the use of additives are indicated to avoid losses during silage fermentation and feed-out phase. Although in some studies associating animal performance and the use of additives in grain silages, the responses of animal performance had not shown consistency and effects are still not clear (Morais, 2016; da Silva, 2016; Santos et al., 2019a; b).

Therefore, the present trial hypothesized that a different pattern of fermentation between high moisture corn and rehydrated corn grain silages could result in the different

performance of dairy cows, and the addition of sodium benzoate, at a specific dosage (0.2% fresh basis) might reduce proteolysis in both silages.

The objective of this study was to evaluate the performance, feeding behavior, and ruminal parameters of Holstein lactating dairy cows fed diets with high moisture corn (HMC) or rehydrated corn grain (RCG), as the main source of starch, with or without sodium benzoate. Also, the objective was to evaluate the chemical and physical composition, the fermentative profile, and the digestibility of HMC or RCG added or not sodium benzoate.

2.2. MATERIALS AND METHODS

The trial was conducted at the University of Sao Paulo in the experimental dairy free stall area of the Luiz de Queiroz College of Agriculture. All animal procedures followed the guidelines and were approved by the Ethics Committee for Animal Use of the University of São Paulo (protocol 2018.5.1093.11.4).

2.2.1. Ensiling

The corn hybrid (30F53, Pioneer, Goias, Brazil) was sown in the same field with 3 weeks of difference in December 2017. The interval was used to harvest the grains in two different moisture content at the same time at the end of April 2018; one on the physiological maturity, i.e. kernel black layer, when reached approximately 35% of moisture; and the other drier with moisture lower than 20%. This strategy was performed aiming to harvest both field plots simultaneously and to match a similar storage interval before offering them to the animals. At ensiling, the high moisture corn (HMC) grains were ground using a roller mill processing system (Silo press Menta, Menta, Cajuru, Brazil), and it was adjusted to crack the grains into 5 to 6 parts before ensiling. The dried grain corn source was harvested with 19% of moisture and dried in a commercial oven overnight until reach 13% of moisture and grounded in a hammer mill using a 5-mm sieve. The target for the reconstitution of the dryer corn source was set to reach the same moisture content as obtained for HMC (33%). Samples of dried grains and HMC were previously set to a lab oven for 24h at 55 ºC to determine the DM content to calculate the amount of water needed for reconstitution. The addition of water was made in a vertical feed wagon (VM4; DeLaval, Tumba, Sweden) and mixed thoroughly for 20 min.

At ensiling, HMC and reconstituted corn grain (RCG) were treated with sodium benzoate (number 532-32-1, Haofei Chemical, Zhengzhou, China) at a concentration of 0.2% (2 kg per ton) of fresh matter. It was diluted in water and added to RCG at reconstitution. The addition of sodium benzoate in HMC was made by mixing in a vertical feed wagon (VM4; DeLaval, Tumba, Sweden), 20 L/ton, for 20 min. To eliminate the interference of mixing, HMC without sodium benzoate treatment was mixed in the same vertical feed wagon with the same amount of water as HMC for 20 min. For each treatment, twenty-four plastic drums (200 L) were prepared totalizing 6,000 kg of silage and six silos per period. The silage density achieved was 1,200 ± 50 kg as fed (mean ± SD). The silages were stored in a plastic bag, positioned inside of the plastic drums and were stored for 80 days before the beginning of the first period of the animal performance trial.

2.2.2. Experimental design

The experimental design was a 4 × 4 Latin square design, balanced for carryover effect, with a 2 × 2 factorial arrangement of treatments (grain silage source and sodium benzoate), with six replicates. Each period corresponded as a 15-d period of adaptation and a 6-d period of sampling (day 16 to day 21), totalizing 21 days for each experimental period. The treatments were: HMC without the addition of sodium benzoate (HMC CON); HMC with sodium benzoate (HMC BEN); RCG without sodium benzoate (RCG CON); RCG with sodium benzoate (RCG BEN). Cows were assigned completely randomized to one of the four experimental diets. The diet composition is shown in table 01.

2.2.3. Animals and housing

Twenty-four Holstein cows were housed in a free-stall barn. The barn had an individual automatic feed system (Intergado Ltda., Contagem, Minas Gerais, Brazil), sand beds, and free access to water. Before the trial, cows showed 30.3 ± 3.8 kg/d of milk yield, 601.6 ± 82.6 kg of BW, and 85 ± 28.7 days in milk (12 primiparous and 12 multiparous). Cows were blocked by milk yield, DIM, and parity. Four multiparous rumen-cannulated cows were blocked as a group with 32 ± 7.5 kg/ d of milk yield and 78 ± 4.1 DIM for rumen parameters evaluation. Feed delivery (0700 and 1800 h) and milking (0600 and 1700 h) were twice daily. Ingredients of the diet were mixed in a vertical feed wagon (VM4; DeLaval, Tumba, Sweden) for 10 min.

Diets were offered to cows allowing 5 to 10% of orts. Dietary ingredient composition is provided in Table 1. Minerals and vitamins were supplemented following the requirements established by NRC (2001).

Table 1. Ingredients and nutrients composition of the experimental diets with corn grain stored as high moisture corn silages (HMC) or reconstituted corn grain silages (RCG), without (CON) or with sodium benzoate (BEN) (mean ± SD; n = 6 cows/ treatment)

	HMC		RCG	
Item	CON	BEN	CON	BEN
Ingredients, % of diet DM				
Corn silage	38.3 ± 0.80	38.3 ± 0.80	38.2 ± 0.80	38.2 ± 0.76
Oat haylage	10.6 ± 0.87	10.6 ± 0.87	10.6 ± 0.86	10.6 ± 0.87
Corn grain source	16.8 ± 0.14	16.9 ± 0.14	16.9 ± 0.15	16.9 ± 0.11
Citrus pulp	12.8 ± 0.08	12.8 ± 0.08	12.7 ± 0.11	12.8 ± 0.11
Soybean meal	16.1 ± 0.11	16.1 ± 0.11	16.1 ± 0.08	16.1 ± 0.11
Rumen protected soybean meal	2.8 ± 0.05	2.8 ± 0.05	2.8 ± 0.05	2.8 ± 0.05
Mineral mix[1]	2.5 ± 0.05	2.5 ± 0.05	2.5 ± 0.04	2.5 ± 0.04
Nutrients, % of DM				
DM, % as fed	48.5 ± 0.31	48.6± 0.31	48.6 ± 0.31	48.6 ± 0.34
CP	16.4 ± 0.19	16.3 ± 0.18	16.5 ± 0.17	16.5 ± 0.20
NDF	37.4 ± 1.27	37.2 ± 1.27	37.2 ± 1.25	37.2 ± 1.25
Ash	7.72 ± 0.095	7.73 ± 0.092	7.71 ± 0.111	7.69 ± 0.110
Ether extract	3.16 ± 0.079	3.23 ± 0.049	3.12 ± 0.118	3.20 ± 0.064
Starch	27.1 ± 1.00	27.7 ± 0.85	27.3 ± 1.14	27.6 ± 0.78
Nonfiber carbohydrates	42.1 ± 0.18	42.1 ± 0.17	42.2 ± 0.18	42.1 ± 0.20
Starch origin, % of total				
Corn Silage	50.8 ± 1.27	49.8 ± 1.91	50.4 ± 2.07	49.8 ± 1.89
Grain silage	44.4 ± 1.69	45.8 ± 2.07	45.0 ± 1.90	45.5 ± 2.07
Penn State Separator sieves, % retained as-fed				
19 mm	20.1 ± 6.6	22.8 ± 6.5	19.8 ± 5.4	20.7 ± 4.3
8 mm	26.5 ± 1.2	24.9 ± 0.8	24.9 ± 1.0	25.1 ± 2.2
1.18 mm	40.9 ± 4.1	41.1 ± 2.7	43.0 ± 2.1	41.1 ± 3.7
Bottom pan	12.5 ± 2.9	11.3 ± 3.5	12.3 ± 3.9	13.2 ± 2.9

[1] Mineral

2.2.4. Silage analyses

Between days 16 through 20 of each period, samples of silages were collected daily from silos of each treatment and frozen to compose a sample per period to each silo. Samples were dried at 55 ºC for 72 h and ground through a 1-mm mesh screen (Wiley mill, Arthur H. Thomas, Philadelphia, PA). Subsamples were analyzed for DM, ether extract (EE), and ash according to the Association of Official Analytical Chemists (AOAC) (1990; methods 934.01, 920.39 and 924.05, respectively). The NDF was analyzed using a fiber analyzer (Tecnal Equipamentos, Piracicaba, Brazil) according to Mertens (2002), using heat-stable amylase and sodium sulfite. Crude protein was analyzed by Dumas method 990.03 (AOAC, 2006) using a nitrogen analyzer (FP-2000A, Leco Corp., St. Joseph, MI) multiplicated by 6.25. Soluble protein was estimated from the difference between the total nitrogen of the samples and the insoluble nitrogen (Krishnamoorthy et al., 1982). Non fiber carbohydrate (NFC) was calculated as NFC = 100 − (CP + EE + ash + NDF) (Hall, 2000). Starch content was analyzed according to Hall (2009).

Twenty-five grams of each silage subsamples were added to 225 g of deionized water and mixed for 4 min in a stomacher. The extract was filtered through 3 layers of cheesecloth to measure the pH (DM 20 pH meter, Digimed Analítica, SP, Brazil). It was centrifuged at 10,000 × g for 15 min at −4°C to quantify lactic acid (Pryce, 1969) and volatile compounds. Volatile fatty acids (VFA), alcohols, and esters concentrations were analyzed using a gas chromatographer coupled to a mass detector (GCMS QP 2010 Plus, Shimadzu, Kyoto, Japan) and separated using a capillary column (Stabilwax, Restek, Bellefonte, PA; 60 m, 0.25 mm, i.d., 0.25 m). The volatile compounds were used to corrected DM content according to the equation proposed by Weissbach (2009): DMcorr (% as fed) = oven DM (% as fed) + n-alcohols (% as fed) + 2,3-butanediol (% as fed) + 0.95 × VFA (% as fed) + 0.77 × 1,2-propanediol (% as fed) + 0.08 × lactic acid (% as fed).

Approximately 400 g of each silage subsample were dried in an air-forced oven at 55 ºC for 72 h for physical analysis. Particle size distribution was measured using a RoTap Shaker (Bertel Ltda., Caieiras, Brazil) with sieves with nominal square apertures of 6.70, 4.75, 3.35, 2.36, 1.70, 1.18, and 0.6 mm and a bottom pan. Geometric mean particle size (GMPS; μm) and surface area (cm^2 /g) were calculated using a log-normal distribution (Baker and Herman, 2002). The percentage of grains below the 4.75-mm screen was also recorded.

Three kilograms of each silage in the respective period were collected and placed loosened into a polyethylene bucket. Silage was exposed to air for 10 days at a room

temperature of 25º C. Silage temperature was monitored with data loggers (Elitech Technology, model RC-5, Milpitas, CA, USA) every 30 min, placed in the geometric center of the silage mass. Silage was considered unstable when the temperature rises 2 ºC above the ambient temperature.

2.2.5. Feeding trial

Dry matter intake (DMI) was obtained during five consecutive days of each sampling period (16d - 20d) by the difference between the amount offered to the cows and orts (DM basis). During the same five days of sampling, milk yield was recorded, and samples for milk composition were collected on the second (17d) and the fourth day (20d) of sampling days for each period. Milk was collected in flasks with bromopol and analyzed for fat, protein, lactose, and urea nitrogen by mid-infrared spectroscopy (Clínica do Leite, Piracicaba, Brazil). Energy corrected milk (ECM) was calculated as ECM = (0.327 × milk yield) + (12.95 × fat yield) + (7.2 × protein yield), according to Tyrrell and Reid (1965). Fat corrected milk (3.5% FCM) was calculated following NRC (2001). Feed efficiency of cows was expressed as FCM by DMI (FCM : DMI).

During days 16 to 20 of each period, samples of orts and ingredients were collected daily and frozen to compose a sample per period. Samples were dried at 55 ºC for 72 h and ground through a 1-mm mesh screen (Wiley mill, Arthur H. Thomas, Philadelphia, PA). Subsamples were analyzed for DM, EE, ash, NDF, CP, and starch as previously described. Indigestible NDF (iNDF) was measured by ruminal *in situ* incubation for 288 h (Huhtanen et al., 1994). The particle size distribution of diets was determined on undried and unground samples using the Penn State Particle Separator (PSPS) as described by Kononnoff et al. (2003).

Fecal samples were collected in three consecutive days (d 18 to d 20) of each period for each cow following the schedule: day 1 – 0500, day 2 – 1300, and day 3- 2100. Samples were composite resulting in one sample per cow per period. Samples were dried and analyzed for DM, NDF, starch, CP, ash, starch, and iNDF as previously described. The total-tract apparent digestibility of DM, OM, NDF, CP, and starch was estimated using iNDF as a marker. Total-tract apparent digestibility was calculated according to the equation: 100 − (TMR iNDF/fecal iNDF) × (fecal nutrient concentration/TMR nutrient concentration).

Urine samples were collected in buckets via peri-vaginal stimulation in the same sampling days of feces to form a composite sample per period per cow. A 10% sulfuric acid

solution was immediately added to the urine samples (1:9) before refrigeration at 4°C. Composite urine samples were diluted 1:5 with distilled water and frozen at -20°C. Urinary creatinine concentration was used as an indicator of urinary output (Chizzotti et al., 2008) and analyzed by laboratory kits (Doles Reagentes e Equipamentos para Laboratórios Ltda, Goiânia, Goiás, Brazil). Allantoin was analyzed as described by Chen and Gomes (1992).

Individual samples of orts for each cow were analyzed for particle size distribution as described by Lammers et al. (1996). Sorting behavior was measured on d 18 of each period according to the method described by Leornardi and Armentano (2003); values equal to 100% indicated the absence of sorting, >100% indicated sorting in favor of the specific particles, and values <100% indicated sorting against the specific particles.

Feeding behavior was obtained on the same day by visual observation of chewing, eating, and ruminating, in intervals of 10 min for 24 h. Chewing time (min/d) was calculated as the sum of eating and ruminating times. A meal was defined by at least 2 consecutive 10-min ingestion events following at least 10 min of water intake, idling, or rumination.

On d 20 and d 21, ruminal fluid samples were collected from four rumen-cannulated cows every 3 h for 24 h. Samples of the solid phase from portions of the ventral rumen sac were collected and squeezed through a cheesecloth into a bucket. Ruminal pH was measure promptly with a portable pH meter (Tecnal, Piracicaba, Brazil) and a ruminal fluid sample was immediately frozen in liquid nitrogen to stop fermentation and stored at −20°C. Subsamples were prepared for VFA analyses according to Ferreira et al. (2016). Ammonia nitrogen concentration was determined by a colorimetric using the method of Chaney and Marbach (1962) adapted by Weatherburn (1967).

2.2.6. Statistical analysis

Data were analyzed using the PROC MIXED procedure of SAS (SAS Studio 3.8, SAS Institute Inc., Cary, NC). The data related to HMC and RCG silages were analyzed using the following model: $Y_{ijk} = \mu + B_i + G_j + A_k + GA_{jk} + e_{ijk}$, where μ = overall mean, B_i = random effect of period (i = 1 to 4), G_j = fixed effect of grain silage source (j = HMC or RCG), A_k = fixed effect of the additive sodium benzoate (k = CON or BEN), GA_{jk} = interaction between G and A, and e_{ijk} = residual error.

The traits related to cows performance were analyzed using the following model: $Y_{ijklm} = \mu + S_i + C_{j(i)} + P_k + G_l + A_m + GA_{lm} + e_{ijklm}$, where μ = overall mean, S_i = fixed

effect of Latin square (i = 1 to 6), Cj(i) = random effect of cow nested in Latin square (j = 1 to 24), Pk = fixed effect of period (k = 1 to 4), Gl = fixed effect of grain silage source (l = HMC or RCG), Am = fixed effect of the additive sodium benzoate (m = CON or BEN), GAlm = interaction between G and A, and eijklm = residual error. Outcomes measured over time were analyzed as repeated measures, such as data obtained from rumen cannulated cows, including hour as a fixed effect, following the model Yijklm = μ + Ci + Pj + Gk + Al + GAkl + H + GHk + AHl + GAHkl + eijkl, where μ = overall mean, Ci = random effect of cow (i = 1 to 4), Pj = fixed effect of period (j = 1 to 4), Gk = fixed effect of grain silage source (k = HMC or RCG), Al = fixed effect of the additive sodium benzoate (l = CON or BEN), GAkl = interaction between G and A, H = fixed effect of hour, GHk = interaction between G and hour, AHl = interaction between A and hour, GAHkl = interaction between G, A, and hour and eijkl = residual error. The mean square of cow, period, and treatment was used as the error to test the treatment effect. Means were considered statistically significant when $P \leq 0.05$, and trends were declared when $P > 0.05$ and ≤ 0.10.

2.3. RESULTS

The DM content corrected for volatile fraction was not different across grain silages and averaged 68.1 ± 0.3 (Table 2). The content of CP for HMC was 8.75% while RCG was 9.60% *(P* = 0.01). Soluble protein content also differed between grain silage sources (HMC = 57.6 % of CP vs. RCG = 51.6 % of CP, *P* = 0.01) and tended to be decreased by 6% with inclusion of sodium benzoate (CON = 56.3 % of CP vs. BEN = 52.9 % of CP, *P* = 0.08). Starch content and FDN did not altered across grain silage sources. Soluble CHO content was higher for RCG silages (0.94% DM) compared to HMC silages (0.71% DM) and decreased for non-treated silages (CON = 0.72% of DM vs. BEN = 0.93% DM).

The addition of sodium benzoate increased (*P* = 0.01) pH (CON = 4.24 vs. BEN = 4.29), and reduced acetic acid concentration by 19% (CON = 0.21% DM vs. BEN = 0.17% DM), ethanol concentration by 57% (CON = 0.21% DM vs. BEN = 0.09% DM), 1,2-propanediol concentration by 46% (CON = 101.7 mg/kg DM vs. BEN = 55.1 mg/kg DM), 2,3-butanediol concentration by 58% (CON = 59.9 mg/kg DM vs. BEN = 24.9 mg/kg DM), propionic acid concentration by 21% (CON = 18.6 mg/kg DM vs. BEN = 14.7 mg/kg DM), ethyl lactate concentration by 59% (CON = 71.7 mg/kg DM vs. BEN = 29.6 mg/kg DM) and

butyric acid concentration by 62% (CON = 11.27 mg/kg DM vs. BEN = 4.35 mg/kg DM) compared with nontreated silages.

Reconstituted corn grain silages increased (P = 0.01) acetic acid concentration (HMC = 0.17 % DM vs. RCG = 0.21% DM), acetone concentration was twice as high (HMC = 11.7 mg/kg DM vs. RCG = 27.2 mg/kg DM), butyric acid concentration tended (P = 0.06) to increase by 60% (HMC = 6.00 mg/kg DM vs. RCG = 9.62 mg/kg DM) and 1-propanol concentration tended (P = 0.07) to increase (HMC = 1.68 mg/kg DM vs. RCG = 2.59 mg/kg DM) compared with HMC silages. Aerobic stability was lower (P = 0.03) for HMC without benzoate (150 hours) while other silages did not differ (228 ± 23 hours).

Table 2. Composition and fermentation profile of high moisture corn silages (HMC) or reconstituted corn grain silages (RCG), without (CON) or with sodium benzoate (BEN)

	HMC		RCG			*P*-value[1]		
Item	CON	BEN	CON	BEN	SEM	G	A	G ×A
Nutrient								
Dmcorr[2], % as fed	67.8	68.1	68.2	68.4	0.18	0.13	0.19	0.84
Crude protein, % DM	8.78	8.73	9.74	9.47	0.137	<0.01	0.12	0.27
Soluble CP, % of CP	60.0	55.2	52.7	50.6	2.98	0.01	0.08	0.48
Starch, % of DM	71.4	73.2	71.4	74.1	1.72	0.79	0.15	0.77
Neutral detergent fiber, % DM	7.91	7.37	7.88	7.44	0.426	0.95	0.11	0.87
WSC[3], %DM	0.54	0.88	0.89	0.99	0.117	0.04	0.05	0.26
Fermentation profile								
pH	4.25	4.30	4.24	4.27	0.019	0.14	0.01	0.27
Lactic acid, % of DM	1.24	1.16	1.34	1.16	0.067	0.47	0.08	0.47
Acetic acid, % of DM	0.18	0.15	0.23	0.18	0.018	<0.01	0.01	0.39
Ethanol, % of DM	0.24	0.11	0.19	0.07	0.045	0.28	0.01	0.90
1,2-Propanediol, mg/kg of DM	91.0	69.6	112.4	40.6	13.08	0.78	<0.01	0.08
2,3-Butanediol, mg/kg of DM	41.0	23.3	78.9	26.5	16.03	0.12	0.01	0.18
Propionic acid, mg/kg of DM	18.5	14.4	18.7	15.0	1.04	0.70	<0.01	0.86
Ethyl lactate, mg/kg of DM	80.1	36.0	63.4	23.2	10.52	0.10	<0.01	0.82
Ethyl acetate, mg/kg of DM	15.46	3.94	15.39	4.28	2.461	0.98	0.10	0.97

Butyric acid, mg/kg of DM	8.16	3.84	14.38	4.87	2.045	0.06	<0.01	0.16
Isobutyric acid, mg/kg of DM	2.83	2.13	2.03	3.60	1.071	0.76	0.70	0.31
1-propanol, mg/kg of DM	1.45	1.92	2.31	2.88	0.553	0.07	0.29	0.92
Acetone, mg/kg of DM	9.20	14.29	23.53	30.81	7.534	<0.01	0.12	0.68
Methanol, mg/kg of DM	25.1	22.7	33.7	23.6	4.70	0.33	0.20	0.42
Isopropyl Alcohol, mg/kg of DM	0.93	0.63	0.67	0.64	0.212	0.54	0.41	0.50
Propyl Acetate, mg/kg of DM	0.69	0.51	0.66	0.65	0.209	0.79	0.64	0.68
Isovaleric acid, mg/kg of DM	3.62	4.64	3.34	4.51	1.143	0.86	0.36	0.95
Valeric acid, mg/kg of DM	3.18	1.54	1.12	0.97	0.521	<0.01	<0.01	<0.01
Aerobic stability, hours	150[b]	>240[a]	205[a]	>240[a]	10.9	0.03	<0.01	0.03

[1]Probabilities for the effects of grain silage source (G), sodium benzoate (A) and the interaction between grain moisture content and sodium benzoate (G × A). Significant differences when $P \leq 0.05$ and trends when $P > 0.05$ and ≤ 0.10.

[2] DMcorr – Dry matter corrected for silage volatile compounds, using Weissbach (2009) equation.

[3] WSC – Water-soluble carbohydrates.

Geometric mean particle size differed ($P < 0.01$) between grain types (HMC = 1909 µm vs. RCG = 1133 µm). The kernel particle size distribution was changed by grain type processing. The percentage of kernel particles retained in the sieves above 2.36 mm was higher ($P < 0.01$) for HMC compared to RCG and for kernel particles below sieve of 2.36 mm the percentage was higher ($P < 0.01$) for RCG compared to HMC.

Table 3. Physical characteristics of high moisture corn silages (HMC) or reconstituted corn grain silages (RCG), without (CON) or with sodium benzoate (BEN)

	HMC		RCG			*P*-value[1]		
Item	CON	BEN	CON	BEN	SEM	G	A	G ×A
Sieve[2],mm								
6.70	3.54	2.92	0.44	0.12	0.418	<0.01	0.28	0.73
4.75	9.48	8.94	0.59	0.58	0.729	<0.01	0.71	0.72
3.35	16.36	16.80	2.75	2.72	1.703	<0.01	0.91	0.89
2.36	24.9	24.0	10.5	11.1	1.86	<0.01	0.95	0.72
1.70	10.54	9.45	14.20	14.29	0.487	<0.01	0.31	0.23
1.18	9.67	9.88	17.86	18.54	0.448	<0.01	0.32	0.60
0.59	12.4	13.6	31.3	31.8	0.60	<0.01	0.06	0.39
Pan	13.00	14.28	22.28	20.84	1.060	<0.01	0.94	0.18
Geometric mean particle size, µm	1953	1866	1124	1142	71.8	<0.01	0.63	0.47
Surface area, sq cm/g	25.5	26.2	32.1	31.7	0.57	<0.01	0.74	0.28
Grains < 4.75 mm, %	86.8	88.1	98.9	99.3	0.97	<0.01	0.42	0.65
Grains < 1.18 mm, %	25.4	27.9	53.6	52.7	1.43	<0.01	0.50	0.14

[1]Probabilities for the effects of grain silage source (G), sodium benzoate (A) and the interaction between grain moisture content and sodium benzoate (G × A). Significant differences when $P \leq 0.05$ and trends when $P > 0.05$ and ≤ 0.10.

[2]Percentage of particles retained on each sieve (DM basis)

Total tract digestibility of DM, OM, CP, and NDF was not altered in diets with different grain silage sources (Table 4).

Table 4. Apparent total-tract digestibility (n = 6 cows/treatment) of nutrients of high moisture corn silages (HMC) or reconstituted corn grain silages (RCG), without (CON) or with sodium benzoate (BEN)

	HMC		RCG			*P*-value[1]		
Item	CON	BEN	CON	BEN	SEM	G	A	G x A
Digestibility, %								
DM	67.7	68.5	67.8	68.2	0.73	0.91	0.32	0.81
OM	71.2	71.3	70.6	71.2	0.82	0.54	0.56	0.74
CP	69.2	68.6	68.4	68.3	1.02	0.52	0.66	0.79
NDF	50.9	51.5	50.3	50.8	1.23	0.52	0.62	0.95
Starch	92.2	92.8	92.3	92.8	1.30	0.83	0.65	0.96
NDT, %	73.0	73.7	73.3	73.6	0.68	0.96	0.30	0.87

[1]Probabilities for the effects of grain silage source (G), sodium benzoate (A) and the interaction between grain moisture content and sodium benzoate (G × A). Significant differences when $P \leq 0.05$ and trends when $P > 0.05$ and ≤ 0.10.

Dry matter intake was not altered across treatments (Table 5). Cows receiving diets with grains added sodium benzoate increased milk yield ($P = 0.05$) by 0.80 kg/d compared to control silages. Although feed efficiency was not different between diets. Fat-corrected milk (kg/d) showed an interaction ($P = 0.03$) in consequence of lower milk fat content for RCG silages with sodium benzoate. Higher milk production for diets with sodium benzoate tended to improve milk protein yield ($P = 0.08$) and total solids in milk ($P = 0.08$).

Table 5. Performance of cows (n = 6 cows/treatment) fed diets with high moisture corn silages (HMC) or reconstituted corn grain silages (RCG), without (CON) or with sodium benzoate (BEN)

	HMC		RCG			*P*-value[1]		
Item	CON	BEN	CON	BEN	SEM	G	A	G x A
DMI, kg/d	23.0	23.8	23.4	23.9	0.64	0.78	0.19	0.96
Milk, kg/d	30.8	31.9	31.4	31.9	0.64	0.20	0.05	0.18
ECM, kg/d	29.4	30.2	30.5	30.2	0.97	0.11	0.34	0.14
Fat corrected milk, kg/d (3.5%)	29.0b	30.6a	30.4a	30.2ab	0.93	0.21	0.07	0.03
Fat, %	3.33	3.42	3.43	3.36	0.103	0.79	0.87	0.15
Fat, kg/d	1.031	1.087	1.080	1.064	0.0373	0.20	0.27	0.23
Protein, %	3.06	3.09	3.07	3.07	0.076	0.79	0.37	0.25
Protein, kg/d	0.943	0.979	0.967	0.971	0.0200	0.48	0.08	0.14
Lactose, %	4.55	4.57	4.57	4.57	0.031	0.64	0.57	0.62
Lactose, kg/d	1.398	1.449	1.443	1.451	0.0336	0.25	0.11	0.25
Solids, %	11.9	12.0	12.0	11.9	0.14	0.87	0.81	0.12
Solids, kg/d	3.661	3.820	3.800	3.787	0.1001	0.22	0.08	0.04
MUN, mg/dL	12.5	12.0	12.0	11.8	0.29	0.15	0.21	0.12
Feed efficiency (FCM : DMI)	1.27	1.28	1.29	1.26	0.038	0.81	0.74	0.59

[1]Probabilities for the effects of grain silage source (G), sodium benzoate (A) and the interaction between grain moisture content and sodium benzoate (G × A). Significant differences when $P \leq 0.05$ and trends when $P > 0.05$ and ≤ 0.10.

The feeding behavior observation (Table 6) revealed that cows fed diets without sodium benzoate spent less time ruminating than cows fed diets with sodium benzoate (CON= 553 min/d vs. BEN= 585 min/d). The time of ingestion was higher ($P < 0.01$) by 11.96% (CON= 242 min/d vs. BEN= 271 min/d) and the number of meals in a day was greater ($P = 0.01$) for diets with the inclusion of sodium benzoate, resulting in a higher ($P = 0.02$) total chewing time

for sodium benzoate diets (control = 822 min/d vs. benzoate = 858 min/d). The meal duration showed a trend to be higher ($P = 0.07$) by 7.77% for RCG diets (40.2 min) compared with HMC diets (37.3 min). Cows on all treatments sorted against larger particles of diet retained on the 19-mm sieve and sorted in favor of smaller particles of diet retained below 8-mm sieve. Cows fed sodium benzoate diets sorted ($P = 0.04$) against particles of 19mm-sieve and tended ($P = 0.07$) to sort in favor particles below 8-mm sieve more than cows fed diets without sodium benzoate.

Table 6. Feeding behavior of cows (n = 6 cows/treatment) fed diets with high moisture corn silages (HMC) or reconstituted corn grain silages (RCG), without (CON) or with sodium benzoate (BEN)

	HMC		RCG			*P*-value[1]		
Item	CON	BEN	CON	BEN	SEM	G	A	G x A
Fecal starch, %	7.92	7.69	7.83	7.70	0.173	0.79	0.27	0.77
Chewing behavior, min/d								
Ingestion	241	267	244	276	11.8	0.52	<0.01	0.72
Rumination	537	587	569	582	17.6	0.34	0.03	0.23
Chewing	808	861	837	855	17.6	0.42	0.02	0.25
Meals, /d	6	7	6	7	0.3	0.95	0.01	0.59
Meal duration, min	36.4	38.3	39.2	41.2	1.51	0.07	0.20	0.98
Particle sorting, %as fed								
>19mm	96.8	97.7	96.1	97.2	0.52	0.28	0.04	0.87
19-8mm	100	100	100	100	0.4	0.12	0.42	0.59
<8mm	102	101	102	101	0.3	0.90	0.07	0.78

[1]Probabilities for the effects of grain silage source (G), sodium benzoate (A) and the interaction between grain moisture content and sodium benzoate (G × A). Significant differences when $P \leq 0.05$ and trends when $P > 0.05$ and ≤ 0.10.

Cows fed HMC tended to have ($P = 0.07$) a higher molar proportion of butyrate in rumen than cows fed RCG by 10.56% and tended ($P = 0.08$) to increase molar proportions of isovalerate by 13.69% (HMC = 7.06 vs. RCG = 6.21; Table 7). Sodium benzoate diets increased total ruminal VFA concentration by 17.75% compared to non-treated silages (CON = 81.7 mmol/L vs. BEN = 96.2 mmol/L). Treatments affected excretion of purines derivatives. Cows fed diets with sodium benzoate increased the ratio allantoin:creatinine ($P = 0.02$) and allantoin

+ uric acid:creatinine (P = 0.04) by 15% compared to silages not treated (CON = 2.72 vs. BEN = 3.14; CON = 3.12 vs. BEN = 3.59; Table 8).

Table 7. Rumen fermentation profile of cows fed diets with high moisture corn silages (HMC) or reconstituted corn grain silages (RCG), without (CON) or with sodium benzoate (BEN)

	HMC		RCG			*P*-value[1]						
Item	CON	BEN	CON	BEN	SEM	G	A	G x A	H	G x H	A x H	G x A x H
Ph	5.98	6.05	6.02	6.06	0.075	0.70	0.47	0.84	<0.01	0.84	0.91	0.90
Ammonia nitrogen, mg/dL	8.86	8.41	9.85	9.06	0.955	0.35	0.47	0.84	<0.01	0.68	0.34	0.38
VFA molar proportion												
Acetate	57.7	57.9	58.0	59.3	1.06	0.42	0.47	0.60	0.43	0.11	0.38	0.18
Propionate	19.1	19.6	20.3	20.0	0.91	0.22	0.87	0.56	0.08	0.47	0.38	0.75
Isobutyrate	0.83	0.86	0.82	0.83	0.073	0.83	0.83	0.87	<0.01	0.93	0.72	0.70
Butyrate	16.6	15.8	15.1	15.2	0.66	0.07	0.41	0.33	0.30	0.48	0.78	0.67
Isovalerate	3.56	3.50	3.40	2.81	0.242	0.08	0.17	0.27	<0.01	0.95	0.90	0.96
Valerate	1.98	1.82	1.95	1.82	0.134	0.91	0.21	0.92	0.80	0.21	0.96	0.87
Acetate:Propionate	3.02	2.93	2.99	3.09	0.190	0.73	0.87	0.39	0.48	0.47	0.36	0.41
Total VFA, mmol/L	82.9	94.0	80.5	98.4	7.92	0.90	0.07	0.67	<0.01	0.82	0.32	0.76

[1]Probabilities for the effects of grain silage source (G), sodium benzoate (A) and the interaction between grain moisture content and sodium benzoate (G × A). Significant differences when $P \leq 0.05$ and trends when $P > 0.05$ and ≤ 0.10.

Table 8. Urinary purine derivatives (n = 6 cows/treatment) of cows fed diets with high moisture corn silages (HMC) or reconstituted corn grain silages (RCG), without (CON) or with sodium benzoate (BEN)

	HMC		RCG			*P*-value[1]		
Item	CON	BEN	CON	BEN	SEM	G	A	G x A
Urine, L/dia	27.4	28.5	29.2	29.4	1.45	0.33	0.63	0.72
Alla/Crea	2.54	3.12	2.89	3.16	0.218	0.30	0.02	0.39
Alla + UA/Crea	2.92	3.55	3.31	3.62	0.231	0.26	0.04	0.68
UA/Crea	0.39	0.44	0.43	0.47	0.033	0.26	0.16	0.96

[1]Probabilities for the effects of grain silage source (G), sodium benzoate (A) and the interaction between grain moisture content and sodium benzoate (G × A). Significant differences when $P \leq 0.05$ and trends when $P > 0.05$ and ≤ 0.10.

Alla = allantoin; UA = uric acid; Crea = creatinine.

2.4. DISCUSSION

The reported results partially refute the null hypothesis; even thought, the addition of sodium benzoate reduced proteolysis in corn grain silages and affected the animal performance by increasing starch digestibility and thus improving milk yield.

A trend has been observed for farmers in Brazil to use a hammer mill on processing dry corn for reconstitution while a roller mill is used for high moisture grains (Santos et al., 2016). Published studies have a variation in mean particle size even with the use of the same screen or rolling process. The GMPS of the grain corn used in the present study is within the range (500 to 4,000 μm) reported in a meta-analysis conducted by Ferraretto et al. (2013). The mean particle size of the present study was considered ground corn according to the classification of grinding degree proposed by Litherland (2006).

The moisture content of grain silage is a key factor that affects fermentation (Buchanan-Smith et al., 2003) and adequate moisture is required to enable microbial growth and activity in grain conservation (Pahlow et al., 2003). The literature has shown a wide range of moisture content for grain conservation resulting in well-fermented silage (Hoffman et al., 2011; Ferraretto et al., 2014; Morais et al., 2017). Although data in the literature suggest that moisture content above 30% is better to ensile grains and allow an increased extension of fermentation, an increment in proteolysis and, an improvement in starch degradability, a more recent target of 35% is considered a better choice of moisture content (Baron et al., 1986;

Benton et al., 2005; Gomes et al., 2020). Based on this information, we aimed to harvest HMC close to 35% moisture, achieving 33.5% ± 0.5. The reconstitution of dry corn matched similar moisture target to HMC silages, reaching 33.0% ± 0.5.

The fermentation profile values of grain silages in all treatments are in agreement with the literature for high-quality grain silages. In general, sodium benzoate decreased microbial activity in fermentation as the concentration of end products decreased. The addition of sodium benzoate resulted in a lower concentration of 2,3-butanediol, which is a product frequently associated with bacterial and clostridia fermentative pathways in silages (McDonald et al., 1991). The lactic acid bacteria ferment soluble sugars and yield lactic acid. The higher content of soluble carbohydrates for sodium benzoate treated silages might be associated with decreased fermentation pattern in the silo and less lactic acid production. The pH was higher in silages treated with sodium benzoate due to a lower concentration of lactic acid, which is a strong acid able to drop the pH of silages (Pahlow et al., 2003).

The occurrence of 1,2-propanediol has been associated with some species of heterofermentative *Lactobacillus* that can use lactate, enabling the formation of acetate and 1,2-propanediol (Oude Elferink et al., 2001). In this trial, the addition of sodium benzoate in silages decreased both 1,2-propanediol and acetic acid concentration since lactic acid tended to be lower for treated silages. Additionally, 1,2-propanediol can be metabolized into 1-propanol and propionic acid by *Lactobacillus diolivorans* (Krooneman et al., 2002), but in this trial, only propionic acid followed with lower concentration for treated silages while 1-propanol did not differ for sodium benzoate treated silages.

Low concentrations of butyric acid indicated less growth of undesirable microorganisms for silages treated with sodium benzoate, as this compound is a typical metabolite of Clostridia pathways (Rooke and Hatfield, 2003). The lower content of ethanol in treated silages might represent a lower yeast count, which is the main microorganism responsible for ethanol production in silages (Pahlow et al., 2003). Using sodium benzoate as an additive for rehydrated sorghum grain silage, Santos et al.(2019a) had less yeast counting and ethanol concentration compared to untreated silages, and the inhibition might be caused by effects on cell wall metabolism and citrate cycle enzymes (Belitz et al., 2009). Ethanol has been positively correlated with ethyl esters due to the spontaneous esterification of organic acids in silages (Weiss et al., 2016). In this trial, the concentration of ethyl lactate was reduced and ethyl acetate tended to reduce because of the lower concentration of ethanol in treated silages.

Drying corn kernels naturally in the field may affect the bacterial community during ensiling and could compromise the fermentation capacity of rehydrated corn grains (Carvalho-

Estrada et al., 2020). In this trial, RCG may have had a higher clostridia activity once butyric acid and acetone increased in comparison to HMC, in which acetone is also considered a clostridia pathway metabolite (Rooke and Hatfield, 2003). In other previous trials (Carvalho-Estrada et al., 2020; Fernandes et al., 2020; Oliveira, 2020) our research team also reported increased levels of butyric acid and other metabolites associated with Clostridia in RGS. It was claimed by (Carvalho-Estrada et al., 2020) that *Lactobacillus* strains are not easily identified in dry grain epiphytic microbial colonization which, in turn, offers the opportunity for undesirable microorganisms to grow, because of the lower natural competition from *Lactobacillus*. The typical water activity (aw) in dry grains is restrictive to *Lactobacillus* colonization. It would be a great result to identify sodium benzoate as an effective tool to lower butyric acid in RGS and offer a lower risk of diet intoxication outbreaks. Even though the concentration of butyric acid was much lower than 0.1% DM for all silages, which is the threshold for safe silages (Jobim et al., 1999).

Valeric acid is a product of protein degradation (Leek, 2006) and differed between treatments. Valeric acid was higher for HMC without the addition of sodium benzoate, which reflects on higher protein solubility for HMC silages in comparison to RCG silages. Also, water-soluble carbohydrate content was lower for HMC silages in comparison to RCG silages, implying that microbial activity in fermentation was higher in HMC silages. It has been suggested that sodium benzoate can mitigate proteolysis in grain silages (Da Silva et al., 2015). However, in sorghum grains silages, the addition of sodium benzoate did not alter protein degradation (Santos et al., 2019b). In this trial, protein solubility tended to decrease for silages with the addition of sodium benzoate.

In addition to the benefits in decrease butyric acid and ethanol concentrations, the antifungal proprieties of sodium benzoate may increase the aerobic stability of treated silages (Morais et al., 2017). In this trial, the addition of sodium benzoate enhanced aerobic stability mainly for HMC silages, even though less content of acetic acid for HMC silages was found, which is the main acid to control the beginning of deterioration because of its antifungal characteristics.

Although DMI of cows were similar across treatments, milk yield was increased for sodium benzoate treated silages by 0.8 kg/day. According to Allen (2000), when the receptors located in the rumen are stimulated by distention and/or have an increase in hepatic oxidation, the DMI can decrease. In the present study, the slightest differences in ration distribution across diets and fermentation of silages would not be enough to have changed the distention of the rumen. Besides, a similar propionate concentration across treatments would not have changed

the hepatic oxidation. Similar DMI and milk yield with low variation resulted in similar feed efficiency among diets.

The lack of difference in milk compounds content for sodium benzoate treated silages agrees with other studies that also had no difference for DMI of treated and non-treated silages (Morais, 2016; Santos et al., 2019a; b). However, the previous studies did not improve milk yield as observed in the current study. The increase in milk solids daily secretion and milk protein daily secretion of cows fed sodium benzoate diets is consistent due to the higher milk yield for the same silages with similar milk compounds content across diets. Even with no difference in milk protein content, higher allantoin : creatinine ratio and allantoin + uric acid : creatinine for diets with sodium benzoate indicates higher microbial protein.

The ruminal pH was not altered by diets even though cows fed diets without sodium benzoate sort against larger particles size (>19-mm sieves) more than cows fed sodium benzoate silages and sorted in favor of smaller particles (<8-mm particle size sieve). High-yielding dairy cows fed energy-dense rations rich in rapidly fermentable starch or sugars at high feed intake levels are particularly susceptible to decrease pH (Dijkstra et al., 2012). For this reason, we speculated that the cows fed all diets would select positively long particles (>19-mm) to attenuate the rapid ruminal digestion of starch from silages and keep the rumen with adequate pH. However, the proportions of TMR particle size at >19-mm sieve for all diets had a higher proportion (21%) compared to guidelines for the particle size of TMR for high producing dairy cows, which suggest having 2 to 8% of the particles retained on the upper sieve (Humer et al., 2018). Thus, cows had long fiber enough to maintain rumen pH even sorting against >19-mm particle size.

The increase in chewing time of cows fed sodium benzoate diets without ruminal pH differences and an increase in ruminal total VFA agrees with the slight increase in starch digestibility. It appears that more starch may have been digested in the rumen of cows fed sodium benzoate diets than cows fed control diets, which increased total VFA production. The accumulation of VFA on rumen will drop the pH and low rumen pH for prolonged periods can negatively impact farm profitability and animal welfare, although to prevent, acids have to be removed from the rumen or be buffered (Dijkstra et al., 2012). In the present study, the increase in total VFA did not affect pH because the acids may have been buffered as the increase in chewing time and rumination for cows eating sodium benzoate silages also increased.

The effect of sodium benzoate on animal performance shows an inconsistency in results in the literature with a small number of reports. The increase in one meal by day reported in this study for cows fed sodium benzoate diets contrasts with the study of Santos et al. (2019a)

who reported that cows fed the control treatment had approximately two more meals per day than the animals receiving sodium benzoate treatment. However, Santos et al. (2019b) reported no effect of sodium benzoate treatment and control treatment on the chewing behavior of cows. In the present study, the increase in one meal by benzoate treatment might have been associated with regulation of intake by the cows, although the lack of effect on DMI suggests that the sodium benzoate can split the meal size.

High moisture corn (HMC) and RCG most of the time are considered synonyms (Sprague, 2006) because moisture and anaerobic environment are present in both and are the keys to improve starch digestibility (Benton et al., 2005). Thus, there is a lack of studies in the literature that compare HMC and RCG regarding animal performance (Galyean and Vasconcelos, 2006). Despite the fermentation had differences between HMC and RCG in this study, in general, it did not affect cow performance. A similar study comparing HMC and RCG on the performance of finishing bulls reported a decrease in DMI for animals fed RCG compared to HMC, with no interference on the average daily weight gain, and consequently, the feed efficiency was slightly higher for RCG (da Silva, 2016). The authors of the previously cited study assign the variances in finishing bulls' performance to a difference in the geometric mean particle size of each silage (HMC and RCG). However, Godoi et al. (2020) reported that the diets based on HMC and RCG showed no differences in performance, with similar intake; rumen digestion kinetics; microbial efficiency; rumen pH; and ruminal, intestinal, and total tract digestibility of nutrients.

2.5. CONCLUSION

Even HMC and RCG differing in fermentation profile, grain moisture at harvesting did not affect dairy cow's performance, being RCG an efficient alternative of HMC. Sodium benzoate reduced protein solubility and fermentation end products, and improve starch digestibility, affecting dairy cow’s chewing behavior and increasing milk yield by 0.8 kg/day.

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3. INFLUENCE OF HEAT-STRESS TEMPERATURE AND ENSILING TEMPERATURE ON GROWING AND PERFORMANCE ON SILAGE OF INOCULANTS

ABSTRACT

Aims: The objectives of this trial were to study the patterns of fermentation between silages inoculated with LAB exposed to high- and low- levels of heat stress and corresponding ensiling temperatures. Methods and Results: Commercial inoculants were heat-stressed at 30 ºC and 40ºC for 24h incubated at 30 ºC and 45ºC to test growth. All inoculants showed significant inhibition of growth in liquid culture at 45˚C compared to growth at 30˚C. Inoculants 2, 3, 4, 6, 7 and 10 grew poorly regardless of prior heat stress treatments while Inoculant 11 Inoculant displayed growth at 45˚C in the HHS treatment but much greater than that of the LHS treatment at 45˚C. Heat-stressed inoculants were ensiled in whole plant corn at temperatures of 30 ºC and 45ºC. The high temperature of ensiling decreased the formation of end products and increased the pH. Inoculant 11 had the lower pH when ensiled at 45ºC and HHS before ensiling was efficient in drop the pH for Inoculants 6, 7, 10 and 11. Conclusions: Overall, prior exposure to heat stress of silage inoculant cultures produced varied effects on the performance of inoculants in culture and in the silo. In particular, prior exposure to high heat stress resulted in lower silage pH values and varied fermentation profiles when compared to LHS or uninoculated controls.

Keywords: acclimatization, heterofermentative microorganisms, homofermentative microorganisms, tropical temperatures

3.1. INTRODUCTION

Ensiling is the process of preserving forage by the conversion of carbohydrates to organic acids. Successful ensiling depends primarily on the epiphytic lactic acid bacteria (LAB) present on the crop (Pahlow et al., 2003), but it is possible to improve fermentation through the application of microbial inoculants. Inoculants facilitate the ensiling process by increasing the likelihood or degree of conservation, accelerating fermentation, increasing nutrient availability to the animal, and improving silage hygiene (Muck et al., 2018).

In the 20th century, silage additives were used largely to ensure a fermentation dominated by LAB and/or improve aerobic stability. The fermentation types of LAB are subject to some debate among microbiologists (Muck et al., 2018). But, for practical purposes, the two main types of silage inoculants include traditional homo-fermenters, such as *Lactobacillus plantarum*, *Pediococcus* species and *Enterococcus faecium*, that convert 6-carbon sugars into

one product, lactic acid. In contrast, hetero-fermentative bacteria, such as *Lactobacillus buchneri* produce multiple products.

Most LAB species found in silages have optimal growth temperatures near 30 ºC and do not grow above 45 ºC (McDonald et al., 1991). Clostridia, common anaerobic spoilage organisms, generally have higher optimum growth temperatures and may grow when temperatures are greater than 45 ºC. The relative growth conditions between desirable LAB and spoilage clostridia raise concerns regarding rising ambient temperatures and the potential effects on ensiling. Higher ensiling temperatures typically lead to a shift from homolactic to heterolactic microbial populations, but the majority of silage microbiological knowledge focuses on optimal fermentation conditions, rather than optimal outcomes under environmental extremes (Wilkinson and Muck, 2018).

Microorganisms grown under heat stress, temperatures greater than optimal, can become acclimated and more thermotolerant (Mulrooney and Kung, 2008). At the same time, higher temperatures may also induce significant cell wall damage and denaturation of ribosomes and proteins (Teixeira et al., 1997). Heat acclimatization and heat stress are not mutually exclusive, and with higher temperatures, diminishing returns from acclimatization are expected as temperatures approach the limit of an organism's heat tolerance plasticity.

Rising global temperatures and the increasing frequency of severe weather events are likely to impact industries that rely on forage preservation. Solutions to this challenge will likely include novel inoculants and procedures. Characterizing the heat tolerance of available commercial inoculants is a logical first step in developing best practices for a changing climate.

We hypothesize that heat-acclimated inoculants would better conserve forages ensiled at similarly high temperatures when compared to inoculants grown with lower levels of heat stress. Freeze-dried LAB were tested for growth at 30 ºC and 45 ºC. Surviving cultures were used to inoculate silage corn incubated at 30 ºC and 40 ºC throughout ensiling. The objectives of this trial were to study the patterns of fermentation between silages inoculated with LAB exposed to high- and low- levels of heat stress and corresponding ensiling temperatures.

3.2. MATERIALS AND METHODS

3.2.1. Inoculant cultivation

The commercial silage inoculants used in this study are described in table 9. All inoculants were purchased independently and kept frozen at -20ºC.

Table 9. Description of inoculants

Legend	Inoculant	Strain	Company	Location
Inoc 1	Pioneer 11A44	*Lactobacillus buchneri*	Pioneer Hi-bred Intl	Johnston, IA
Inoc 2	Pioneer 1132	*Lactobacillus plantarum* *Enterococcus faecium*	Pioneer Hi-bred Intl	Johnston, IA
Inoc 3	Biomax MP	*Lactobacillus plantarum* *Pediococcus pentosaceus*	Chr. Hansen Biosystems	Milwaukee, WI
Inoc 4	Ecosyl MTD/1	*Lactobacillus plantarum* *MTD/1 NCIMB 40027*	Ecosyl Products Ltd.	Stokesley, UK
Inoc 5	Crop-N-Rich	*Lactobacillus plantarum* MTD/1 NCIMB 40027	Vita Plus Corporation	Madison, WI
Inoc 6	Pioneer 11C33	*Lactobacillus buchneri* *Enterococcus faecium,* *Lactobacillus plantarum*	Pioneer Hi-bred Intl	Johnston, IA
Inoc 7	Pioneer 1174	*Lactobacillus plantarum* *Enterococcus faecium*	Pioneer Hi-bred Intl	Johnston, IA
Inoc 8	PowerStart Bacteria	*Lactobacillus lactis* *Lactobacillus plantarum* *(ABERF-1 & L-54)*	ABS Global	DeForest, WI
Inoc 9	Sunisil	*Lactobacillus plantarum* *MA1815U* *Lactobacillus buchneri NCIMB 40788*	Lallemand Animal Nutrition	France

Inoc 10	Biotal Plus	*Pediococcus pentosaceus NCIMB 12455* *Lactobacillus plantarum NCIMB 12422* *Propionobacterium fruedenreichii*	Lallemand Animal Nutrition	Milwaukee, WI
Inoc 11	Biotal Buchneri 500	*Pediococcus pentosaceus* 12455 *Lactobacillus buchneri* 40788	Lallemand Animal Nutrition	Milwaukee, WI
Inoc 12	Biotal Plus II	*Pediococcus pentosaceus NCIMB 12455* *Propionobacterium fruedenreichii NCIMB R2453*	Lallemand Animal Nutrition	Milwaukee, WI

Freeze-dried inoculants were hydrated in four mL of ultra-pure dH20, following the recommendation of each manufacturer, and vortexed to mix. After 30 min, 100 µL were transferred to two milliliters of autoclaved MRS broth in quadruplicate. Inoculants composed of more than one species of LAB were treated identically to single-species inoculants and grown in co-culture. The tubes were incubated at 25 ˚C on a shaker at 150 rpm overnight. The cultures were diluted 10^4-fold and made a plate for each tube. The plates were incubated at 25 ˚C and after 72 h colonies were checked for morphological features consistent with LAB, and then were streaked for isolation on subsequent plates.

Single colonies from each isolation streak plate, or consortia for multi-species inoculants, were picked and placed in 15 mL MRS broth and incubated at temperatures 30 ˚C, 35 ˚C, 40 ˚C, and 45 ˚C and pH values of 6 and 4. Inoculants selected were those that grew well at 40 ˚C.

3.2.2. Heat-stress procedures

Selected colonies of consortia were grown in 6 replicate MRS broth tubes and incubated at 25 ˚C with 150 rpm shaking for 72 h. 100 µl of each culture was transferred to 15 mL of MRS broth in preparation for the heat stress portion of the experiment. Cultures were

exposed to two heat-stress temperatures for 24h before application to silage. Three tubes of each culture were heat-acclimated at 30˚C for the low heat-stress (LHS) treatment. The remaining three tubes of each culture were heat-acclimated at 40˚C for the high heat-stress (HHS) treatment. Cultures were incubated for 24 hours at their respective temperatures in an orbital shaker at 150 rpm.

Following heat-stress incubation, 200 µl of each culture were divided into duplicate 10 mL tubes of MRS broth to test the growth of both LHS and HHS cultures at higher and lower temperatures. All cultures were incubated at both 30˚C and 45˚C for 24h in orbital shakers at 150 rpm. Optical density at 600nm (OD600) was determined by spectrophotometry (Spectronic 21, Bausch & Lomb, Canada) to estimate bacterial cell density.

3.2.3. Effects of cryopreservation on HHS culture performance

To ascertain the effects of cryopreservation on any potential heat acclimatization of HHS cultures, one milliliter of each culture was collected and preserved in 20% glycerol and stored at -80 ˚C. After 30 days at -80 ˚C, cultures were revived in 10 ml MRS media broth and incubated at 45˚C. OD 600 was determined after 24 h for comparison to growth kinetics prior to cryopreservation.

3.2.4. Ensiling

Silage corn used for ensiling was the variety Dairyland 3508RA grown at the USDFRC Research Farm in Prairie du Sac, WI and was harvested at 38% dry matter. Chopped fresh corn was inoculated with 100 µL each LHS and HHS inoculant. Silos were prepared in duplicate, one stored at 30˚C and other at 45˚C. Laboratory silos were prepared in 20 mL screw top vials packed with approximately 20 g of inoculated corn each for 30 days. Ensiling treatments were: 1) LHS inoculant ensiled at 30˚C, 2) HHS inoculant ensiled at 30˚C, 3) LHS inoculant ensiled at 45˚C, 4) HHS inoculant ensiled at 45˚C, with three replicates per treatment. Additionally to commercial inoculants, a control silage was prepared with no inoculant added. All vials were weighed before and after storage to calculate dry matter loss by the difference of weight.

After 30 days, silos were opened and a subsample was collected and dried in a forced-air oven for 72 h at 55 ˚C to measure dry matter (AOAC, 1990; method 934.01). 5 g subsample

were weighed, added to 55 g of deionized water, and mixed for 1 min in a blender. The resulting water extracts were filtered with a Whatman P8 filter and the pH was measured on an Orion economy pH meter (Thermofisher, Waltham, MA, USA). Water extracts were also prepared for HPLC (Shimadzu, Kyoto, Kyoto, Japan) determination of fermentation end products analysis including lactic acid, acetic acid, propionic acid, butyric acid, ethanol, 1,2 propanediol, and isobutyrate (modified from Siegfried et al., 1984). To determine the degree of protein hydrolysis, the ninhydrin colorimetric method was employed to quantify amines in conjunction with a standard leucine standard (Winters et al., 2002). Degree of proteolysis was calculated from the difference in detected amines before and after ensiling.

3.2.5. Statistical analyses

Inoculant growth was evaluated via ANOVA, with three factors (heat-stress temperature: 30 ˚C or 40 ˚C (S); incubation temperature: 30 ˚C or 45 ˚C (IT); inoculants (I)) using the program R (RStudio, version 1.3.959, agricolae package). The cryopreservation for treatment HHS incubated at 45˚C was included in the model considering a heat-stress factor. The statistical analyses for ensiling parameters were evaluated via ANOVA with three factors ((heat-stress temperature: 30 ˚C or 40 ˚C (S); ensiling temperature: 30 ˚C or 45 ˚C (E); inoculants (I)). All means were tested by Tukey's and the significance was considered at $P < 0.05$. The figures were created using the ggplot2 package from R.

3.3. RESULTS

The inoculants selected to test acclimatization was based on inoculants that grew strongly at 40 ˚C as shown in Table 10 (Inoculants 1 through 12).

Table 10. Evaluation of growth at different temperatures and pH

Inoculant	1	2	3	4	5	6	7	8	9	10	11	12	Control
30˚C	+	+	+	+	+	+	+	+	+	+	+	+	-
35˚C	+	+	+	+	+	+	+	+	+	+	+	+	-
40˚C	+	+	+	+	W	+	+	W	W	+	+	W	-
45˚C	+	W	W	W	-	W	W	-	-	W	+	-	-
pH 6 @ 40˚C	+	+	+	W	W	+	+	W	W	+	+	W	-
pH 4 @ 30˚C	+	+	+	+	+	+	+	+	+	+	+	+	-

+, 90% or more of the strains positive; -, 90% or more of the strains negative; W, weakly positive.

As expected, inoculants performed better when incubated at 30˚C than when incubated at 45˚C. Cell density of LAB after 24 hours growth among treatments and following cryopreservation of HHS cultures are compared in Figure 01. All inoculants showed significant inhibition of growth in liquid culture at 45˚C compared to growth at 30˚C. However, there is a slight numerical trend for inoculants 1, 3, 4, and 11 of increased growth at 30˚C after HHS. Growth patterns for heat-stress by growth temperature interactions are more complex. Inoculants 2, 3, 4, 6, 7 and 10 grew poorly regardless of prior heat stress treatments. Inoculant 1 tolerated 45˚C cultivation with higher growth than previously mentioned inoculants, but no difference between HHS and LHS treatments. Inoculant 11 displayed growth at 45˚C similar to it's performance at 30˚C in the HHS treatment, but much greater than that of the LHS treatment. HHS culture samples cryopreserved in 20% glycerol at -80˚C revived and cultured in MRS for 24 h at 45˚C performed similarly to HHS cultures prior to cryopreservation, including Inoculant 11.

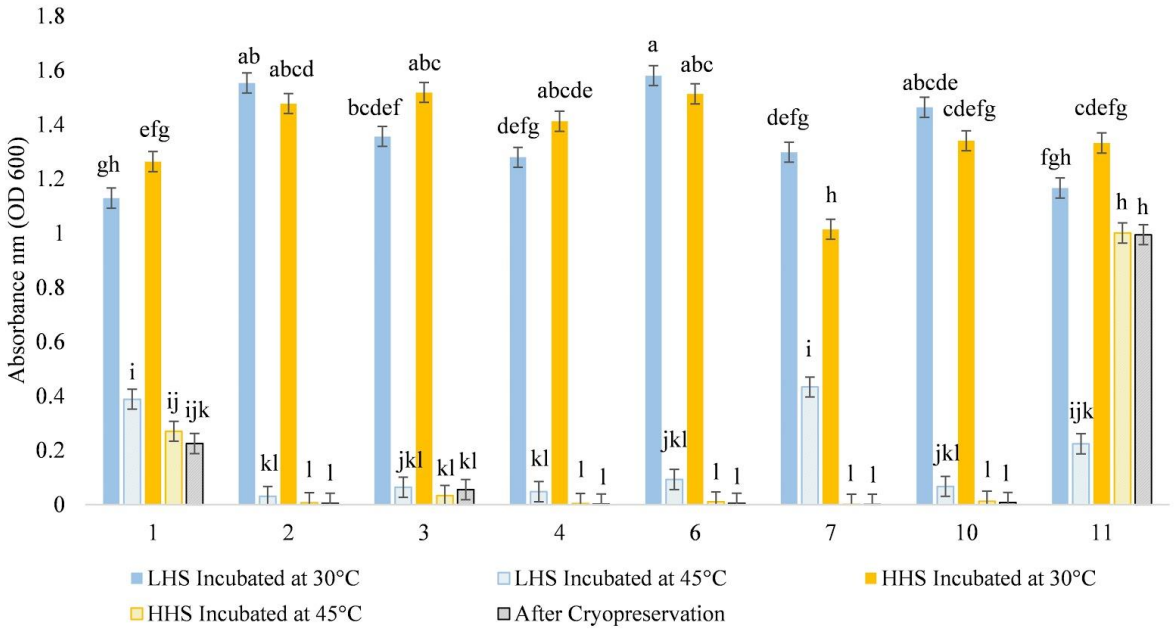

Figura 1. Effect of low (30°C) and high (40°C) heat-stress on microbial cell density as determined by absorbance of inoculant cultures at 600nm (OD600) following incubation at 30°C or 45°C after 24h. Gray bars represent the cell density of freeze-dried HHS samples revived and cultured directly into 45°C MRS broth. $^{*}p < 0.01$ for inoculant × heat stress temperature x incubation temperature interaction. Standard error of the mean = 0.11.

Silages showed clear differences in quality between ensiling temperatures of 30°C and 45°C (Figure 02). Forages ensiled at 45°C showed a nearly uniform response of significant increases in final pH and DM loss during ensiling. Notably, samples inoculated with HHS, Inoculant 11, and ensiled at 45°C showed equivalent pH and DM loss values as seen when ensiled at 30°C. Silage pH values between HHS and LHS inoculants were largely indistinguishable at 30°C, but at 45°C HHS inoculants 6, 7, 10, and 11 silages had significantly lower pH values. Dry matter loss among samples showed the most variability from inoculant to inoculant, with some non-systematic differences by ensiling temperature. HHS inoculants 1, 4, and 7 produced significantly improved DM recovery in silages at 45°C when compared to LHS inoculated silages. LHS inoculants 1 and 7 showed a significant increase in DM loss when compared to ensiling at 30°C.

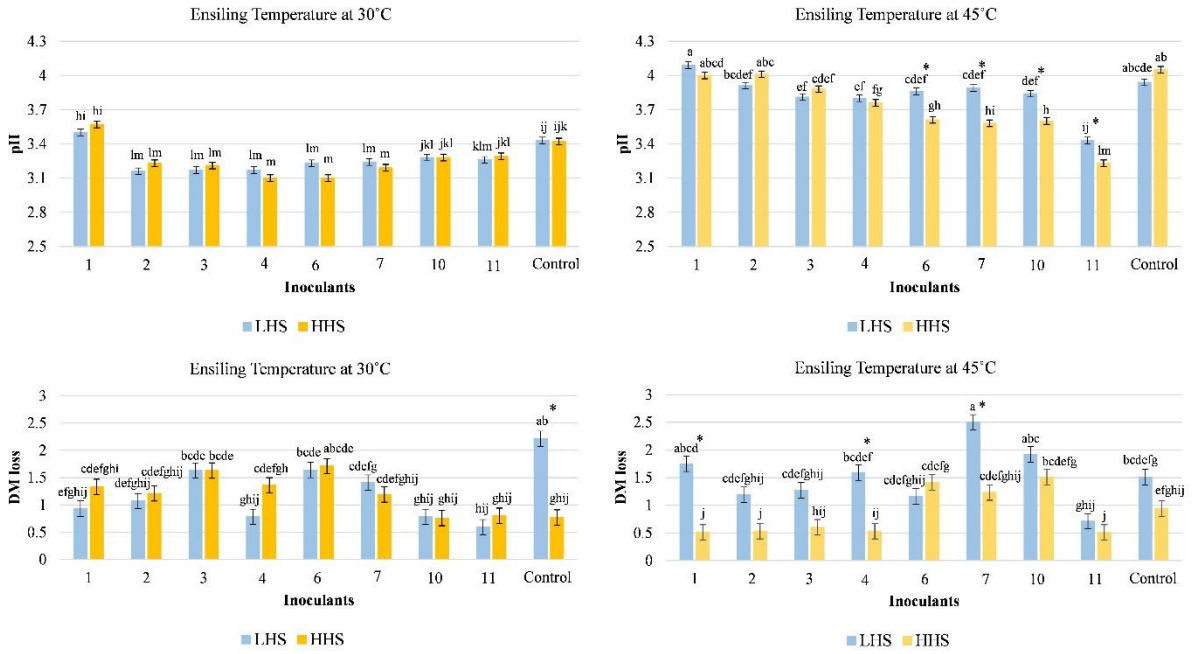

Figura 2. Effects of low (30°C) and high (40°C) heat-stress on silage pH and DM loss when ensiled at 30°C or 45°C after 30 days. pH: p <0.01 for inoculant × heat stress temperature x ensiling temperature interaction. Standard error of the mean = 0.03. DM loss: p <0.01 for inoculant × heat stress temperature x ensiling temperature interaction. Standard error of the mean = 0.14. Asterisks denote statistical differences between LHS and HHS responses for each inoculant.

Fermentation profiles of inoculated silages revealed significant shifts across heat-stress treatment of inoculants, ensiling temperature, and inoculants (Table 11). Overall, ensiling at 45°C uniformly decreased concentrations of lactic acid, acetic acid, and ethanol as determined by HPLC. Lactic:acetic acid ratios were variable in the study, with variation driven by changes in both lactic and acetic acid concentrations. HHS inoculants 1, 7, and 11 revealed estimates of protein degradation via ninhydrin assay were increased over T0 control values. Overall, patterns of difference across ensiling temperatures and heat-stress treatments were not uniform. In general, LHS and HHS inoculants produced similar ninhydrin values at 45°C, but HHS inoculants 1, 2, 3, 6, and 7 all displayed significantly lower ninhydrin values at 30°C.

Table 11. Effects of low (30˚C) and high (45˚) heat-stress on silage quality when inoculants were ensiled at 30˚C or 45˚C after 30 days.

Inoculant	Ensiling Temperature	*Heat-stress	‡Lactic acid, %DM	†Acetic acid, %DM	Propionic acid, %DM	†Isobutyrate, g·kg-1 DM	†Ethanol, %DM	‡⊿Ninhydrin, g·kg-1 DM
		LHS	2.95j	5.81a	0.10	0.03f	0.12efgh	2.69abc
	30˚C	HHS	2.85j	4.30b	0.03	0.01jklm	0.12efgh	1.13defg
		LHS	0.72k	0.63efgh	0.13	0.02hijkl	0.03hf	1.36defg
1	45˚C	HHS	1.02k	0.48efghijk	0.02	0.05e	0.04gh	1.04defg
		LHS	6.13a	0.63efg	0.15	0.01lmn	0.46b	3.05[a]
	30˚C	HHS	5.13bcdef	0.63efgh	0.10	0.03fgh	0.33bcd	0.70fg
		LHS	1.05k	0.35jk	0.13	0.01lmn	0.04gh	1.64bcdef
2	45˚C	HHS	1.06k	0.35jk	0.04	0.05de	0.04gh	1.33defg
		LHS	5.18abcdef	0.66ef	0.26	0.03fghi	0.62a	2.72ab
	30˚C	HHS	4.24fgh	0.66ef	0.07	0.02ghij	0.34bcd	0.77efg
		LHS	1.29k	0.33jk	0.14	0n	0.03h	1.32defg
3	45˚C	HHS	1.06k	0.24jk	0.05	0.06cd	0.04gh	1.06defg
		LHS	6.04ab	0.57efghij	0.13	0.01jklm	0.44b	0.65fg
	30˚C	HHS	5.32abcde	0.71e	0.07	0.01jklm	0.38bc	0.87efg
		LHS	1.29k	0.32jk	0.14	0.02ghijk	0.03h	0.90efg
4	45˚C	HHS	0.75k	0.45fghijk	0.07	0.02ghijkl	0.02h	1.02defg
		LHS	5.57abc	0.54efghijk	0.18	0.03fghi	0.45b	1.88bcde
	30˚C	HHS	5.66abc	0.61efghi	0.03	0.02ghijk	0.26cde	0.61fg
		LHS	1.04k	0.33jk	0.15	0.03f	0.03h	0.89efg
6	45˚C	HHS	1.33k	0.33jk	0.03	0.05de	0.03h	1.08defg
		LHS	4.57defgh	0.50efghijk	0.11	0.01jklm	0.42b	1.62bcdef
7	30˚C	HHS	4.82cdefgh	0.51efghijk	0.13	0.03f	0.46b	0.31g
		LHS	0.82k	0.40ghijk	0.08	0.02ijkl	0.05fgh	1.57cdef
	45˚C	HHS	1.30k	0.31k	0.12	0.06bc	0.04gh	0.81efg
		LHS	5.50abcd	0.66ef	0.07	0.01mn	0.37bc	1.64bcdef
	30˚C	HHS	4.11gh	0.67ef	0.08	0.03fg	0.15efgh	0.61fg
		LHS	1.23k	0.42fghijk	0.11	0.02hijkl	0.04fgh	1.17defg
10	45˚C	HHS	1.16k	0.39ghijk	0.03	0.06bcd	0.03gh	1.25defg
		LHS	5.06cdefg	2.02c	0.11	<0.01mn	0.26cde	2.10abcd
	30˚C	HHS	4.23fgh	0.73e	0.04	0.01jklm	0.20def	1.19defg
		LHS	2.51ij	0.48efghijk	0.11	0.03f	0.05fgh	0.84efg
11	45˚C	HHS	3.13j	0.38hijk	0.03	0.07ab	0.03h	1.52def
		LHS	4.50efgh	1.30d	0.05	0.01klmn	0.19defg	0.87efg
	30˚C	HHS	4.06hi	1.14d	0.03	0.01jklm	0.15efgh	0.90efg
		LHS	0.86k	0.37ijk	0.10	0.03f	0.04gh	0.97defg
Control	45˚C	HHS	0.80k	0.43fghijk	0.03	0.08a	0.03h	1.26defg

SEM		0.173	0.140	0.030	0.002	0.028	0.205
	I x S	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	I x E	<0.01	<0.01	0.43	<0.01	<0.01	<0.01
	S x E	<0.01	<0.01	0.62	<0.01	<0.01	<0.01
	I x S x						
P-value	E	0.07	<0.01	0.59	<0.01	<0.01	0.06

*LHS = low heat-stress (30˚C). HHS = high heat-stress (40˚C).

† Data are means of three samples, means in the same column that share letters are not significantly different (P < 0.05) for interaction of inoculant, heat-stress temperature and ensiling temperature.

‡ Data are means of three samples, means in the same column that share letters are not significantly different (lactic acid: p = 0.07; Ninhydrin difference: p = 0.06)) for the interaction of inoculant (I), heat-stress temperature (S) and ensiling temperature (E).

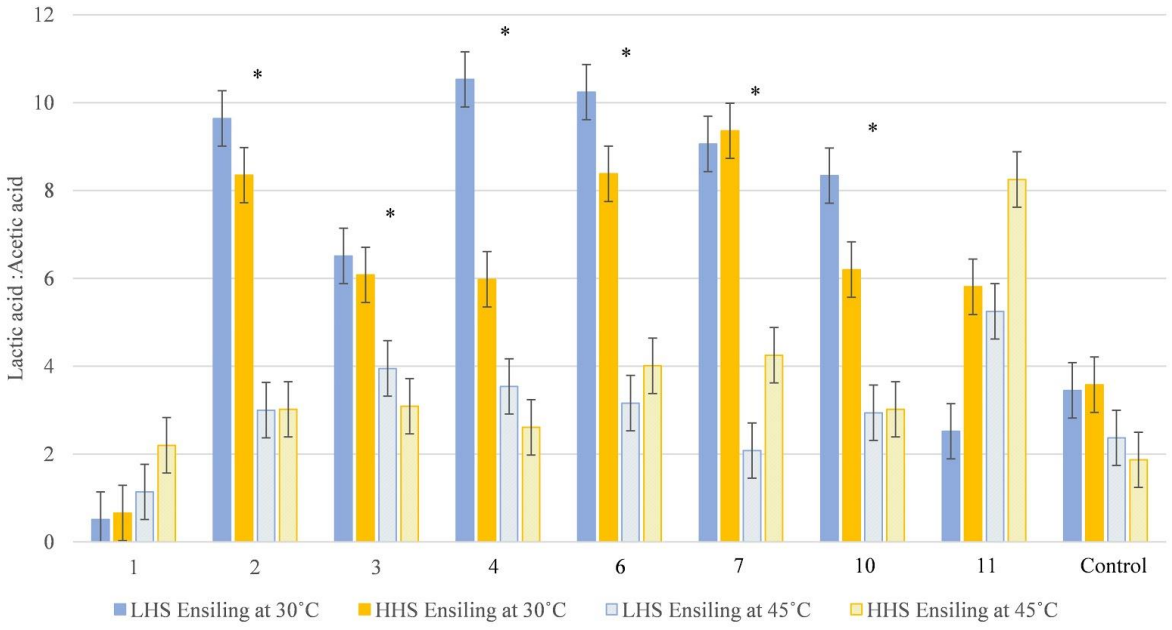

Figura 3. Effects of low (30°C) and high (40°C) heat-stress on silage lactic acid:acetic acid ratio when ensiled at 30°C or 45°C after 30 days. Asterisks denote statistical significance for interaction Inoculants x Ensiling temperature (p <0.01). p-values were: p = 0.21 for Inoculant x Heat-stress temperature x Ensiling temperature interaction; p <0.01 for Inoculant x Heat-stress temperature interaction; p <0.01 for Inoculant x Ensiling temperature interaction; p <0.01 for Heat-stress temperature x Ensiling temperature interaction.

3.4. DISCUSSION

High temperature is an important factor limiting the benefits of silage LAB inoculation in tropical and subtropical locations due to heat stress inhibition (McDonald et al., 1991). Successful use of inoculants is dependent on the species/strain chosen and dosage of viable

bacteria (Mulrooney and Kung, 2008). The temperature within a silage pile, particularly in the tropics and subtropics, may rise to more than 40°C at the start of ensiling due to oxygen contained within the forage matrix supporting plant and microbial respiration (Bernardes et al., 2018). Higher ensiling temperatures decrease silage quality, typically by limiting acidification through decreased organic acid production and increased volatilization (Kim and Adesogan, 2006). These conditions promote spoilage losses and are particularly favorable to clostridial growth (McDonald et al., 1966), which can have impacts on animal health, production, and human food safety. Ohmomo et al. (1996) suggested that poor silage quality, even after LAB inoculation, may be due to high temperatures (42°C or above) reached during the early stages of ensiling since rapid early fermentation and acidification is critical to final silage quality (Muck et al., 2003).

Heat-resistant LAB have been proposed as a method to enhance silage fermentation in warmer climates due to the impared growth of many LAB at higher temperatures (Chen et al.,2013; Gulfam et al., 2017). Acclimatization, or prior exposure to stress, can change the response of microorganisms to subsequent stress events (Mulrooney and Kung, 2008). The mechanism of induced thermotolerance is unknown, but growth conditions, such as pH play a role in determining response to heat stress (Ahmad, Smith and Mahboob, 2002). At temperatures above optimum, bacteria respond to thermal stress by rapid induction of heat-shock proteins to help with adaptation (Gould, 1989). Despite many lactic acid bacteria having growth temperature optima between 25°C and 40°C (Pahlow et al. 2003), specific data on thermotolerance of silage inoculant bacteria are lacking.

LAB grown at higher temperatures in the present study showed generally lower growth in MRS broth, likely due to heat-induced cell death or injury, but the extent varied by inoculant. The effects of prior heat-stress exposure and subsequent growth in liquid culture reveal complex interactions with temperature. For the majority of inoculants, growth at 45˚C was poor regardless of prior heat stress, but inoculants 1, 7, and 11 were notable exceptions. Inoculant 7 showed somewhat improved performance at 45˚C in the LHS treatment over HHS. In contrast, inoculant 11 appeared to benefit strongly from prior HHS exposure with significantly higher growth than LHS cultures at either 30˚C or 45˚C. These shifts may be illustrative of differences in heat acclimatization and tolerance strategies of the cultured organisms.

Inoculants 1, 7, and 11 showed a higher tolerance for growth at 45˚C. Inoculants 1 and 11 share *L. buchneri* as a component species, however, inoculant 6 also contains *L. buchneri* and did not show a similar effect. Chen et al. (2013) observed higher tolerance to high temperature in heterofermentative strains, like *L. buchneri*, but this does not explain the

discrepancies of inoculants 6 and 7, which contains no heterofermenters. Mulrooney and Kung (2008) found both *L. plantarum* (MTD/1) and *L. buchneri* 40788 appeared to have better heat tolerance after heat shock (45˚C) than the other organisms, but similar results were not seen uniformly in this study.

Interestingly, HHS inoculants performed identically prior to and following cryopreservation. In the case of inoculant 11, this is particularly significant due to its unique performance after HHS treatment. The industry standard for inoculant processing and storage is freeze-drying. The response of inoculant 11 following cryopreservation provides initial evidence that the effects of heat acclimatization may be transferable after processing and storage. The effects of freeze-drying and time in storage on these effects should be the subject of future work.

Small decreases in viability of LAB would most likely result in the inability of the added LAB to dominate a silage fermentation process (Mulrooney and Kung, 2008). Growth in liquid culture did not appear to be predictive of poor performance of silage fermentation. In particular, even with a decrease in cell density for HHS cultures incubated at 45°C, Inoculant 7 was able to promote a good fermentation with lower pH and DM loss than LHS cultures incubated at 45°C. The lactic acid : acetic acid ratio close to 1 : 1 suggested heterolactic fermentation (Zhou et al., 2016). But the lower values for silages ensiling at 45°C in this study is associated with the decrease in lactic acid production in these silages. Adesogan (2006) also reported that corn silage stored at 40°C underwent a restricted fermentation with more proteolysis and lower lactic:acetic ratio than silage stored at 20°C. Inoculant 11 was an exception to this, as the amount of lactic acid decreased less than other inoculants, which performed similarly to ensiling at 30°C.

Temperature can play an important role in the fermentation profile of silages (Bernardes et al., 2018). In literature, high ensiling temperatures have been shown to limit fermentation (Zhang et al., 2000, Zhou et al., 2016, Guan et al., 2020). These results were consistent with the present study, in which the 45°C silages produced less lactic acid, resulting in a higher pH compared to 30°C silages. High ensiling temperatures can also result in butyric acid fermentation, which is also an indicator of clostridial fermentation, and increased proteolysis in silage (Wieringa 1960; Rooke and Hatfield, 2003; Zhang et al. 2010; Liu et al. 2011). Decreased ethanol in warmer silages could represent lower yeast content, but also may be associated with volatilization or drier environment conditions that were not appropriate for yeasts. (Pahlow et al., 2003)

3.5. CONCLUSION

Overall, prior exposure to heat stress of silage inoculant cultures produced varied effects on the performance of inoculants in culture and in the silo. In particular, prior exposure to high heat stress resulted in lower silage pH values and varied fermentation profiles when compared to LHS or uninoculated controls. Heat acclimatization is a largely unexplored facet for silage inoculant optimization. However, the results of the current study provide initial evidence that expanded, and more in-depth future work is warranted and of both industrial and scientific value.

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