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EFFECT OF *Lactobacillus* AND *Bacillus subtilis* ON THE FERMENTATIVE PROCESS OF CORN SILAGE AND PERFORMANCE OF BEEF CATTLE AND SHEEP

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Advisor: Prof. Dr. Ricardo Andrade Reis

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CERTIFICADO DE APROVAÇÃO

TÍTULO: EFFECT OF *Lactobacillus* AND *Bacillus subtilis* ON THE FERMENTATIVE PROCESS OF CORN SILAGE AND PERFORMANCE OF BEEF CATTLE AND SHEEP

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Jaboticabal, 26 de agosto de 2016.

DADOS CURRICULARES DO AUTOR

CARLOS HENRIQUE SILVEIRA RABÊLO - filho de Carlos Henrique Rabelo e Maria Ambrosina Silveira Rabelo, nasceu em Três Pontas - MG, em 09 de setembro de 1987. Ingressou no curso de Zootecnia no ano de 2006 pela Universidade José do Rosário Vellano/UNIFENAS, Instituto de Ciências Agrárias, *campus* de Alfenas. Foi coordenador do Núcleo de Estudos em Pastagem e Forragicultura/NEPAR (2009 a 2010), bolsista do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) durante o período de Agosto de 2007 a Julho de 2010, obtendo o título de Zootecnista em dezembro de 2010. Em março de 2011 ingressou no curso de Pós-graduação, Mestrado em Zootecnia, pela Universidade Estadual Paulista "Júlio de Mesquita Filho"/UNESP, Faculdade de Ciências Agrárias e Veterinárias, *campus* de Jaboticabal - SP, obtendo o título de Mestre em Zootecnia em fevereiro de 2013. Durante este período, o aluno foi bolsista do CNPq e, posteriormente da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). Em março do mesmo ano ingressou no curso de Pós-graduação, Doutorado em Zootecnia, pela UNESP, Faculdade de Ciências Agrárias e Veterinárias, *campus* de Jaboticabal - SP, onde atualmente é bolsista da FAPESP. Durante um período do curso de Doutorado (01 de outubro de 2014 a 30 de maio de 2015), o aluno realizou estágio no Agriculture and Agri-Food Canada (Lethbridge, Canada) pelo programa de Bolsa Estágio de Pesquisa no Exterior (BEPE) concedido pela FAPESP.

DEDICATÓRIA

Dedico esta tese como parte dos meus esforços e de minha vida a Deus, em razão Dele, em sua imensa bondade, permitir que seu único filho Jesus Cristo concedesse sua vida para que toda humanidade tivesse o privilégio de viver. Dedico a Deus também por me conceder uma vida plena e por iluminar meu caminho, concedendo-me inúmeras bênçãos ao longo de minha vida.

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A todos vocês, meu MUITO OBRIGADO!

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto intitulado **"Action of lactic acid bacteria and *Bacillus subtilis* on the fermentative process of corn silages and their effect on the animal performance"**, protocolo nº 8.002/16, sob a responsabilidade do Prof. Dr. Ricardo Andrade Reis, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de junho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 06 de julho de 2016.

Vigência do Projeto	01/05/2013 a 31/10/2016
Espécie / Linhagem	Bovinos de corte cruzados / Ovinos
Nº de animais	36 / 6
Peso / Idade	316 - 2 anos / 74,5 – 4 anos
Sexo	Machos
Origem	Animais adquiridos em propriedade privada

Jaboticabal, 06 de julho de 2016.

Profª Drª Lizandra Amoroso
Coordenadora – CEUA

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EFFECT OF *Lactobacillus* AND *Bacillus subtilis* ON THE FERMENTATIVE PROCESS OF CORN SILAGE AND PERFORMANCE OF BEEF CATTLE AND SHEEP

ABSTRACT - Our objective was to determine the impact of *Lactobacillus* and *Bacillus subtilis* as silage additives on the quality of corn silage and their effects on the performance of beef cattle and sheep. For that, three studies were carried out. The first study investigated the effect of *L. buchneri* as a silage inoculant or probiotic on in vitro ruminal fermentation of corn silage. The experiment was carried out under a 2 × 3 factorial arrangement, with two silages (untreated or treated with *L. buchneri*) as substrate combined with three ruminal fluids obtained from the wethers consuming the three different diets described in the second study. The results appointed that *L. buchneri* as a silage inoculant alters fermentation patterns of corn silage leading to an increase in gas production over time; however, this increase is not accompanied by increased organic matter digestibility or total volatile fatty acids concentration. Conversely, the utilization of *L. buchneri* as a probiotic appears to have a greater impact on fermentation end products than in vitro gas production, particularly during earlier stages of fermentation (i.e., up to 9 h of fermentation). The second study investigated the effect of *L. buchneri* as a silage inoculant or probiotic in wethers. Six cannulated-wethers were arranged in a double 3 × 3 Latin square of three 19-d periods, and they were fed a total mixed ration *ad libitum* using a 70:30 forage:concentrate ratio. Diets were composed of 1) untreated corn silage; 2) inoculated corn silage; and 3) untreated corn silage with a daily dose of *L. buchneri* applied directly into the rumen of the wethers. *L. buchneri* as a silage inoculant led to changes in fermentation and chemical composition of corn silage, and increased dry matter intake with additional minor shifts in the relative proportion of *Ruminococcus flavefaciens* and ruminal fermentation of wethers. Conversely, *L. buchneri* as a probiotic led only to a minimal shift in the relative proportion of *R. flavefaciens*. The third study investigated the effect of combining *Lactobacillus plantarum* either with *Lactobacillus buchneri* (LBLP silage) or *B. subtilis* (BSLP silage) for corn silage on the growth performance of finishing feedlot beef cattle. Thirty six Nellore × Brown Swiss crossbred bulls (initial body weight of 316 ± 33.9 kg) were fed a total mixed ration *ad libitum* using a 40:60 forage:concentrate ratio for 89 d post-adaptation. Bulls (n = 12) were distributed in a completely randomized design for one of three diets containing untreated, LBLP and BSLP corn silage. Silage inoculation unaffected feed intake and growth performance of bulls, but depressed apparent digestibility of the diets. Overall, the results of the three experiments revealed that bacterial inoculants were not able to improve the fermentation process of corn silage and the performance of animals fed corn silage-based diet.

Keywords: aerobic stability, animal performance, bacterial inoculants, silage quality

EFEITO DO *Lactobacillus* E *Bacillus subtilis* SOBRE O PROCESSO FERMENTATIVO DE SILAGEM DE MILHO E DESEMPENHO DE GADO DE CORTE E OVINOS

RESUMO - O nosso objetivo foi determinar o impacto do *Lactobacillus* e *Bacillus subtilis* como aditivos sobre a qualidade da silagem de milho e desempenho de gado de corte e ovinos. Para isso, três experimentos foram conduzidos. O primeiro estudo investigou o efeito do *L. buchneri* como inoculante para silagem ou probiótico sobre a fermentação ruminal da silagem de milho em condições in vitro. O experimento foi conduzido em um esquema fatorial 2 × 3, sendo duas silagens (não inoculada ou inoculada com *L. buchneri*) como substratos combinadas com três fluidos ruminais obtidos a partir dos ovinos que consumiram as três diferentes dietas descritas no segundo estudo. Os resultados apontaram que o uso do *L. buchneri* como inoculante para silagem alterou os padrões de fermentação da silagem de milho aumentando a produção de gás em todos os tempos avaliados. No entanto, este acréscimo não foi acompanhado do aumento na digestibilidade da matéria orgânica ou concentração total de ácidos graxos voláteis. Por outro lado, a utilização do *L. buchneri* como probiótico parece ter um maior impacto sobre os produtos finais da fermentação in vitro do que sobre a produção de gás, sendo estes resultados mais contundentes em tempos mais curtos de fermentação (até 9 horas de fermentação). O segundo estudo investigou o efeito do *L. buchneri* como inoculante para silagem ou probiótico em ovinos. Seis ovinos canulados foram utilizados em um duplo quadrado latino 3 × 3 contendo três períodos de 19 dias cada, e os ovinos foram alimentados *ad libitum* com uma dieta total usando a relação volumoso:concentrado 70:30. As dietas foram compostas de 1) silagem de milho não inoculada; 2) silagem de milho inoculada; e 3) silagem de milho não inoculada com uma dose de *L. buchneri* aplicada diretamente dentro do rúmen dos ovinos. *L. buchneri* quando usado como inoculante para silagem modificou a fermentação e composição química da silagem de milho e, aumentou o consumo de matéria seca em ovinos com uma pequena alteração na população relativa de *Ruminococcus flavefaciens* e na fermentação ruminal. Por outro lado, *L. buchneri* como probiótico somente refletiu em uma mínima alteração na proporção relativa de *R. flavefaciens*. O terceiro estudo investigou o efeito da combinação do *Lactobacillus plantarum* com *Lactobacillus buchneri* (silagem LBLP) ou *B. subtilis* (silagem BSLP) em silagem de milho sobre o desempenho de gado de corte terminado em confinamento. Trinta e seis tourinhos cruzados Nelore × Pardo Suíço (peso vivo inicial de 316 ± 33,9 kg) foram alimentados *ad libitum* com uma dieta total usando a relação volumoso:concentrado de 40:60 durante 89 dias após adaptação. Os tourinhos (n = 12) foram distribuídos para uma das três dietas contendo silagem de milho não tratada, LBLP e BSLP em delineamento inteiramente ao acaso. A inoculação bacteriana da silagem de milho não afetou o consumo e o desempenho dos animais, mas reduziu a digestibilidade aparente das dietas. Em geral, os resultados dos três experimentos revelaram que os inoculantes bacterianos não foram hábeis em melhorar o processo fermentativo em silagens de milho e o desempenho dos animais alimentados com estas silagens.

Palavras-chave: desempenho animal, estabilidade aeróbia, inoculantes bacterianos, qualidade da silagem

CHAPTER 1 - GENERAL CONSIDERATIONS

1. INTRODUCTION

Corn silage (*Zea mays* L.) is the forage source most widely used in dairy and feedlot cattle systems in Brazil (BERNARDES; RÊGO, 2014; COSTA et al., 2013; OLIVEIRA; MILLEN, 2014). Whole-crop corn has several advantages during the ensiling process, such as an adequate content of water soluble carbohydrates (WSC) that allows a more efficient fermentation, a low buffering capacity, and large forage yields (up to 20 t/ha on dry matter (DM) basis). However, corn silages produced under tropical climate may have a low nutritive value compared to those produced under temperate climate, and may have a low aerobic stability during feed-out phase (BERNARDES; ADESOGAN, 2012).

The use of lactic-acid bacteria (LAB) as silage inoculants (i.e., homo- (LAB^{ho}) and heterofermentative LAB (LAB^{he})) may improve the silage fermentation process and ensure a better nutritive value of silages (MCDONALD; HENDERSON; HERON, 1991). The first generation of inoculants consisted of LAB^{ho} and the facultative heterofermentative LAB (LAB^{fh}; mainly *Lactobacillus plantarum*), which promote a rapid decline in post-ensiling pH (FILYA, 2003). Later, *L. buchneri* (an obligate LAB^{he}) was developed as a second-generation inoculant to produce acetic acid and improve the aerobic stability of silage by inhibiting spoilage microorganisms (KLEINSCHMIT; KUNG, 2006). Certain *L. buchneri* strains were further developed as third-generation inoculants to produce fibrolytic enzymes, which potentially increase forage digestibility (ADDAH et al., 2011).

The LAB are commonly used as silage inoculants in many parts of the world, but other microorganisms such as *Bacillus subtilis* have been further proposed as silage additives (BASSO et al., 2012a; LARA et al., 2015; PHILLIP; FELLNER, 1992) due to their ability to produce enzymes and antifungal compounds (DONAGHY; KELLY; MCKAY, 1998; TODOVORA; KOZHUHAROVA, 2010; ZUBER; NAKANO; MARAHIEL, 1993). Antifungal compounds may inhibit the growth of yeasts (BASSO et al., 2012a) and a large number of fungi (MUNIMBAZI; BULLERMAN, 1998). In particular, *B. subtilis* may be considered a second- and third-generation inoculant

due to its ability to enhance aerobic stability of silage (BASSO et al., 2012a) and its capacity to produce enzymes (e.g., amylase and ferulic acid esterase (FAE)) (DONAGHY; KELLY; MCKAY, 1998). *B. subtilis* is further used as a direct-fed microbial (DFM) in ruminant diets (JENNY; VANDIJK; COLLINS, 1991) to offer nutritional and health benefits to livestock by modifying the microbial ecology of the digestive tract (BRASHEARS; AMEZQUITA; JARONI, 2005). The use of *B. subtilis* as a DFM may, thus, result in improved animal productivity (TELLES et al., 2011) and act as a probiotic (i.e., fourth generation of silage inoculants).

Combining LAB^{fh} either with LAB^{he} or *B. subtilis* may ensure good silage quality and may increase dry matter intake (DMI) and weight gain for animals fed inoculated corn silage, as previously reported for other silage inoculants (BASSO et al., 2014; BAYATKOUHSAR; TAHMASEBI; NASERIAN, 2011).

Although the literature on the use of silage inoculants is expense, data in particular with regard to potential effects of LAB combined with *B. subtilis* on ruminal fermentation characteristics and animal productivity is lacking. Therefore, the studies outlined in this dissertation aim to understand the mechanisms of action of silage inoculants and, ultimately, improve livestock production in tropical agricultural areas.

2. LITERATURE REVIEW

2.1. Species and classification of bacilli and lactobacilli with regard to their fermentation characteristics

Lactobacillus plantarum and *L. buchneri* are gram-positive LAB typically found in fermented feed. Lactobacilli are typically classified by their fermentation characteristics of hexose to lactic acid, which can be homofermentative (i.e., yielding mainly lactic acid) or heterofermentative (i.e., yielding additional products such as ethanol or acetic acid and CO_2) (PAHLOW et al., 2003). Both fermentation pathways can occur simultaneously during silage making.

However, other classification for lactobacilli species has been proposed, and it can be classified into three groups based on the presence or absence of the enzymes aldolase (i.e., fructose-1,6-bisphosphate-aldolase) and phosphoketolase (KANDLER; WEISS, 1986). Group I consists of obligate LAB^{ho}, and hexoses are

almost exclusively (>85%) fermented to lactic acid, yet are unable to ferment pentoses or gluconates due to the lack of phosphoketolase. Group II consists of LAB^{fh} (e.g., *L. plantarum*), which typically ferment hexoses homofermentatively to lactic acid, but, under certain conditions where WSC is lacking, they can ferment hexoses heterofermentatively to lactic acid, ethanol (or acetic acid) and CO_2. In addition, acetic acid production can occur under conditions in which nicotinamide adenine dinucleotide (NAD^+) can be regenerated without the formation of ethanol, for instance, through the reduction of fructose or molecular oxygen. Group II microorganisms secrete phosphoketolase enzymes, and thus, are able to ferment pentoses to lactic and acetic acid (HOLZER et al., 2003). Group III consists of LAB^{he} (e.g., *L. buchneri*), which ferment hexoses to lactic acid, ethanol (or acetic acid in the presence of an alternative electron acceptor), and CO_2, whereas pentoses are converted to lactic and acetic acid (HOLZER et al., 2003).

B. subtilis are aerobic gram-positive, endospore-forming, rod-shaped bacteria commonly found in soil, water sources, and in association with plants (KUNST et al., 1997; NAKANO; ZUBER, 1998). *B. subtilis* can also grow anaerobically on crop forages in the presence of nitrate (NO_3) as an electron acceptor (KUNST et al., 1997). Even though *Bacillus* spp. is scarce in fresh plant material, it can be present in silages and is not suppressed by fermentation products or by a low silage pH (PAHLOW et al., 2003). *Bacillus* spp. were found to produce lactic and acetic acid (although in lower amount than LAB), and occasionally, butyric acid (MCDONALD; HENDERSON; HERON, 1991). Under laboratory conditions (i.e., medium prepared for incubation), *B. subtilis* had a very inefficient or entirely lacked a glucose fermentation pathway, but it grew anaerobically by fermentation either when both glucose and pyruvate were provided or when glucose and amino acids were present likely by stimulating genes involved in pyruvate catabolism (NAKANO et al., 1997). Moreover, *B. subtilis* can produce ammonia-N by converting NO_3 (HOFFMANN et al., 1998), ethanol (ROMERO et al., 2007), as well as small amounts of acetoin and 2,3 butanediol (NAKANO et al., 1997). In general, acetic acid and ethanol are the main fermentation end products associated with *B. subtilis* metabolism (NAKANO et al., 1997; ROMERO et al., 2007). Normally, elevate temperatures is favorable for growth of *B. subtilis*, since the optimal temperature for *B. subtilis* growth is 30–37°C. In this

regard, *B. subtilis* may be more active in silages produced under tropical climate compared with those produced under temperate climate. However, the action of mechanism of *B. subtilis* during ensiling is yet unclear.

2.2. Fermentation of whole-crop corn silages and addition of silage inoculants

Corn silage is widely used in ruminant diets in many parts of the world as it has relatively stable forage yields under a wide variety of environmental and agronomical conditions, a high energy content and suitable ensiling characteristics (KHAN et al., 2015). Maturity stage at harvest is a major factor in determining the nutritive value of corn silage (FILYA, 2004).Typically, corn is harvested between 30 and 37% of DM (NUSSIO, 2001) because within this DM range the whole-crop plant has a high WSC content (~10% of DM), low buffering capacity, good digestibility and can be easily packed for silo filling. These characteristics are required for a successful fermentation by avoiding the undesirable action of enterobacteria, clostridia, certain bacilli, and yeasts (PAHLOW et al., 2003).

Corn silages produced under tropical climate may have a lower nutritive value (i.e., high fiber content) than corn silage produced under to temperate climate (BERNARDES; ADESOGAN, 2012). Epiphytic LAB require carbohydrates as energy and carbon sources but, due to a lack of an enzymatic complex to metabolize polysaccharides, they require the presence of substantial amounts of readily fermentable sugars (i.e., nonstructural carbohydrates; notably WSC) to produce organic acids (ROOKE; HATFIELD, 2003). In addition, high temperatures in tropical agricultural areas typically promote the growth of yeasts an, thus, reduce the aerobic stability of silages (ASHBELL et al., 2002).

Lactobacilli as silage inoculants were proposed and initially used in Europe and North America, to improve silage fermentation. Due to their quick growth, LAB^{ho} and LAB^{fh} compete for substrate with the existent epiphytic microorganisms present in forage (Table 1), in particular with enterobacteria (PAHLOW et al., 2003). Thanks to recent advances in molecular biology, a higher fermentation rate and a more extensive silage fermentation may be expected (ARASU et al., 2015) from cloned LAB able to express amylolytic activity (e.g., *L. amylovorus*) (FITZSIMONS et al., 1994) or able to use fructan (e.g., *L. casei*) (MERRY et al., 1995).

Table 1. Typical populations of bacterial and fungal groups on plants prior to ensiling.

Group	Population (cfu/g of crop)
Total aerobic bacteria	>10 000 000
Lactic acid bacteria	10-1 000 000
Enterobacteria	1000-1 000 000
Yeasts and yeasts-like fungi	1000-100 000
Molds	1000-10 000
Clostridia (endospores)	100-1000
Bacilli (endospores)	100-1000
Acetic acid bacteria	100-1000
Propionic acid bacteria	10-100

Source: Pahlow et al. (2003)

Under unfavorable ensiling conditions (e.g., low DM and WSC contents and high buffering capacity), certain undesirable microorganisms may predominate over LAB, resulting in increased DM and energy losses (Table 2). Typically, enterobacteria are dominant in the beginning phase of the fermentation process due to the presence of oxygen in the silo (MCDONALD; HENDERSON; HERON, 1991). The major losses are associated with yeasts and clostridia, which may be active during the secondary fermentation process under unfavorable ensiling conditions (e.g., high-moisture silages) (PAHLOW et al., 2003). Yet, clostridial fermentation is effectively avoided by a low silage pH (<4.2) and yeast growth is inhibited by undissociated acids, in particular acetic acid (MOON, 1983; PAHLOW et al., 2003).

Table 2. Metabolic pathways, fermentation end products and DM and energy recovery for various microorganisms.

Metabolic pathway	End products	Recovery (%)	
		DM	Energy
Homolactic (glucose)	Lactic acid	100	99
Heterolactic (glucose)	Lactic acid, ethanol, CO_2	76	98
Heterolactic (fructose)	Lactic acid, acetic acid, mannitol, CO_2	95	99
Yeasts (glucose)	Ethanol, CO_2	51	99
Clostridium (glucose and lactic acid)	Butyric acid, CO_2	49	82

Source: McDonald; Henderson; Heron (1991)

The use of first-generation silage inoculants may, thus, reduce fermentative losses (FILYA, 2003) as lactic acid is produced in large amounts and silage pH is quickly reduced. First-generation silage inoculants mainly consist of pediococci and lactobacilli ssp., with pediococci dominating the initial fermentation phase in which lactobacilli merely slowly produce lactic acid at a typical silage pH of around 6.0 (FITZSIMONS et al., 1992).

The use of silage inoculants such as LAB^{ho} and LAB^{fh} may further contribute to improve silage fermentation by increasing the silage fermentation rate (i.e., higher ratio between lactic and acetic acid), reducing proteolysis and forage protein deamination, and improving nutrients preservation and silage quality through a more efficient use of WSC (ADDAH et al., 2011; HENDERSON, 1993). Under conditions in which sugars are not limiting, the use of LAB^{ho} and LAB^{fh} results in smaller DM and energy losses. The homolactic pathway is more efficient by yielding lactic acid (i.e., one mole of hexose is converted to two moles of lactic acid, decreasing the fermentative losses by CO_2). In contrast, LAB^{he} and LAB^{fh} under certain conditions where WSC is lacking yield additional end products, such as ethanol or acetic acid and CO_2, and lead increased DM losses during fermentation (NISHINO et al., 2003), and thus, heterolactic pathway is less efficient (MCDONALD; HENDERSON; HERON, 1991) (Table 2). Despite the small (1-2%) and similar energy losses with heterolactic compared with homolactic fermentation (Table 2), DM losses are variable and depend on substrate and on the metabolic pathway of LAB^{he} (KUNG; STOKES; LIN, 2003a). Heterolactic fermentation of fructose results in 5% DM losses, whereas heterolactic fermentation of glucose results in 24% DM losses (MCDONALD; HENDERSON; HERON, 1991).

Well-fermented silage typically has low aerobic stability due to large amounts of lactic acid and residual WSC (FILYA, 2002; MCDONALD; HENDERSON; HERON, 1991). Lactic acid no has antifungal properties *per se* (MOON, 1983) and may be even contributing to spoilage by serving as a growth substrate for yeasts (ADESOGAN, 2014). Therefore, the use of LAB^{he} as second-generation silage inoculants may be necessary. In particular, *L. buchneri* has been found to improve aerobic stability of widely varied silages (BASSO et al., 2012b; KLEINSCHMIT; SCHMIDT; KUNG, 2005; TAYLOR et al., 2002). *L. buchneri* converts glucose and

fructose to lactic and acetic acid (MCDONALD; HENDERSON; HERON, 1991). Moreover, these bacteria are able to anaerobically convert lactic acid to acetic acid, which can greatly inhibit yeast growth (MOON, 1983). Further end products are formed, namely 1,2-propanediol, traces of ethanol and CO_2 (OUDE ELFERINK et al., 2001), as well as propionic acid and 1-propanol from the conversion of 1,2-propanediol in case *Lactobacillus diolivorans* is present in the plant material (KROONEMAN et al., 2002). In addition to yeast inhibition, *L. buchneri* can produce bacteriocins (e.g., buchnericin LB), which may avoid or reduce the growth of spoilage microorganisms (YILDIRIM, 2001).

Certain bacteria species (e.g., *L. plantarum*, *L. buchneri* and *B. subtilis*) can produce fibrolytic enzymes with the potential to increase forage digestibility (third-generation inoculants) (ADDAH et al., 2011; COMINO et al., 2014; DONAGHY; KELLY; MCKAY, 1998). In particular, there is increasing interest in the effect of FAE as it was found to improve ruminal digestion and increase the susceptibility of cell-walls to ruminal digestion by complete or partial hydrolysis of ferulic acid linkages (NSEREKO et al., 2008; KANG et al., 2009). However, the fermentation process is complex and results on the use of FAE are contradicting (LYNCH; BAAH; BEAUCHEMIN, 2015).

Silage inoculants such as LAB are widely used, but there is increasing interest in further microorganisms. While the *Bacillus* genus is often associated with the second aerobic deterioration phase (i.e., the post-initial action of yeasts and acetic acid bacteria) in corn silages, *B. subtilis* is not associated with this process (PAHLOW et al., 2003). *B. subtilis* is able to use sugars in anaerobic conditions and the inherent enzymatic complex provides different metabolic pathways resulting in the production of various end products, such as lactic, acetic and succinate acids, next to ethanol and 2,3-butanediol (NAKANO et al., 1997). Consequently, fermentative losses may increase, although no increased losses were reported for corn silages inoculated with various levels of *B. subtilis* (BASSO et al., 2012a). *B. subtilis* further produces a variety of enzymes, including amylase, xylanase and FAE (DONAGHY; KELLY; MCKAY, 1998; SÁ-PEREIRA et al., 2002; VAN SOEST; ROBERTSON; LEWIS, 1991), dextrinase, cellobiase, polymethylgalacturonase and polygalacturonase (APIRAKSAKORN; BUWJOOM; NITISINPRASERT, 2006). These

enzymes may act on complex polysaccharides and provide more WSC to be fermented by LAB within the silo (PHILLIP; FELLNER, 1992). In addition, *B. subtilis* may produce a large amount of antifungal compounds (TODOVORA; KOZHUHAROVA, 2010; ZUBER; NAKANO; MARAHIEL, 1993), likely during the anaerobic and feed-out phase, and may, thus, inhibit the growth of yeasts (BASSO et al., 2012a) and a large number of fungi (MUNIMBAZI; BULLERMAN, 1998). Therefore, corn silages produced under tropical climate conditions had a greater aerobic stability when *B. subtilis* was added compared with untreated corn silages (BASSO et al., 2012a).

2.3. Aerobic stability and spoilage process of silage

The detrimental effect of air on silage quality is manifested in two ways, where the first is associated to deterioration of surface layers during the storage period, often visible as a result of growth of molds and yeasts. This problem is particularly relevant for silage with a large surface/volume ratio (i.e., stack silos). Secondly, aerobic instability during feed-out phase inevitably results in a certain degree of spoilage and may result in substantial DM losses (PAHLOW et al., 2003; WOOLFORD, 1990). A similar situation occurs if the silage is poorly packed or if the silo face is poorly managed and air enters into the silo. Indicators for spoilage process are increasing temperature and pH, DM losses, loss of available nutrients, growth of mold on the surface and feed refusals by the animal (HOLZER et al., 2003). These factors are generally used to predict the aerobic stability of silages (PITT; MUCK; PICKERING, 1991), despite the complexity of spoilage process depending on a great number of factors (PAHLOW; MUCK, 2009). Yet, in Brazil a great part of production systems do not consider losses during the feed-out phase (SIQUEIRA; BERNARDES; REIS, 2005).

During aerobic exposure, fungi (in particular yeasts) are the key microorganisms responsible for initiating aerobic deterioration (PAHLOW et al., 2003), which results in increased production of CO_2 from the metabolism of sugars and may result in less organic acids (in particular lactic acid), ultimately increasing silage pH (MUCK; PITT; LEIBENSPERGER, 1991). In general, silages containing populations of yeasts above 10^5 colony-forming units (cfu) per gram are more

susceptible to aerobic spoilage. However, spoilage level depends vastly on the type of yeast present in the silage (WOOLFORD, 1990). Lactate-assimilating yeasts (*Candida, Endomycopsis, Hansenula* and *Pichia*) are most abundant in silages and differ from non-lactate assimilating yeasts (*Torulopsis*) (WOOLFORD, 1990). Although corn silages typically have a low pH (3.8 to 4.2), spoilage can still occur as yeasts can develop within a wide range of pH (2.5 to 8.5) (ORIJ; BRUL; SMITS, 2011).

In addition to yeasts, acetic acid bacteria can also initiate aerobic deterioration in corn silages as they are tolerant to low pH and have the ability to oxidize various sugars and ethanol to acetic acid (MAMLOUK; GULLO, 2013; PAHLOW et al., 2003). The initiation of aerobic deterioration typically results in losses of residual WSC and increased production of ammonia-N and CO_2, which greatly compromises the nutritive value of silages (WOOLFORD, 1990). Moreover, increased silage pH promotes growth of molds and aerobic bacteria. Molds degrade a wide range of nutrients, including protein, WSC, and structural carbohydrates. Spoilage may imply large DM losses and reduction of digestibility (WOOLFORD, 1990) and the nutritive value of silage (MUCK; PITT; LEIBENSPERGER, 1991; O'KIELY; MUCK; O'CONNOR, 1986). Aerobic stability of silages is a major problem in tropical agricultural areas regarding silage management because spoilage microorganisms find ideal growth conditions at high temperatures (ASHBELL et al., 2002).

To improve aerobic stability of silages, research focused on the use of LAB^{he} as silage inoculants, in particular *L. buchneri* (BASSO et al., 2012b; KUNG et al., 2003b; KUNG et al., 2007; MARI et al., 2009; NISHINO; TOUNO, 2005). Inoculation with *L. buchneri* commonly reduced yeasts due to increased acetic acid production, which results in improved aerobic stability of silages (DANNER et al., 2003; RANJIT; KUNG, 2000). A meta-analysis based on 23 studies comprising 43 experiments showed that *L. buchneri* reduced lactic acid concentrations, and increased acetic acid concentrations and aerobic stability of corn, wheat, barley and grass silages due to inhibition of yeasts (KLEINSCHMIT; KUNG, 2006).

Acetic acid concentrations are supposed to be the major determinant to enhance aerobic stability of silages (DANNER et al., 2003) by reducing yeasts and molds activity (MOON, 1983). At low silage pH (i.e., <4.2), the acetic acid (pKa 4.73)

is present in its undissociated form, and can, thus, cross the membrane of microorganisms into the cell via passive transport. Within the cell, the acetic acid is present in its dissociated form ($RCOO^-$ + H^+) due to high intracellular pH (approximately 7), resulting in increased release of H^+ ions and, ultimately, decreased intracellular pH. To sustain a constant intracellular pH, the microorganisms must eliminate H^+ ions, which, however, results in increased energy losses, reduced microbial growth and, in some cases, reduced lifespan of microorganisms (DAVIDSON, 1997; Figure 1).

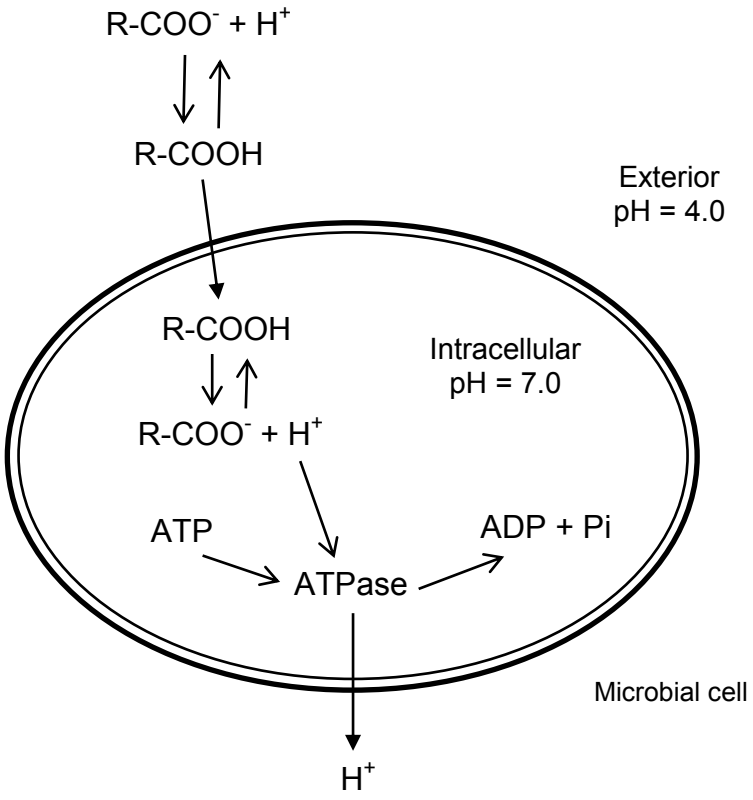

Figure 1. Fate of an organic acid molecule in a low pH environment in the presence of a microbial cell (from DAVIDSON, 1997).

Next to acetic acid, LAB may effectively control the development of spoilage microorganisms as some LAB species can produce bacteriocins with antifungal properties. In particular, *L. buchneri* is able to produce the bacteriocin buchnericin LB, which can control the growth of spoilage microorganisms associated with silage deterioration (YILDIRIM, 2001).

The antimicrobial properties of *B. subtilis* are widely used in the food industry to protect food against spoilage (WILSON et al., 1991). *B. subtilis* can effectively control aerobic deterioration of corn silage under tropical climate (BASSO et al., 2012a). In fact, *B. subtilis* can produce a large amount of enzymes and may be, therefore, also ideal to combine with other silage inoculants (PHILLIP; FELLNER, 1992). Nevertheless, there is little information on the use of *B. subtilis* as silage inoculant and on its mechanism of action. Knowledge is limited so far on its antifungal properties. Antifungal peptides produced by *B. subtilis* may inhibit the growth of yeasts (BASSO et al., 2012a) and a large number of fungi, such as *Aspergillus*, *Penicillium* and *Fusarium* species (CHITARRA et al., 2003; MUNIMBAZI; BULLERMAN, 1998), which greatly affect food security and silage quality (RICHARD et al., 2009; WOOLFORD, 1990).

Aerobic stability of silages is important because during feed-out declines on the nutritive value and safety of silages can occur leading direct effects on milk yield and meat quality of animals fed those silages. Molds and clostridia that growth during silage making or feed-out due to poor silage management practices may result in increased mycotoxins post-ensiling (ensilage-derived mycotoxins) (DRIEHUIS, 2011; 2013). Mycotoxins in silages are a major safety issue for animals and on-farm staff as well as for milk and meat products. Moreover, *Listeria monocytogenes* and *Bacillus* species (there is no relationship with *B. subtilis*) present in poor-quality silages may affect animal and human health (WOOLFORD, 1990), in particular *L. monocytogenes*, which has been a great concern to public health in Europe (DRIEHUIS, 2013).

The use of *L. buchneri* and *B. subtilis* as silage inoculants can control growth of molds and reduce the production of mycotoxins in poor-quality silages and can be, thus, of great importance to livestock production, animal health, and product safety.

2.4. Impact of silage inoculants on ruminal fermentation, an *in vitro* approach

Some of the effects of silage inoculants on silage fermentation may also occur in an anaerobic ruminal environment. Since *in vivo* studies not always report the effects of silage inoculants on ruminal fermentation, *in vitro* studies may be useful in this aspect. *In vitro* gas production techniques were originally developed to predict

ruminal fermentation characteristics and screen the quality of feedstuffs (RYMER et al., 2005). The technique is essentially a batch culture system and consists of inoculating feedstuff (e.g., corn silage treated with a specific LAB strain) in buffered rumen fluid and measure the accumulation of gas production, as well as fermentation end products such as VFA, ammonia-N, and microbial biomass yield (MBY) (BLÜMMEL et al., 1997). Gas production reflects the fermentation of digestible carbohydrates and, to a lesser extent, that of dietary protein and fat. *In vitro* organic matter digestibility (IVOMD), metabolizable energy and, thanks to recent automated gas production systems, *in vitro* methane production can be further measured (PELLIKAAN et al., 2011). *In vitro* gas production techniques may, thus, be a potential method to study effects of LAB on ruminal fermentation characteristics.

In a review on the effects of LAB^{ho} and LAB^{fh} (basically about *L. plantarum* strain MTD1) on animal productivity, Kung and Muck (1997) indicated that silage inoculation affects not only silage fermentation, but also growth performance of cattle as shown by increased milk yield, weight gain, or feed intake, or both. Effects on animal productivity did not strictly coincide with changes in silage fermentation, which may be due to the probiotic effects of silage inoculants in the rumen (WEINBERG; MUCK, 1996). Various *in vitro* studies showed that LAB are able to survive in rumen fluid at least for 96 h (WEINBERG; MUCK; WEIMER, 2003; WEINBERG; CHEN; GAMBURG, 2004). These studies further showed the LAB may modify ruminal *in vitro* fermentation characteristics and the VFA composition. Moreover, glucose addition markedly enhanced the survival of LAB in the rumen fluid, suggesting that LAB can effectively compete with ruminal microflora in the presence of exogenous glucose (WEINBERG; MUCK; WEIMER, 2003). Further *in vitro* study showed that LAB appeared to minimize the inhibitory effect of starch on neutral detergent fiber (NDF) digestibility (WEINBERG et al., 2007). Specific strains of LAB are able to produce FAE enzymes, which enhance ruminal NDF degradability (NSEREKO et al., 2008). It can also be hypothesized that LAB from silage inoculants might compete with efficient lactate-producing ruminal microorganisms (e.g., *Ruminobacter amylophilus* and *Streptococcus bovis*) on essential compounds such as mono- and disaccharides released from starch hydrolysis, ammonia, vitamins, and essential minerals (WEINBERG et al., 2007). This competition might reduce rate of lactate

production by ruminal bacteria and avoid a drop in ruminal pH, which may result increased activity of cellulolytic ruminal microorganisms (WEINBERG et al., 2007). Whereas LAB are generally not important for animals fed forage-based diets, except for when large amounts of soluble carbohydrates or starch are available (VAN SOEST, 1994), the use of LAB as silage inoculants may be a suitable strategy to reduce the negative effects of high-grain diets on rumen acidosis often observed in feedlot cattle (MCALLISTER et al., 1998; WEINBERG et al., 2007).

As described above, silage inoculants have antibacterial properties, which may be associated with a variety of antimicrobial substances, such as bacteriocins (AMADO et al., 2012; MULLER; BEHRENDT; MULLER, 1996; VANDENBERGH, 1993). For instance, *Lactococcus lactis* produces nisin, which has a similar effect on ruminal fermentation as monensin (CALLAWAY; MELLO; RUSSEL, 1997). The antibacterial activity is specific for each LAB strain and depends on the environmental conditions in the silage, such as water activity, pH, and presence of VFA (GOLLOP; ZAKIN; WEINBERG, 2005). Antimicrobial activity may be also expected for ingested LAB present in the rumen fluid (WEINBERG; MUCK; WEIMER, 2003). Further research is required as the antibacterial compounds produced by LAB can also act against other (beneficial) LAB (OHMOMO et al., 2000).

Positive effects of LAB inoculants on *in vitro* gas production and VFA composition (BAYATKOUHSAR; TAHMASBI; NASERIAN, 2012; HAGHPARVAR et al., 2012; LIMA et al., 2010), silage digestibility (RABELO et al., 2014) and *in situ* degradability (ADDAH et al., 2012a) have been reported. Even without such effects, silage inoculants may still increase MBY compared to untreated silages as certain microbial inoculants can affect ruminal microbial biomass (CONTRERAS-GOVEA et al., 2011; 2013). Silage inoculants may also help preserving true protein during silage fermentation (CHARMLEY, 2001; CONTRERAS-GOVEA et al., 2011). However, silage inoculants were also found to have contradictory effects (MUCK; FILYA; CONTRERAS-GOVEA, 2007) or no effects on *in vitro* ruminal gas and VFA production (MUCK; WEINBERG; CONTRERAS-GOVEA, 2013). Further research should focus on *in vitro* gas production techniques to study effects of LAB as silage inoculants on *in vitro* ruminal fermentation characteristics.

Despite a lack of information on the effects of *B. subtilis* on fermentation silages, it can be assumed this microorganism produces antifungal volatile metabolites *in vitro* (FIDDAMAN; ROSSALL, 1993). The source of substrate determines the production of antifungal metabolites by *B. subtilis*; for instance, although starch and cellulose can be used by *B. subtilis*, glucose is more efficient in increasing the production of antifungal metabolites (FIDDAMAN; ROSSALL, 1994). *B. subtilis* further produces the enzymes catalase and subtilisin (a bacterial proteinase), which may support growth and viability of lactobacilli (HOSOI et al., 2000). Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen. It essentially acts like a defense system against accumulation of hydrogen peroxide produced by LAB in the presence of oxygen in the silo or rumen, yet toxic to LAB (PAHLOW et al., 2003), and promotes, thus, growth of LAB (HOSOI et al., 2000), in particular during the feed-out phase. From a nutritional point of view, the use of *B. subtilis* alone or combined with *L. plantarum* may improve the *in vitro* dry matter digestibility (IVDMD) and IVOMD of corn silages produced under tropical climate (LARA et al., 2015). However, IVOMD was also found to decrease for similar corn silages inoculated with *B. subtilis* combined with *L. plantarum* (BASSO et al., 2013). In view of the limited amount of available studies and the controversial results reported in literature, further research is required to investigate potential effects of *B. subtilis* as silage inoculant on ruminal fermentation and on the underlying mechanism of action.

2.5. Probiotic effects of silage inoculants and effects on animal productivity

Next to improved ensiling fermentation and aerobic stability, animal productivity is an important issue related to the use of LAB and *B. subtilis* as silage inoculants. Although increased animal performance has been found less frequently than changes in fermentation owing the silage inoculants, enhances on performance has been substantial (WEINBERG; MUCK, 1996). The *L. plantarum* strain MTD1 increased carcass gain of beef cattle by 11 kg compared with beef cattle fed untreated grass silage and this effect persisted over the entire experimental period of 77 d (KEADY; STEEN, 1994; KEADY et al., 1994). In a review of 14 studies on alfalfa, corn, and grass silages inoculated with the *L. plantarum* strain MTD1, DMI

(+4.8%) and milk yield (+4.6%) increased for dairy cows fed inoculated silage compared to that fed untreated silage (MORAN; OWEN, 1994). Likewise, a later review reported that DMI increased by 4.8% for 28% of the studies included in the review, and that, both ADG and milk yield increased by 4.6% for 53 and 47% of the studies, respectively (KUNG; MUCK, 1997).

Some studies conducted under tropical climate reported beneficial effects of adding LAB^{ho} and LAB^{fh} on ruminal fermentation characteristics of beef cattle, dairy cows, and lambs fed corn silage, along with increased ADG in beef cattle and lambs (ANDRADE et al., 2016; BASSO et al., 2014; RABELO et al., 2016; ZANETTE et al., 2011). Although the number of studies concerning growth performance of animals fed inoculated silage in Brazil is still quite low, the magnitude of the effects of silage inoculants may be similar than that reported for inoculated silages produced under temperate climate (ZOPOLLATTO; DANIEL; NUSSIO, 2009).

Effects on animal productivity are typically associated with changes in the nutritive value of silages (NSEREKO et al., 2008), which, in turn, may be affected by using silage inoculants. However, as previously described, effects on animal productivity might be also related to effects of silage inoculants on ruminal fermentation characteristics, ruminal digestibility, and the ruminal microbial community. Because LAB may survive in the rumen fluid as suggested by *in vitro* studies (WEINBERG; MUCK; WEIMER, 2003; WEINBERG et al., 2004), LAB may also have probiotic properties within the digestive tract by providing microbial biomass directly to the animal and interacting with ruminal microorganisms to improve ruminal functionality and growth performance (MOHAMMED et al., 2012; WEINBERG et al., 2004). A recent study demonstrated the viability of LAB within the rumen and gut of dairy cows (HAN et al., 2014). Silage inoculants present in the rumen fluid may, thus, affect microbial protein synthesis (BASSO et al., 2014) fermentation end products (JATKAUSKAS; VROTNIAKIENE, 2007), which likely explains the observed beneficial effects on animal productivity. The probiotic properties of LAB might, in theory, improve digestibility and inhibit acidosis (WEINBERG et al., 2007), improve feed intake efficiency (MOHAMMED et al., 2012; WEINBERG et al., 2007) and, post-ruminally, improve the intestinal microbial balance (FULLER, 1989).

Improved fiber digestibility of LAB inoculated silage was reported for cattle (MUCK, 1993). The buffering effect in the rumen caused by LAB (as previously discussed) may be a possible explanation, as growth of ruminal fibrolytic bacteria is inhibited at pH < 6 (WEIMER, 1996). Moreover, FAE produced by certain LAB strains act on the arabinoxylans presents in cell walls and release ferulic-acid (BARTOLOME et al., 1995; DONAGHY; KELLY; MCKAY, 1998), rendering the fiber fraction more susceptible to ruminal fibrolytic enzymes, which may explain observed effects on improved fiber digestibility.

Studies showed that *L. buchneri* increased fiber degradability through increased production of FAE (NSEREKO et al., 2008; ADDAH et al., 2012b), with potential beneficial effects on DMI. However, animal productivity was not improved by *L. buchneri* for dairy cows (ARRIOLA et al., 2011), heifers (SALVO et al., 2013), and lambs (ADESOGAN et al., 2003). The combined use of LAB^{fh} and LAB^{he} may, however, result in improved animal productivity due to its beneficial effects on the nutritive value of silages, as well as on aerobic stability of silages, as shown for lambs (BASSO et al., 2014). Yet, information on the effects of a combined use of *L. buchneri* and *L. plantarum* as silage inoculants on animal productivity under tropical climate conditions is scarce, in particular for corn silage-fed animals (BASSO et al., 2014; SALVO et al., 2013).

Likewise, little is known on the effects of *B. subtilis* as silage inoculant on animal productivity of corn silage-fed animals. Based on studies on the use of *B. subtilis* as a DFM in ruminant diets (JENNY; VANDIJK; COLLINS, 1991; SUN; WANG; ZHANG, 2011), we know that spores of *B. subtilis* are resistant to acid and oxygen and may reach the intestine, where they might induce beneficial effects through the secretion of active substances by germinated cells (HOSOI et al., 2000), along with benefits for rumen development in dairy calves (SUN; WANG; ZHANG, 2011). It was further reported that spores of *B. subtilis* administered orally to broilers, mouse, and sows positively affected the intestinal morphology and bacterial flora (ALIAKBARPOUR et al., 2012; MARUTA et al., 1996a, b; HOSOI et al., 1999), improved recovery from diarrhea in piglets (MARUTA et al., 1996b), and increased body weight gain and improved feed efficiency in turkeys (JIRAPHOCAKUL; SULLIVAN; SHAHANI, 1990). Moreover, *B. subtilis* administered orally increased the

number of fecal LAB in mice depending on the type of diet (HOSOI et al., 1999), and also enhanced the growth of lactobacilli in a co-cultured aerobically *in vitro* (HOSOI et al., 2000).

As previously discussed, *B. subtilis* produce amylase (VAN SOEST; ROBERTSON; LEWIS, 1991), which can improve the efficiency of silage utilization, in particular for corn silage, either by increased fermentation rate due to degradation of starch to WSC (PAHLOW et al., 2003), or increased corn grain digestibility (CROSBY et al., 2012). As a result, the use of *B. subtilis* as a DFM improved digestibility of organic matter and non-fiber carbohydrates for young Nellore bulls (TELLES et al., 2011).

The mechanism of action of LAB and *B. subtilis* on ruminal fermentation characteristics and, thus, animal productivity is still unclear. Further research should focus on *in vitro* and *in vivo* techniques to investigate the fate of LAB and *B. subtilis* in the digestive tract, in particular with regard to changes in fermentation end products and ruminal microorganisms (HAN et al., 2014; MOHAMMED et al., 2012; WEINBERG; MUCK; WEIMER, 2003).

2.6. Effect of silage inoculants on carcass and meat traits

In the last years, consumers increasingly demand a high product quality, which can be achieved by feeding high-quality diets to beef cattle. Corn silage is typically included in feedlot diets (OLIVEIRA; MILLEN, 2014) and the use of silage inoculants may contribute to achieve high-quality silage, in particular under difficult ensiling conditions typically found in tropical agricultural areas.

However, information on the effect of silage inoculants on meat quality is scarce, in particular for tropical agricultural areas. Carcass traits and meat quality of young crossbred (Nellore x Angus) bulls were unchanged by adding a blend of LAB^{ho} and LAB^{fh} in corn silage (FUGITA et al., 2012). Carcass traits and dressing percentage of finishing steers were likewise not affected by adding LAB^{he} and LAB^{fh} in barley silage (ADDAH et al., 2014). Similarly, carcass traits of lambs were not improved by adding an unspecified commercial inoculant in whole-crop corn silage (HAFEZ; ABEDO; KHALIFA, 2012). In contrast, a study on whole-crop corn silage inoculated with LAB^{he} (*L. buchneri*) reported increased concentrations of

polyunsaturated fatty acids (PUFA) and the ratio of PUFA to saturated fatty acids in the meat of young Nellore bulls finished in feedlot (RABELO et al., 2016).

Further research is required to investigate potential effects of silage additives during silage making on carcass traits and meat quality of beef cattle.

3. RESEARCH OBJECTIVES

The objective of this dissertation was to investigate the effects of *Lactobacillus* and *B. subtilis* as silage inoculants on 1) the fermentation and aerobic stability of corn silage; 2) the *in vitro* and *in vivo* ruminal fermentation characteristics, along with ruminal bacteria community; 3) the productivity of beef cattle under tropical climate conditions; 4) the feed intake and apparent digestibility in wethers.

4. REFERENCES

ADDAH, W.; BAAH, J.; GROENEWEGEN, P.; OKINE, E. K.; MCALLISTER, T. A. Comparison of the fermentation characteristics, aerobic stability and nutritive value of barley and corn silages ensiled with or without a mixed bacterial inoculant. **Canadian Journal of Animal Science**, Ottawa, v. 91, p. 133-146, 2011.

ADDAH, W.; BAAH, J.; OKINE, E. K.; MCALLISTER, T. A. A third-generation esterase inoculant alters fermentation pattern and improves aerobic stability of barley silage and the efficiency of body weight gain of growing feedlot cattle. **Journal of Animal Science**, Albany, v. 90, p. 1541-1552, 2012b.

ADDAH, W.; BAAH, J.; OKINE, E. K.; MCALLISTER, T. A. Use of thermal imaging and the *in situ* technique to assess the impact of an inoculant with feruloyl esterase activity on the aerobic stability and digestibility of barley silage. **Canadian Journal of Animal Science**, Ottawa, v. 92, p. 381-394, 2012a.

ADDAH, W.; BAAH, J.; OKINE, E. K.; OWENS, F. N.; MCALLISTER, T. A. Effects of chop-length and a ferulic acid esterase-producing inoculant on fermentation and aerobic stability of barley silage, and growth performance of finishing feedlot. **Animal Feed Science and Technology**, Amsterdam, v. 197, p. 34-46, 2014.

ADESOGAN, A. T. Avoiding the two greatest silage problems. In: **Proceedings…** Gainesville: 50th Florida Dairy Production Conference, 2014. p. 9-17.

ADESOGAN, A. T.; SALAWU, M. B.; ROSS, A. B.; DAVIES, D. R.; BROOKS, A. E. Effect of *Lactobacillus buchneri*, *Lactobacillus fermentum*, *Leuconostoc mesenteroides* inoculants, or a chemical additive on the fermentation, aerobic stability, and nutritive value of crimped wheat grains. **Journal of Dairy Science**, Champaign, v. 86, p. 1789-1796, 2003.

ALIAKBARPOUR, H. R.; CHAMANI, M.; RAHIMI, G.; SADEGHI, A. A.; QUJEQ, D. The *Bacillus subtilis* and lactic acid bacteria probiotics influences intestinal mucin gene expression, histomorphology and growth performance in broilers. **Asian-Australasian Journal of Animal Science**, Seoul, v. 25, p. 1285-1293, 2012.

ANDRADE, F. L.; RODRIGUES, J. P. P.; DETMANN, E.; VALADARES FILHO, S. C.; CASTRO, M. M. D.; TRECE, A. S.; SILVA, T. E.; FISCHER, V.; WEISS, K.; MARCONDES, M. I. Nutritional and productive performance of dairy cows fed corn silage or sugarcane silage with or without additives. **Tropical Animal Health and Production**, v. 48, p. 747-753, 2016.

AMADO, I. R.; FUCIÑOS, C.; FAJARDO, P.; GUERRA, N. P.; PASTRANA, L. Evaluation of two bacteriocin-producing probiotic lactic acid bacteria as inoculants for controlling *Listeria monocytogenes* in grass and maize silages. **Animal Feed Science and Technology**, Amsterdam, v. 175, p. 137-149, 2012.

APIRAKSAKORN, J.; BUWJOOM, T.; NITISINPRASERT, S. Characterization of grass degrading bacteria active on β-1,3-1,4-D-glucans from *Bacillus subtilis* GN156 potential use for grass silage-making. **Kasetsart Journal**, Bangkok, v. 40, p. 136-147, 2006.

ARASU, M. V.; JUNG, M. W.; KIM, D. H.; PARK, H. S.; ILAVENIL, S.; AL-DHABI, N. A.; CHOI, K. C. Identification and phylogenetic characterization of novel *Lactobacillus plantarum* species and their metabolite profiles in grass silage. **Annals of Microbiology**, Heidelberg, v. 65, p. 15-25, 2015.

ARRIOLA, K. G.; KIM, S. C.; STAPLES, C. R.; ADESOGAN, A. T. Effect of applying bacterial inoculants containing different types of bacteria to corn silage on the performance of dairy cattle. **Journal of Dairy Science**, Champaign, v. 94, p. 3973-3979, 2011.

ASHBELL, G.; WEINBERG, Z. G.; HEN, Y.; FILYA, I. The effects of temperature on the aerobic stability of wheat and corn silages. **Journal of Indusexperiment Microbiology & Biotechnology**, Basingstoke, v. 28, p. 261-263, 2002.

BARTOLOME, B.; FAULDS, C. B.; TUOHY, M.; HAZLEWOOD, G. P.; GILBERT, H. J. WILLIAMSON, G. Influence of different xylanases on the activity of ferulic acid esterase of wheat bran. **Biotechnology and Applied Biochemistry**, San Diego, v. 22, p. 65-73, 1995.

BASSO, F. C.; ADESOGAN, A. T.; LARA, E. C.; RABELO, C. H. S.; BERCHIELLI, T. T.; TEIXEIRA, I. A. M. A.; SIQUEIRA, G. R.; REIS, R. A. Effects of feeding corn silage inoculated with microbial additives on the ruminal fermentation, microbial protein yield, and growth performance of lambs. **Journal of Animal Science**, Albany, v. 92, p. 5640-5650, 2014.

BASSO, F. C.; BERNARDES, T. F.; ROTH, A. P. T. P.; LODO, B. N.; BERCHIELLI, T. T.; REIS, R. A. Short Communication: Fermentation and aerobic stability of corn

silage inoculated with *Lactobacillus buchneri*. **Revista Brasileira de Zootecnia**, Viçosa, v. 41, n. 7, p. 1789-1794, 2012b.

BASSO, F. C.; LARA, E. C.; ASSIS, F. B.; RABELO, C. H. S.; MORELLI, M.; REIS, R. A. Características da fermentação e estabilidade aeróbia de silagens de milho inoculadas com *Bacillus subtilis*. **Revista Brasileira de Saúde e Produção Animal**, Salvador, v. 13, n. 4, p. 1009-1019, 2012a.

BASSO, F. C.; RABELO, C. H. S.; LARA, E. C.; JORGE, L. G. O.; SIQUEIRA, G. R.; SADER, A. P. O.; BERCHIELLI, T. T.; REIS, R. A. *In vitro* ruminal fermentation, gas production and true digestibility of the corn silages inoculated with microbial additives. In: III International Symposium on Forage Quality and Conservation. **Proceedings…** Piracicaba, 2013.

BAYATKOUHSAR, J.; TAHMASBI, A. M.; NASERIAN, A. A. Effects of microbial inoculant on composition, aerobic stability, *in situ* ruminal degradability and *in vitro* gas production of corn silage. **International Journal of AgriScience**, Kassel, v. 2, p. 774-786, 2012.

BAYATKOUHSAR, J.; TAHMASBI, A. M.; NASERIAN, A. A. The effects of microbial inoculation of corn silage on performance of lactating dairy cows. **Livestock Science**, Amsterdam, v. 142, p. 170-174, 2011.

BERNARDES, T. F.; ADESOGAN, A. T. Aerobic deterioration of silages in warm climates. In: **Symposium on strategic management of pastures**. 6ed. Viçosa, 2012. p. 249-268.

BERNARDES, T. F.; RÊGO, A. C. Study on the practices of silage production and utilization on Brazilian dairy farms. **Journal of Dairy Science**, Champaign, v. 97, p. 1852-1861, 2014.

BLÜMMEL, M.; MAKKAR, H. P. S.; BECKER, K. *In vitro* gas production: a technique revisited. **Journal of Animal Physiology and Animal Nutrition**, Berlin, v. 77, p. 24–34, 1997.

BRASHEARS, M. M.; AMEZQUITA, A.; JARONI, D. Lactic acid bacteria and their uses in animal feeding to improve food safety. **Advances in Food and Nutrition Research**, Amsterdam, v. 50, p. 1-31, 2005.

CALLAWAY, T. R.; CARNEIRO DE MELLO, A. M. S.; RUSSEL, J. B. The effect of Nisin and Monensin on ruminal fermentations *in vitro*. **Current Microbiology: an International Journal**, New York, v. 35, p. 90-96, 1997.

CHARMLEY, E. Towards improved silage quality – A review. **Canadian Journal of Animal Science**, Ottawa, v. 81, p. 157-168, 2001.

CHITARRA, G. S.; BREEUWER, P.; NOUT, M. J. R.; VAN AELST, A. C.; ROMBOUTS, F. M.; ABEE, T. An antifungal compound produced by *Bacillus subtilis* YM 10–20 inhibits germination of *Penicillium roqueforti* conidiospores. **Journal of Applied Microbiology**, Oxford, v. 94, p. 159-166, 2003.

COMINO, L.; TABACCO, E.; RIGHI, F.; REVELLO-CHION, A.; QUARANTELLI, A.; BORREANI, G. Effects of an inoculant containing a *Lactobacillus buchneri* that produces ferulate-esterase on fermentation products, aerobic stability, and fibre digestibility of maize silage harvested at different stages of maturity. **Animal Feed Science and Technology**, Amsterdam, v. 198, p. 94-106, 2014.

CONTRERAS-GOVEA, F. E.; MUCK, R. E.; BRODERICK, G. A.; WEIMER, P. J. *Lactobacillus plantarum* effects on silage fermentation and *in vitro* microbial yield. **Animal Feed Science and Technology**, Amsterdam, v. 179, p. 61-68, 2013.

CONTRERAS-GOVEA, F. E.; MUCK, R. E.; MERTENS, D. R.; WEIMER, P. J. Microbial inoculants effects on silage and in vitro ruminal fermentation, and microbial biomass estimation for alfalfa, bmr corn, and corn silages. **Animal Feed Science and Technology**, Amsterdam, v. 163, p. 2-10, 2011.

COSTA, J. H. C.; HOTZEL, M. J.; LONGO, C.; BALCÃO, L. F. A survey of management practices that influence production and welfare of dairy cattle on family farms in southern Brazil. **Journal of Dairy Science**, Champaign, v. 96, p. 307-317, 2013.

CROSBY, M.; MENDOZA, G. D.; BONOLA, I.; PLATA, F. X.; SANDOVAL, H.; MELGOZA, L. M. Slow-release amylase increases *in vitro* ruminal digestion of maize and sorghum grain. **South African Journal of Animal Science**, Pretoria, v. 42, p. 33-37, 2012.

DANNER, H.; HOLZER, M.; MAYRHUBER, E.; BRAUN, R. Acetic acid increases stability of silage under aerobic conditions. **Applied and Environmental Microbiology**, Washington, v. 69, n. 1, p. 562-567, 2003.

DAVIDSON, P. M. Chemical preservatives and natural antimicrobial compounds. In: DOYLE, M. P.; BEUCHAT, L. R.; MONTEVILLE, T. J. (Eds.). **Food Microbiology: Fundamentals and Frontiers**. Washington: ASM Press, 1997. p. 520-556.

DONAGHY, J.; KELLY, P. F.; MCKAY, A. M. Detection of ferulic acid esterase production by *Bacillus* spp. and *Lactobacilli*. **Applied Microbiology and Biotechnology**, New York, v. 50, n. 2, p. 257-260, 1998.

DRIEHUIS, F. Occurrence of mycotoxins in silage. In: INTERNATIONAL SYMPOSIUM ON FORAGE QUALITY AND CONSERVATION, II, 2011, São Pedro. **Proceedings…** São Pedro: Forage Quality and Conservation, 2011. 20 p.

DRIEHUIS, F. Silage and the safety and quality of dairy foods: a review. **Agricultural and Food Science**, Helsinki, v. 22, p. 16-34, 2013.

FIDDAMAN, P. J.; ROSSALL, S. The production of antifungal volatiles by *Bacillus subtilis.* **Journal of Applied Bacteriology**, London, v. 74, p. 119-126, 1993.

FIDDAMAN, P. J.; ROSSALL, S. Effect of substrate on the production of antifungal volatiles from *Bacillus subtilis*. **Journal of Applied Bacteriology**, London, v. 76, p. 395-405, 1994.

FILYA, I. Nutritive value and aerobic stability of whole crop maize silage harvested at four stages of maturity. **Animal Feed Science and Technology**, Amsterdam, v. 116, p. 141-150, 2004.

FILYA, I. The effect of *Lactobacillus buchneri* and *Lactobacillus plantarum* on the fermentation, aerobic stability, and ruminal degradability of low dry matter corn and sorghum silages. **Journal of Dairy Science**, Champaign, v. 86, p. 3575-3581, 2003.

FILYA, I. The effects of lactic acid bacterial inoculants on the fermentation, aerobic stability, and in situ rumen degradability characteristics of maize and sorghum silages. **Turkish Journal of Veterinary and Animal Sciences**, Ankara, v. 26, p. 815-823, 2002.

FITZSIMONS, A.; DUFFNER, F.; CURTIN, D.; BROPHY, G.; O'KIELY, P.; O'CONNELL, M. Assessment of *Pediococcus acidilactici* as a potential silage inoculant. **Applied and Environmental Microbiology**, Washington, v. 58, p. 3047-3052, 1992.

FITZSIMONS, A.; HOLS, P.; JORE, J.; LEER, R. J.; O'CONNELL, M.; DELCOUR, J. Development of an amylolytic *Lactobacillus plantarum* silage strain expressing the *Lactobacillus amylovorus* a-amylase gene. **Applied and Environmental Microbiology**, Washington, v. 60, p. 3529-3535, 1994.

FUGITA, C. A.; PRADO, I. N.; JOBIM, C. C.; ZAWADZKI, F.; VALERO, M. V.; PIRES, M. C. O.; PRADO, R. M.; FRANÇOZO, M. C. Corn silage with and without enzyme-bacteria inoculants on performance, carcass characteristics and meat quality in feedlot finished crossbred bulls. **Revista Brasileira de Zootecnia**, Viçosa, v. 41, n. 1, p. 154-163, 2012.

FULLER, R. A review: Probiotics in man and animals. **Journal of Applied Bacteriology**, London, v. 66, p. 365-378, 1989.

GOLLOP, N.; ZAKIN, V.; WEINBERG, Z. G. Antibacterial activity of lactic acid bacteria included in inoculants for silage and in silages treated with these inoculants. **Journal of Applied Microbiology**, Oxford, v. 98, p. 662-666, 2005.

HAFEZ, Y. H.; ABEDO, A. A.; KHALIFA, E. I. Effect of microbial inoculation of whole plant corn silage on growth performance and carcass characteristics of Rahmani lambs. **Egyptian Journal of Sheep and Goat Sciences**, Giza, v. 7, p. 17-29, 2012.

HAGHPARVAR, R.; SHOJAIAN, K.; ROWGHANI, E.; PARSAEI, S.; YOUSEF ELLAHI, M. The effects of *Lactobacillus plantarum* on chemical composition, rumen degradability, *in vitro* gas production and energy content of whole-plant corn ensiled at different stages of maturity. **Iranian Journal of Veterinary Research**, Shiraz, v. 13, p. 8-15, 2012.

HAN, H.; OGATA, Y.; YAMAMOTO, Y.; NAGAO, S.; NISHINO, N. Identification of lactic acid bacteria in the rumen and feces of dairy cows fed total mixed ration silage

to assess the survival of silage bacteria in the gut. **Journal of Dairy Science**, Champaign, v. 97, p. 5754-5762, 2014.

HENDERSON, N. Silage additives. **Animal Feed Science and Technology**, Amsterdam, v. 45, n. 1, p. 35-56, 1993.

HOFFMANN, T.; FRANKENBERG, N.; MARINO, M.; JAHN, D. Ammonification in *Bacillus subtilis* utilizing dissimilatory nitrite reductase is dependent on *resDE*. **Journal of Bacteriology**, Washington, v. 180, p. 186-189, 1998.

HOLZER, M.; MAYRHUBER, E.; DANNER, H.; BRAUN, R. The role of *Lactobacillus buchneri* in forage preservation. **TRENDS in Biotechnology**, Amsterdam, v. 21, p. 282-287, 2003.

HOSOI, T.; AMETANI, A.; KIUCHI, K.; KAMINOGAWA, S. Changes in fecal microflora induced by intubation of mice with *Bacillus subtilis* (*natto*) spores are dependent upon dietary components. **Canadian Journal of Microbiology**, Ottawa, v. 45, p. 59-66, 1999.

HOSOI, T.; AMETANI, A.; KIUCHI, K.; KAMINOGAWA, S. Improved growth and viability of lactobacilli in the presence of *Bacillus subtilis* (*natto*), catalase, or subtilisin. **Canadian Journal of Microbiology**, Ottawa, v. 46, p. 892-897, 2000.

JENNY, B. F.; VANDIJK, H. J.; COLLINS, J. A. Performance and fecal flora of calves fed a *Bacillus subtilis* concentrate. **Journal of Dairy Science**, Champaign, v. 74, P. 1968-1973, 1991.

JIRAPHOCAKUL, S.; SULLIVAN, T. W.; SHAHANI, K. M. Influence of a dried *Bacillus subtilis* culture and antibiotics on performance and intestinal microflora in turkeys. **Poultry Science**, Oxford, v. 69, p. 1966-1973, 1990.

KANDLER, O.; WEISS, N. Genus *Lactobacillus* Beijerinck 1901, 212^{AL}. In: SNEATH, P. H. A. et al. (Ed.) **Bergey's manual of systematic bacteriology**. Baltimore: MD, 1986. p. 1209-1234.

KANG, T. W.; ADESOGAN, A. T.; KIM, S. C.; LEE, S. S. Effects of an esterase-producing inoculant on fermentation, aerobic stability, and neutral detergent fiber digestibility of corn silage. **Journal of Dairy Science**, Champaign, v. 92, p. 732-738, 2009.

KEADY, T. W. J.; STEEN, R. W. J.; KILPATRICK, D. J.; MAYNE, C.S. Effects of inoculant treatment on silage fermentation, digestibility and intake by growing cattle. **Grass and Forage Science**, Oxford, v. 49, n. 2, p. 284-294, 1994.

KEADY, T. W. J.; STEEN, W. J. The effects of treating low dry-matter, low digestibility grass with bacterial inoculant on the intake and performance of beef cattle, and studies of its mode of action. **Grass and Forage Science**, Oxford, v. 50, p. 217-226, 1994.

KHAN, N. A.; YU, P.; ALI, M.; CONE, J. W.; HENDRIKS, W. H. Nutritive value of maize silage in relation to dairy cow performance and milk quality. **Journal of the Science of Food and Agriculture**, London, v. 95, p. 238-252, 2015.

KLEINSCHIMIT, D. H.; KUNG JR., L. A meta-analysis of the effects of *Lactobacillus buchneri* on the fermentation and aerobic stability of corn and grass and small-grain silages. **Journal of Dairy Science**, Champaign, v. 89, p. 4005-4013, 2006.

KLEINSCHIMIT, D. H.; SCHMIDT, R. J.; KUNG JR., L. The effects of various antifungal additives on the fermentation and aerobic stability of corn silage. **Journal of Dairy Science**, Champaign, v. 88, p. 2130-2139, 2005.

KROONEMAN, J.; FABER, F.; ALDERKAMP, A. C.; OUDE ELFERINK, S. J. H. W.; DRIEHUIS, F.; CLEENWERCK, I.; SWINGS, J.; GOTTSCHAL, J. C.; VANCANNEYT, M. *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. **International Journal of Systematic and Evolutionary Microbiology**, London, v. 52, p. 639-646, 2002.

KUNG JR., L.; MUCK, R. E. Animal Response to Silage Additives. In: Silage: Field to Feedbunk. North American Conference, Pennsylvania, 1997. **Proceedings…** Pennsylvania: ASAS, 1997. p. 200-210.

KUNG JR., L.; SCHMIDT, R. J.; EBLING, T. E.; HU, W. The effect of *Lactobacillus buchneri* 40788 on the fermentation and aerobic stability of ground and whole high-moisture corn. **Journal of Dairy Science**, Champaign, v. 90, p. 2309-2314, 2007.

KUNG JR., L.; STOKES, M. R.; LIN, C. J. Silage additives. In: BUXTON, D. R.; MUCK, R. E.; HARRISON, J. H. (Eds.). **Silage Science and Technology**. Madison, USA: American Society of Agronomy, 2003a. p. 305-360.

KUNG JR., L.; TAYLOR, C. C.; LYNCH, M. P.; NEYLON, J. M. The effect of treating alfalfa with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for lactating Dairy cows. **Journal of Dairy Science**, Champaign, v. 86, p. 336-343, 2003b.

KUNST, F.; OGASAWARA, N.; MOSZER, I.; ALBERTINI, A. M.; ALLONI, G.; AZEVEDO, V.; BERTERO, M. G.; BESSIÈRES, P.; BOLOTIN, A.; BORCHERT, S.; BORRISS, R.; BOURSIER, L.; BRANS, A.; BRAUN, M.; BRIGNELL, S. C.; BRON, S.; BROUILLET, S.; BRUSCHI, C. V.; CALDWELL, B.; CAPUANO, V.; CARTER, N. M.; CHOI, S. K.; CODANI, J. J.; CONNERTON, I. F.; CUMMINGS, N. J.; DANIEL, R. A.; DENIZOT, F.; DEVINE, K. M.; DÜSTERHÖFT, A.; EHRLICH, S. D.; EMMERSON, P. T.; ENTIAN, K. D.; ERRINGTON, J.; FABRET, C.; FERRARI, E.; FOULGER, D.; FRITZ, C.; FUJITA, M.; FUJITA, Y.; FUMA, S.; GALIZZI, A.; GALLERON, N.; GHIM, S. Y.; GLASER, P.; GOFFEAU, A.; GOLIGHTLY, E. J.; GRANDI, G.; GUISEPPI, G.; GUY, B. J.; HAGA, K.; HAIECH, J.; HARWOOD, C. R.; HÉNAUT, A.; HILBERT, H.; HOLSAPPEL, S.; HOSONO, S.; HULLO, M. F.; ITAYA, M.; JONES, L.; JORIS, B.; KARAMATA, D.; KASAHARA, Y.; KLAERR-BLANCHARD, M.; KLEIN, C.; KOBAYASHI, Y.; KOETTER, P.; KONINGSTEIN, G.; KROGH, S.; KUMANO, M.; KURITA, K.; LAPIDUS, A.; LARDINOIS, S.; LAUBER, J.;

LAZAREVIC, V.; LEE, S. M.; LEVINE, A.; LIU, H.; MASUDA, S.; MAUËL, C.; MÉDIGUE, C.; MEDINA, N.; MELLADO, R. P.; MIZUNO, M.; MOESTL, D.; NAKAI, S.; NOBACK, M.; NOONE, D.; O'REILLY, M.; OGAWA, K.; OGIWARA, A.; OUDEGA, B.; PARK, S. H.; PARRO, V.; POHL, T. M.; PORTETELLE, D.; PORWOLLIK, S.; PRESCOTT, A. M.; PRESECAN, E.; PUJIC, P.; PURNELLE, B.; RAPOPORT, G.; REY, M.; REYNOLDS, S.; RIEGER, M.; RIVOLTA, C.; ROCHA, E.; ROCHE, B.; ROSE, M.; SADAIE, Y.; SATO, T.; SCANLAN, E.; SCHLEICH, S.; SCHROETER, R.; SCOFFONE, F.; SEKIGUCHI, J.; SEKOWSKA, A.; SEROR, S. J.; SERROR, P.; SHIN, B. S.; SOLDO, B.; SOROKIN, A.; TACCONI, E.; TAKAGI, T.; TAKAHASHI, H.; TAKEMARU, K.; TAKEUCHI, M.; TAMAKOSHI, A.; TANAKA, T.; TERPSTRA, P.; TOGNONI, A.; TOSATO, V.; UCHIYAMA, S.; VANDENBOL, M.; VANNIER, F.; VASSAROTTI, A.; VIARI, A.; WAMBUTT, R.; WEDLER, E.; WEDLER, H.; WEITZENEGGER, T.; WINTERS, P.; WIPAT, A.; YAMAMOTO, H.; YAMANE, K.; YASUMOTO, K.; YATA, K.; YOSHIDA, K.; YOSHIKAWA, H. F.; ZUMSTEIN, E.; YOSHIKAWA, H.; DANCHIN, A. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. **Nature**, London, v. 390, p. 249-256, 1997.

LARA, E. C.; BASSO, F. C.; ASSIS, F. B.; SOUZA, F. A.; BERCHIELLI, T. T.; REIS, R. A. Changes in the nutritive value and aerobic stability of corn silages inoculated with *Bacillus subtilis* alone or combined with *Lactobacillus plantarum*. **Animal Production Science**, 2015. doi: 10.1071/AN14686

LIMA, R.; LOURENÇO, M.; DÍAZ, R. F.; CASTRO, A.; FIEVEZ, V. Effect of combined ensiling of sorghum and soybean with or without molasses and lactobacilli on silage quality and in vitro rumen fermentation. **Animal Feed Science and Technology**, Amsterdam, v. 155, p. 122-131, 2010.

LYNCH, J. P.; BAAH, J.; BEAUCHEMIN, K. A. Conservation, fiber digestibility, and nutritive value of corn harvested at 2 cutting heights and ensiled with fibrolytic enzymes, either alone or with a ferulic acid esterase-producing inoculant. **Journal of Dairy Science**, Champaign, v. 98, p. 1214-1224, 2015.

MAMLOUK, D.; GULLO, M. Acetic acid bacteria: physiology and carbon sources oxidation. **Indian Journal of Microbiology**, v. 53, n. 4, p. 377-384, 2013.

MARI, L. J.; SCHMIDT, R. J.; NUSSIO, L. G.; HALLADA, C. M.; KUNG JR., L. Short communication: An evaluation of the effectiveness of *Lactobacillus buchneri* 40788 to after fermentation and improve the aerobic stability of corn silage in farm silos. **Journal of Dairy Science**, Champaign, v. 92, p. 1174-1176, 2009.

MARUTA, K.; MIYAZAKI, H.; MASUDA, S.; TAKAHASHI, M.; MARUBASHI, T.; TADANO, Y.; TAKAHASHI, H. Exclusion of intestinal pathogens by continuous feeding with *Bacillus subtilis* C-3102 and its influence on the intestinal microflora in broilers. **Animal Science and Technology (Japan)**, v. 67, p. 273-280, 1996a.

MARUTA, K.; MIYAZAKI, H.; TADANO, Y.; MASUDA, S.; SUZUKI, A.; TAKAHASHI, H.; TAKAHASHI, M. Effects of *Bacillus subtilis* C-3102 intake on fecal flora of sows and on diarrhea and mortality rate of their piglets. **Animal Science and Technology (Japan)**, v. 67, p. 403-409, 1996b.

MCALLISTER, T. A.; FENIUK, R.; MIR, Z.; MIR, P.; SELINGER, L. B.; CHENG, K. -J. Inoculants for alfalfa silage: effects on aerobic stability, digestibility and the growth performance of feedlot steers**. Livestock Production Science**, Amsterdam, v. 53, p. 171-181, 1998.

MCDONALD, P.; HENDERSON, A. R.; HERON S. J. E. **The biochemistry of silage**. Marlow: Chalcomb Publications, 1991. 340 p.

MERRY, R. I.; WINTERS, A. L.; THOMAS, P. I.; MULLER, M.; MULLER, T. Degradation of fructans by epiphytic and inoculated LAB and by plant enzymes during ensilage of normal and sterile hybrid ryegrass. **Journal of Applied Bacteriology**, London, v. 79, p. 583-591, 1995.

MOHAMMED, R.; STEVENSON, D. M.; BEAUCHEMIN, K. A.; MUCK, R. E.; WEIMER, P. J. Changes in ruminal bacterial community composition following feeding of alfalfa ensiled with a lactic acid bacterial inoculant. **Journal of Dairy Science**, Champaign, v. 95, n. 1, p. 328-339, 2012.

MOON, N. J. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. **Journal of Applied Bacteriology**, London, v. 55, p. 453-460, 1983.

MORAN, J. P.; OWEN, T. R. The effects of Ecosyl treated silage on milk production by lactating cows. In: NATIONAL CONFERENCE ON FORAGE QUALITY, EVALUATION AND UTILIZATION, 1994, Lincoln. **Proceedings...** Lincoln: University of Nebraska, 1994. p. 126.

MUCK, R. E. The role of silage additives in making high quality silage. In: **Silage Production from Seed to Animal**. Syracuse, NY: NRAES-67, Northeast Regional Agricultural Engineering Service, 1993. p. 106-116.

MUCK, R. E.; FILYA, I.; CONTRERAS-GOVEA, F. E. Inoculant effects on alfalfa silage: In vitro gas and volatile fatty acid production. **Journal of Dairy Science**, Champaign, v. 90, p. 5115-5125, 2007.

MUCK, R. E.; PITT, R. E.; LEIBENSPERGER, R. Y. A model of aerobic fungal growth in silage. Microbial characteristics. **Grass and Forage Science**, Oxford, v. 46, n. 3, p. 283-290, 1991.

MUCK, R. E.; WEINBERG, Z. G.; CONTRERAS-GOVEA, F. E. Silage extracts used to study the mode of action of silage inoculants in ruminants. **Agricultural and Food Science**, Helsinki, v. 22, p. 108-114, 2013.

MULLER, T.; BEHRENDT, U.; MULLER, M. Antagonistic activity in plant-associated lactic acid bacteria. **Microbiological Research**, Jena, v. 151, p. 63-70, 1996.

MUNIMBAZI, C.; BULLERMAN, L. B. Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus*. **Journal of Applied Microbiology**, Oxford, v. 84, p. 959-968, 1998.

NAKANO, M. M.; DAILLY, Y. P.; ZUBER, P.; CLARK, D. P. Characterization of anaerobic fermentative growth of *Bacillus subtilis*: identification of fermentation end products and genes required for growth. **Journal of Bacteriology**, Washington, v. 179, p. 6749-6755, 1997.

NAKANO, M. M.; ZUBER, P. Anaerobic growth of a “strict aerobe” (*Bacillus subtilis*). **Annual Review of Microbiology**, Palo Alto, v. 52, p. 165-190, 1998.

NISHINO, N.; TOUNO, E. Ensiling characteristics and aerobic stability of direct-cut and wilted grass silages inoculated with *Lactobacillus casei* or *Lactobacillus buchneri*. **Journal of the Science of Food and Agriculture**, London, v. 85, p. 1882-1888, 2005.

NSEREKO, V. L.; SMILEY, B. K.; RUTHERFORD, W. M.; SPIELBAUER, A.; FORRESTER, K. J.; HETTINGER, G. H.; HARMAN, E. K.; HARMAN, B. R. Influence of inoculating forage with lactic acid bacterial strains that produce ferulate esterase on ensilage and ruminal degradation of fiber. **Animal Feed Science and Technology**, Amsterdam, v. 145, p. 122-135, 2008.

NUSSIO, L. G. Cultura de milho para produção de silagem de alto valor alimentício. In: **Simpósio sobre nutrição de bovinos**. Piracicaba, 1991. p. 59-168.

O’KIELY, P.; MUCK, R. E.; O’CONNOR. **Aerobic deterioration of alfalfa and maize silage**. In: AMERICAN SOCIETY OF AGRICULTURAL ENGINEERING, 1986. n. 86-1526.

OHMOMO, S.; MURATA, S.; KATAYAMA, N.; NITISINPRASART, S.; KOBAYASHI, M.; NAKAJIMA, T.; YAJIMA, M. Purification and some characteristics of enterocin ON-157, a bacteriocins produced by *Ent. faecium* NIAI 157. **Journal of Applied Microbiology**, Oxford, v. 88, p. 81-89, 2000.

OLIVEIRA, C. A.; MILLEN, D. D. Survey of the nutritional recommendations and management practices adopted by feedlot cattle nutritionists in Brazil. **Animal Feed Science and Technology**, Amsterdam, v. 197, p. 64-75, 2014.

ORIJ, R.; BRUL, S.; SMITS, G. J. Intracellular pH is a tightly controlled signal in yeast. **Biochimica et Biophysica Acta**, Amsterdam, v. 1810, p. 933-944, 2011.

OUDE ELFERINK, S. J. W. H.; KROONEMAN, J.; GOTTSCHAL, J. C.; SPOELSTRA, S. F.; FABER, F.; DRIEHUIS, F. Anaerobic conversion of lactic acid to acetic acid and 1,2 propanediol by *Lactobacillus buchneri*. **Applied and Environmental Microbiology**, Washington, v. 67, p. 125-132, 2001.

PAHLOW, G.; MUCK R. E. Managing for improved aerobic stability. In: BRODERICK G. A. (Ed.) **Proceedings of the 15th International Silage Conference**, Madison, Wisconsin, 2009. p. 77-90.

PAHLOW, G.; MUCK, R. E.; DRIEHUIS, F.; OUDE ELFERINK, S. J. W. H.; SPOELSTRA, S. F. Microbiology of ensiling. In: BUXTON, D. R.; MUCK, R. E.;

HARRISON, J. H. (Eds.). **Silage Science and Technology**. Madison, USA: American Society of Agronomy, 2003. p. 31-94.

PELLIKAAN, W. F.; STRINGANO, E.; LEENAARS, J.; BONGERS, D. J. G. M.; LAAR-VAN SCHUPPEN, S. V.; PLANT, J.; MUELLER-HARVEY, I. Evaluating effects of tannins on extent and rate of in vitro gas and CH_4 production using an automated pressure evaluation system (APES). **Animal Feed Science and Technology**, Amsterdam, v. 166-167, p. 377-390, 2011.

PHILLIP, L. E.; FELLNER, V. Effects of bacterial inoculation of high-moisture ear corn on its aerobic stability, digestion, and utilization for growth by beef steers. **Journal of Animal Science**, Albany, v. 70, n. 10, p. 3178-3187, 1992.

PITT, R. E.; MUCK, R. E.; PICKERING, N. B. A model of aerobic fungal growth in silage.2. Aerobic stability. **Grass and Forage Science**, Oxford, v. 46, n. 3, p. 301-312, 1991.

RABELO, C. H. S.; BASSO, F. C.; MCALLISTER, T. A.; LAGE, J. F.; GONÇALVE, G. S.; LARA, E. C.; OLIVEIRA, A. A.; BERCHIELLI, T. T.; REIS, R. A. Influence of *Lactobacillus buchneri* as silage additive and forage:concentrate ratio on the growth performance, fatty acid profile in longissimus muscle and meat quality of beef cattle. **Canadian Journal of Animal Science**, Ottawa, 2016. doi: 10.1139/CJAS-2015-0161

RABELO, C. H. S.; REZENDE, A. V.; RABELO, F. H. S.; NOGUEIRA, D. A.; SENEDESE, S. S.; VIEIRA, P. F.; BERNARDES, C. L.; CARVALHO, A. Silagens de milho inoculadas microbiologicamente em diferentes estádios de maturidade: perdas fermentativas, composição bromatológica e digestibilidade *in vitro*. **Ciência Rural**, Santa Maria, v. 44, n. 2, p. 368-373, 2014.

RANJIT, N. K.; KUNG JR., L. The effect of *Lactobacillus buchneri, Lactobacillus plantarum*, or a chemical preservative on the fermentation and aerobic stability of corn silage. **Journal of Dairy Science**, Champaign, v. 83, n. 3, p. 526-535, 2000.

RICHARD, E.; HEUTTE, N.; BOUCHART, V.; GARON, D. Evaluation of fungal contamination and mycotoxin production in maize silage. **Animal Feed Science and Technology**, Amsterdam, v. 148, p. 309-320, 2009.

ROMERO, S.; MERINO, E.; BOLÍVAR, F.; GOSSET, G.; MARTINEZ, A. Metabolic engineering of *Bacillus subtilis* for ethanol production: lactate dehydrogenase plays a key role in fermentative metabolism. **Applied and Environmental Microbiology**, Washington, v. 73, p. 5190-5198, 2007.

ROOKE, J. A.; HATFIELD, R. D. **Biochemistry of ensiling**. In: BUXTON, D. R.; MUCK, R. E.; HARRISON, J. H. (Eds.). **Silage Science and Technology**. Madison, USA: American Society of Agronomy, 2003. p. 95-140.

RYMER, C.; HUNTINGTON, J. A.; WILLIAMS, B. A.; GIVENS, D. I. *In vitro* cumulative gas production techniques: History, methodological considerations and challenges. **Animal Feed Science and Technology**, Amsterdam, p. 123-124, 2005.

SALVO, P. A. R.; BASSO, F. C.; RABELO, C. H. S.; OLIVEIRA, A. A.; SADER, A. P.; CASAGRANDE, D. R.; BERCHIELLI, T. T.; REIS, R. A. Características fermentativas e nutricionais de silagens de milho inoculadas com *Lactobacillus buchneri* e/ou *Lactobacillus plantarum*. **Archivos de Zootecnia**, Córdoba, v. 62, p. 1-12, 2013.

SÁ-PEREIRA, P.; MESQUITA, A.; DUARTE, J. C.; BARROS, M. R. A.; COSTA-FERREIRA, M. Rapid production of thermostable cellulase-free xylanase by a strain of *Bacillus subtilis* and its properties. **Enzyme and Microbial Technology**, Amsterdam, v. 30, p. 924-933, 2002.

SIQUEIRA, G. R.; BERNARDES, T. F.; REIS, R. A. Instabilidade aeróbia de silagens: efeitos e possibilidades de prevenção. In: SIMPÓSIO SOBRE VOLUMOSOS NA PRODUÇÃO DE RUMINANTES, 10., 2005, Jaboticabal. **Anais...** Jaboticabal: Funep, 2005. p. 25-60.

SUN, P.; WANG, J.-Q.; ZHANG, H.-T. Effects of supplementation of *Bacillus subtilis natto* Na and N1 strains on rumen development in dairy calves. **Animal Feed Science and Technology**, Amsterdam, v. 164, p. 154-160, 2011.

TAYLOR, C. C.; RANJIT, N. J.; MILLS, J. A.; NEYLON, J. M.; KUNG JR., L. The effect of treating whole-plant barley with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for dairy cows. **Journal of Dairy Science**, Champaign, v. 85, p. 1793-1800, 2002.

TELLES, R. R. T.; VIEIRA, B. R.; NAVES, V. V.; CARVALHO, I. P. C.; REZENDE, C. F.; ROCHA, L. B.; REIS, R. A. Efeito da inclusão de *Bacillus subtilis* na dieta de novilhos de corte sobre a digestibilidade aparente dos nutrientes. In: XXI CONGRESSO BRASILEIRO DE ZOOTECNIA, 21., 2011, Maceió. **Anais…** Maceió: Associação Brasileira de Zootecnia, 2011. CD-ROM.

TODOVORA, S; KOZHUHAROVA, L. Characteristics and antimicrobial activity of *Bacillus subtilis* strains isolated from soil. **World Journal of Microbiology and Biotechnology**, v. 26, p. 1207-1216, 2010.

VAN SOEST, P. J. Function of the ruminant forestomach. In: VAN SOEST, P. J. (Ed.). **Nutritional Ecology of the Ruminant**. Ithaca: Cornell University Press, 1994. p. 230-252.

VAN SOEST, P. J.; ROBERTSON, J. B.; LEWIS, B. A. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. **Journal of Dairy Science**, Champaign, v. 74, p. 3583-3597, 1991.

VANDENBERGH, P. A. Lactic acid bacteria, their metabolic products and interference with microbial growth. **FEMS Microbiology Reviews**, v. 12, p. 221-238, 1993.

WEIMER, P. J. Why don't ruminal bacteria digest cellulose faster? **Journal of Dairy Science**, Champaign, v. 79, p. 1496-1502, 1996.

WEINBERG, Z. G.; MUCK, R. E. New trends and opportunities in the development and use of inoculants for silage. **FEMS Microbiology**, Amsterdam, v. 19, p. 53-68, 1996.

WEINBERG, Z. G.; MUCK, R. E.; WEIMER, P. J. The survival of silage inoculant lactic acid bacteria in rumen fluid. **Journal of Applied Microbiology**, Oxford, v. 94, p. 1066-1071, 2003.

WEINBERG, Z. G.; MUCK, R. E.; WEIMER, P. J.; CHEN, Y.; GAMBURG, M. Lactic acid bacteria used in inoculants for silage as probiotics for ruminants. **Applied Biochemistry and Biotechnology**, Clifton, v. 118, p. 1-9, 2004.

WEINBERG, Z. G.; SHATZ, O.; CHEN, Y.; YOSEF, E.; NIKBAHAT, M.; BEN-GHEDALIA, D.; MIRON, J. Effect of lactic acid bacteria inoculants on *in vitro* digestibility of wheat and corn silages. **Journal of Dairy Science**, Champaign, v. 90, p. 4754-4762, 2007.

WILSON, C. L.; WISNIEWSKI, M. E.; BILES, C. L.; MCLAUGHLIN, R.; CHALUTZ, E.; DROBY, S. Biological control of post-harvest diseases of fruits and vegetables: alternatives to synthetic fungicides. **Crop Protection**, Amsterdam, v. 10, p. 172-177, 1991.

WOOLFORD, M. K. The detrimental effects of air on silage. **Journal of Applied Bacteriology**, London, v. 68, p. 101-116, 1990.

YILDIRIM, M. Purification of buchnericin LB produced by *Lactobacillus buchneri* LB. **Turkish Journal of Biology**, Ankara, v. 25, p. 59-65, 2001.

ZANETTE, P. M.; NEUMANN, M.; SANDINI, I.; MARAFON, F.; MARIA, F. N.; POCZYNEK, M. Características da carcaça de bovinos e digestibilidade de silagens de milho (Zea mays L.) com adição de açúcar ou inoculante enzimo-bacteriano. **Revista Brasileira de Milho e Sorgo**, Sete Lagoas, v. 10, n. 3, p. 235-246, 2011.

ZOPOLLATTO, M.; DANIEL, J. L. P.; NUSSIO, L. G. Aditivos microbiológicos em silagens no Brasil: revisão dos aspectos da ensilagem e do desempenho de animais. **Revista Brasileira de Zootecnia**, Viçosa, v. 38, p. 170-189, 2009. (supl. especial)

ZUBER, P., NAKANO, M. M., AND MARAHIEL, M. A. Peptide Antibiotics. In: SONENSHEIN, A. L.; HOCH, J. A.; LOSICK, R. (Eds.). ***Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics**. Washington: American Society for Microbiology, 1993. p. 897-916.

CHAPTER 2

The paper was written following the guidelines for authors of *Animal Feed Science and Technology*, with exception of tables and figures position.

Effects of *Lactobacillus buchneri* as a silage inoculant or probiotic on in vitro organic matter digestibility, gas production, and fermentation end products of corn silage

ABSTRACT

Our objective was to investigate the effects of *Lactobacillus buchneri* as a silage inoculant or probiotic on in vitro OM digestibility, gas production, and fermentation end products of corn silage. Whole-crop corn forage was chopped (279 g/kg DM) and ensiled without inoculant (untreated) and with *L. buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage (inoculated). Three runs of in vitro gas production were carried out, and untreated and inoculated corn silages (wet-ground) were incubated with three different ruminal inoculums, in a 2 × 3 factorial arrangement (n = 8). Ruminal fluids were collected from cannulated wethers consuming 1) untreated corn silage (RF-U); 2) inoculated corn silage (RF-I); and 3) untreated corn silage with a daily dose of *L. buchneri* CNCM I-4323 administered directly into the rumen (1×10^7 cfu/g of supplied silage [LB-probiotic]). In comparison to untreated silage, inoculation of corn silage with *L. buchneri* effectively altered carbohydrate fractionation by increasing the concentrations of acetic acid (P = 0.0063; from 28.9 to 54.4 g/kg DM) and residual water-soluble carbohydrates (P = 0.0189; from 12.2 to 18.9 g/kg DM), and reducing lactic acid concentration (P = 0.0265; from 66.1 to 45.3 g/kg DM). Gas production was consistently higher (P < 0.05) when inoculated silage was used as the substrate of fermentation, as compared to the untreated silage. In addition, inoculated silage had a higher degradation rate (P = 0.0385) than untreated silage (0.038% and 0.035%/h, respectively). When untreated silage was used as the substrate of fermentation, the molar proportion of methane (CH_4) was reduced (P = 0.0302) after 9 h of fermentation by using RF-I and LB-probiotic inoculums compared to RF-U inoculum (33.4 and 33.5 mM/100 mM vs. 34.7 mM/100 mM, respectively). When untreated silage was used as the substrate, total VFA concentration was higher when the RF-I and LB-probiotic inoculums were used as compared to the RF-U inoculum at 9 h (P = 0.0083) and at 48 h of fermentation (P = 0.0023). After 48 h of fermentation, OM digestibility was reduced (P = 0.0042) when inoculated silage was incubated with the LB-probiotic inoculum (556 g/kg OM) as compared to all other

treatments. In conclusion, the utilization of *L. buchneri* as a silage inoculant is more efficient than a probiotic because it alters silage quality by increasing in vitro gas production.

Keywords: lactic acid bacteria, methane, silage quality, volatile fatty acid

1. Introduction

Lactobacillus buchneri is a heterofermentative lactic-acid bacteria (^{he}LAB) largely used to enhance the aerobic stability of silages by producing high concentrations of acetic acid under anaerobic conditions (Driehuis et al., 1999). In some cases, animals consuming silage inoculated with *L. buchneri* had higher levels of DM intake, feed efficiency, and weight gain than animals fed untreated silage (Basso et al., 2014; Nkosi et al., 2009; Schmidt et al., 2014).

As primarily hypothesized for homolactic inoculants, enhanced growth performance of animals consuming silages inoculated with ^{he}LAB likely arises from improvements in silage quality, along with a possible probiotic effect of lactic-acid bacteria (LAB) (Weinberg and Muck, 1996; Weinberg et al., 2003). In addition, a recent study has indicated that increased microbial protein synthesis in the rumen due to LAB inoculation of corn silage could be the reason for enhanced growth performance in lambs (Basso et al., 2014). However, the effects of silage inoculants and their interactions with the ruminal bacterial community remain unclear.

In many opportunities, the known mechanisms of action of silage inoculants in the rumen have been established by in vitro techniques (Contreras-Govea et al., 2011; Muck et al., 2007; Weinberg et al., 2003). In these techniques, the gas production during in vitro fermentation has been positively correlated with volatile fatty acids (VFA) produced by ruminal microorganisms (Blümmel et al., 1997a). In vitro studies also found increased microbial biomass yield (MBY) by LAB inoculation of silages even with no differences in gas production and VFA concentration (Contreras-Govea et al., 2011; 2013). However, in vitro responses concerning effect of silage inoculants are widely varied and depending of the microorganism, strain identify, and substrate utilized (Ellis et al., 2016; Muck et al., 2007). Recently, one study demonstrated that LAB added as a probiotic or silage inoculant may elicit different in vitro responses, depending on the strain or concentration added in the

former case, and the strain and specific substrate in the latter (Ellis et al., 2016). The same study indicated that increased OM digestibility of rye grass silage due to LAB inoculation led to increased methane (CH_4) emission. Nevertheless, reductions in CH_4 emissions following LAB inoculation have been reported both with and without improvements in digestibility of total mixed ration silage prepared with whole crop rice and vegetable residue silage, respectively (Cao et al., 2010; 2011).

In addition, in vitro studies have reported that the lag time of a feedstuff is affected by inoculums during incubation and different responses likely arises from differences in microbial activity (Aiple et al., 1992; Mauricio et al., 2001). Consequently, in vitro gas production and OM digestibility might be impacted as well. In this regard, *L. buchneri* has the ability to produce bacteriocins (Yildirim, 2001) that might be act on sensitive gram-positive bacteria (Klaenhammer, 1993). Therefore, OM digestibility and fermentation end products under conditions of in vitro incubation might also be affected. However, to our best knowledge, ruminal fluids collected from animals consuming untreated or inoculated silages and their effects as inoculums on in vitro measurements has been not investigated. Thus, our objective was to investigate the effects of *L. buchneri* as a silage inoculant or probiotic on in vitro OM digestibility, gas production, and fermentation end products of corn silage.

2. Material and Methods

2.1. Ensiling procedure

Flint corn - *Zea mays* (hybrid Impacto Víptera, Syngenta, Matão, SP, Brazil) was harvested at 1/3 milk line (279 g/kg DM) using a Premium Flex forage harvester (Menta Mit, Cajuru, SP, Brazil) and chopped to a length of 10 mm. The Impacto Víptera is a hybrid recommended to produce grains and also for ensilage, which has 55,000–60,000 plants/ha and a productivity of approximately 50 ton/ha. Four piles of whole-crop corn forage was treated with water (5 L/t) (untreated), and other four piles were treated with *L. buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage (inoculated) (Lallemand Animal Nutrition, Goiânia, GO, Brazil). The inoculant was dissolved in distilled water (5 L/t) and sprayed onto fresh forage during silo filling. Viability and label levels of the bacteria in the inoculant were confirmed prior to use.

Eight concrete pipe silos were each filled with approximately 350 kg of corn forage on the same day. Four silos were filled with inoculated forage and the other four were filled with untreated forage. To avoid a possible cross contamination, untreated forage was ensiled first followed by inoculated forage. Forage packing was achieved with the application of human pressure, and the bulk density among the silos was assumed to be the same by the end of filling. Silos were sealed with black-on-white polyethylene film (200-μm thick) (Electro Plastic, São Paulo, SP, Brazil), and stored at an ambient temperature for 229 d. Four fresh samples of corn forage and silage were collected from each silo during filling and after silos were opened, and stored at -20°C for further analysis.

2.2. In vitro gas production

The kinetics of in vitro gas production (Theodorou et al., 1994) was evaluated over three runs using an adapted semi-automatic system (Mauricio et al., 1999). Wet-ground silages were used during incubation because dried samples might mask the effects of LAB occurring in an in vivo situation (Muck et al., 2007). Thus, a portion each of untreated and inoculated corn silage (~100 g) was wet-ground for 1 min in a Phillips Walita mixer (Walita, Varginha, MG, Brazil) to a particle size of approximately 1–4 mm (Muck et al., 2007). For each run, 1.0 g of the respective wet-ground silage was weighed, placed in a 115-mL serum bottle, and refrigerated at 4°C, overnight.

In vitro analysis and preparation of the ruminal inoculums followed the procedures described by Muck et al. (2007) and Contreras-Govea et al. (2011). On the day of inoculation, the Marten and Barnes (1979) buffer solution was continuously purged with CO_2 in volumetric flasks kept in a water bath at 39°C for 30 min. After this time, a reducing solution was added to the volumetric flasks under continuous CO_2 injection. Ruminal fluid used for the in vitro analysis was collected from six cannulated Santa Inês × Dorper crossbred wethers (three years of age and body weight = 74.5 ± 4.48 kg) arranged in a double 3 × 3 Latin square. Wethers were individually housed in 0.9 × 2.0 m pens, fitted with individual feed bunks and water bowls, and fed ad libitum once a day (0800) a mixed ration of 70% corn silage and 30% concentrate (24.6% ground corn, 3.4% soybean meal, 0.5% urea, and 1.5% mineral and vitamins supplement), on a DM basis. Wethers were adapted over three

19-d periods and were assigned to one of three diets: 1) untreated corn silage (RF-U); 2) inoculated corn silage (RF-I); and 3) untreated corn silage supplemented with a daily dose of *L. buchneri* CNCM I-4323 administered directly into the rumen (1×10^7 cfu/g of supplied silage [LB-probiotic]). In the third diet, the silage inoculant containing *L. buchneri* was diluted with distilled water and administered prior to feeding every day. The silages used to feed wethers were the same described earlier. Animal care and handling procedures in the present study were in accordance with the Brazilian College of Animal Experimentation (COBEA - Colégio Brasileiro de Experimentação Animal) guidelines and were approved by the Ethics, Bioethics and Animal Welfare Committee (CEBEA - Comissão de Ética e Bem Estar Animal) of the FCAV/UNESP - Jaboticabal campus, Brazil. Prior to feeding, pooled ruminal fluid (n = 2) from each diet was collected in pre-warmed thermos flasks, squeezed through four layers of cheesecloth, and mixed (1:4 v/v) with the aforementioned buffer solution in three volumetric flasks under continuous CO_2 injection. Thereafter, 60 mL of buffered ruminal inoculum was added to each bottle using an automatic dispenser. Bottles (n = 96) were then sealed with a rubber stopper and aluminum crimp cap and stored in a water bath at 39°C.

The gas pressure within the bottles was measured at 3, 6, 9, 12, 24, and 48 h of fermentation, using a pressure transducer connected to a visual display (data logger pressure model press DATA 800, MPL, Piracicaba, SP, Brazil). The pressure value readings were converted to volumes of gas, using the following equation (Eq. 1) specific to our laboratory conditions:

$$V\,(mL) = (5.4766 \times P) + 0.0934 \quad (1)$$

where V is the volume of gas and P is the measured pressure (psi).

The raw gas production was adjusted by deducting gas production from blank bottles (containing only buffer, reducing solution, and ruminal inoculum; two for each treatment and each run) and adjusting proportionately by using the gas production from a dried Tifton hay (two samples for each run) based on prior analyses. Gas production was expressed as mL/g of OM. Relative gas production was calculated by dividing gas production at a given time by the gas production after 48 h of fermentation. Degradability rate and lag time were estimated by the one-pool logistic

model proposed by Schofield et al. (1994) using non-linear least squares regression of the NLIN procedure of SAS (v. 9.4 SAS Institute Inc., Cary, NC).

2.3. End-products of in vitro fermentation

After 9 and 48 h of fermentation, 48 bottles were removed from the water bath and immersed in ice water to inhibit microbial activity. The pH of the ruminal inoculum was immediately measured using a pH meter (MA522 model, Marconi Laboratory Equipment, Piracicaba, SP, Brazil). Following pH measurement, 1 mL of H_2SO_4 (1:1) was added to the ruminal fluid, and aliquots of 45 mL were collected and stored at -20°C for subsequent determination of VFA. Measurements at 9 and 48 h of fermentation were selected based on an earlier in vitro study with undried silage where 9 h of fermentation was approximately the end of linear gas production, and 48 h of fermentation represented 95–98% of the gas produced at 96 h of fermentation (Muck et al., 2007).

2.4. Sample preparation and chemical analyses

A water extract was produced from fresh silage samples as described by Kung et al. (1984), and silage pH was measured using a pH meter (MA522 model, Marconi Laboratory Equipment, Piracicaba, SP, Brazil). Lactic acid was measured using a high performance liquid chromatography (HPLC) (Shimadzu model SIL-20A, Shimadzu Corp., Kyoto, Japan) equipped with a UV/VIS detection system and a refractive index detector (SPD-20A). An apolar column (Shimadzu CLC-ODS; 4.6 mm × 25 cm) was used for chromatographic separation at 30°C. The polar mobile phase consisted of a 20 mM monosodium phosphate solution with a flow rate of 1.25 mL/min. The acid was detected by UV absorbance (210 nm). VFAs were measured using a gas chromatograph (Shimadzu model GC2014, Shimadzu Corp., Kyoto, Japan) equipped with a capillary column (HP-INNOWax 30 m × 0.32 mm; Agilent Technologies, Colorado, USA) set at an initial temperature of 80°C for 3 min, followed by heating at a rate of 20°C/min until a final temperature of 240°C was achieved. Water-soluble carbohydrates (WSC) were determined following the Nelson-Somogyi method (Nelson, 1944). Ammonia-N was determined by distillation (AOAC, 1996; method no. 941.04), and expressed as g/kg of total N (TN).

For microbiological analyses, fresh silage samples (25 g) from each replicate were homogenized in 225 mL of autoclaved saline solution (0.85% NaCl) for 1 min. An aliquot (1 mL) of this solution was transferred into tubes containing 9 mL of saline solution, and thereafter, 1 mL of this mixture was plated onto Petri plates after serial dilutions of 10^{-1}–10^{-9}. Man, Rogosa, and Sharpe (MRS) agar was used to count LAB with pour plate, whereas potato dextrose agar (PDA) was used to count yeasts and molds by spread-plate. Both MRS and PDA plates were incubated at 28°C. LAB and yeasts were counted after 2 d, and molds were counted after 5 d, respectively. All microbiological data were $\log_{10}$-transformed.

Forage and silage samples were oven dried (at 55°C for 72 h) and processed in a knife mill before being ground through a 1-mm screen and analyzed for DM (105°C for 12 h) and ash (500°C for 5 h). OM was calculated as 1000 - ash. Ether extract (EE) was determined according to the procedures described by AOAC (1996; method no. 920.39). The total nitrogen (TN) was measured by rapid combustion using an analyzer (LECO model F528 N, LECO Corp., St. Joseph, MI, USA), and crude protein (CP) was calculated as TN × 6.25. Neutral detergent fiber (aNDF) was measured using a heat stable amylase without sodium sulfite in a fiber analyzer (ANKOM 2000, ANKOM Technologies, Macedon, NY, USA) following the procedures described by Mertens (2002). The aNDF was expressed inclusive of residual ash. Lignin was sequentially measured after hydrolysis of the acid detergent fiber residual in 72% H_2SO_4 (Van Soest and Robertson, 1985). Carbohydrate fractionation was calculated as total carbohydrates (CHOT); indigestible fiber (CC); digestible fiber (CB3); non-fiber carbohydrates (NFC); VFA (CA1); lactic acid (CA2); organic acids (CA3, assumed to be 0); sugars (CA4); starch (CB1); and soluble fiber (CB2) according to the procedures of Lanzas et al. (2007).

2.5. Calculations

DM as determined in a forced air oven (DM_{oven}) was corrected (DM_{corr}) for volatile compounds, using an adaptation (Eq. 2) from the original equation proposed by Weißbach and Strubelt (2008):

$$DM_{corr}\ (g/kg\ as\ fed) = DM_{oven} + 0.95*VFA + 0.08*lactic\ acid \quad (2)$$

where all volatile compounds are expressed in g/kg of fresh matter.

All variables regarding chemical composition and fermentation products of silage were expressed on DM_{corr}.

Starch was estimated using the following equation (Eq. 3) proposed by Mertens (2005):

$$Starch\ (g/kg\ DM) = -60 + 7.6*NFC \quad (3)$$

where non-fiber carbohydrates are expressed in g/kg DM and were previously calculated as NFC = 1000 - (aNDF + ash + CP + EE).

Since *in vitro* gas production reflects the content of digestible carbohydrates more than that of protein or fat, we used a multiple regression analysis (Eq. 4) proposed by Menke et al. (1979) to calculate the OM digestibility as follows:

$$OM\ digestibility\ (g/kg\ DM) = (13.3*gp) - (0.05*gp^2) + (511*CP) + (76*EE) + 91.2 \quad (4)$$

where *gp* is the gas production over 24 h (mL/0.2 g DM), and the *CP* and *EE* contents are expressed in g/kg DM.

In vitro estimations of the molar proportions of CO_2 (Eq. 5) and CH_4 (Eq. 6) were derived using equations proposed by Wolin (1960) as follows:

$$Fermentative\ CO_2\ (mol) = A/2 + P/4 + 1.5*B \quad (5)$$

where *A*, *P*, and *B* are mol of acetic, propionic, and butyric acids respectively.

$$Fermentative\ CH_4\ (mol) = (A + 2*B) - CO_2 \quad (6)$$

where *A* and *B* are mol of acetic and butyric acid, respectively, and CO_2 is mol of carbon dioxide calculated from the previous equation.

2.6. Statistical analyses

Silage data were analyzed as a completely randomized design using the MIXED procedure of SAS. Silage was considered a fixed effect and error, a random effect. Concrete pipe silos were considered the experimental unit for silage measurements (n = 4).

Each in vitro measurement was also analyzed as a completely randomized design using the MIXED procedure of SAS in a 2 (silage) × 3 (rumen inoculum) factorial design. Run was included in the model as random using the RANDOM statement of the MIXED procedure of SAS. Silage and ruminal inoculums were considered a fixed effect and error, a random effect. Bottles were considered the experimental unit for the in vitro assay (n = 8).

Differences between silage means were determined using an F test, whereas significant means for in vitro ruminal inoculums were compared using the PDIFF option of LSMEANS. When significant interactions between silage and ruminal inoculums occurred, means were separated using a Fisher´s *F*-protected least significant difference test. Significant differences were declared at $P \leq 0.05$.

3. Results

3.1. Fermentation, microbial profile, and chemical composition of corn silage

In comparison to untreated silage, inoculation of corn silage with *L. buchneri* increased silage pH ($P = 0.0077$) and the concentration of ammonia-N ($P = 0.0019$) by 120% (Table 1). In contrast, inoculation reduced ($P = 0.0034$) CP content by 12.6%, when compared to untreated silage. The CA1 fraction was increased ($P = 0.0266$) by 64% as a result of inoculation of the corn silage. Among the VFAs that comprised the CA1 fraction, only acetic acid was affected by inoculation and it increased ($P = 0.0063$) by 88% compared to untreated silage (Fig. 1). The CA2 fraction was reduced ($P = 0.0265$) by 31.5% in inoculated silage compared to untreated silage. In addition, the CA4 fraction was increased ($P = 0.0189$) by 55% in inoculated silage. Contents of DM_{oven}, DM_{corr}, OM, EE, CHOT, and other carbohydrates fractions (CC, CB1, CB2, CB3, and NFC) were unaffected by inoculation of corn silage ($P > 0.05$).

Inoculation of corn silage with *L. buchneri* increased ($P = 0.0054$) the LAB count by 0.52 cfu/g of fresh silage, and reduced ($P = 0.0371$) the yeast count by 0.95 cfu/g of fresh silage as compared to untreated silage (Table 1). The mold count was unaffected ($P = 0.2201$) by inoculation of corn silage.

Table 1
Fermentation, chemical composition, and microbial profile of corn silage untreated and inoculated with *Lactobacillus buchneri* in concrete pipe silos after 229 d of ensilage (n = 4; data are given in g/kg DM, unless otherwise stated).[a]

Item	Untreated	Inoculated	SEM	*P*-value
Chemical composition				
DM_{oven}, g/kg as fed	248	260	7.51	0.2723
DM_{corr}, g/kg as fed	258	271	8.24	0.2875
OM	968	967	0.93	0.6747
EE	39.4	35.9	1.18	0.0555
CP	88.2[a]	77.1[b]	2.22	0.0034
Ammonia-N, g/kg of TN	24.6[b]	54.2[a]	5.49	0.0019
pH	4.15[b]	4.30[a]	0.03	0.0077
Carbohydrate fractionation[b]				
CHOT	802	790	7.12	0.2801
CC	82.9	106	9.47	0.1101
CB3	178	155	21.12	0.4482
NFC	529	527	15.93	0.9368
CA1	37.2[b]	61.0[a]	6.79	0.0266
CA2	66.1[a]	45.3[b]	5.23	0.0265
CA3	0	0		
CA4	12.2[b]	18.9[a]	1.78	0.0189
CB1	342	341	12.10	0.9365
CB2	105	82	16.22	0.3363
Microbial profile				
LAB, log_{10} cfu/g[c]	6.82[b]	7.34[a]	0.11	0.0054
Yeasts, log_{10} cfu/g	5.91[a]	4.96[b]	0.29	0.0371
Molds, log_{10} cfu/g	4.60	4.17	0.23	0.2201

[a-b]Means in the same row with different superscripts differed ($P < 0.05$), and do not include a comparison with the forage prior to ensiling.
[a] Corn silage was either untreated or treated at ensiling with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage (inoculated) (Lallemand Animal Nutrition, Milwaukee, WI, USA).
[b] Carbohydrates fractionation proposed by Lanzas et al. (2007), where CHOT = total carbohydrates; CC = indigestible fiber; CB3 = digestible fiber; NFC = non-fiber carbohydrates; CA1 = volatile fatty acids; CA2 = lactic acid; CA3 = organic acids; CA4 = water-soluble carbohydrates; CB1 = starch; CB2 = soluble fiber.
[c] LAB = lactic-acid bacteria; DM = dry matter; OM = organic matter; EE = ether extract; CP = crude protein; TN = total nitrogen.

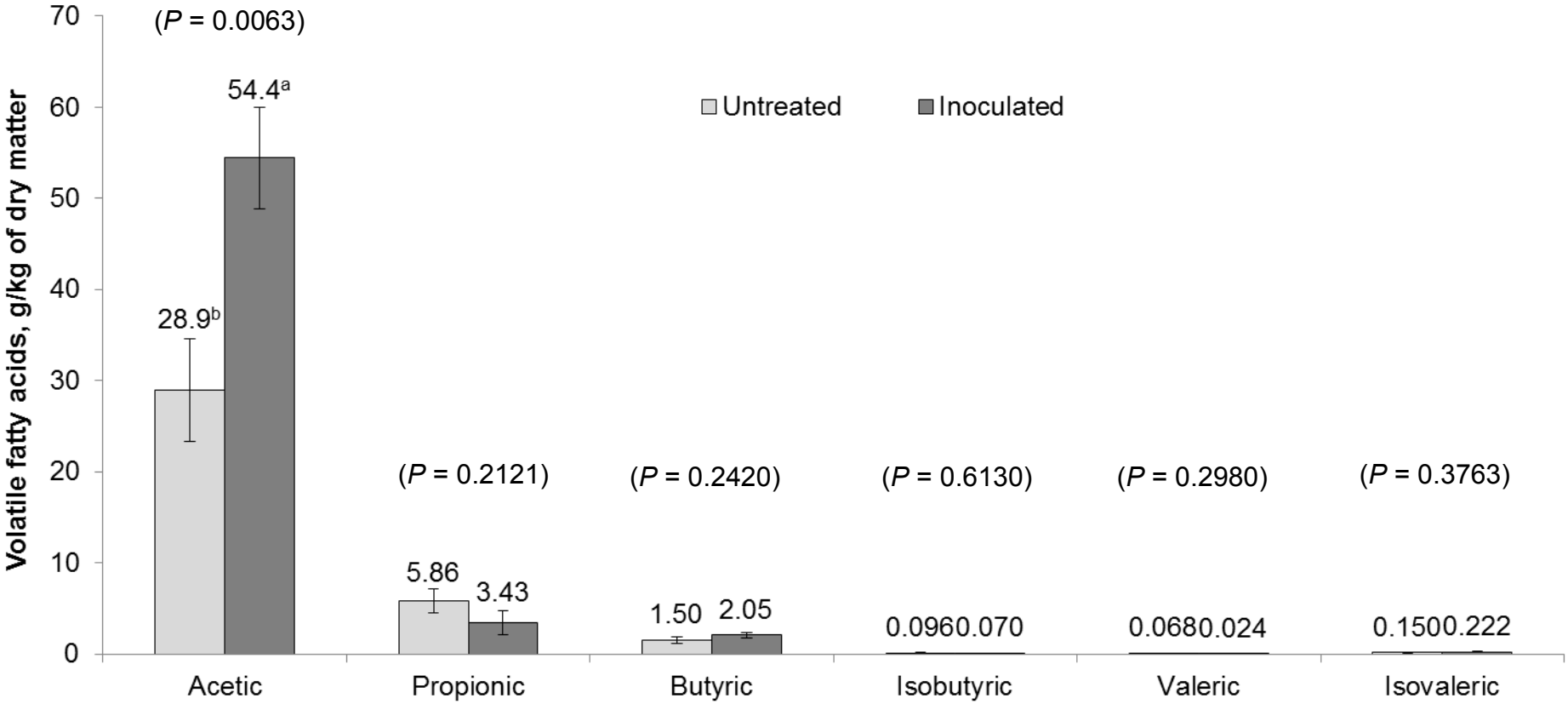

Fig. 1. Volatile fatty acids (g/kg of DM) of corn silage untreated and inoculated with *Lactobacillus buchneri* in concrete pipe silos after 229 d of ensilage (n = 4).

3.2. In vitro gas production

No significant interactions ($P > 0.05$) were noted between silage and ruminal inoculum at any interval during fermentation (Table 2). But gas production was consistently higher ($P < 0.05$) when inoculated silage was used as the substrate, with values increasing by 11.2%, 6.8%, 7.9%, 7.4%, 8.6%, and 5.6% at 3, 6, 9, 12, 24, and 48 h of fermentation, respectively. Regarding the effects of ruminal inoculum, gas production was consistently lower ($P < 0.01$) within the first 9 h of fermentation when RF-U was used, as compared to RF-I and LB-probiotic inoculums.

Silage used as the substrate of fermentation affected relative gas production only at 3 h ($P = 0.0002$) and 12 h ($P = 0.0102$) of fermentation; however, the data were inconsistent, since inoculated silage yielded relatively higher and lower relative gas production at 3 and 12 h of fermentation, respectively, when compared to untreated silage (Table 2). Nevertheless, relative gas production was consistently lower ($P < 0.01$) at all fermentation times when the RF-U inoculum was used during incubation as compared to RF-I and LB-probiotic inoculums. With the RF-I and LB-probiotic inoculums, approximately 24%, 35%, and 43% of 48-h gas production occurred by 6, 9, and 12 h of fermentation, respectively.

Table 2
In vitro gas production (mL/g OM) and relative gas production (fraction of 48-h production) at various incubation times using corn silage untreated and inoculated with *Lactobacillus buchneri* as substrates in combination with three ruminal inoculums (n = 8).

Silage[a]	Untreated			Inoculated			SEM	*P*-value[c]		
Ruminal inoculum[b]	RF-U	RF-I	LB-probiotic	RF-U	RF-I	LB-probiotic		S	RI	S × RI
Gas production										
3 h	37.9	43.4	40.9	39.6	48.0	48.3	2.56	0.0004	<0.0001	0.1637
6 h	45.0	49.4	49.1	45.6	53.8	53.9	12.68	0.0332	0.0009	0.4640
9 h	64.9	68.8	70.8	66.2	80.0	74.5	17.24	0.0064	0.0003	0.2587
12 h	87.1	88.1	86.4	90.2	95.5	95.2	18.43	0.0019	0.4161	0.4794
24 h	164	156	168	172	177	181	19.59	<0.0001	0.1351	0.2858
48 h	214	201	209	223	217	219	20.73	0.0025	0.1118	0.7518
Relative gas production										
3 h	0.159	0.197	0.186	0.168	0.204	0.212	0.009	0.0002	<0.0001	0.0572
6 h	0.207	0.247	0.240	0.201	0.236	0.237	0.041	0.3071	<0.0001	0.8402
9 h	0.295	0.350	0.356	0.288	0.337	0.349	0.055	0.2303	<0.0001	0.9164
12 h	0.395	0.449	0.436	0.393	0.422	0.415	0.050	0.0102	<0.0001	0.2315
24 h	0.765	0.798	0.809	0.776	0.803	0.811	0.017	0.2787	<0.0001	0.8063

[a] Corn silage was either untreated or treated at ensiling with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage (inoculated) (Lallemand Animal Nutrition, Milwaukee, WI, USA).
[b] RF-U = rumen fluid collected from wethers fed the untreated silage; RF-I = rumen fluid collected from wethers fed the inoculated silage; LB-probiotic = rumen fluid collected from wethers fed the untreated silage supplemented with a daily dose of *L. buchneri* CNCM I-4323 administered directly into the rumen (1×10^7 cfu/g of supplied silage).
[c] S = silage; RI = ruminal inoculum; S × RI = interaction between silage and ruminal inoculum.

3.3. In vitro fermentation products

3.3.1. 9 h of fermentation

Significant interactions were noted between silage used as the fermentation substrate and ruminal inoculum used during incubation for CH_4 (P = 0.0302), total VFA concentration (P = 0.0083), molar proportion of acetic (P = 0.0126) and isovaleric acids (P = 0.0016), and acetic:propionic acid ratio (P = 0.0297) (Table 3). RF-I and LB-probiotic inoculums increased the total VFA concentration and reduced the molar proportions of acetic acid and CH_4, and the acetic:propionic acid ratio as compared to the RF-U inoculum when untreated silage was used as the substrate. However, similar results were not observed for inoculated silage. When compared to the RF-U inoculum, only RF-I inoculum reduced the molar proportion of acetic acid when inoculated silage was the substrate. The molar proportion of isovaleric acid was reduced when RF-I and LB-probiotic inoculums were used with inoculated silage as the substrate.

Furthermore, the pH of the ruminal inoculums were lower (P < 0.01) with inoculated silage than with untreated silage (6.59 and 6.63, respectively) (Table 3). The ruminal inoculums used during incubation also affected (P = 0.0027) the pH, as RF-U, RF-I, and LB-probiotic inoculums having values of 6.63, 6.59, and 6.61, respectively. The molar proportion of CO_2 was only affected by ruminal inoculums (P < 0.01), and the RF-I inoculum had the greatest value (58.7 mM/100 mM). The molar proportions of propionic (P = 0.0045) and butyric (P < 0.01) acids increased with the use of the RF-I inoculum (propionic acid = 19.1 mM/100 mM; butyric acid = 15.4 mM/100 mM). The molar proportion of isobutyric acid reduced (P < 0.01) when inoculated silage was used as the substrate in comparison to untreated silage (1.45 and 1.94 mM/100 mM, respectively); however, the molar proportion of valeric acid increased (P < 0.01; 0.651 and 0.446 mM/100 mM, respectively). For both isobutyric (P = 0.0015) and valeric acids (P = 0.0198), incubation using the LB-probiotic inoculum yielded the greatest values (1.83 and 0.585 mM/100 mM, respectively).

Table 3
Characteristics of rumen fluid after 9 h of in vitro fermentation and methane estimation using corn silage untreated and inoculated with *Lactobacillus buchneri* as substrates in combination with three ruminal inoculums (n = 8; data are given in mM/100 mM, unless otherwise stated).

Silage[a]	Untreated			Inoculated			SEM	*P*-value[c]		
Ruminal inoculum[b]	RF-U	RF-I	LB-probiotic	RF-U	RF-I	LB-probiotic		S	RI	S × RI
pH	6.65	6.62	6.62	6.61	6.55	6.60	0.09	<0.0001	0.0027	0.1081
CO_2	56.6	58.5	56.2	57.0	58.9	56.8	0.89	0.1581	<0.0001	0.9225
CH_4	34.7^{a}	33.4^{b}	33.5^{b}	34.4^{a}	34.0^{ab}	34.4^{a}	0.50	0.0414	0.0046	0.0302
Total VFA, mM	54.7^{c}	73.6^{a}	61.7^{b}	63.4^{b}	66.2^{b}	61.8^{b}	2.43	0.8249	0.0001	0.0083
Molar proportion										
Acetic acid	65.3^{a}	61.3^{c}	63.4^{b}	64.4^{ab}	62.2^{c}	64.9^{a}	0.45	0.1590	<0.0001	0.0126
Propionic acid	17.9	19.5	19.2	18.2	18.7	18.4	0.79	0.1037	0.0045	0.0979
Acetic:propionic acid ratio	3.63^{a}	3.16^{d}	3.30^{c}	3.53^{b}	3.26^{c}	3.54^{b}	0.12	0.1199	<0.0001	0.0297
Butyric acid	13.0	15.3	13.1	13.5	15.4	13.2	0.83	0.5014	<0.0001	0.8410
Isobutyric acid	1.86	1.83	2.14	1.55	1.28	1.52	0.25	<0.0001	0.0015	0.1205
Valeric acid	0.404	0.427	0.506	0.604	0.686	0.663	0.11	<0.0001	0.0198	0.1576
Isovaleric acid	1.61^{a}	1.60^{a}	1.65^{a}	1.62^{a}	1.52^{b}	1.39^{c}	0.06	0.0004	0.0540	0.0016

[a-c]Means in the same row with different superscripts differed ($P < 0.05$).
[a] Corn silage was either untreated or treated at ensiling with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage (inoculated) (Lallemand Animal Nutrition, Milwaukee, WI, USA).
[b] RF-U = rumen fluid collected from wethers fed the untreated silage; RF-I = rumen fluid collected from wethers fed the inoculated silage; LB-probiotic = rumen fluid collected from wethers fed the untreated silage supplemented with a daily dose of *L. buchneri* CNCM I-4323 administered directly into the rumen (1×10^7 cfu/g of supplied silage).
[c] S = silage; RI = ruminal inoculum; S × RI = interaction between silage and ruminal inoculum.

3.3.2. 48 h of fermentation

Significant interactions were noted between silage used as a substrate and ruminal inoculum used during incubation for OM digestibility (P = 0.0042), total VFA concentration (P = 0.0023), and molar proportion of butyric acid (P = 0.0456) (Table 4). OM digestibility was reduced (556 g/kg OM) when the LB-probiotic inoculum was incubated with inoculated silage, as compared to all other treatments. Total VFA concentration increased when RF-I and LB-probiotic inoculums were used, as compared to the RF-U inoculum, although this was observed only when untreated silage was the substrate. In addition, incubation of inoculated silage with the LB-probiotic inoculum reduced the total VFA concentration and the molar proportion of butyric acid, in comparison to RF-U and RF-I inoculums, when inoculated silage was the substrate.

There was a small but significant difference (P = 0.0385) in the degradability rate, and inoculated silage had higher values than untreated silage (0.038% and 0.035%/h, respectively) (Table 4). The degradability rate was also affected (P = 0.0373) by ruminal inoculums, with higher values for the RF-I and LB-probiotic inoculums (0.040% and 0.039%/h, respectively) as compared to the RF-U inoculum (0.036%/h). The pH of the ruminal inoculums were lower (P = 0.0008) during incubation of inoculated silage as compared to untreated silage (6.53 and 6.58, respectively). In addition, pH was also affected (P = 0.0021) by the ruminal inoculum used during incubation, as LB-probiotic inoculum having a comparatively lower value (6.53). The molar proportion of CO_2 was reduced ($P < 0.01$) when the LB-probiotic inoculum was used during incubation (56.6 mM/100 mM) as compared to other ruminal inoculums; however CH_4 was unaffected by silage treatment (P = 0.9508) or ruminal inoculum (P = 0.5452). The molar proportion of acetic acid was lower (P = 0.0082) when the RF-I inoculum was used during incubation (58.8 mM/100 mM). The molar proportions of isobutyric (P = 0.0017) and isovaleric (P = 0.0427) acids were increased when RF-I and LB-probiotic inoculums were used during incubation.

Table 4
Organic matter digestibility, characteristics of rumen fluid after 9 h of in vitro fermentation, and methane estimation using corn silage untreated and inoculated with *Lactobacillus buchneri* as substrates in combination with three ruminal inoculums (n = 8; data are given in mM/100 mM, unless otherwise stated).

Silage[a]	Untreated			Inoculated			SEM	*P*-value[c]		
Ruminal inoculum[b]	RF-U	RF-I	LB-probiotic	RF-U	RF-I	LB-probiotic		S	RI	S × RI
OM digestibility, g/kg of OM	711[a]	686[a]	709[a]	728[a]	731[a]	556[b]	38.64	0.2402	0.0124	0.0042
Degradability rate, %/h	0.035	0.033	0.037	0.036	0.037	0.041	0.002	0.0385	0.0373	0.5050
Lag time, h	1.59	0.68	1.29	2.43	2.26	2.25	1.56	0.0650	0.7186	0.8452
pH	6.59	6.59	6.56	6.58	6.53	6.49	0.08	0.0008	0.0021	0.0846
CO_2	57.5	57.6	56.3	56.9	57.8	56.8	1.10	0.9745	<0.0001	0.0589
CH_4	32.5	32.3	32.3	32.5	32.2	32.4	0.25	0.9508	0.5452	0.9389
Total VFA, mM	82.6[b]	104[a]	102[a]	112[a]	108[a]	87.7[b]	26.42	0.2173	0.1676	0.0023
Molar proportion										
Acetic acid	59.8	59.1	60.1	60.0	58.5	59.4	0.69	0.2786	0.0082	0.3862
Propionic acid	19.4	19.8	19.6	19.4	19.6	19.4	0.35	0.6416	0.6529	0.9697
Acetic:propionic acid ratio	3.15	2.99	3.08	3.11	3.02	3.08	0.06	0.9250	0.1906	0.8393
Butyric acid	15.2[a]	15.4[a]	14.3[b]	14.7[ab]	15.7[a]	14.8[ab]	0.94	0.5472	<0.0001	0.0456
Isobutyric acid	1.55	1.64	1.72	1.53	1.62	1.70	0.11	0.5378	0.0017	0.9937
Valeric acid	1.46	1.45	1.57	1.52	1.53	1.61	0.06	0.0223	0.0035	0.8776
Isovaleric acid	2.79	2.87	3.00	2.85	2.98	3.00	0.13	0.3196	0.0427	0.7294

[a-b]Means in the same row with different superscripts differed ($P < 0.05$).
[a] Corn silage was either untreated or treated at ensiling with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage (inoculated) (Lallemand Animal Nutrition, Milwaukee, WI, USA).
[b] RF-U = rumen fluid collected from wethers fed the untreated silage; RF-I = rumen fluid collected from wethers fed the inoculated silage; LB-probiotic = rumen fluid collected from wethers fed the untreated silage supplemented with a daily dose of *L. buchneri* CNCM I-4323 administered directly into the rumen (1×10^7 cfu/g of supplied silage).
[c] S = silage; RI = ruminal inoculum; S × RI = interaction between silage and ruminal inoculum.

The molar proportion of valeric acid was increased (*P* = 0.0223) when inoculated silage was the substrate, as compared to untreated silage (1.55 and 1.49 mM/100 mM, respectively) (Table 4). Valeric acid was also affected (*P* = 0.0035) by ruminal inoculum, and the incubation using LB-probiotic inoculum increased the molar proportion (1.59 mM/100 mM) compared to RF-U and RF-I inoculums (1.49 mM/100 mM for both).

4. Discussion

4.1. Fermentation, microbiology profile, and chemical composition of corn silage

As expected, inoculation of corn silage with *L. buchneri* reduced lactic acid while increased acetic acid concentration compared to untreated silage. This response occurred because the ability of *L. buchneri* to convert lactic acid to acetic acid and 1,2-propanediol under anaerobic conditions (Driehuis et al., 1999; Oude Elferink et al., 2001). Considering the stoichiometry of the complete degradation of lactic acid (Oude Elferink et al., 2001), inoculated corn silage had a concentration of acetic acid (54.4 g/kg DM) that was slightly higher than the expected value (51.1 g/kg DM). However, this small difference of 3.3 g/kg DM likely may be attributed to the action of other LAB and enterobacteria, since both are able to produce acetic acid during fermentation (Pahlow et al., 2003). Furthermore, inoculation of corn silage increased LAB count while decreased yeast count compared to untreated silage. The LAB count increased in response to the addition of *L. buchneri* during ensiling, whereas yeast growth was inhibited because of the increased concentration of acetic acid and its antifungal properties (Moon, 1983). Inoculated silage also had a greater WSC concentration (CA4 fraction of carbohydrates) as compared to untreated silage. This response could be attributed to the inhibition of yeasts (that primarily use soluble carbohydrates for growth) during fermentation (Pahlow et al., 2003).

Our results are consistent with those of a previous report of meta-analysis, in which *L. buchneri* consistently elevated the acetic acid concentration of several types of crops, reduced the yeast count, and enhanced the aerobic stability of silages (Kleinschmit and Kung, 2006). In addition, previous studies on silages inoculated with *L. buchneri* have reported enhanced preservation of WSC during fermentation (Nkosi et al., 2009; 2012).

Inoculation of corn silage with *L. buchneri* increased the pH from 4.15 in untreated silage to 4.30 after 229 d of ensilage. This response was expected, because *L. buchneri* converts lactic acid (pK_a of 3.86) to the weaker acetic acid (pK_a of 4.73), resulting in an increase in silage pH. In addition, CP content was reduced whereas ammonia-N was increased in inoculated silage, as compared to untreated silage. Increased ammonia-N concentration has been observed in *L. buchneri*-treated silages stored for a prolonged time because this bacterium can remain fairly active for prolonged periods of time (up to a year) (Der Bedrosian et al., 2012; Kleinschmit and Kung, 2006), and LAB are relatively acid tolerant and possess a wide variety of extracellular proteases and intracellular peptidases (Kunji et al., 1996). Therefore, although the fermentation process might be altered after a prolonged period by inoculation with *L. buchneri* (i.e., higher acetic acid concentration), likely the chronic proteolytic activity played by LAB and other microorganisms or even by some plant proteases has a more important role on the degradation of proteins and production of ammonia-N (Hoffman et al., 2011).

4.2. In vitro gas production

Gas production was consistently higher at all fermentation times, when inoculated silage was used as the substrate, as compared to untreated silage. Since gas production is basically the result of digestible carbohydrates fermentation (Menke et al., 1979), our results could be attributed to enhanced WSC preservation in inoculated silage compared to untreated silage. Insofar as inoculated silage had a greater gas production, we could also expect increased VFA production by ruminal microorganisms, as suggested by Blümmel et al. (1997a). However, no differences in total VFA concentrations were noted between untreated and inoculated silage at 9 and 48 h of fermentation. Gas production arises directly from microbial degradation of feed, and indirectly from the buffering of acids generated by fermentation (Getachew et al., 2004). Moreover, additional gas is produced simply by contact between VFAs produced in the silo and the buffer inoculum (Palmer et al., 2005). Therefore, indirect gas production from VFAs produced in the silo and during in vitro fermentation might explain the lack of a positive relationship between gas production and VFA concentration in the present study. Indeed, interactions between the

amount of gas produced and fermentation end products are very complex (Beuvink and Spoelstra, 1992). However, Muck et al. (2007) incubated alfalfa silages treated with several LAB inoculants and demonstrated that increased gas production is not always accompanied by increased VFA concentrations.

Blümmel et al. (1997b) indicated that a greater in vitro gas production was positively correlated with DM digestibility. In the present study, OM digestibility was estimated at 48 h of fermentation; however, as observed for total VFA, apparently there was not any relationship with gas produced. The lack of a relationship between OM digestibility and gas production found in our study could be due to feed constituents, such as fat and protein that produce little or no gas, but are degraded in vitro. This finding was also reported by Getachew et al. (2004). However, the results of the present study revealed that increased gas production led to a higher degradability rate. The constant degradation of silage should also constantly produce gas, and this trend was consistently observed for inoculated silage, over 48 h of fermentation.

Even without variations in the total VFA concentration, the pH at 9 and 48 h of fermentation was slightly lower in inoculated silage as compared to untreated silage. The relatively small, but significant difference in pH likely arose from alterations in the molar proportions of VFAs. The concentration of valeric acid was consistently increased in inoculated silage at 9 and 48 h of fermentation as compared to untreated silage. Increased concentration of valeric acid can be indicative of an increased cellulose digestion (Cline et al., 1958), even though none alteration had been observed in fiber fraction of corn silage caused by inoculation.

Despite of using *L. buchneri* as a probiotic in the ruminal inoculum, in the first 9 h of fermentation the gas production was higher by using RF-I and LB-probiotic inoculums compared to RF-U inoculum, but not thereafter. In a similar manner, the relative gas production was consistently higher up to 24 h of fermentation when RF-I and LB-probiotic inoculums were used during incubation, as compared to the RF-U inoculum. Our results indicate that approximately 41–45% of the total gas produced during in vitro fermentation occurred during the first 12 h when RF-I and LB-probiotic inoculums were used. Incubation with RF-U led to a lesser relative gas production at 12 h of fermentation (39%). This response is most likely related to the specific

microbial populations that are predominant during fermentation, since LAB and native microorganisms in the ruminal fluid have preferences for specific substrates (Ellis et al., 2016) and the rate of LAB growth depends of the substrate (Carvalho et al., 2011). Moreover, the relative gas production observed after 9 h of fermentation in the present study was much lower than that observed by Muck et al. (2007). In this study, the authors reported that 65–70% of the gas produced during in vitro ruminal fermentation of alfalfa silages treated with several LAB inoculants, occurred within the first 9–10 h of incubation. However, this response is likely crop-specific and dependent of the different ruminal inoculums prepared for in vitro fermentation.

The degradability rate increased, whereas the pH declined at 48 h of fermentation when the LB-probiotic inoculum was used during incubation. As discussed earlier, LAB and native microorganisms in the ruminal fluid have preferences for specific substrates (Ellis et al., 2016); therefore, differences in the rate of feed degradation could be expected. In addition, after 48 h of fermentation, the concentration of isobutyric and isovaleric acids increased when RF-I and LB-probiotic inoculums were used during incubation as compared to the RF-U inoculum. High concentrations of isoacids can positively influence the MBY (Andries et al., 1987). Although the MBY was not estimated in the present study, earlier in vitro studies have reported increased MBY following inoculation of silages with *L. plantarum* and *Lactococcus lactis* (Contreras-Govea et al., 2011; 2013).

Some interactions were noted between silage used as a substrate and the ruminal inoculum used during incubation. Only when untreated silage was used as the substrate, the total VFA concentration was increased with RF-I and LB-probiotic inoculums at 9 and 48 h of fermentation, as compared to the RF-U inoculum. In addition, after 9 h of fermentation, incubation of untreated silage combined with RF-I and LB-probiotic inoculums reduced acetic acid concentration and increased propionic acid, leading to a reduction in the acetic:propionic acid ratio and the molar proportion of CH_4. *L. buchneri* has the ability to produce bacteriocin (Yildirim, 2001). The activity of bacteriocins might be directed towards sensitive gram-positive bacteria (Klaenhammer, 1993), with a possible reduction in cellulolytic activity in the rumen (Wolin et al., 1997). As has been proposed for other LAB (Contreras-Govea et al., 2011), *L. buchneri* could possibly influence some microflora in the rumen, or

otherwise confer some unknown silage characteristic or combination of characteristics that might directly enhance ruminal microbial biomass production. This hypothesis could explain why the concentration of acetic acid was reduced with RF-I and LB-probiotic inoculums, as compared to the RF-U inoculum. Furthermore, the metabolic pathway used by ruminal bacteria to produce acetic acid involves the formation of CO_2 and H_2, but these products are not formed during the fermentation of carbohydrates to propionic acid (Church, 1993). Both CO_2 and H_2 are used by methanogenic bacteria to form CH_4 (Church, 1993), and this explains the reduction in the molar proportion of CH_4 with RF-I and LB-probiotic inoculums, as compared to the RF-U inoculum. The reduction in the molar proportion of CH_4 observed in the present study is consistent with the findings of Cao et al. (2010), who also reported reductions in CH_4 emissions following LAB inoculation, without any alterations in the digestibility of vegetable residue silage.

Although the inoculation of corn silage with *L. buchneri* increased gas production after 48 h of fermentation, OM digestibility was reduced when inoculated silage was combined with the LB-probiotic inoculum in comparison to all other treatments. However, the reasons behind this finding remain unclear.

In summary, in comparison to untreated silage, the use of *L. buchneri* as a silage inoculant affected the fermentation of corn silage by preserving a larger proportion of WSC and increasing in vitro gas production and the degradability rate. Conversely, the increased gas production was not accompanied by increases in OM digestibility or total VFA. In contrast, the results of the present study revealed that there is some interaction between substrate and inoculum. Furthermore, *L. buchneri* when used as a probiotic, consistently increased total VFA concentrations at 9 and 48 h of fermentation when untreated corn silage was used as a substrate; however, similar results were not observed for inoculated silage. In addition, the molar proportion of CH_4 was reduced and gas production was increased after 9 h of fermentation, when *L. buchneri* was used as a probiotic in combination with untreated silage used as the substrate; however, similar results were not observed after 48 h of fermentation. In other words, *L. buchneri* appears to have different mechanisms of action depending on its use as a silage inoculant or probiotic. As has been previously reported, this bacteria has a greater ability to convert lactic acid to acetic acid and

1,2-propanediol at a pH of 3.8, as compared to that of 5.8 (Oude Elferink et al., 2001). Thus, the different responses observed in the present study likely reflects the capacity of *L. buchneri* to grow and produce metabolites that act more efficiently as silage inoculants (pH of silage generally around 4.2) than probiotics (pH of ruminal inoculum generally around 6.5). Our results and those of previous studies (Contreras-Govea et al., 2011; Muck et al., 2007) demonstrate that in vitro gas production is greatly affected by the substrate used during incubation. In a similar manner, the results of the present study corroborate the assumptions of Ellis et al. (2016), who reported that the responses observed following the use of LAB as a probiotic might be also substrate-specific. In addition, our results revealed that fermentation end products were affected to a greater extent than gas production when *L. buchneri* was used as a probiotic.

5. Conclusions

L. buchneri is more efficient as a silage inoculant than a probiotic because it alters fermentation patterns of corn silage leading an increased gas production over time, even though without any benefits on OM digestibility.

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References

Aiple, K.P., Steingass, H., Menke, K.H., 1992. Suitability of a buffered faecal suspension as the inoculum in the Hohenheim gas test. J. Anim. Physiol. Nutr. 67, 57–66.

Andries, J.I., Buysse, F.X., Brabander, D.L., Cottyn, B.G., 1987. Isoacids in ruminant nutrition: their role in ruminal and intermediary metabolism and possible influences on performance - A review. Anim. Feed Sci. Technol. 18, 169-180.

Association of Official Analytical Chemist, AOAC, Official Methods of Analysis, 16 ed., 1996, AOAC; Washington DC, USA.

Basso, F.C., Adesogan, A.T., Lara, E.C., Rabelo, C.H.S., Berchielli, T.T., Teixeira, I.A.M.A., Siqueira, G.R., Reis R.A., 2014. Effects of feeding corn silage inoculated with microbial additives on the ruminal fermentation, microbial protein yield, and growth performance of lambs. J. Anim. Sci. 92, 5640–5650.

Beuvink, J.M.W., Spoelstra, S.F., 1992. Interactions between substrate, fermentation end-products, buffering systems and gas production upon fermentation of different carbohydrates by mixed rumen microorganisms in vitro. Appl. Microbiol. Biot. 37, 505–509.

Blümmel, M., Makkar, H.P.S., Becker, K., 1997a. In vitro gas production: a technique revisited. J. Anim. Physiol. Anim. Nutr. 77, 24–34.

Blümmel, M., Steingass, H., Becker, K., 1997b. The relationship between in vitro gas production, in vitro microbial biomass yield and 15N incorporation and its implications for the prediction of voluntary feed intake of roughages. Br. J. Nutr. 77, 911–921.

Cao, Y., Cai, Y., Takahashi, T., Yoshida, N., Tohno, M., Uegaki, R., Nonaka, K., Terada, F., 2011. Effect of lactic acid bacteria inoculant and beet pulp addition on fermentation characteristics and in vitro ruminal digestion of vegetable residue silage. J. Dairy Sci. 94, 3902–3912.

Cao, Y., Takahashi, T., Horiguchi, K., Yoshida, N., Cao, Y., 2010. Effect of adding lactic acid bacteria and molasses on fermentation quality and in vitro ruminal digestion of total mixed ration silage prepared with whole crop rice. Grass Sci. 56, 19–25.

Carvalho, I.P.C., Detmann, E., Mantovani, H.C., Paulino, M.F., Valadares Filho, S.C., Costa, V.A.C., Gomes, D.I., 2011. Growth and antimicrobial activity of lactic acid bacteria from rumen fluid according to energy or nitrogen source. R. Bras. Zootec. 40, 1260–1265.

Church D.C., 1993. The ruminant animal – Digestive physiology and nutrition. Waveland Press, Inc., Illinois – USA.

Cline, J.H., Hershberger, T.V., Bentley, O.G., 1958. Utilization and/or synthesis of valeric acid during the digestion of glucose, starch and cellulose by rumen micro-organisms. J. Anim. Sci. 17, 284–292.

Contreras-Govea, F.E., Muck, R.E., Broderick, G.A., Weimer, P.J., 2013. *Lactobacillus plantarum* effects on silage fermentation and in vitro microbial yield. Anim. Feed Sci. Technol. 179, 61–68.

Contreras-Govea, F.E., Muck, R.E., Mertens, D.R., Weimer, P.J., 2011. Microbial inoculant effects on silage and in vitro ruminal fermentation, and microbial biomass estimation for alfalfa, bmr corn, and corn silages. Anim. Feed Sci. Technol. 163, 2–10.

Der Bedrosian, M.C., Nestor, K.E., Jr., Kung, L., Jr., 2012. The effects of hybrid, maturity, and length of storage on the composition and nutritive value of corn silage. J. Dairy Sci. 95, 5115–5126.

Driehuis, F., Oude Elferink, S.J.W.H., Spoelstra, S.F., 1999. Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. J. Appl. Microbiol. 87, 583–594.

Ellis, J.L., Bannink, A., Hindrichsen, I.K., Kinley, R.D., Pellikaan, W.F., Milora, N., Dijkstra, J., 2016. The effect of lactic acid bacteria included as a probiotic or silage inoculant on in vitro rumen digestibility, total gas and methane production. Anim. Feed Sci. Technol. 211, 61–74.

Getachew, G., Robinson, P.H., DePeters, E.J., Taylor, S.J., 2004. Relationships between chemical composition, dry matter degradation and in vitro gas production of several ruminant feeds. Anim. Feed Sci. Technol. 111, 57–71.

Gollop, N., Zakin, V., Weinberg, Z.G., 2005. Antibacterial activity of lactic acid bacteria included in inoculants for silage and in silages treated with these inoculants. J. Appl. Microbiol. 98, 662–666.

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

Klaenhammer, T.R., 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12, 39–86.

Kleinschmit, D.H., Kung, L., Jr., 2006. A meta-analysis of the effects of *Lactobacillus buchneri* on the fermentation and aerobic stability of corn and grass and small-grain silages. J. Dairy Sci. 89, 4005–4013.

Kung, L., Jr., Grieve, D.B., Thomas, J.W., Huber, J.T., 1984. Added ammonia or microbial inoculant for fermentation and nitrogenous compounds of alfalfa ensiled at various percents of dry matter. J. Dairy Sci. 67, 299–306.

Kunji, E.R.S., Mierau, I., Hagting, A., Poolman, B., Konings, W.N., 1996. The proteolytic systems of lactic acid bacteria. Antonie van Leeuwenhoek 70, 187–221.

Lanzas, C., Sniffen, C.J., Seo, S., Tedeschi, L.O., Fox, D.G., 2007. A revised CNCPS feed carbohydrate fractionation scheme for formulating rations for ruminants. Anim. Feed Sci. Technol. 136, 167–190.

Marten, G.C., Barnes, R.F., 1979. Prediction of energy digestibility of forages with in vitro rumen fermentation and fungal enzymes systems. In: Pigden, W.J., Balch, C.C., Graham, M. (Eds.). Standardization of analytical methodology for feeds. International Development Research Center; Ottawa, Canada, pp. 61–128.

Mauricio, R.M., Mould, F.L., Dhanoa, M.S., Owen, E., Channa, K.S., Theodorou, M.K., 1999. A semi-automated in vitro gas production technique for ruminant feedstuff evaluation. Anim. Feed Sci. Technol. 79, 321–330.

Mauricio, R.M., Owen, E., Mould, F.L., Givens, I., Theodorou, M.K., France, J., Davies, D.R., Dhanoa, M.S., 2001. Comparison of bovine rumen liquor and bovine faeces as inoculum for an in vitro gas production technique for evaluating forages. Anim. Feed Sci. Technol. 89, 33–48.

Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, D., Schneider, W., 1979. The estimation of the digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor in vitro. J. Agric. Sci. 93, 217–222.

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

Mertens, D.R., 2005. Particle size, fragmentation index, and effective fiber: Tools for evaluating the physical attributes of corn silages. In: Proc. Four-State Dairy Nutr. & Mgmt. Conf. MWPS-4SD18. Dubuque, IA, pp. 211–220.

Moon, N., 1983. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. J. Appl. Bacteriol. 55, 453–460.

Muck, R.E., Filya, I., Contreras-Govea, F.E., 2007. Inoculant effects on alfalfa silage: In vitro gas and volatile acid production. J. Dairy Sci. 90, 5115–5125.

Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153, 375–380.

Nkosi, B.D., Meeske, R., Palic, D., Langa, T., Leeuwa, K.J., Groenewald, I.B., 2009. Effects of ensiling whole crop maize with bacterial inoculants on the fermentation, aerobic stability, and growth performance of lambs. Anim. Feed Sci. Technol. 154, 193–203.

Nkosi, B.D., Vadlani, P.V., Brijwani, K., Nanjunda, A., Meeske, R., 2012. Effects of bacterial inoculants and an enzyme on the fermentation quality and aerobic stability of ensiled whole-crop sweet sorghum. S. Afr. J. Anim. Sci. 42, 323–240.

Oude Elferink, S.J.W.H., Krooneman, J., Gottschal, J.C., Spoelstra, S.F., Faber, F., Driehuis, F., 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2 propanodiol by *Lactobacillus buchneri*. Appl. Environ. Microbiol. 67, 125–132.

Pahlow, G., Muck, R.E., Driehuis, F., Oude-Elferink, S.J.W.H., Spoelstra, S.F., 2003. Microbiology of ensiling. In: Buxton, D.R., Muck, R.E., Harrison, J.H. (Eds.). Silage Science and Technology. American Society of Agronomy, Madison, WI, pp. 31–93.

Palmer, M.J.A., Jessop, N.S., Fawcett, R., Illius A.W., 2005. Interference of indirect gas produced by grass silage fermentation acids in an in vitro gas production technique. Anim. Feed Sci. Technol. 123–124, 185–196.

Schmidt, P., Nussio, L.G., Queiroz, O.C.M., Santos, M.C., Zopollatto, M., Toledo Filho, S.G., Daniel, J.L.P., 2014. Effects of *Lactobacillus buchneri* on the nutritive value of sugarcane silage for finishing beef bulls. R. Bras. Zootec. 43, 8–13.

Schofield, P., Pitt, R.E., Pell, A.N., 1994. Kinetics of fiber digestion from in-vitro gas-production. J. Anim. Sci. 72, 2980–2991.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., Mcallan, A.B., France, J., 1994. A simple gas-production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48, 185–197.

Van Soest, P.J., Robertson, J.B., 1985. Analysis of forages and fibrous foods, Cornell University, Ithaca.

Weißbach, F., Strubelt, C., 2008. Correcting the dry matter content of maize silages as a substrate for biogas production. Landtechnik 63, 82–83.

Weinberg, Z.G., Muck, R.E., 1996. New trends and opportunities in the development and use of inoculants for silage. FEMS Microbiol. Rev. 19, 53–68.

Weinberg, Z.G., Muck, R.E., Weimer, P.J., 2003. The survival of silage inoculant lactic acid bacteria in rumen fluid. J. Appl. Microbiol. 94, 1066–1071.

Wolin, M.J., 1960. A theoretical rumen fermentation balance. J. Dairy Sci. 43, 1452-1459.

Wolin, M.J., Miller, T.L., Stewart, C.S., 1997. Microbe-microbe interactions. In: Hobson, P.N., Stewart C.S. (Eds.). The rumen microbial ecosystem. 2.ed. London: Chapman Hall, pp. 467–491.

Yildirim, M., 2001. Purification of Buchnericin LB Produced by *Lactobacillus buchneri* LB. Turk. J. Biol. 25, 59–65.

CHAPTER 3

The paper was written following the guidelines for authors of *Livestock Science*, with exception of tables and figures position.

Effects of *Lactobacillus buchneri* as a silage inoculant or probiotic on feed intake, apparent digestibility, fermentation end products, and ruminal bacteria in wethers feeding corn silage

ABSTRACT

Our objective was to investigate the effects of *Lactobacillus buchneri* as a silage inoculant or probiotic on feed intake, apparent digestibility, fermentation end products, and relative proportion of ruminal microorganisms in wethers fed corn silage. Whole-crop corn forage was chopped (279 g/kg DM), ensiled in concrete pipe silos without inoculant (untreated), and with *L. buchneri* CNCM I-4323 at a rate of 1 × 10^5 cfu/g of fresh forage (inoculated), for use in the feeding program. Six cannulated Santa Inês × Dorper crossbred wethers (body weight = 74.5 ± 4.48 kg) were arranged in a double 3 × 3 Latin square and assigned to one of three diets: 1) untreated corn silage (untreated); 2) inoculated corn silage (inoculated); and 3) untreated corn silage with a daily dose of *L. buchneri* applied directly into the rumen at a rate of 1 × 10^7 cfu/g of provided silage (LB-probiotic). In comparison to untreated silage, the lactic acid concentration of *L. buchneri*-inoculated corn silage was significantly reduced (71.6 vs. 44.1 g/kg; P = 0.0376), whereas that of acetic acid was significantly increased (34.3 vs. 67.8 g/kg; P = 0.0008). In comparison to untreated silage, inoculated corn silage significantly reduced (P = 0.0037) aNDF content by 15.2%. Wethers fed the inoculated diet had a greater (P = 0.0500) DM intake (1.30% body weight [BW]), compared to wethers fed untreated and LB-probiotic diets (1.17% and 1.18% BW, respectively). Apparent digestibility was unaffected (P > 0.05) by the experimental diets. The relative proportions of *Ruminococcus flavefaciens* in wethers fed inoculated and LB-probiotic diets (both 0.42%) tended towards a reduction (P = 0.0980), in comparison to those fed the untreated diet (0.83%), regardless of the time of evaluation. Other ruminal bacteria evaluated, remained unaffected (P > 0.05) by the diets. Despite of the ruminal fermentation, only the acetic acid concentration was reduced (P = 0.0063) in the ruminal fluid of wethers fed the inoculated diet (71.6 mM/100 mM), compared to those fed the untreated and LB-probiotic diets (73.2 and 73.7 mM/100 mM, respectively). *L. buchneri* is more efficient when used as a silage

inoculant than a probiotic because it had a greater impact on the performance of wethers.

Keywords: lactic-acid bacteria, ruminal bacterial community, silage quality, volatile fatty acid

1. Introduction

Lactobacillus buchneri is a heterofermentative lactic-acid bacteria (LAB) used to enhance the aerobic stability of silages by anaerobically converting lactic acid to acetic acid (Driehuis et al., 1999; Oude Elferink et al., 2001). The ability of *L. buchneri* to produce antifungal byproducts, such as acetic acid and bacteriocins (Yildirim, 2001), could be particularly beneficial in preventing the spoilage of tropical silages (Ashbell et al., 2002; Bernardes and Adesogan, 2012). In addition, some studies have revealed that animals fed silages inoculated with *L. buchneri* have increased dry matter intake (DMI), feed efficiency, and growth performance as compared to those fed untreated silages (Basso et al., 2014; Nkosi et al., 2009; Schmidt et al., 2014). Enhanced growth performance could likely be attributed to improvements in silage quality, along with possible probiotic effects from LAB inoculation of silage (Weinberg and Muck, 1996).

By definition, the term probiotic means ‘a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance’ (Fuller, 1989). However, the probiotic effects of LAB inoculation of silage could also confer advantages in the rumen, such as improvements in digestibility and prevention of acidosis. Thus, the hypothesis regarding the probiotic effect of LAB inoculation could be supported by: 1) the ability of LAB to survive in ruminal fluid (Weinberg et al., 2003); 2) the bacteriocin-like antibacterial activity exhibited by some LAB (Gollop et al., 2005); 3) in vivo and in vitro studies that reveal the interaction between LAB and ruminal microorganisms with shifts in the ruminal bacterial community, microbial N supply, and ruminal fermentation (Basso et al., 2014; Mohammed et al., 2012; Weinberg et al., 2007); and 4) enhanced animal performance following LAB inoculation even without alterations to silage fermentation (Weinberg and Muck, 1996).

Nevertheless, the term ‘probiotic’ has not been used appropriately to explain enhanced growth performance in animals consuming inoculated silage. The inclusion of LAB as a silage inoculant or probiotic in the diet, could shift ruminal fermentation in various ways, as was demonstrated in a recent study under in vitro conditions (Ellis et al., 2016). Moreover, a series of experiments carried out in Northern Ireland demonstrated increased DMI and enhanced growth performance in beef cattle, owing to the addition of *Lactobacillus plantarum* as a silage inoculant to grass (Keady and Steen, 1994; 1995). However, positive changes in the same variables (DMI and enhanced growth performance) were not observed following the addition of *L. plantarum* to grass silage as a probiotic, immediately before feeding (Keady and Steen, 1996). To our best knowledge, no previous reports have compared LAB as a silage inoculant with its use as a probiotic, and its effects on feed intake and the ruminal bacterial community of animals. Therefore, our objective was to investigate the effects of *L. buchneri* as a silage inoculant or probiotic on feed intake, apparent digestibility, ruminal fermentation end products, and relative proportion of ruminal microorganisms in wethers fed corn silage.

2. Material and Methods

2.1. Ethics statement

Animal care and handling procedures used in the present study were in accordance with the Brazilian College of Animal Experimentation (COBEA, Colégio Brasileiro de Experimentação Animal) guidelines and was approved by the Ethics, Bioethics and Animal Welfare Committee (CEBEA, Comissão de Ética e Bem Estar Animal) of the São Paulo State University, Brazil.

2.2. Ensiling procedure

Flint corn - *Zea mays* (hybrid Impacto Víptera, Syngenta, Matão, SP, Brazil) was harvested at 1/3 milk line (279 g DM/kg as fed) using a Premium Flex forage harvester (Menta Mit, Cajuru, SP, Brazil) and chopped to a length of 10 mm. The Impacto Víptera is a hybrid recommended to produce grains and also for ensilage, which has 55,000–60,000 plants/ha and a productivity of approximately 50 ton/ha. Four piles of whole-crop corn forage was treated with water (5 L/t) (untreated), and

other four piles were treated with *L. buchneri* CNCM I-4323 at 1 × 10^5 cfu/g of fresh forage (inoculated) (Lallemand Animal Nutrition, Goiânia, GO, Brazil). The inoculant was dissolved in distilled water (5 L/t) and sprayed onto fresh forage during silo filling. Viability and label levels of the bacteria in the inoculant were confirmed prior to use.

Eight concrete pipe silos were each filled with approximately 350 kg of corn forage on the same day. Four silos were filled with inoculated forage and the other four were filled with untreated forage. To prevent cross contamination, untreated forage was ensiled first, followed by inoculated forage. Forage packing was performed by the application of human pressure. Silos were sealed with black-on-white polyethylene film (200-µm thick) (Electro Plastic, São Paulo, SP, Brazil), and stored at ambient temperature for 229 d. Four fresh samples of corn forage were collected from each silo during filling to determine the chemical composition. During the feedout phase, silage samples were collected from each silo weekly, and stored at -20°C until further analysis. Thereafter, silage samples were processed to form composite samples per three 19-d period where wethers were fed experimental diets (n = 3).

2.3. Animal study

Six ruminally cannulated Santa Inês × Dorper crossbred wethers (three years of age and body weight [BW] = 74.5 ± 4.48 kg), each fitted with a silicone, 2.5-inch ruminal cannula were used in a double 3 × 3 Latin square design. Each wether was housed individually in 0.9 × 2.0 m pens, fitted with individual feed bunks and water bowls. Wethers were fed a total mixed ration (TMR) of 70% corn silage and 30% concentrate *ad libitum* once a day (0800), on a DM basis (Table 1). Diets comprised: 1) untreated corn silage (untreated); 2) inoculated corn silage (inoculated); and 3) untreated corn silage with a daily dose of *L. buchneri* applied directly into the rumen at a rate of 1 × 10^7 cfu/g of provided silage (LB-probiotic). To establish the dose of *L. buchneri* applied directly into the rumen of wethers in the third diet, we aimed at having *L. buchneri* at a rate of 1 × 10^6 cfu/mL. We assumed that good silage should contain approximately a population of LAB at a rate of 1 × 10^6 cfu/g, and wethers (rumen with capacity for 30 L) should receive daily 3 kg of corn silage (70% of TMR).

Therefore, the dose of *L. buchneri* applied directly into the rumen at a rate of 1×10^7 cfu/g of provided silage was our starting point. In the third diet, the silage inoculant containing *L. buchneri* was diluted with distilled water and administered every day immediately prior to feeding.

2.4. Feed intake, apparent digestibility, and ruminal fermentation

The experiment was carried out over three 19-d periods, and during each interval the wethers were given 12 d to adapt to the diets. Feed intake and apparent digestibility were determined from days 13–17. Orts were weighed daily before the morning feeding to calculate DMI.

Apparent digestibility was calculated indirectly using the indigestible neutral detergent fiber (iNDF) as a marker to estimate fecal output (Valente et al., 2011). Fecal grab samples were collected daily from each wether on days 13–17 (Pina et al., 2006). Samples of silage, concentrate, and orts were also collected daily during this period and stored at -20°C until further analysis.

Table 1
Ingredient proportions and chemical composition of total mixed rations containing corn silage and *Lactobacillus buchneri* as a silage inoculant or probiotic[a] (data are given in g/kg of DM, unless otherwise stated).

Item	Untreated	Inoculated	LB-probiotic
Ingredient proportion			
Corn silage	700	700	700
Ground corn (2 mm)	246	246	246
Soybean meal	34.0	34.0	34.0
Urea	5.00	5.00	5.00
Mineral supplement[b]	15.0	15.0	15.0
Chemical composition[c]			
DM (g/kg as fed)	444	452	444
OM	956	955	956
CP	121	119	121
EE	44.5	41.4	44.5
CHOT	791	795	791
aNDF	250	238	250
ADF	138	133	138
NFC	544	562	544

[a] Wethers were fed diets containing 1) untreated corn silage (untreated); 2) corn silage inoculated with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh

forage (inoculated) (Lallemand Animal Nutrition, Milwaukee, WI, USA); and 3) untreated corn silage with a daily dose of *L. buchneri* applied directly into the rumen at 1×10^7 cfu/g of provided silage (LB-probiotic).
[b] Mineral supplement was composed of 70 g/kg P; 160 g/kg Ca; 100 g/kg Na; 5 g/kg Mg; 40 g/kg S; 2560 mg/kg Zn; 690 mg/kg Cu; 530 mg/kg Mn; 41 mg/kg Co; 51 mg/kg I; 700 mg/kg F; and 13 mg/kg Se.
[c] DM = dry matter; OM = organic matter; EE = ether extract; CP = crude protein; CHOT = total carbohydrates; aNDF = neutral detergent fiber (assayed with a heat stable amylase and expressed inclusive of residual ash); ADF = acid detergent fiber (expressed inclusive of residual ash); NFC = non-fiber carbohydrates.

On day 18, a 50-mL sample of ruminal fluid was collected from each wether before feeding (0 h), and at 6, 12, and 18 h post-feeding. Ruminal fluid was squeezed through four layers of cheesecloth and its pH was immediately measured using a pH meter (MA522 model, Marconi Laboratory Equipment, Piracicaba, SP, Brazil). A volume of 1 mL of H_2SO_4 (1:1) was then added to the ruminal fluid and the resulting solution was stored at -20°C until further analysis of volatile fatty acids (VFA). On day 19, ruminal fluid was collected for in vitro measurements (chapter 2).

2.5. DNA extraction and quantification

Ruminal samples (50 mL) were collected at 6 and 12 h post-feeding on day 18, and immediately treated with phosphate buffered saline (1% Tween, pH 7.4). The buffered solution was then vigorously stirred for 3 min and filtered with a mesh fabric (100 microns). The filtrate was centrifuged at 16,000× *g* for 10 min at 4°C, and bacterial pellets were formed. Pellets were resuspended in 4 mL of 10:1 TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0), and centrifuged again at 16,000× *g* for 10 min. The supernatant was discarded and the bacterial pellet was frozen at -20°C until DNA extraction.

DNA extraction was performed using the Qiagen DNA stool kit (Qiagen, Hilden, Germany) according to the manufacturer guidelines. A 200-µL buffer solution of low recovering and quantity of salts was used to separate the DNA from the silica membrane of the extraction column.

2.6. Real-time PCR (qPCR)

The relative quantification of bacteria was performed using q-PCR, the ABI Prism® 7500 detection system (Applied Biosystems), and SYBR technology

(Invitrogen®). The amplifications were performed in duplicate and negative controls were run in the assay (excluding total DNA). The qPCR was carried out using 1 µL of total DNA in reaction with 1× SYBR Green (Applied Biosystems), 300 nM of each primer pair, and 6.6 µL of nuclease free H_2O, resulting in a final volume of 20 µL. The primers used in the present study are presented in Table 2. Real-time PCR amplification included an initial denaturation cycle at 95°C for 10 min; followed by 44 cycles at 95°C for 15 s; 55°C for 30 s; and final extension at 72°C for 30 s. A dissociation curve of the reaction products was used to analyze the specificity of amplification for each bacteria (*Fibrobacter succinogenes*, *L. buchneri*, *R. flavefaciens*, *Selenomonas ruminantium*, and *Streptococcus bovis*).

Relative quantification was used to determine species proportion, and the results were expressed as 16S rDNA ratios of general bacteria, following the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

2.7. Sample preparation and chemical analyses

A water extract was produced from fresh silage samples, as described by Kung et al. (1984), and silage pH was measured with a pH meter. Lactic acid was measured using a high performance liquid chromatography (HPLC) autosampler (Shimadzu model SIL-20A, Shimadzu Corp., Kyoto, Japan) equipped with a UV/VIS detection system and a refractive index detector (SPD-20A). An apolar column from Shimadzu (CLC-ODS; 4.6 mm × 25 cm) was used at 30°C for chromatographic separation. The polar mobile phase consisted of a 20 mM monosodium phosphate solution with a flow rate of 1.25 mL/min. Acids were detected by UV absorbance (210 nm). VFAs were measured using a gas chromatograph (Shimadzu model GC2014, Shimadzu Corp., Kyoto, Japan) equipped with an HP-INNOWax capillary column (30 m × 0.32 mm) (Agilent Technologies, Colorado, USA) at an initial temperature of 80°C for 3 min, followed by heating at a rate of 20°C/min, until a final temperature of 240°C was achieved. Water soluble carbohydrates (WSC) were determined following the Nelson-Somogyi method (Nelson, 1944). Ammonia-N was determined by distillation (AOAC, 1996; method no. 941.04), and expressed as g/kg of total N (TN).

Table 2
Target primers used in the relative quantification of *Fibrobacter succinogenes*, *Lactobacillus buchneri*, *Ruminobacter flavefaciens*, *Selenomonas ruminantium*, and *Streptococcus bovis* by q-PCR.

Target bacteria	Sequence (5′ - 3′)[a]	Bp[b]	Reference
General bacteria	F: GTGSTGCAYGGYTGTCGTCA	147	Maeda et al. (2003)
	R: ACGTCRTCCMCACCTTCCTC		
Fibrobacter succinogenes	F: GGTATGGGATGAGCTTGC	445	Tajima et al. (2001)
	R: GCCTGCCCCTGAACTATC		
Lactobacillus buchneri	F: GGACCAATGCAGCAACTGAA	72	Stevenson et al. (2006)
	R: AGATTACTGACGCATTGGTTACCA		
Ruminococcus flavefaciens	F: TCTGGAAACGGATGGTA	259	Koike and Kobayashu (2001)
	R: CCTTTAAGACAGGAGTTTACAA		
Selenomonas ruminantium	F: CAATAAGCATTCCGCCTGGG	82	Stevenson and Weimer (2007)
	R: TTCACTCAATGTCAAGCCCTGG		
Streptococcus bovis	F: TTCCTAGAGATAGGAAGTTTCTTCGG	127	Hino et al. (1994)
	R: ATGATGGCAACTAACAATAGGGGT		

[a] Forward and reverse primers.
[b] Amplicon size in base pairs.

For microbiological analyses, fresh silage samples (25 g) from each replicate were homogenized in 225 mL of autoclaved saline solution (0.85% NaCl) for 1 min. An aliquot (1 mL) of this solution was transferred into tubes containing 9 mL of saline solution, and 1 mL of this mixture was then plated onto Petri plates after serial dilutions of 10^{-1}–10^{-9}. Man, Rogosa, and Sharpe (MRS) agar was used to count LAB with the pour plate method, whereas potato dextrose agar (PDA) was used to count yeasts and molds by the spread plate method. Both MRS and PDA plates were incubated at 28°C. LAB and yeasts were counted after 2 d, and molds were counted after 5 d. All microbiological data were $\log_{10}$-transformed.

Forage and silage samples were oven dried (55°C for 72 h) and processed in a knife mill, before being ground through a 1-mm screen and analyzed for DM (105°C for 12 h) and ash (500°C for 5 h). OM was calculated as 1000 - ash. Ether extract (EE) was determined according to the procedures described by AOAC (1996; method no. 920.39). The TN was measured by rapid combustion using a LECO analyzer (model F528 N, LECO Corp., St. Joseph, MI, USA), and crude protein (CP) was calculated as TN × 6.25. Neutral detergent fiber (aNDF) and acid detergent fiber (ADF) were determined in an ANKOM 2000 Fiber Analyzer (ANKOM Technologies, Macedon, NY, USA) following the procedures described by Mertens (2002). The aNDF was measured using a heat stable amylase without sodium sulfite, and both aNDF and ADF were expressed inclusive of residual ash. The iNDF was determined using F57 bags (ANKOM®) containing silage samples that were incubated in the rumen of two Nellore steers for 264 h (Casali et al., 2008). Samples were then removed from the rumen of the steers and analyzed for aNDF. Total carbohydrate (CHOT) and non-fiber carbohydrate (NFC) contents of the corn silages were determined according to the procedures of Sniffen et al. (1992).

Aliquots of strained ruminal fluid were thawed in a refrigerator overnight and centrifuged at 20,000× *g* for 30 min at 4°C. The supernatant was analyzed for VFA by gas chromatography according to the aforementioned procedures.

2.8. Calculations

The DM determined in forced air oven (DM_{oven}) was corrected (DM_{corr}) for volatile compounds, using an adaptation (Eq. 1) from the original equation proposed by Weißbach and Strubelt (2008) as follows:

DM_{corr} (g/kg as fed) = DM_{oven} + 0.95 × VFA + 0.08 × lactic acid (1)

where all volatile compounds are expressed in g/kg of fresh matter.

All variables regarding chemical composition and fermentation products of silage were expressed as DM_{corr}.

The fecal output and apparent digestibility were calculated using Eqs. 2 and 3, respectively, as follows:

Fecal yield = iNDF intake (g) / iNDF fecal (%) × 100 (2)

Apparent digestibility = [DMI (g) - fecal yield (g)] / DMI (g) × 100 (3)

2.9. Statistical analyses

Silage data were analyzed as repeated measures over time in a completely randomized design using the MIXED procedure of SAS (v. 9.4 SAS Institute Inc., Cary, NC). Silage and sampling time were considered a fixed effect and error as random effect. Concrete pipe silos were considered the experimental unit for silage measurements (n = 4).

Feed intake and apparent digestibility were analyzed in a double 3 × 3 Latin square design, and the relative proportion of ruminal microorganisms and ruminal fermentation were analyzed as repeated measures over time. All variables were analyzed using the MIXED procedure of SAS. Several covariance structures were tested and those that generated the lowest corrected Akaike information criterion (AICc) and Bayesian information criterion (BIC) were selected. Diet was considered a fixed effect, and wethers and period as random effects.

Differences among silage means were determined using an F test, and differences between diet means were determined using the PDIFF option of LSMEANS in SAS. Significant differences were declared at $P \leq 0.05$, and trends discussed at $0.05 > P \leq 0.10$.

3. Results

3.1. Fermentation, microbial profile, and chemical composition of corn silage

In comparison to untreated silage, inoculation of corn silage with *L. buchneri* reduced (P = 0.0376) the concentration of lactic acid by 38.4% and increased that of acetic acid (P = 0.0008) by 97.7% (Table 3). The concentrations of butyric and isobutyric acids were reduced (P < 0.01) by 62.5% and 58.5%, respectively, in the inoculated silage. In addition, inoculation of corn silage with *L. buchneri* increased (P < 0.05) the concentration of ammonia-N from 26.1 to 43.8 g/kg of TN, and that of valeric acid, from 0.240 to 0.807 g/kg of DM. Silage pH, WSC, concentrations of propionic and isovaleric acid, and counts of LAB, yeasts, and molds were unaffected (P > 0.05) by inoculation of the corn silage.

Inoculation of the silage with *L. buchneri* increased DM_{oven} content (P = 0.0480) and DM_{corr} content (P = 0.0057) by 5.7% and 8.1%, respectively (Table 3). In contrast, inoculation reduced EE, CP, and aNDF contents by 8.7%, 11.2%, and 15.2%, respectively (P < 0.05). OM, CHOT, iNDF, ADF, and NFC were unaffected (P > 0.05) by inoculation of corn silage.

Table 3
Fermentation, microbial profile, and chemical composition of corn silage untreated or inoculated with *Lactobacillus buchneri* in concrete pipe silos after 229 d of ensilage (n = 4; data are given in g/kg DM, unless otherwise stated).

Item	Forage	Silage[a]		SEM	*P*-value[b]		
		Untreated	Inoculated		S	T	S × T
Fermentation profile							
Ammonia-N, g/kg TN	-	26.1[b]	43.8[a]	4.43	0.0112	0.0980	0.4328
WSC, mg glucose/g[c]	-	13.8	15.7	1.54	0.3962	0.7894	0.5842
pH	-	4.29	4.33	0.03	0.3561	0.0028	0.0020
Lactic acid	NA	71.6[a]	44.1[b]	7.29	0.0376	0.9062	0.7435
Acetic acid	NA	34.3[b]	67.8[a]	6.07	0.0008	0.0139	0.1332
Propionic acid	NA	5.70	4.99	0.74	0.5147	<0.0001	0.4685
Butyric acid	NA	6.06[a]	2.27[b]	0.82	0.0036	0.0024	0.0005
Isobutyric acid	NA	0.193[a]	0.080[b]	0.03	0.0074	0.0018	0.0003
Valeric acid	NA	0.240[b]	0.807[a]	0.15	0.0372	0.2311	0.4111
Isovaleric acid	NA	0.358	0.286	0.06	0.4266	0.0084	0.0926
Microbial profile							
LAB, $\log_{10}$ cfu/g	-	7.23	7.20	0.12	0.8348	0.0028	0.4355
Yeasts, $\log_{10}$ cfu/g	-	5.02	5.04	0.24	0.9522	0.0010	0.8707
Molds, $\log_{10}$ cfu/g	-	4.44	4.09	0.21	0.2795	0.5941	0.4932
Chemical composition							
DM_{oven}, g/kg as fed	279	247[b]	261[a]	4.71	0.0480	0.0205	0.7605
DM_{corr}, g/kg as fed	-	259[b]	280[a]	4.72	0.0057	0.0018	0.7473
OM	966	967	966	0.90	0.1827	0.0428	0.3382
EE	-	38.1[a]	34.8[b]	0.89	0.0147	0.1954	0.4980
CP	108	86.4[a]	76.8[b]	1.73	0.0008	0.4308	0.2930
CHOT	-	796	783	6.34	0.1598	0.0148	0.3129
aNDF	330	302[a]	256[b]	9.56	0.0037	0.7537	0.2530
iNDF, g/kg aNDF	-	319	328	11.70	0.6122	0.3860	0.6190
ADF	160	165	153	4.66	0.0951	0.8404	0.3176
NFC	-	521	551	12.09	0.1002	0.1013	0.1279

[a-b]Means in the same row with different superscripts differed significantly ($P < 0.05$), and exclude comparisons with forage prior to ensiling.
[a] Corn silage was treated at ensiling either without (untreated), or with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage (inoculated) (Lallemand Animal Nutrition, Milwaukee, WI, USA).
[b] S = silage; T = time; S × T = interaction between silage and time.
[c] WSC = water-soluble carbohydrate; LAB = lactic-acid bacteria; DM = dry matter; OM = organic matter; EE = ether extract; CP = crude protein; CHOT = total carbohydrates; aNDF = neutral detergent fiber (assayed with a heat stable amylase and expressed inclusive of residual ash); ADF = acid detergent fiber (expressed inclusive of residual ash); NFC = non-fiber carbohydrates; iNDF = indigestible neutral detergent fiber.

NA = not applicable.

3.2. Feed intake and apparent digestibility

Wethers fed the inoculated silage diet had a greater DMI (P = 0.0500) as expressed in % BW, compared to those fed the untreated and LB-probiotic diets (Table 4). In addition, wethers fed the inoculated diet had a tendency towards an increased DMI (P = 0.0955) as expressed in kg/d, and consumed 83 and 100 g DM more than wethers fed the untreated and LB-probiotic diets, respectively. However, OM, CP, and aNDF intake were unaffected (P > 0.05) by the use of *L. buchneri* as a silage inoculant or probiotic.

Table 4
Feed intake and apparent digestibility in wethers fed diets containing corn silage and *Lactobacillus buchneri* as a silage inoculant or probiotic[a].

Item	Untreated	Inoculated	LB-probiotic	SEM	*P*-value
Intake, kg/day					
DM[b]	0.885	0.968	0.868	0.10	0.0955
DM, % BW	1.17[b]	1.30[a]	1.18[b]	0.12	0.0500
OM	0.839	0.916	0.823	0.10	0.1003
CP	0.095	0.099	0.091	0.01	0.3003
aNDF	0.214	0.215	0.204	0.03	0.4795
Apparent digestibility, g/kg of DM					
DM	879	885	887	0.03	0.9792
OM	883	890	898	0.03	0.9387
CP	835	840	852	0.03	0.9469
aNDF	653	681	655	0.09	0.9668

[a-b]Means in the same row with different superscripts differed significantly (P < 0.05).
[a] Wethers were fed diets containing 1) untreated corn silage (untreated); 2) corn silage inoculated with *Lactobacillus buchneri* CNCM I-4323 at 1 × 10^5 cfu/g of fresh forage (inoculated) (Lallemand Animal Nutrition, Milwaukee, WI, USA); and 3) untreated corn silage with a daily dose of *L. buchneri* applied directly into the rumen at 1 × 10^7 cfu/g of provided silage (LB-probiotic).
[b] DM = dry matter; BW = body weight; OM = organic matter; CP = crude protein; aNDF = neutral detergent fiber (assayed with a heat stable amylase and expressed inclusive of residual ash).

The apparent digestibility of DM, OM, CP, and aNDF remained unaltered (P > 0.05) by the use of *L. buchneri* as a silage inoculant or probiotic in the diets containing corn silage (Table 4).

3.3. Relative proportion of ruminal microorganisms

The relative proportion of *R. flavefaciens* in the rumen of wethers fed the inoculated and LB-probiotic diets tended (P = 0.0980) to be reduced, in comparison to those fed the untreated diet, regardless of the time of evaluation (Table 5). However, relative proportions of *L. buchneri*, *F. succinogenes*, *S. ruminantium*, and *S. bovis* were unaffected by the treatments (P > 0.05).

3.4. Ruminal fermentation end-products

The concentration of acetic acid was reduced (P = 0.0063) in the ruminal fluid of wethers fed the inoculated silage diet, as compared to those fed the untreated and LB-probiotic diets (Table 6). Ruminal pH, the concentrations of total VFA, propionic, butyric, isobutyric, valeric, and isovaleric acids, and the acetic:propionic acid ratio were all unaffected (P > 0.05) by the experimental diets. However, the intervals at which ruminal fluid was sampled affected some variables. The total VFA concentration was increased (P = 0.0053) from 52.2 mM prior to feeding, to 70.5 mM at 12 h post-feeding (Fig. 1a). Ruminal pH was reduced (P < 0.01) from 6.57 prior to feeding, to 6.01 at 12 h post-feeding (Fig. 1b). Similarly, the concentration of propionic acid was lower (P = 0.0012) prior to feeding than it was 12 h post-feeding (11.0 vs. 14.4 mM/100 mM; Fig. 2a). The acetic:propionic acid ratio (6.37) was also higher (P = 0.0165) prior to feeding than it was 12 h post-feeding (5.33; Fig. 2b). Isobutyric and isovaleric acids yielded the highest concentrations (P < 0.01) prior to feeding (1.06 and 1.92 mM/100 mM, respectively) compared to subsequent sampling intervals (Fig. 3).

Table 5
Relative proportion of ruminal microorganisms at 6 and 12 h post-feeding in wethers fed diets containing corn silage and *Lactobacillus buchneri* as a silage inoculant or probiotic.

Diet[a]	Untreated		Inoculated		LB-probiotic		SEM	*P*-value[b]		
Time post-feeding	6 h	12 h	6 h	12 h	6 h	12 h		D	T	D × T
Fibrobacter succinogenes	1.00	1.59	0.47	0.73	0.42	1.05	1.02	0.4576	0.2754	0.9158
Lactobacillus buchneri	1.00	1.11	0.83	1.70	4.23	1.42	0.76	0.1592	0.8194	0.1674
Ruminococcus flavefaciens	1.00	0.65	0.32	0.52	0.42	0.42	0.67	0.0980	0.9414	0.3791
Selenomonas ruminantium	1.00	1.06	1.34	1.42	1.00	1.15	0.31	0.2006	0.5418	0.9687
Streptococcus bovis	1.00	1.06	1.03	0.93	0.65	0.81	0.90	0.4719	0.8307	0.8816

[a] Wethers were fed diets containing 1) untreated corn silage (untreated); 2) corn silage inoculated with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage (inoculated) (Lallemand Animal Nutrition, Milwaukee, WI, USA); and 3) untreated corn silage with a daily dose of *L. buchneri* applied directly into the rumen at 1×10^7 cfu/g of provided silage (LB-probiotic).
[b] D = diet; T = time; D × T = interaction between diet and time.

Table 6
Ruminal fermentation in wethers fed diets containing corn silage and *Lactobacillus buchneri* as a silage inoculant or probiotic.

Item	Diet[a]			SEM	*P*-value[b]		
	Untreated	Inoculated	LB-probiotic		D	T	D × T
pH	6.21	6.16	6.15	0.20	0.5254	<0.0001	0.9885
Total VFA, mM	58.8	60.0	56.5	6.97	0.4214	0.0053	0.3913
Molar proportion, mM/100 mM							
Acetic acid	73.2[a]	71.6[b]	73.7[a]	1.02	0.0063	0.0563	0.2171
Propionic acid	12.5	14.3	12.5	1.39	0.2054	0.0012	0.9599
Acetic:propionic acid ratio	5.91	5.53	6.31	1.25	0.3476	0.0165	0.2407
Butyric acid	11.2	10.9	10.6	0.47	0.5853	0.0910	0.1044
Isobutyric acid	0.839	0.766	0.722	0.04	0.1597	<0.0001	0.8033
Valeric acid	0.877	0.856	0.816	0.06	0.5511	0.7110	0.8913
Isovaleric acid	1.58	1.54	1.55	0.14	0.9440	0.0002	0.4130

[a-b]Means in the same row with different superscripts differed significantly ($P < 0.05$).
[a] Wethers were fed diets containing 1) untreated corn silage (untreated); 2) corn silage inoculated with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage (inoculated) (Lallemand Animal Nutrition, Milwaukee, WI, USA); and 3) untreated corn silage with a daily dose of *L. buchneri* applied directly into the rumen at 1×10^7 cfu/g of provided silage (LB-probiotic).
[b] D = diet; T = time; D × T = interaction between diet and time.
[c] L = linear; Q = quadratic; C = cubic.

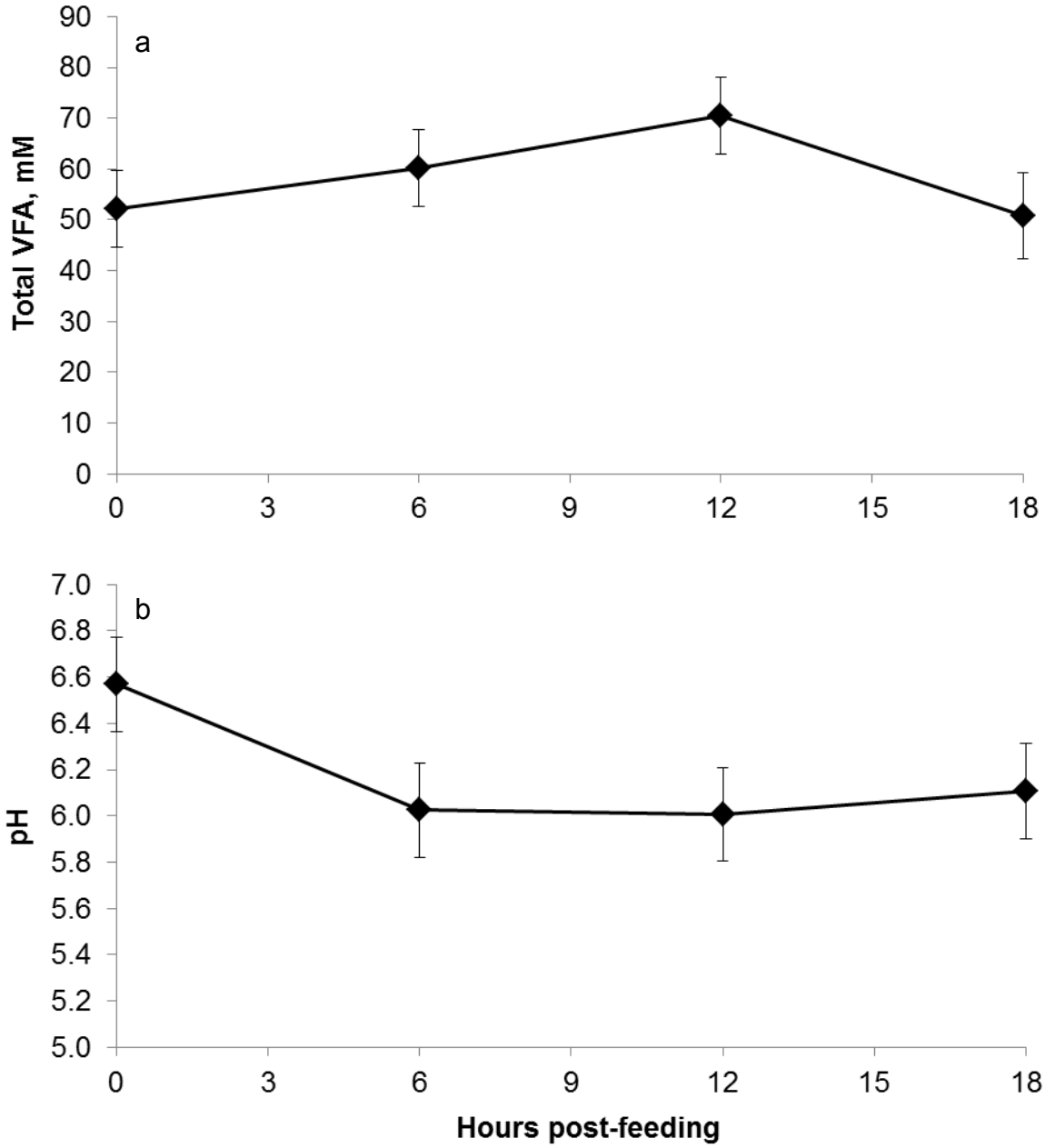

Fig. 1. Total VFA concentration (a) and pH (b) in the ruminal fluid of wethers fed diets containing corn silage and *Lactobacillus buchneri* as a silage inoculant or a probiotic at different intervals post-feeding.

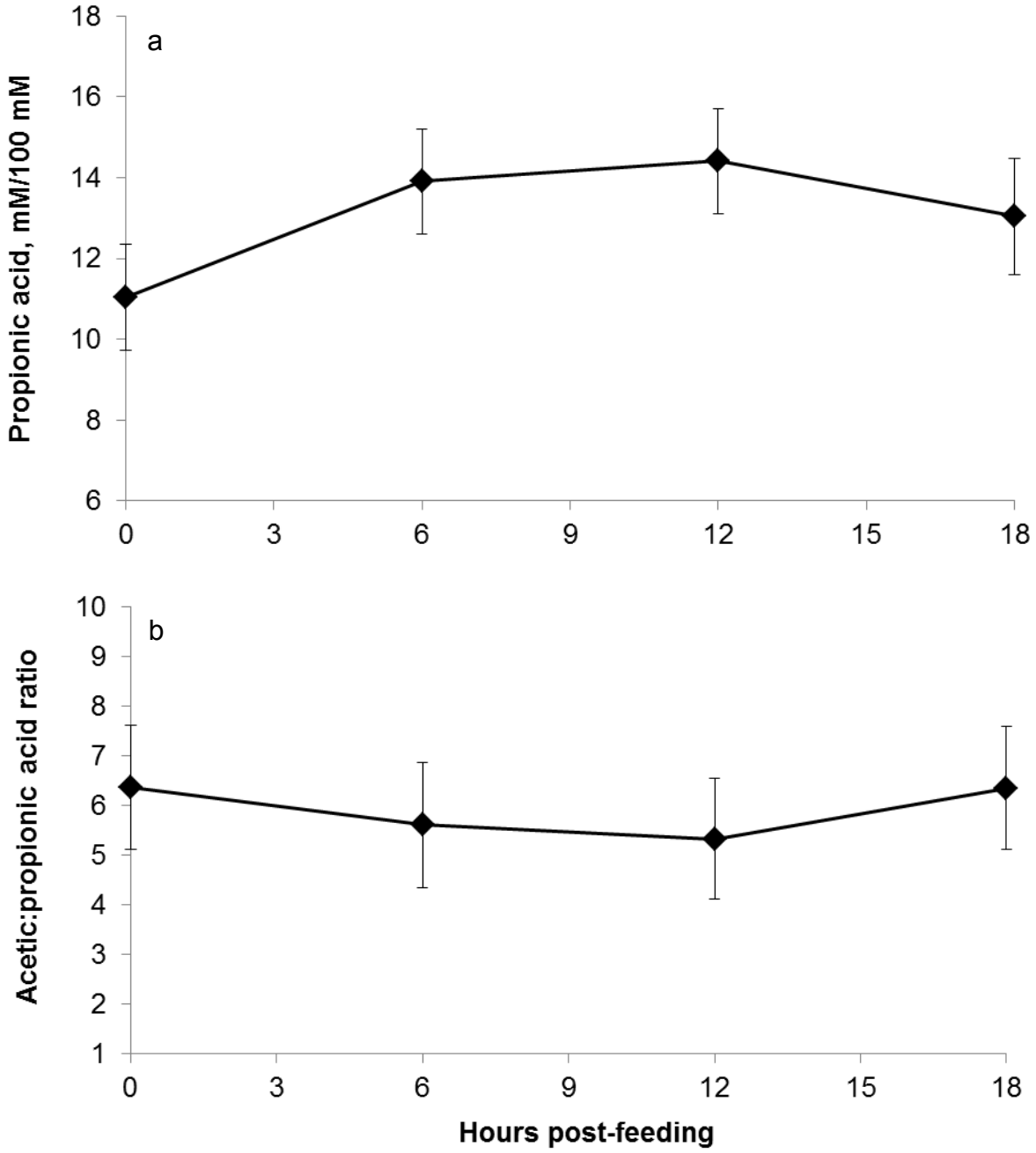

Fig. 2. Propionic acid concentration (a) and acetic:propionic acid ratio (b) in the ruminal fluid of wethers fed diets containing corn silage and *Lactobacillus buchneri* as a silage inoculant or probiotic at different intervals post-feeding.

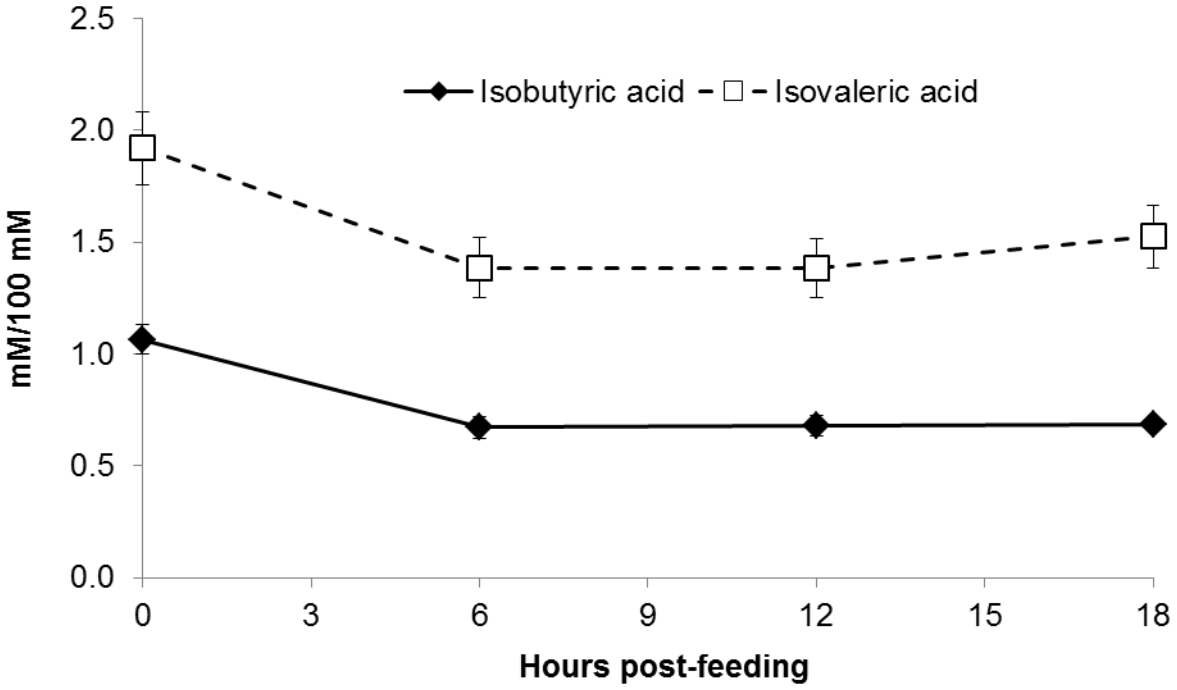

Fig. 3. Concentrations of isobutyric and isovaleric acids in the ruminal fluid of wethers fed diets containing corn silage and *Lactobacillus buchneri* as a silage inoculant or probiotic at different intervals post-feeding.

4. Discussion

An elevated concentration of acetic acid (instead of lactic acid) was observed in our study for corn silage treated with *L. buchneri* after 229 d of ensilage. *L. buchneri* is a heterofermentative LAB used as a silage inoculant to enhance the aerobic stability of silage owing to a shift in metabolism toward heterolactic fermentation (Driehuis et al., 1999). This bacteria has the ability to convert lactic acid to acetic acid and 1,2-propanediol in anaerobic conditions (Oude Elferink et al., 2001), a response often observed when the primary fermentation phase of ensiled crop has ended. Therefore, our results are consistent with previous reports, in which *L. buchneri* typically altered the fermentation process by increasing the concentration of acetic acid (reviewed by Kleinschmit and Kung, 2006).

An unexpected reduction in the concentration of butyric acid was observed, due to inoculation of the corn silage. This response could likely be attributed to alterations in the products formed by *L. buchneri* (i.e., acetic acid and bacteriocin) during fermentation that could inhibit clostridial growth. Furthermore, CP content was

reduced, whereas that of ammonia-N was increased in the inoculated silage. A previous study also reported an elevated concentration of ammonia-N in corn silage inoculated with *L. buchneri*, in comparison to untreated silage after 92 d of ensilage (Driehuis et al., 1999). Indeed, increased ammonia-N concentration has been observed in *L. buchneri*-treated silages stored for a prolonged time because this bacterium can remain fairly active for prolonged periods of time (up to a year) (Der Bedrosian et al., 2012; Kleinschmit and Kung, 2006), and LAB are relatively acid tolerant and possess a wide variety of extracellular proteases and intracellular peptidases (Kunji et al., 1996). Therefore, although the fermentation process might be altered after a prolonged period by inoculation with *L. buchneri* (i.e., higher acetic acid concentration), likely the chronic proteolytic activity played by LAB and other microorganisms or even by some plant proteases has a more important role on the degradation of proteins and production of ammonia-N (Hoffman et al., 2011).

Increases in DM content and a reduction in aNDF have also been reported in response to the inoculation of corn silage with *L. buchneri*. The strain of *L. buchneri* used in the present study no holds fibrolytic activity as the production of ferulate esterase enzyme. Thus, even with no significant difference in the yeast and mold counts, these results suggest that aerobic deterioration of the inoculated silage occurred to a lesser extent during feed-out. This occurred because similar counts of spoilage microorganisms do not necessarily imply similar levels of deterioration activity. Evidence of this has been revealed in previous reports of increased aerobic stability, even in silages treated with *L. buchneri* that yield similar yeasts counts to those of untreated silage (Mari et al., 2009). In addition, the aNDF reduction observed in the present study is consistent with previous studies that report a reduction in NDF content in corn silages treated with *L. buchneri* (Arriola et al., 2011; Nkosi et al., 2011).

Because of the VFAs formed during fermentation, the voluntary intake of silage has been recognized to a lesser extent than that of the same forage that has not undergone fermentation (Charmley, 2001). For instance, levels of acetic acid above 50 g/kg DM negatively affect silage intake (Muck, 2010). In this context, concerns regarding a depression in DMI are taken in account when animals are fed silages treated with *L. buchneri*. However, despite the increased concentration of acetic acid

(67.8 g/kg DM) in silage treated with *L. buchneri* in the present study, wethers fed inoculated silage had an increased DMI (as expressed in % BW). This response most likely stemmed from the reduced aNDF content observed in the inoculated silage, in comparison to the untreated silage. Fiber is known to have a negative effect on DMI (Jung and Allen, 1995). Shifts in the chemical composition of corn silage treated with *L. buchneri* have also been attributed to an increased DMI in growing lambs (Basso et al., 2014; Nkosi et al., 2009), and is often one of the main reasons for improved growth performance (Weinberg and Muck, 1996).

Enhanced animal performance is closely linked to improved silage digestibility caused by LAB inoculation (Muck, 1993). Thus, an increase in DMI is expected to lead to improved silage digestibility in wethers fed inoculated silage; however, this response was not observed. Mature wethers were used in the present study, and these animals, as mentioned earlier, typically have a low DMI (< 1.5% BW) that basically provides maintenance requirements, without expending energy for growth (NRC, 2007). In addition, a low DMI suggests a relatively low passage rate of the total mixed ration among all treatments. A low passage rate of the digesta through the gastrointestinal tract facilitates greater microbial colonization of the feedstuff, and consequently, increase digestibility (Church, 1993; Sniffen et al., 1992). Thus, this hypothesis could explain the lack of differences in digestibility, as well as the elevated values observed for DM digestibility (> 870 g/kg) among all treatments. Furthermore, the lack of positive results from the use of *L. buchneri* as a probiotic has been supported by Keady and Steen (1996), who reported no improvements in silage digestibility following the addition of a homofermentative LAB (*L. plantarum*) to grass silage immediately before feeding to beef cattle.

In many cases, improvements in growth performance have been attributed to a possible probiotic effect caused by LAB inoculation of silages (Weinberg and Muck, 1996). This is based on the interaction between LAB and ruminal microorganisms with shifts in the ruminal bacterial community, microbial N supply, and ruminal fermentation (Basso et al., 2014; Mohammed et al., 2012; Weinberg et al., 2007). However, results of the present study showed little changes in ruminal fermentation and the ruminal bacterial community. Only the molar proportion of acetic acid was affected by the experimental treatments without any increases in the total VFA

concentration. Wethers fed the inoculated silage diet had a lower concentration of acetic acid than those fed the untreated and LB-probiotic diets. This response could be attributed to the lower aNDF content reported in the inoculated silage. Fiber degradation reportedly increases the production of acetic acid in the rumen (Church, 1993).

L. buchneri has the ability to produce bacteriocin, more specifically the LB buchnericin (Yildirim, 2001). The activity of bacteriocins might target sensitive gram-positive bacteria (Klaenhammer, 1993) that exhibit cellulolytic activity. In this context, *F. succinogenes*, *R. albus*, and *R. flavefaciens* are recognized as the major cellulolytic bacterial species in the rumen (Church, 1993). Since some LAB are able to survive and grow in ruminal fluid (Weinberg et al., 2003), we expected an increase in the relative proportion of *L. buchneri* in wethers fed the inoculated and LB-probiotic diets. However, this response was not observed. The results of our study revealed a trend towards a consistent reduction in the relative proportion of *R. flavefaciens* in wethers fed diets containing *L. buchneri*, regardless of whether it was used as a silage inoculant or probiotic. These results suggest that products formed by *L. buchneri* would be microorganism-specific, once any shift was observed for *F. succinogenes*, another gram-positive bacterium. Amylolytic bacteria are gram-negative and *L. buchneri* is not expected to exhibit antibacterial activity against these microbes (Klaenhammer, 1993; Yildirim, 2001). Thus, as expected, the relative proportions of *S. ruminantium* and *S. bovis* were unaffected by the use of *L. buchneri* as a silage inoculant or probiotic. As previously reported, *L. buchneri* has a greater capacity to convert lactic acid to acetic acid and 1,2-propanediol at a pH of 3.8, as compared to that of 5.8 (Oude Elferink et al., 2001). Thus, the minimal effects of *L. buchneri* on animal performance when used as a probiotic in the diet, likely reflects its capacity to grow and produce metabolites that act more efficiently as a silage inoculant (silage pH in our study was 4.3) than a probiotic (pH of ruminal inoculum in our study ranged from 6.0 to 6.6). In addition, a lack of significant shifts in the relative proportions of the bacterial community in the rumen has been previously reported by Mohammed et al. (2012). In that study, the authors observed that dairy cows fed alfalfa silage inoculated with *L. plantarum* had a greater relative proportion of *L.*

plantarum in the rumen; however, no similar shifts were observed in the relative proportion of *Megasphaera elsdenii*.

Since the probiotic effect has been one of the main causes of enhanced growth performance (Weinberg and Muck, 1996), further studies should focus on the ruminal bacterial community of animals fed LAB-treated silages. The actual scenario in vivo remains unclear; however, the results of the present study combined with those of previous studies (Gollop et al., 2005; Han et al., 2014; Mohammed et al., 2012) certainly indicate that enhanced growth performance might arise from shifts in the ruminal bacterial community, and these responses might be LAB- and crop-specific and also dose dependent.

5. Conclusions

L. buchneri is more efficient when used as a silage inoculant than a probiotic because it led to changes in fermentation and chemical composition of corn silage, and positively affected DMI with additional minor shifts in the relative proportion of *R. flavefaciens* and ruminal fermentation.

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References

Arriola, K.G., Kim, S.C., Staples, C.R., Adesogan, A.T., 2011. Effect of applying bacterial inoculants containing different types of bacteria to corn silage on the performance of dairy cattle. J. Dairy Sci. 94, 3973–3979.

Ashbell, G., Weinberg, Z.G., Hen, Y., Filya, I., 2002. The effects of temperature on the aerobic stability of wheat and corn silages. J. Ind. Microbiol. Biotechnol. 28, 261–263.

Association of Official Analytical Chemist, AOAC, Official Methods of Analysis, 16 ed., 1996, AOAC; Washington DC, USA.

Basso, F.C., Adesogan, A.T., Lara, E.C., Rabelo, C.H.S., Berchielli, T.T., Teixeira, I.A.M.A., Siqueira, G.R., Reis R.A., 2014. Effects of feeding corn silage inoculated

with microbial additives on the ruminal fermentation, microbial protein yield, and growth performance of lambs. J. Anim. Sci. 92, 5640–5650.

Bernardes, T.F., Adesogan, A.T., 2012. Aerobic deterioration of silages in warm climates. In: Proceedings of the 6ed Symposium on Strategic Management of Pastures, Federal University of Viçosa, MG, pp. 249–268.

Casali, A.O., Detmann, E., Valadares Filho, S.C., Pereira, J.C., Henriques, L.T., Freitas, S.G., Paulino, M.F., 2008. Influence of incubation time and particles size on indigestible compounds contents in cattle feeds and feces obtained by in situ procedures. R. Bras. Zootec. 37, 335–342.

Charmley, E., 2001. Towards improved silage quality – A review. Can. J. Anim. Sci. 81, 157–168.

Church D.C., 1993. The ruminant animal – Digestive physiology and nutrition. Waveland Press, Inc., Illinois – USA.

Der Bedrosian, M.C., Nestor, K.E., Jr., Kung, L., Jr., 2012. The effects of hybrid, maturity, and length of storage on the composition and nutritive value of corn silage. J. Dairy Sci. 95, 5115–5126.

Driehuis, F., Oude Elferink, S.J.W.H., Spoelstra, S.F., 1999. Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. J. Appl. Microbiol. 87, 583–594.

Ellis, J.L., Bannink, A., Hindrichsen, I.K., Kinley, R.D., Pellikaan, W.F., Milora, N., Dijkstra, J., 2016. The effect of lactic acid bacteria included as a probiotic or silage inoculant on in vitro rumen digestibility, total gas and methane production. Anim. Feed Sci. Technol. 211, 61–74.

Fuller, R., 1989. Probiotics in man and animal. J. Appl. Bacteriol. 66, 365–378.

Gollop, N., Zakin, V., Weinberg, Z.G., 2005. Antibacterial activity of lactic acid bacteria included in inoculants for silage and in silages treated with these inoculants. J. Appl. Microbiol. 98, 662–666.

Han, H., Ogata, Y., Yamamoto, Y., Nagao, S., Nishino, N., 2014. Identification of lactic acid bacteria in the rumen and feces of dairy cows fed total mixed ration silage to assess the survival of silage bacteria in the gut. J. Dairy Sci. 97, 5754–5762.

Hino, T., Shimada, K., Maruyama, T., 1994. Substrate preference in a strain of *Megasphaera elsdenii*, a ruminal bacterium, and its implications in propionate production and growth competition. Appl. Environ. Microbiol. 60, 1827–1831.

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

Jung, H.G., Allen, M.S., 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. J. Anim. Sci. 73, 2774–2790.

Keady, T.W.J., Steen, R.W.J., 1994. Effects of treating low dry matter grass with a bacterial inoculant on the intake and performance of beef cattle and studies on its mode of action. Grass For. Sci. 49, 438–446.

Keady, T.W.J., Steen, R.W.J., 1995. The effects of treating low dry matter, low digestibility grass with a bacterial inoculant on the intake and performance of beef cattle, and studies on its mode of action. Grass For. Sci. 50, 217–226.

Keady, T.W.J., Steen, R.W.J., 1996. Effects of applying a bacterial inoculant to silage immediately before feeding on silage intake, digestibility, degradability and rumen volatile fatty acid concentrations in growing beef cattle. Grass For. Sci. 51, 155–162.

Klaenhammer, T.R., 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. 12, 39–86.

Kleinschmit, D.H., Kung, L., Jr., 2006. A meta-analysis of the effects of *Lactobacillus buchneri* on the fermentation and aerobic stability of corn and grass and small-grain silages. J. Dairy Sci. 89, 4005–4013.

Koike, S., Kobayashi, Y., 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. FEMS Microbiol. Lett. 204, 361–366.

Kung, L., Jr., Grieve, D.B., Thomas, J.W., Huber, J.T., 1984. Added ammonia or microbial inoculant for fermentation and nitrogenous compounds of alfalfa ensiled at various percents of dry matter. J. Dairy Sci. 67, 299–306.

Kunji, E.R.S., Mierau, I., Hagting, A., Poolman, B., Konings, W.N., 1996. The proteolytic systems of lactic acid bacteria. Antonie van Leeuwenhoek 70, 187–221.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_T$ method. METHODS 25, 402–408.

Maeda, H., Fujimoto, C., Haruki, Y., Maeda, T., Kokeguchi, S., Petelin, M., Arai, H., Tanimoto, I., Nishimura, F., Takashiba, S., 2003. Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, tetQ gene and total bacteria. FEMS Immunol. Med. Mic. 39, 81–86.

Mari, L.J., Schmidt, R.J., Nussio, L.G., Hallada, C.M., Kung, L., Jr., 2009. Short communication: An evaluation of the effectiveness of *Lactobacillus buchneri* 40788 to alter fermentation and improve the aerobic stability of corn silage in farm silos. J. Dairy Sci. 92, 1174–1176.

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

Mohammed, R., Stevenson, D.M., Beauchemin, K.A., Muck, R.E., Weimer, P.J., 2012. Changes in ruminal bacterial community composition following feeding of alfalfa ensiled with a lactic acid bacterial inoculant. J. Dairy Sci. 95, 328–339.

Muck, R.E., 2010. Silage microbiology and its control through additives. R. Bras. Zootec. 39, 183–191.

National Research Council (NRC), 2007. Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids. National Academy Press, Washington, DC, USA.

Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153, 375–380.

Nkosi, B.D., Meeske, R., Langa, T., Thomas, R.S., 2011. Effects of bacterial silage inoculants on whole-crop maize silage fermentation and silage digestibility in rams. S. Afr. J. Anim. Sci. 41, 350–359.

Nkosi, B.D., Meeske, R., Palic, D., Langa, T., Leeuwa, K.J., Groenewald, I.B., 2009. Effects of ensiling whole crop maize with bacterial inoculants on the fermentation,

aerobic stability, and growth performance of lambs. Anim. Feed Sci. Technol. 154, 193–203.

Oude Elferink, S.J.W.H., Krooneman, J., Gottschal, J.C., Spoelstra, S.F., Faber, F., Driehuis, F., 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2 propanediol by *Lactobacillus buchneri*. Appl. Environ. Microbiol. 67, 125–132.

Pina, D.S., Valadares Filho, S.C., Detmann, E., Valadares, R.F.D., Campos, J.M.S., Moraes, K.A.K., Oliveira, A.S., Paixão, M.L., 2006. Comparison of internal markers and extent of total fecal collection to estimate nutrient digestibility and energy value of feeds on dairy cows fed different protein sources. R. Bras. Zootec. 35, 2461–2468.

Schmidt, P., Nussio, L.G., Queiroz, O.C.M., Santos, M.C., Zopollatto, M., Toledo Filho, S.G., Daniel, J.L.P., 2014. Effects of *Lactobacillus buchneri* on the nutritive value of sugarcane silage for finishing beef bulls. R. Bras. Zootec. 43, 8–13.

Sniffen, C.J., O'Connor, J.D., Van Soest, P.J., Fox, D.G., Russell, J.B., 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. J. Anim. Sci. 70, 3562–3577.

Stevenson, D.M., Muck, R.E., Shinners, K.J., Weimer, P.J., 2006. Use of real time PCR to determine population profiles of individual species of lactic acid bacteria in alfalfa silage and stored corn stover. Appl. Microbiol. Biotechnol. 71, 329–338.

Stevenson, D.M., Weimer, P.J., 2007. Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Appl. Microbiol. Biotechnol. 75, 165–174.

Tajima, K., Aminov, R.I., Nagamine, T., Matsui, H., Nakamura, M., Benno, Y., 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Environ. Microbiol. 67, 2766–2774.

Valente, T.N.P., Detmann, E., Valadares Filho, S.C., Cunha, M., Queiroz, A.C., Sampaio, C.B., 2011. In situ estimation of indigestible compounds contents in cattle feed and feces using bags made from different textiles. R. Bras. Zootec. 40, 666–675.

Weinberg, Z.G., Muck, R.E., 1996. New trends and opportunities in the development and use of inoculants for silage. FEMS Microbiol. Rev. 19, 53–68.

Weinberg, Z.G., Muck, R.E., Weimer, P.J., 2003. The survival of silage inoculant lactic acid bacteria in rumen fluid. J. Appl. Microbiol. 94, 1066–1071.

Weinberg, Z.G., Shatz, O., Chen, Y., Yosef, E., Nikbahat, M., Ben-Ghedalia, D., Miron, J., 2007. Effect of lactic acid bacteria inoculants on in vitro digestibility of wheat and corn silages. J. Dairy Sci. 90, 4754–4762.

Weißbach, F., Strubelt, C., 2008. Correcting the dry matter content of maize silages as a substrate for biogas production. Landtechnik 63, 82–83.

Yildirim, M., 2001. Purification of Buchnericin LB Produced by *Lactobacillus buchneri* LB. Turk. J. Biol. 25, 59–65.

CHAPTER 4

The paper was written following the guidelines for authors of *Animal Production Science*, with exception of tables and figures position.

Feed intake and growth performance of finishing feedlot beef cattle fed diets containing maize silages inoculated with lactic-acid bacteria and *Bacillus subtilis*

Abstract.

Our objective was to evaluate the effect of lactic-acid bacteria and *Bacillus subtilis* as silage additives on feed intake and growth performance of finishing feedlot beef cattle. Whole-maize forage was ensiled either with distilled water (untreated), or inoculated with *Lactobacillus buchneri* and *L. plantarum* at a rate 1×10^5 cfu/g fresh forage for each bacteria (LBLP); or inoculated with *B. subtilis* and *L. plantarum* at a rate 1×10^5 cfu/g fresh forage for each bacteria (BSLP). Thirty-six Nellore × Brown Swiss crossbred bulls (316 ± 33.9 kg) were used in the feedlot program for 110 d, and they were assigned (*n* = 12) to one of three diets containing untreated, LBLP, or BSLP silages in a 40:60 forage:concentrate ratio. Dry matter (DM) intake, average daily gain (ADG), and dressing percentage of bulls were unaffected by silage inoculation. Conversely, bulls fed the BSLP silage lowered DM, organic matter (OM), and crude protein (CP) digestibility compared to bulls fed untreated silage. Bulls fed both inoculated silages had a reduction of approximately 12% in neutral detergent fibre (NDF) and acid detergent fibre (ADF) digestibility compared to that in bulls fed untreated silage. Bulls fed the LBLP silage spent more time chewing (496 min/d) than bulls fed untreated silage. Ruminal fermentation was little affected by silage inoculation, but bulls fed the inoculated silages had a lower concentration of ammonia-N. In conclusion, adding *L. plantarum* combined with *L. buchneri* or *B. subtilis* to maize silage do not improve the growth performance of finishing feedlot beef cattle.

Additional keywords: digestibility, ingestive behaviour, *Lactobacillus* spp., ruminal fermentation, silage inoculant.

1. Introduction

Bacterial inoculants are the most common additives used in the world to improve preservation of ensiled crops. Homofermentative and heterofermentative facultative

lactic-acid bacteria (LAB), such as *Lactobacillus plantarum*, are used to 1) ensure rapid production of lactic acid and pH decline of the ensiled crop, 2) avoid the growth of undesirable microorganisms (e.g., enterobacteria and clostridia), 3) improve preservation of water-soluble carbohydrates (WSC), and 4) enhance dry matter (DM) recovery during fermentation (McDonald *et al.* 1991). Addition of homofermentative inoculants, however, can reduce the aerobic stability of silages during feed-out, because the lactic acid they produce is used as a growth substrate by yeasts that initiate spoilage (Adesogan 2014). This problem is particularly acute in silages produced under tropical conditions (Ashbell *et al.* 2002; Bernardes and Adesogan 2012). Hence, *L. buchneri*, an obligatory heterofermentative LAB, has been successfully used to enhance aerobic stability of silages (Kleinschmit and Kung 2006) because of its ability to convert lactic acid to acetic acid and 1,2-propanediol under anaerobic conditions (Oude Elferink *et al.* 2001). Acetic acid protects the silage against spoilage by aerobic microorganisms because of its antifungal nature (Moon 1983). Furthermore, *Bacillus subtilis*, a gram-positive bacterium, is known to produce amylase and antifungal compounds (Van Soest *et al.* 1991; Zuber *et al.* 1993). Recently, this bacterium has been used as a silage inoculant, and a reduction in yeast growth as well as increased *in vitro* silage digestibility and aerobic stability of maize silages inoculated with *B. subtilis* has been reported (Basso *et al.* 2012; Lara *et al.* 2015). In this context, combining *L. plantarum* with *L. buchneri* or *B. subtilis* may confer enhanced fermentation, nutritive value, and aerobic stability on silages (Filya 2003; Basso *et al.* 2014; Lara *et al.* 2015).

Improvements in silage quality caused by LAB inoculation, along with a possible probiotic effect from LAB inoculation of silage, have been proposed as the reasons for enhanced growth performance of animals consuming inoculated silage (Weinberg and Muck 1996; Weinberg *et al.* 2003; Mohammed *et al.* 2012). Increased feed intake, digestibility, and average daily gain (ADG) have been noted in large ruminants fed silages treated with *L. plantarum* and *L. buchneri* as a single inoculant (Keady and Steen 1995; Rabelo *et al.* 2016), and also in small ruminants when these bacteria are combined (Nkosi *et al.* 2009; Basso *et al.* 2014). However, few general studies have investigated the effect of bacteria combinations on growth performance of finishing feedlot beef cattle.

Regarding *B. subtilis*, this bacterium can form spores that are resistant to acid and oxygen, and many live spores administered orally may reach the intestine and then induce some beneficial effects through secretion of active substances by germinated cells (Hosoi *et al.* 2000). Previous studies reported benefits from the oral administration of *B. subtilis* on increased ADG in calves (Sun *et al.* 2010), and increased organic matter (OM) digestibility in Nellore bulls fed total mixed rations containing maize silage (Telles *et al.* 2011). To the best of our knowledge, however, there are no studies concerning the effect of *B. subtilis* as a single or combined silage inoculant on ruminant performance. Therefore, our objective was to evaluate the impact of LAB and *B. subtilis* as silage additives on feed intake and growth performance of finishing feedlot beef cattle.

2. Material and methods

2.1. *Ethics statement*

Animal care and handling procedures used in this study were in accordance with the Brazilian College of Animal Experimentation (COBEA, Colégio Brasileiro de Experimentação Animal) guidelines and were approved by the Ethics, Bioethics, and Animal Welfare Committee (CEBEA, Comissão de Ética e Bem Estar Animal) of São Paulo State University, Brazil.

2.2. *Silage production*

The flint maize (*Zea mays*) hybrid 'Impacto Víptera' (Syngenta, Matão, SP, Brazil) was harvested at 2/3 of the milk line (354 g DM/kg as fed) using a Premium Flex forage harvester (Menta Mit, Cajuru, SP, Brazil) and chopped to a length of 10 mm. The Impacto Víptera is a hybrid recommended to produce grains and also for ensilage, which has 55,000–60,000 plants/ha and a productivity of approximately 50 ton/ha. Whole-crop maize forage was treated either with distilled water (5 L/t) (designated as 'untreated'); or with *L. buchneri* CNCM I-4323 (1×10^5 cfu/g of fresh forage) and *L. plantarum* MA18/5U (1×10^5 cfu/g of fresh forage) (designated as 'LBLP'); or with *B. subtilis* AT553098 (1×10^5 cfu/g of fresh forage) and *L. plantarum* MA18/5U (1×10^5 cfu/g of fresh forage) (designated as 'BSLP') (Lallemand Animal Nutrition, Milwaukee, WI, USA; Fatec Animal Nutrition, São Paulo, SP, Brazil). The

inoculants were dissolved in distilled water (5 L/t) and sprayed on fresh forage during silo filling. Viability and label levels of the bacteria in the inoculant were confirmed prior to use.

Three stack silos (*n* = 1) were filled in two days with approximately 40 tons each of the respective maize forage (untreated, LBLP, and BSLP). To avoid possible cross contamination, the untreated forage was ensiled first, followed by the inoculated forages. Once the silage mass reached approximately 1 m in height, twelve net bags with fresh well mixed forage (~5 kg) and known DM content (Ashbell and Weinberg 1992) were placed on the top of the silos. An additional 0.5 m of forage was then placed on the top of each silo. Silos were sealed with 200-µm thick black-on-white polyethylene film (Electro Plastic, São Paulo, SP, Brazil). At ensiling, twelve fresh samples of maize forage were collected from each silo to determine chemical composition.

Silos were stored at ambient temperature for 88 d. Approximately 13 cm/d of silage was removed from the face of each silo to provide forage for the bulls. The silage was removed from the silo face using a fork, and the implanted bags were recovered as they were encountered for measurement of DM recovery. Each bag was immediately weighed and sampled to estimate DM content of the silage it contained. Silage samples were collected weekly and stored at -20°C for subsequent analyses.

2.3. *Aerobic stability*

An aerobic stability assay was carried out three times during feed-out as follows. Four silage samples (~3 kg) from each farm silo were placed in plastic buckets of 5 L capacity and kept at ambient temperature. The silage temperature was measured every half hour by a datalogger placed in the centre of the mass for 5 d. The ambient temperature was also measured every half hour by two dataloggers distributed near the buckets. Aerobic stability was defined as the number of hours that the silage temperature remained stable before increasing 2°C above the ambient temperature (Moran *et al.* 1996).

2.4. *Animal performance study*

Thirty-six Nellore × Brown Swiss crossbred bulls (initial BW: 316 ± 33.9 kg) were housed randomly in individual stalls (4.5 m^2) located in a well-ventilated covered feedlot, with each stall being equipped with a feed bunk and water trough. Bulls were randomly assigned (*n* = 12) to one of three diets containing untreated, LBLP, and BSLP silages. Diets were composed of 40% the respective maize silage and 60% concentrate on a DM basis (Table 1), and were balanced to meet the nutrient requirements of beef cattle gaining 1.4 kg/d (National Research Council 2000). Bulls were fed for *ad libitum* intake (approximately 10% orts) once daily (0700), and provided with free access to water. Orts were weighed daily before the morning feeding. Samples of offered feed and orts were collected twice weekly and stored at -20°C for later analyses. Orts were used to calculate DM intake (DMI) daily, and their chemical composition was also taken into consideration when digestibility was calculated.

Table 1. Ingredient proportions and chemical composition of total mixed rations containing maize silage inoculated with lactic-acid bacteria and *Bacillus subtilis* (data are given in g/kg of dry matter unless otherwise stated)

Item	Untreated[A]	LBLP	BSLP
	Ingredient proportion		
Maize silage	400	400	400
Ground maize (2 mm)	492	492	492
Soybean meal	68.0	68.0	68.0
Urea	10.0	10.0	10.0
Mineral supplement[B]	30.0	30.0	30.0
	Chemical composition		
Dry matter (g/kg as fed)	666	673	685
Organic matter	937	938	946
Crude protein	150	151	152
Ether extract	45.9	45.6	46.9
Total carbohydrates	741	742	747
Neutral detergent fibre	221	229	226
Acid detergent fibre	110	119	106
Non-fibre carbohydrates	531	524	529

[A]Maize silage was either untreated, or treated at ensiling with *L. buchneri* CNCM I-4323 (1 × 10^5 cfu/g of fresh forage) and *L. plantarum* MA18/5U (1 × 10^5 cfu/g of fresh forage) (LBLP); or with *B. subtilis* AT553098 (1 × 10^5 cfu/g of fresh forage) and

L. plantarum MA18/5U (1 × 10^5 cfu/g of fresh forage) (BSLP) (Lallemand Animal Nutrition, Milwaukee, WI, USA; Fatec Animal Nutrition, São Paulo, SP, Brazil).
[B]Mineral supplement was composed of 70 g/kg P, 160 g/kg Ca, 100 g/kg Na, 5 g/kg Mg, 40 g/kg S, 2560 mg/kg Zn, 690 mg/kg Cu, 530 mg/kg Mn, 41 mg/kg Co, 51 mg/kg I, 700 mg/kg F, and 13 mg/kg Se, on a DM basis.

Bulls were adapted to the diets for 21 d, with a gradual increase in concentrate levels during this period (i.e., bulls were located in the feedlot and adapted feeding maize silage for 3 d, and thereafter we increased 10% concentrate in the diet for each 3 d of adaptation until reach the roughage:concentrate ratio of 40:60). After 21-d adaptation, we carried out the experiment for 89 d and bulls were weighted in intervals of approximately 30 d during this period without fasting. The initial and final body weights (BW) were measured after a 16-h fast, and the total BW gain during the experiment length (89 d) was calculated by subtracting initial BW from final BW. The ADG was calculated by subtracting initial BW from final BW and dividing the difference by the experiment length of 89 d post-adaptation. The gain efficiency (gain:feed) was determined by dividing ADG by DMI.

2.5. *Digestibility study*

In vivo apparent digestibility was calculated indirectly using indigestible neutral detergent fibre (iNDF) as a marker to estimate faecal output (Valente *et al.* 2011). Faecal grab samples were collected from each bull every 26 h for three-consecutive days in the middle of the experiment (Pina *et al.* 2006). Samples of silage, concentrate, and refusals were also collected daily during this period.

2.6. *Evaluation of ingestive behaviour*

Eating and ruminating activities were monitored visually for individual bulls over three consecutive 24 h periods from feedlot day 79 to day 82 post-adaptation, and noted every 10 min. The average intake during the third experimental period (last 30 d of feedlot) was used to estimate time spent eating or ruminating per kilogram of DM, neutral detergent fibre (NDF), acid detergent fibre (ADF), and iNDF intake. Total time spent chewing was calculated as the sum of total time spent eating and ruminating (Maekawa *et al.* 2002). Total time spent resting was calculated as 24 h minus the total time spent chewing.

2.7. *Slaughter, carcass trait data collection, and sampling procedures*

Bulls (final BW: 449 ± 37.7 kg) were slaughtered at a commercial beef plant (Minerva®, Barretos, SP, Brazil) after feedlot day 110 with a 16-h fast, the carcass was split in two, and hot carcass weight was measured. All carcasses were refrigerated at 4°C for approximately 24 h. Dressing percentage was calculated by dividing hot carcass weight by fasted body weight and multiplying by 100. The 12^{th} rib fat thickness (RFT) and 12^{th} rib *longissimus* muscle area (LMA) was also recorded on the left side of each carcass. The LMA was traced on transparencies and subsequently measured with a calliper, and RFT measurements were taken 3/4 the length ventrally over the *longissimus* muscle using a digital paquimeter (Greiner *et al.* 2003).

2.8. *Determination of ruminal fermentation products*

Six ruminally cannulated Nellore bulls (initial BW: 479 ± 47.9 kg), each fitted with a silicone 10.2-cm ruminal cannula, were used in a double 3 × 3 Latin Square. Each animal was housed individually in pens (4.5 m^2) equipped with a feed bunk and water trough. Bulls were fed for *ad libitum* intake (approximately 10% orts) once a day (0700), and the diets used were the same as those used in the feedlot program.

Ruminal measurements were taken over three 21-d periods, each period consisting of 20 d to adapt to the diets and 1 d for rumen fluid collection. A 50-mL sample of ruminal fluid was collected from the reticulum and the ventral, caudal, and dorsal-ventral sac of the reticulorumen of each bull immediately before feeding (0 h) and at 3, 6, 9, 12, and 24 h post-feeding. Ruminal fluid was squeezed through two layers of cheesecloth and the pH was immediately measured using a pH meter (MA522 model, Marconi Laboratory Equipment, Piracicaba, SP, Brazil). Subsequently, 1 mL H_2SO_4 (1:1) was added to the ruminal fluid and the solution was stored at -20°C for analyses of ammonia-N and volatile fatty acids (VFAs).

2.9. *Sample preparation and chemical analyses*

A water extract was produced from fresh silage samples as described by Kung *et al.* (1984), and silage pH was measured with a pH meter. Lactic acid was determined by

the colorimetric method (Pryce, 1969). VFAs were measured using a gas chromatograph (Shimadzu model GC2014, Shimadzu Corp., Kyoto, Japan) equipped with an HP-INNOWax capillary column (30 m × 0.32 mm; Agilent Technologies, Colorado, USA) at an initial temperature of 80°C for 3 min followed by heating at a rate of 20°C/min until a final temperature of 240°C was achieved. Water-soluble carbohydrates (WSC) were determined following the Nelson-Somogyi method (Nelson 1944). Ammonia-N was measured by distillation (AOAC 1996; method no. 941.04), and expressed as g/kg of total N (TN).

For microbiological analyses, fresh silage samples (25 g) from each replicate were homogenized in 225 mL of autoclaved saline solution (0.85% NaCl) for 1 min. An aliquot (1 mL) of this solution was transferred into tubes containing 9 mL of saline solution, and then 1 mL of this mixture was plated onto Petri plates after serial dilutions of 10^{-1}–10^{-9}. Man, Rogosa, and Sharpe (MRS) agar was used to count LAB using pour-plates, and potato dextrose agar (PDA) was used to count yeasts and moulds using spread-plates. Both MRS and PDA plates were incubated at 28°C. LAB and yeasts were counted after 2 d, and moulds were counted after 5 d. All microbiological data were $\log_{10}$-transformed.

Forage and silage samples were oven dried (55°C for 72 h) and processed in a knife mill before being ground through a 1-mm screen and analysed for DM (105°C for 12 h) and ash (500°C for 5 h). OM was calculated as 1000 – ash. Ether extract (EE) was determined following the procedures described by AOAC (1996; method no. 920.39). The TN was measured by rapid combustion using a LECO Analyzer (model F528 N, LECO Corp., St. Joseph, MI, USA), and crude protein (CP) was calculated as TN × 6.25. The NDF and ADF were determined in an ANKOM 2000 Fiber Analyzer (ANKOM Technologies, Macedon, NY, USA) following the procedures described by Mertens (2002). NDF was measured using a heat stable amylase without sodium sulphite, and both NDF and ADF were expressed exclusive of residual ash. Lignin was sequentially measured after hydrolysis of the ADF residual in 72% H_2SO_4 (Van Soest and Robertson 1985). The iNDF was determined by incubating F57 bags (ANKOM) containing silage samples in the rumen of two Nellore steers for 264 h (Casali *et al.* 2008). Afterward, samples were removed from the rumen of the Nellore steers and analysed for NDF. The total carbohydrate (CHOT)

and non-fibre carbohydrate (NFC) contents of the maize silages were determined according to Sniffen *et al.* (1992).

Ruminal ammonia-N was determined by distillation with 2N KOH according to Fenner (1965). Aliquots of strained ruminal fluid collected were thawed in a refrigerator overnight and centrifuged at 4°C and 20,000 × g for 30 min, and the supernatant was analysed for VFA by gas chromatography as described earlier.

2.10. *Calculations*

DM values determined in the forced air oven (DM_{oven}) were corrected (DM_{corr}) for volatile compounds using an adaptation (Eq. 1) from the original equation proposed by Weißbach and Strubelt (2008).

DM_{corr} (g/kg as fed) = DM_{oven} + 0.95*VFA + 0.08*lactic acid (1)

where all volatile compounds are expressed in g/kg of fresh matter.

All variables regarding chemical composition and fermentation products of silage were expressed in terms of DM_{corr}.

Faecal output and apparent digestibility were calculated using Eqs. 2 and 3, respectively.

Faecal yield = iNDF intake (g)/iNDF faecal (%) * 100 (2)

Apparent digestibility = [DMI (g) – faecal yield (g)]/DMI (g) * 100 (3)

2.11. *Statistical analyses*

Statistical analyses of silage data were precluded because there was only one farm silo replicate for each treatment. Growth performance, apparent digestibility, ingestive behaviour, and carcass traits were analysed using the MIXED procedure of SAS (v. 9.4 SAS Institute Inc., Cary, NC) in a completely randomized design (n = 12). Diet was considered as a fixed effect and error as a random effect. Ruminal fermentation was carried out under a double 3 × 3 Latin Square design and analysed as repeated measures over time. All variables were analysed using the MIXED procedure of SAS. Several covariance structures were tested and those that generated the lowest corrected Akaike information criterion (AICc) and Bayesian information criterion (BIC) were selected. Diet was considered as a fixed effect and bulls and period as random effects. Differences between diets were determined using

the PDIFF option of LSMEANS. Significant differences were declared at $P \leq 0.05$ and trends discussed at $0.05 > P \leq 0.10$.

3. Results

3.1. *Fermentation, microbial profile, and chemical composition of maize silage*

The LBLP silage had lower DM recovery and higher aerobic stability compared to untreated and BSLP silages (Table 2). In addition, all maize silages had similar chemical composition except with respect to DM and ash content, which were higher and lower, respectively, in the BSLP silage.

Table 2. Fermentation, microbial profile, and chemical composition of maize silages inoculated with lactic-acid bacteria and *Bacillus subtilis* in stack silos after 88 days of ensiling (mean ± standard error of the mean)

Item	Forage	Silages[A]		
		Untreated	LBLP	BSLP
Fermentative profile (g/kg of dry matter)				
Ammonia-N (g/kg total N)	-	15.2 ± 2.24	12.7 ± 2.12	14.4 ± 2.77
WSC[B] (mg glucose/g silage)	-	20.3 ± 1.08	12.0 ± 1.78	19.2 ± 2.18
pH	-	3.97 ± 0.03	4.10 ± 0.03	4.17 ± 0.04
Lactic acid	NA	49.8 ± 2.45	32.1 ± 3.31	34.8 ± 1.69
Acetic acid	NA	20.8 ± 2.56	21.9 ± 3.44	19.4 ± 2.54
Lactic:acetic acid ratio	NA	2.87 ± 0.36	1.48 ± 0.07	1.83 ± 0.12
Propionic acid	NA	0.19 ± 0.08	0.17 ± 0.09	0.15 ± 0.24
Butyric acid	NA	0.29 ± 0.06	0.35 ± 0.37	0.56 ± 0.20
Isobutyric acid	NA	0.02 ± 0.01	0.01 ± 0.02	0.03 ± 0.01
Valeric acid	NA	0.07 ± 0.03	0.09 ± 0.15	0.01 ± 0.01
Isovaleric acid	NA	0.33 ± 0.25	0.15 ± 0.31	0.33 ± 0.11
Dry matter recovery	NA	921 ± 19.7	852 ± 83.9	903 ± 69.3
Aerobic stability (h)	NA	14.4 ± 1.86	93.7 ± 12.5	12.4 ± 0.98
Microorganisms profile (log_{10} cfu/g of fresh silage)				
Lactic-acid bacteria	-	6.68 ± 0.27	7.43 ± 0.10	7.48 ± 0.11
Yeasts	-	6.64 ± 0.30	7.15 ± 0.16	6.63 ± 0.29
Moulds	-	5.06 ± 0.16	5.36 ± 0.32	6.32 ± 0.11
Chemical composition (g/kg of dry matter)				
Dry matter oven (g/kg as fed)	354	346 ± 1.03	362 ± 2.47	392 ± 3.81
Dry matter corrected (g/kg as fed)	NA	364 ± 3.63	388 ± 2.79	411 ± 3.67
Ash	32.9	51.7 ± 4.93	49.9 ± 2.07	29.6 ± 0.47
Ether extract	-	34.6 ± 0.82	33.9 ± 0.72	37.1 ± 0.91
Crude protein	115	92.7 ± 0.82	93.4 ± 1.28	96.8 ± 0.72
Total carbohydrates	-	821 ± 3.49	818 ± 4.92	836 ± 1.46
Neutral detergent fibre	-	331 ± 3.89	340 ± 7.17	353 ± 3.76
Acid detergent fibre	149	203 ± 4.37	224 ± 4.23	194 ± 3.02
Lignin	48.2	49.9 ± 4.36	62.7 ± 4.13	29.9 ± 2.19
Non-fibre carbohydrates	-	492 ± 5.11	468 ± 6.74	485 ± 3.77

[A]Maize silage was either untreated, or treated at ensiling with *L. buchneri* CNCM I-4323 (1×10^5 cfu/g of fresh forage) and *L. plantarum* MA18/5U (1×10^5 cfu/g of fresh forage) (LBLP); or with *B. subtilis* AT553098 (1×10^5 cfu/g of fresh forage) and *L. plantarum* MA18/5U (1×10^5 cfu/g of fresh forage) (BSLP) (Lallemand Animal Nutrition, Milwaukee, WI, USA; Fatec Animal Nutrition, São Paulo, SP, Brazil).

[B]WSC = water soluble carbohydrates.

NA = not applicable.

3.2. *Feed intake, apparent digestibility, ingestive behaviour, growth performance, and carcass traits*

Bulls fed the LBLP silage consumed 9.6% and 10.5% more ADF (P = 0.0089) than bulls fed the untreated and BSLP silages, respectively (Table 3). However, DM, OM, CP, and NDF intake was unaffected (P > 0.05) by the treatments.

Apparent digestibility of DM, OM, and CP was reduced (P < 0.05) by 9%, 7.7%, and 8.8%, respectively, when bulls were fed BSLP silage compared to untreated silage (Table 3). NDF and ADF digestibility decreased (P < 0.05) by approximately 12.4% under both inoculation treatments (LBLP and BSLP) compared to untreated silage.

Bulls fed LBLP and BSLP silages spent more time eating (39 and 53 min/d, respectively) compared to bulls fed untreated silage (P = 0.0032), even when expressed per kilogram of DM (P = 0.0089; Table 3). Conversely, bulls fed LBLP silage spent more time ruminating (P = 0.0073) compared to bulls fed untreated and BSLP silages; but, when rumination was expressed per kilogram of DM (P = 0.0237) and NDF (P = 0.0192), bulls fed the LBLP silage spent more time ruminating only as compared to the bulls fed BSLP silage. Rumination expressed per kilogram of ADF and iNDF was unaffected (P > 0.05) by the treatments. Bulls fed LBLP silage spent more time chewing (P = 0.0198) and less time resting (P = 0.0243) as compared to the bulls fed untreated silage.

The treatments had no effect (P < 0.05) on initial and final BW, ADG, gain efficiency, dressing percentage, and RFT (Table 3). However, there was a trend (P = 0.0863) towards reduced LMA in bulls fed LBLP silage compared to bulls fed untreated and BSLP silages.

Table 3. Feed intake, apparent digestibility, ingestive behaviour, growth performance, and carcass traits of finishing beef cattle fed total mixed rations containing maize silage inoculated with lactic-acid bacteria and *Bacillus subtilis* for 89 days of feedlot post-adaptation (*n* = 12)

Item	Untreated[A]	LBLP	BSLP	SEM	*P*-value
Feed intake (kg/d)					
Dry matter	8.76	8.96	8.95	0.31	0.8736
Dry matter (% body weight)	2.34	2.34	2.32	0.07	0.9702
Organic matter	8.73	8.93	8.93	0.27	0.7431
Crude protein	1.41	1.43	1.45	0.05	0.8698
Neutral detergent fibre	2.12	2.24	2.19	0.06	0.3754
Acid detergent fibre	1.15^{b}	1.26^{a}	1.14^{b}	0.03	0.0089
Apparent digestibility (%)					
Dry matter	80.1^{a}	76.5^{ab}	72.9^{b}	1.42	0.0066
Organic matter	84.5^{a}	81.8^{ab}	78.0^{b}	1.28	0.0064
Crude protein	82.0^{a}	77.4^{ab}	74.8^{b}	1.54	0.0176
Neutral detergent fibre	62.5^{a}	55.4^{b}	54.0^{b}	1.90	0.0121
Acid detergent fibre	57.2^{a}	50.1^{b}	50.1^{b}	2.09	0.0476
Ingestive behaviour					
Eating					
(min/d)	131^{b}	170^{a}	184^{a}	10.34	0.0032
(min/kg of dry matter)	15.2^{b}	19.2^{a}	20.6^{a}	1.21	0.0089
Rumination					
(min/d)	281^{b}	326^{a}	258^{b}	14.46	0.0073
(min/kg of dry matter)	32.8^{ab}	36.5^{a}	28.9^{b}	1.86	0.0237
(min/kg of neutral detergent fibre)	136^{ab}	146^{a}	116^{b}	7.24	0.0192
(min/kg of acid detergent fibre)	248	258	222	12.64	0.1323
(min/kg of iNDF)[B]	500	441	404	29.41	0.1145
Total chewing (min/d)	413^{b}	496^{a}	441^{ab}	20.12	0.0198
Resting (min/d)	1026^{a}	944^{b}	994^{ab}	20.31	0.0243
Growth performance and carcass traits					
Initial body weight (kg)	305	315	322	9.30	0.4553
Final body weight (kg)	442	452	453	11.11	0.7350
Average daily gain (kg/d)	1.45	1.54	1.47	0.09	0.7622
Gain efficiency	0.17	0.17	0.16	0.01	0.4305
Dressing percentage (%)	60.7	58.1	57.0	2.11	0.4368
Rib fat thickness (mm)	3.64	2.88	3.71	0.34	0.2252
Longissimus muscle area (cm^2)	83.0	73.5	83.6	3.05	0.0863

[a-b]Means in the same row with different superscripts were significantly different ($P < 0.05$).

[A]Maize silage was either untreated, or treated at ensiling with *L. buchneri* CNCM I-4323 (1×10^5 cfu/g of fresh forage) and *L. plantarum* MA18/5U (1×10^5 cfu/g of fresh forage) (LBLP); or with *B. subtilis* AT553098 (1×10^5 cfu/g of fresh forage) and *L. plantarum* MA18/5U (1×10^5 cfu/g of fresh forage) (BSLP) (Lallemand Animal Nutrition, Milwaukee, WI, USA; Fatec Animal Nutrition, São Paulo, SP, Brazil).
[B]Indigestible neutral detergent fibre.

3.4. *Ruminal fermentation*

Bulls fed LBLP and BSLP silages had lower ($P = 0.0023$) concentrations of ammonia-N in the rumen compared to bulls fed untreated silage (Table 4). Rumen fluid pH, total VFA concentration, molar proportions of butyric and isovaleric acids, and acetic:propionic acid ratios were unaffected ($P > 0.05$) by treatments.

All variables regarding ruminal fermentation were altered by time post-feeding, and an interaction between silage and time post-feeding was observed for acetic, propionic, isobutyric, and valeric acid. The concentration of total VFA was higher ($P < 0.01$) 9 and 12 h post-feeding (104 and 106 mM, respectively; Fig. 1a). Rumen fluid pH was lower ($P < 0.01$) 9 and 12 h post-feeding (5.82 and 5.79, respectively; Fig. 1b). There was an observed peak ($P < 0.01$) in the ammonia-N concentration 3 h post-feeding (18.6 mg/dL; Fig. 2). At 9 and 12 h post-feeding, bulls fed LBLP and untreated silage had a higher concentration of acetic acid ($P = 0.0023$; Fig. 3a) and propionic acid ($P = 0.0023$; Fig. 3a), respectively. With exception of 12 h post-feeding, bulls fed BSLP silage had slight higher ($P = 0.0348$) concentrations of isobutyric acid at all times evaluated (Fig. 3c). The concentration of valeric acid was also higher ($P = 0.0020$) in bulls fed BSLP silage at 3, 9, and 12 h post-feeding (Fig. 3d). As observed for pH, the acetic:propionic acid ratio was lower ($P < 0.01$) 9 and 12 h post-feeding (4.7 and 4.4; Fig. 4). Butyric acid had a higher ($P < 0.01$) concentration 9 and 12 h post-feeding (12.1 and 12.3 mM/100 mM, respectively; Fig. 5a), whereas the higher ($P < 0.01$) concentration of isovaleric acid occurred 3 h post-feeding (2.6 mM/100 mM; Fig. 5b).

Table 4. Ruminal fermentation in cannulated beef cattle fed total mixed rations containing maize silage inoculated with lactic-acid bacteria and *Bacillus subtilis* (*n* = 6)

Item	Silages[A]			SEM	*P*-value[B]		
	Untreated	LBLP	BSLP		S	T	S × T
pH	6.31	6.16	6.27	0.10	0.1914	<0.0001	0.6546
Ammonia-N (mg/dL)	16.2[a]	12.9[b]	11.5[b]	1.78	0.0023	<0.0001	0.0984
Total VFA (mM)	82.5	88.4	90.2	5.28	0.4160	<0.0001	0.9882
	Molar proportion (mM/100 mM)						
Acetic acid	69.7	70.4	70.1	1.02	0.9019	0.0005	0.0023
Propionic acid	14.8	14.2	14.1	0.82	0.8250	0.0003	0.0003
Acetic:propionic acid ratio	4.92	5.10	5.14	0.47	0.9220	<0.0001	0.1955
Butyric acid	11.3	10.9	11.2	0.72	0.7943	<0.0001	0.1755
Isobutyric acid	0.895	0.891	1.01	0.04	0.0129	<0.0001	0.0348
Valeric acid	1.18	1.22	1.30	0.07	0.4840	<0.0001	0.0020
Isovaleric acid	2.24	2.26	2.31	0.16	0.9380	<0.0001	0.2278

[a-b]Means in the same row with different superscripts are significantly different ($P < 0.05$).

[A]Maize silage was either untreated, or treated at ensiling with *L. buchneri* CNCM I-4323 (1×10^5 cfu/g of fresh forage) and *L. plantarum* MA18/5U (1×10^5 cfu/g of fresh forage) (LBLP); or with *B. subtilis* AT553098 (1×10^5 cfu/g of fresh forage) and *L. plantarum* MA18/5U (1×10^5 cfu/g of fresh forage) (BSLP) (Lallemand Animal Nutrition, Milwaukee, WI, USA; Fatec Animal Nutrition, São Paulo, SP, Brazil).

[B]S = silage; T = time; S × T = interaction between silage and time.

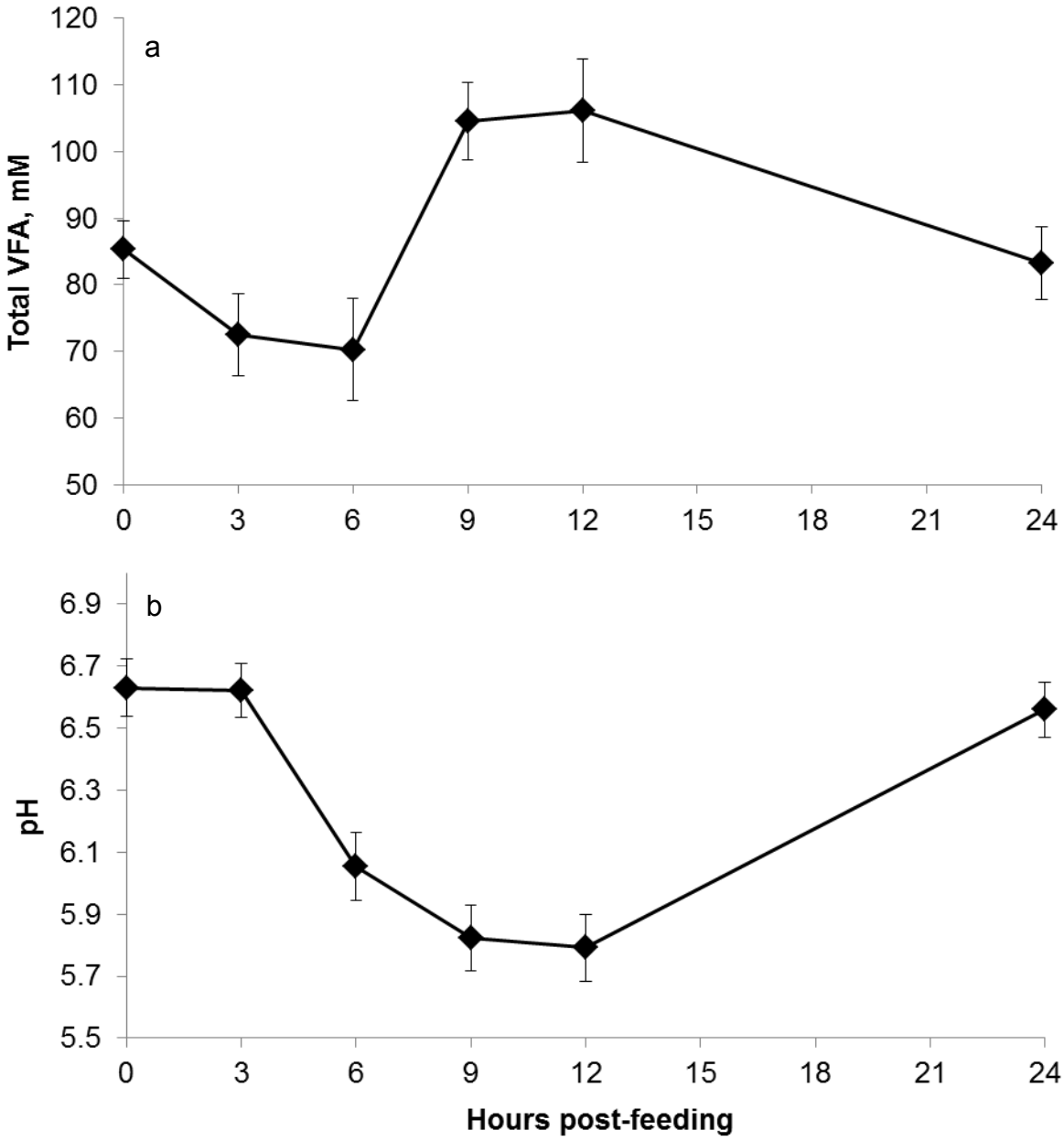

Fig. 1. Overall means for total VFA concentration (a) and ruminal pH (b) during 24 h post-feeding in cannulated Nellore fed total mixed rations containing maize silage inoculated with lactic-acid bacteria and *Bacillus subtilis*.

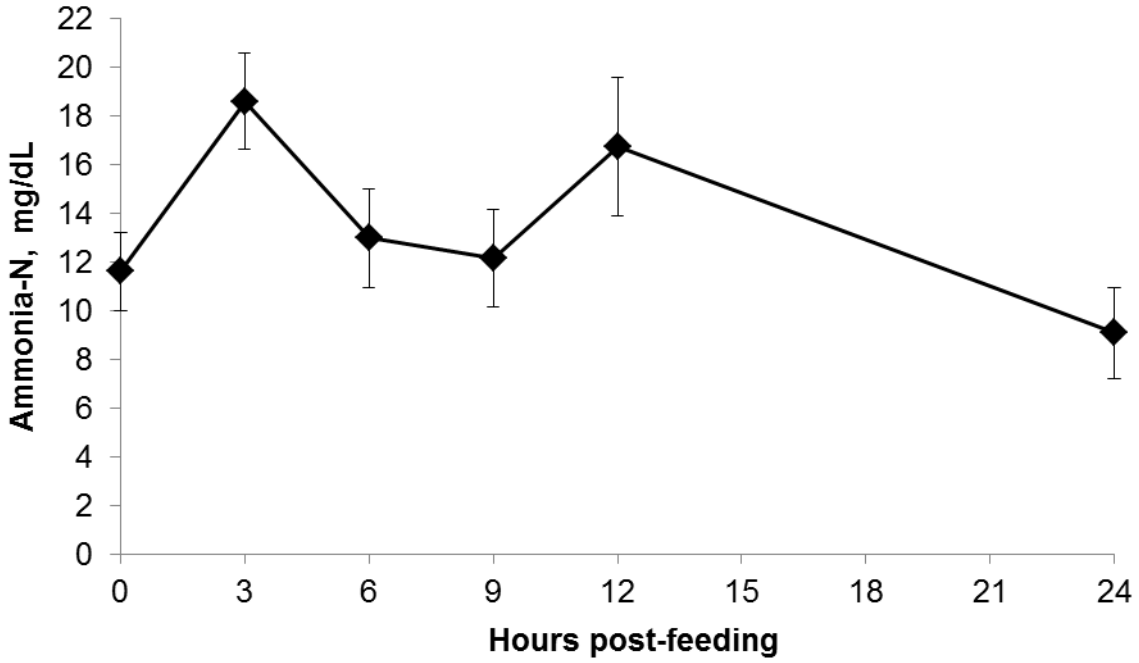

Fig. 2. Overall means for ammonia-N concentration during 24 h post-feeding in cannulated Nellore fed total mixed rations containing maize silage inoculated with lactic-acid bacteria and *Bacillus subtilis*.

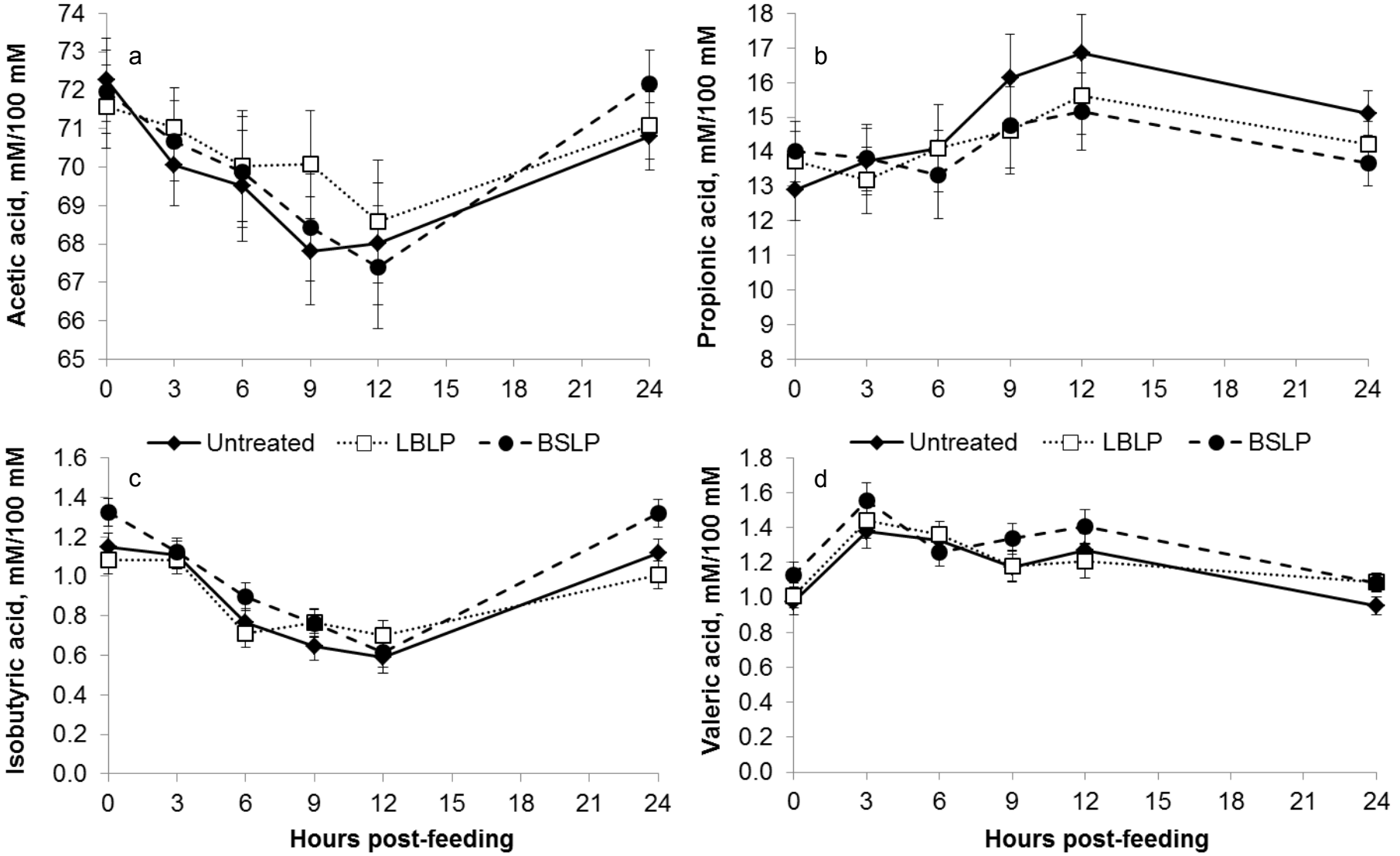

Fig. 3. Concentrations of acetic (a), propionic (b), isobutyric (c), and valeric acids (d) during 24 h post-feeding in cannulated Nellore fed total mixed rations containing maize silage inoculated with lactic-acid bacteria and *Bacillus subtilis* (untreated, maize silage without inoculant; LBLP, maize silage treated with *L. buchneri* CNCM I-4323 (1×10^5 cfu/g of fresh forage) and *L. plantarum*

MA18/5U (1×10^5 cfu/g of fresh forage); BSLP, maize silage treated with *B. subtilis* AT553098 (1×10^5 cfu/g of fresh forage) and *L. plantarum* MA18/5U).

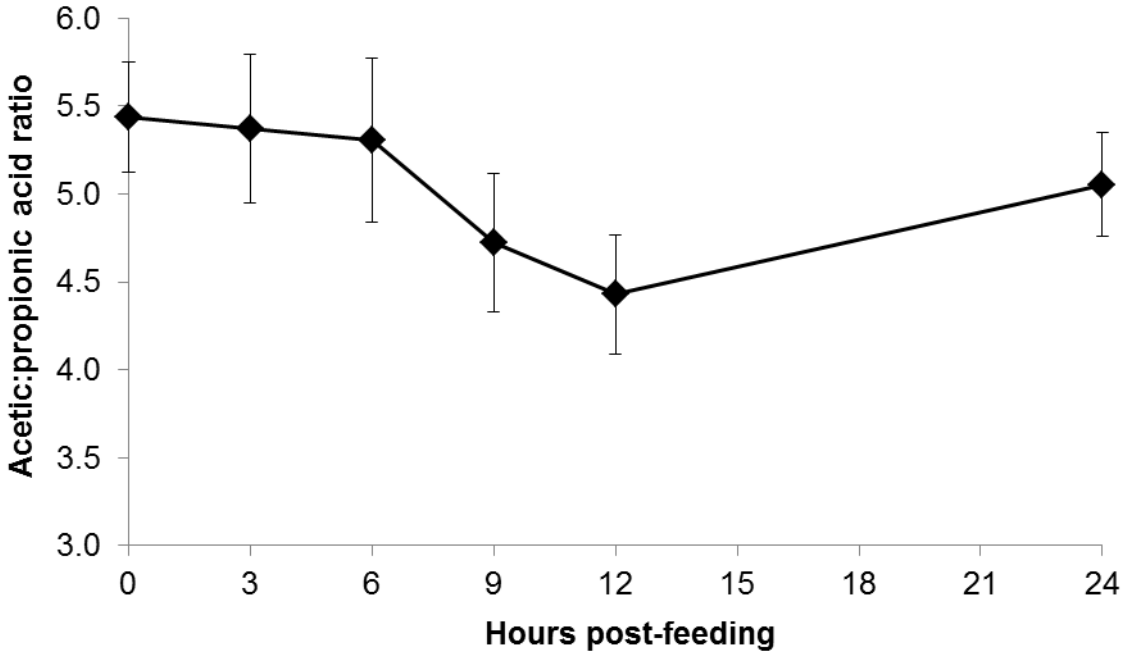

Fig. 4. Overall means for the acetic:propionic acid ratio during 24 h post-feeding in cannulated Nellore fed total mixed rations containing maize silage inoculated with lactic-acid bacteria and *Bacillus subtilis*.

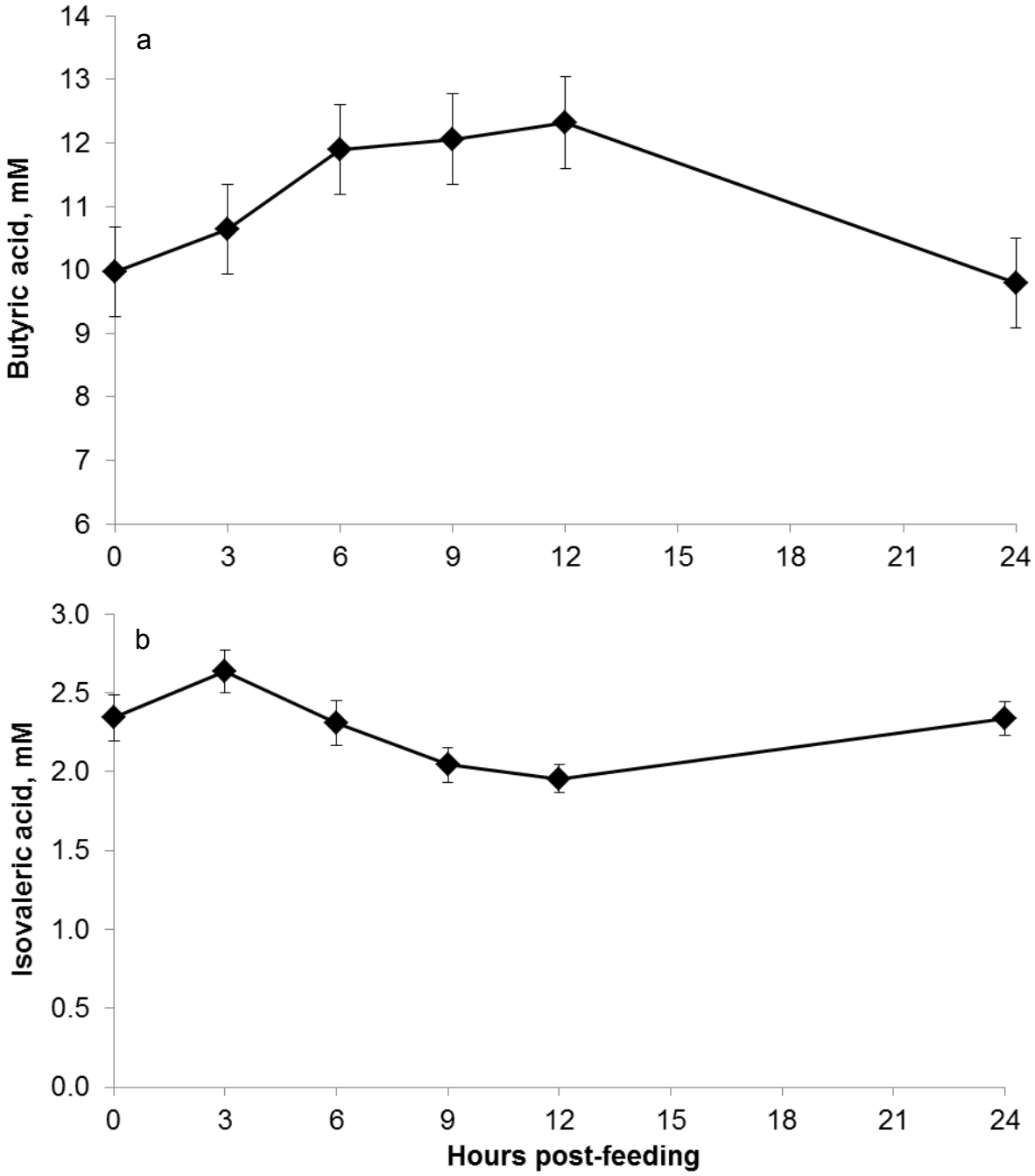

Fig. 5. Overall means for concentrations of butyric (a) and isovaleric acids (b) during 24 h post-feeding in cannulated Nellore fed total mixed rations containing maize silage inoculated with lactic-acid bacteria and *Bacillus subtilis*.

4. Discussion

Bacterial inoculation of silage has led to enhanced silage intake and growth performance in some studies, a response likely arising from improved silage quality along with the possible probiotic effect caused by inoculation (Weinberg and Muck

1996; Weinberg *et al.* 2003; Mohammed *et al.* 2012). Nevertheless, the effects on animal performance of combining different microorganisms, including *B. subtilis*, as silage inoculants have not been previously investigated.

In the present study, although statistical analysis of the silage data was precluded by a lack of silo replicates in the field (n = 1), clear trends from bacterial inoculation were observed and they needed to be considered because it affected some responses of the bulls. LBLP silage had lower WSC content and less DM recovery compared to untreated silage. Reduced sugar preservation accompanied by increased DM loss has been reported in *L. buchneri*-treated silages (Weinberg *et al.* 2002; Filya 2003; Hu *et al.* 2009). *L. buchneri* is an obligate heterofermentative LAB and its metabolic pathway involves CO_2 yield (Nishino *et al.* 2003), which explains the low DM recovery in LBLP silage. Otherwise, aerobic stability increased from 14 h in the untreated silage to 94 h in the LBLP silage. *L. buchneri*-treated silage may be most stable when exposed to air because of the additional acetic acid produced instead of lactic acid (Kleinschmit and Kung 2006). *L. buchneri* has the ability to convert lactic acid to acetic acid and 1,2-propanediol when the primary fermentation is ended (Oude Elferink *et al.* 2001). Although acetic acid slightly increased in LBLP silage compared to untreated silage, the lactic:acetic acid ratio strongly decreased, and this likely conferred a higher aerobic stability on LBLP silage. Furthermore, in contrast to the enhanced aerobic stability of maize silage treated with *B. subtilis* as a single inoculant or combined with *L. plantarum* that has been reported in previous studies (Basso *et al.* 2012; Lara *et al.* 2015), in our study the aerobic stability of BSLP silage was similar to untreated silage. The concentration of antifungal acids (i.e., acetic and propionic; Moon 1983) in BSLP silage was similar to untreated silage. Thus, the similar aerobic stability suggests no production of antifungal compounds by *B. subtilis*, a factor previously attributed to the increased aerobic stability of *B. subtilis*-treated silages (Basso *et al.* 2012; Lara *et al.* 2015).

Alterations to the fermentation process (amount of organic acids and ammonia-N produced) and to the chemical composition of silages may have a direct relationship with feed intake and may explain animal performance (McAllister *et al.* 1995; Charmley 2001). However, even after altering silage fermentation and chemical composition, in general bacterial inoculation of maize silage did not affect

feed intake, with the exception of ADF intake, which was higher in bulls fed LBLP silage. Enhanced feed intake due to inoculants is difficult to envision, since factors such as the species and the strain of the microorganisms and their interaction with the ensiled crop, as well as alterations caused in silage or ruminal fermentation, can have different impacts. Indeed, bacterial inoculation often shifts silage fermentation, as observed in our study, but it affects animal performance less frequently (Weinberg and Muck 1996). The lack of positive effects from bacterial inoculation of silages on feed intake in finishing feedlot beef cattle has been extensively reported around the world (Fugita *et al.* 2012; Addah *et al.* 2014, 2015). Conversely, the increased ADF intake in bulls fed LBLP silage is likely related to the higher ADF content of this silage resulting from extensive sugar metabolism with a low DM recovery.

In the present study, bulls fed LBLP silage spent more time chewing compared to bulls fed untreated silage. To our knowledge, no studies suggest a possible direct influence of the microorganisms in the inoculants on feeding behaviour. In this regard, changes in feeding behaviour should be more closely linked with physical and chemical alterations in the diet fed to bulls. Previously Beauchemin (1991) proposed that time spent chewing increases with an increasing proportion of fibre in the diet. Moreover, Addah *et al.* (2015) reported that bacterial inoculation decreased feeding duration when steers consumed diets formulated with short-chop (1.0 cm) whole-crop barley silage, but not when steers were fed long-chop (2.0 cm). In our study, even though NDF was similar among diets (~225 g/kg DM), the LBLP diet had slightly increased ADF content. This fact, accompanied by a higher ADF intake, likely contributed to the observation that bulls fed LBLP silage spent more time chewing.

Inoculation of maize silage with bacterial inoculants decreased NDF and ADF digestibility of the diets. Previous studies have shown that inoculants increase (Nkosi *et al.* 2010; Salvo *et al.* 2013), have no effect (Rowghani *et al.* 2008; Arriola *et al.* 2011), or decrease (Arriola *et al.* 2011; Basso *et al.* 2014; Rabelo *et al.* 2016) fibre digestibility of potato hash and maize silages. Obtaining enhanced fibre digestibility by using silage inoculants is difficult to envision because the microorganisms used in this study do not possess or produce the enzymes required for cell-wall hydrolysis of maize plants. Nevertheless, the reduced fibre digestibility observed in bulls fed LBLP silage is probably more closely linked with the increased lignin content of this silage.

The negative effect of lignin on fibre digestibility is recognized and well-documented (Jung and Allen 1995). In addition, bulls fed BSLP silage had lowered acetic acid concentration in the rumen at 12 h post-feeding compared to bulls fed untreated silage. In the present study, ruminal pH was similar among the diets, suggesting that factors other than low pH in the rumen may be responsible for depressing fibrolytic activity, with consequent reduction in fibre digestibility in bulls fed BSLP silage.

Overall, silage inoculants had little effect on ruminal fermentation, but bulls fed LBLP and BSLP silage had lower ammonia-N concentration. Increased ammonia-N in the rumen of animals fed silage-based diets may mean higher protein solubility caused by proteolysis within the silo (Charmley 2001). Indeed, the ammonia-N concentration of inoculated silages was lower than that of untreated silage. In the present study, we added *L. plantarum* combined with *L. buchneri* or *B. subtilis* to the ensiled maize. *L. plantarum* is known to produce high concentrations of lactic acid leading to a quicker reduction in silage pH, thereby avoiding the higher proteolysis caused by undesirable microorganisms (e.g., clostridia) (McDonald *et al.* 1991).

Adding *B. subtilis* as a direct-fed microbial (DFM) to diets of calves and Nellore bulls resulted in enhanced growth performance (Sun *et al.* 2010) and increased OM digestibility (Telles *et al.* 2011), respectively. *B. subtilis* when administered orally may reach the intestine and then induce some beneficial effects through secretion of active substances by germinated cells (Hosoi *et al.* 2000). However, the possible benefits claimed to the host from *B. subtilis* supplementation did not occur in our study for its use as a silage inoculant, and the apparent digestibility of BSLP diet was impaired, as earlier discussed. Furthermore, alterations in feeding behaviour (i.e., bulls fed BSLP silage spent more time eating compared to bulls fed untreated silage) appeared to be most closely linked with altered fermentation and chemical composition of maize silage than with the own microorganisms in the inoculant.

Bacterial inoculation of maize silage was unable to alter ADG, feed efficiency, or dressing percentage of bulls. Greater ADG may be partially attributed to an increase in feed intake or improved feed efficiency, along with increased digestibility of the diet (McAllister *et al.* 1995). In this regard, the lack of differences in feed intake and feed efficiency, accompanied by reduced digestibility, explain the similar ADG values in our study. The results of this study are in accordance with Fugita *et al.* (2012) and

Addah *et al.* (2014), who assessed the bacterial inoculation of maize and barley silage, respectively, on growth performance of finishing feedlot beef cattle and did not report benefits for ADG or carcass traits.

In sum, our results corroborated the majority of literature on silage inoculants (i.e., only LAB) that has found no beneficial effects of bacterial inoculation on animal performance. Furthermore, to the best of our knowledge the present study is the first to assess growth performance of bulls fed maize silage containing *B. subtilis* as a silage inoculant. However, the mechanisms of action of *B. subtilis* in ruminants are still little known, and this makes it difficult to account for animal performance. In this regard, further studies are needed to identify whether responses to *B. subtilis*-treated silage are dose-dependent and strain-specific, as well as the impact this inoculant has on animal performance using silage-based diets.

5. Conclusions

Combining *L. plantarum* with *L. buchneri* decreased DM recovery, but significantly increased the aerobic stability of maize silages. The results of this study indicate that *L. plantarum* combined with either *L. buchneri* or *B. subtilis* did not improve feed intake and growth performance of Nellore × Brown Swiss crossbred bulls.

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References

Addah W, Baah J, Okine EK, McAllister TA (2015) Effect of barley silage chop length and inoculation on growth performance, feeding behavior, and ruminal acidosis in finishing feedlot steers. *Journal of Animal Science* **93**, 2309–2321. doi: 10.2527/jas.2014-8247

Addah W, Baah J, Okine EK, Owens FN, McAllister TA (2014) Effects of chop-length and a ferulic acid esterase-producing inoculant on fermentation and aerobic stability of barley silage, and growth performance of finishing feedlot steers.

Animal Feed Science and Technology **197**, 34–46. doi: 10.1016/j.anifeedsci.2014.07.012

Adesogan AT (2014) Avoiding the two greatest silage problems. In 'Proceedings 50th Florida Dairy Production Conference' pp. 9-17. (University of Florida: Gainesville FL)

Arriola KG, Kim SC, Staples CR, Adesogan AT (2011) Effect of applying bacterial inoculants containing different types of bacteria to corn silage on the performance of dairy cattle. *Journal of Dairy Science* **94**, 3973–3979. doi: 10.3168/jds.2010-4070

Ashbell G, Weinberg ZG (1992) Top silage losses in horizontal silos. *Canadian Agricultural Engineering* **34**, 171–175.

Ashbell G, Weinberg ZG, Hen Y, Filya I (2002) The effects of temperature on the aerobic stability of wheat and corn silages. *Journal of Indusexperiment Microbiology & Biotechnology* **28**, 261–263. doi: 10.1038/sj/jim/7000237

Association of Official Analytical Chemists (AOAC) (1996) 'Official methods of analysis.' 16th edn. (AOAC: Arlington, VA)

Basso FC, Adesogan AT, Lara EC, Rabelo CHS, Berchielli TT, Teixeira IAMA, Siqueira GR, Reis RA (2014) Effects of feeding corn silage inoculated with microbial additives on the ruminal fermentation, microbial protein yield, and growth performance of lambs. *Journal of Animal Science* **92**, 5640–5650. doi: 10.2527/jas.2014-8258

Basso FC, Lara EC, Assis FB, Morelli M, Rabelo CHS, Reis RA (2012) Características da fermentação e estabilidade aeróbia de silagens de milho inoculadas com *Bacillus subtilis*. *Revista Brasileira de Saúde e Produção Animal* **13**, 1009–1019.

Beauchemin KA (1991) Ingestion and mastication of feed by dairy cattle. *The Veterinary Clinics of North America. Food Animal Practice* **7**, 439–463.

Bernardes TF, Adesogan AT (2012) Aerobic deterioration of silages in warm climates. In 'Proceedings of the 6th Symposium on Strategic Management of Pastures' pp. 249-268. (Federal University of Viçosa: Viçosa)

Casali AO, Detmann E, Valadares Filho SC, Pereira JC, Henrique LT, Freitas SG, Paulino MF (2008) Influência do tempo de incubação e do tamanho de partículas

sobre os teores de compostos indigestíveis em alimentos e fezes bovinas obtidos por procedimentos *in situ*. *Revista Brasileira de Zootecnia* **37**, 335–342.

Charmley E (2001) Towards improved silage quality – A review. *Canadian Journal of Animal Science* **81**, 157–168. doi: 10.4141/A00-066

Fenner H (1965) Method for determining total volatile bases in rumen fluid by stem distillation. *Journal of Dairy Science* **48**, 249–251.

Filya I (2003) The effect of *Lactobacillus buchneri* and *Lactobacillus plantarum* on the fermentation, aerobic stability, and ruminal degradability of low dry matter corn and sorghum silages. *Journal of Dairy Science* **86**, 3575–3581. doi: 10.3168/jds.S0022-0302(03)73963-0

Fugita CA, Prado IN, Jobim CC, Zawadzki F, Valero MV, Pires MCO, Prado RM, Françozo MC (2012) Corn silage with and without enzyme-bacteria inoculants on performance, carcass characteristics and meat quality in feedlot finished crossbred bulls. *Revista Brasileira de Zootecnia* **41**, 154–163. doi: 10.1590/S1516-35982012000100023

Greiner SP, Rouse GH, Wilson DE, Cundiff LV, Wheeler TL (2003) Prediction of retail product weight and percentage using ultrasound and carcass measurements in beef cattle. *Journal of Animal Science* **81**, 1736–1742. doi: 2003.8171736x

Hosoi T, Ametani A, Kiuchi K, Kaminogawa S (2000) Improved growth and viability of lactobacilli in the presence of *Bacillus subtilis* (*natto*), catalase, or subtilisin. *Canadian Journal of Microbiology* **46**, 892–897. doi: 10.1139/w00-070

Hu W, Schmidt RJ, McDonell EE, Klingerman CM, Kung LJr (2009) The effect of *Lactobacillus buchneri* 40788 or *Lactobacillus plantarum* MTD-1 on the fermentation and aerobic stability of corn silages ensiled at two dry matter contents. *Journal of Dairy Science* **92**, 3907–3914. doi: 10.3168/jds.2008-1788

Jung HG, Allen MS (1995) Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. *Journal of Animal Science* **73**, 2774–2790. doi: 1995.7392774x

Keady TWJ, Steen RWJ (1995) The effects of treating low dry matter, low digestibility grass with a bacterial inoculant on the intake and performance of beef cattle, and studies on its mode of action. *Grass and Forage Science* **50**, 217–226. doi: 10.1111/j.1365-2494.1995.tb02317.x

Kleinschmit DH, Kung LJr. (2006) A meta-analysis of the effects of *Lactobacillus buchneri* on the fermentation and aerobic stability of corn and grass and small-grain silages. *Journal of Dairy Science* **89**, 4005–4013. doi: 10.3168/jds.S0022-0302(06)72444-4

Kung LJr., Grieve DB, Thomas JW, Huber JT (1984) Added ammonia or microbial inoculant for fermentation and nitrogenous compounds of alfalfa ensiled at various percents of dry matter. *Journal of Dairy Science* **67**, 299–306.

Lara EC, Basso FC, Assis FB, Souza FA, Berchielli TT, Reis RA (2015) Changes in the nutritive value and aerobic stability of corn silages inoculated with *Bacillus subtilis* alone or combined with *Lactobacillus plantarum*. *Animal Production Science*. doi: 10.1071/AN14686

Maekawa M, Beauchemin KA, Christensen DA (2002) Effect of concentrate level and feeding management on chewing activities, saliva production, and ruminal pH of lactating dairy cows. *Journal of Dairy Science* **85**, 1165–1175. doi: 10.3168/jds.S0022-0302(02)74179-9

McAllister TA, Selinger LB, McMahon LR, Bae HD, Lysyk TJ, Oosting SJ-, Cheng K-J (1995) Intake, digestibility and aerobic stability of barley silage inoculated with mixtures of *Lactobacillus plantarum* and *Enterococcus faecium*. *Canadian Journal of Animal Science* **75**, 425–432. doi: 10.4141/cjas95-062

McDonald P, Henderson AR, Heron SJE (1991) 'The biochemistry of silage.' (Chalcomb Publications: Marlow, Bucks UK)

Mertens DR (2002) Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. *Journal of AOAC International* **85**, 1217–1240.

Mohammed R, Stevenson DM, Beauchemin KA, Muck RE, Weimer PJ (2012) Changes in ruminal bacterial community composition following feeding of alfalfa ensiled with a lactic acid bacterial inoculant. *Journal of Dairy Science* **95**, 328–339. doi: 10.3168/jds.2011-4492

Moon N (1983) Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. *Journal of Applied Bacteriology* **55**, 453–460. doi: 10.1111/j.1365-2672.1983.tb01685.x

Moran JP, Weinberg ZG, Ashbell G, Hen Y, Owen TR (1996) A comparison of two methods for the evaluation of the aerobic stability of whole crop wheat silage. In 'Proceedings of the 11th International Silage Conference'. pp. 162–163. (University of Wales: Aberystwyth)

National Research Council (2000) 'Nutrient requirements of beef cattle.' 7th edn. (National Academies Press, Washington, DC)

Nelson N (1944) A photometric adaptation of the Somogyi method for the determination of glucose. *The Journal of Biological Chemistry* **153**, 375–386.

Nishino N, Yoshida M, Shiota H, Sakaguchi E (2003) Accumulation of 1,2-propanediol and enhancement of aerobic stability in whole crop maize silage inoculated with *Lactobacillus buchneri*. *Journal of Applied Microbiology* **94**, 800–807. doi: 10.1046/j.1365-2672.2003.01810.x

Nkosi BD, Meeske R, Palic D, Langa T, Leeuwa KJ, Groenewald IB (2009) Effects of ensiling whole crop maize with bacterial inoculants on the fermentation, aerobic stability, and growth performance of lambs. *Animal Feed Science and Technology* **154**, 193–203. doi: 10.1016/j.anifeedsci.2009.09.009

Nkosi BD, Meeske R, van der Merwec HJ, Groenewald IB (2010) Effects of homofermentative and heterofermentative bacterial silage inoculants on potato hash silage fermentation and digestibility in rams. *Animal Feed Science and Technology* 157, 195–200. doi: 10.1016/j.anifeedsci.2010.03.008

Oude Elferink SJWH, Krooneman J, Gottschal JC, Spoelstra SF, Faber F, Driehuis F (2001) Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. *Applied and Environmental Microbiology* **67**, 125–132. doi: 10.1128/AEM.67.1.125-132.2001

Pina DS, Valadares Filho SC, Detmann E, Valadares RFD, Campos JMS, Moraes KAK, Oliveira AS, Paixão ML (2006) Efeitos de indicadores e dias de coleta na digestibilidade dos nutrientes e nas estimativas do valor energética de alimentos para vacas alimentadas com diferentes fontes de proteína. *Revista Brasileira de Zootecnia* **35**, 2461–2468.

Pryce JD (1969) A modification of the Barker -Summerson method for the determination of lactic acid. *Analyst* **94**, 1151–1152. doi: 10.1039/AN9699401151

Rabelo CHS, Basso FC, McAllister TA, Lage JF, Gonçalves GS, Lara EC, Oliveira AA, Berchielli TT, Reis RA (2016) Influence of *Lactobacillus buchneri* as silage additive and forage:concentrate ratio on the growth performance, fatty acid profile in longissimus muscle and meat quality of beef cattle. *Canadian Journal of Animal Science*. (in press)

Rowghani E, Zamiri MJ, Khorvash M, Abdollahipanah A (2008) The effects of *Lactobacillus plantarum* and *Propionibacterium acidipropionici* on corn silage fermentation, ruminal degradability and nutrient digestibility in sheep. *Iranian Journal of Veterinary Research* **9**, 308–315.

Salvo PAR, Basso FC, Rabelo CHS, Oliveira AA, Sader AP, Casagrande DR, Berchielli TT, Reis RA (2013) Características de silagens de milho inoculadas com *Lactobacillus buchneri* e *L. plantarum*. *Archivos de Zootecnia* **62**, 1–12. doi: 10.4321/S0004-05922013000300006

Sniffen CJ, O'Connor JD, Van Soest PJ, Fox DG, Russell JB (1992) A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. *Journal of Animal Science* **70**, 3562–3577. doi: 1992.70113562x

Sun P, Wang JQ, Zhang HT (2010) Effects of *Bacillus subtilis natto* on performance and immune function of preweaning calves. *Journal of Dairy Science* **93**, 5851–5855. doi: 10.3168/jds.2010-3263

Telles RRT, Vieira BR, Naves VV, Carvalho IPC, Rezende CF, Rocha LB, Reis RA (2011) Efeito da inclusão de *Bacillus subtilis* na dieta de novilhos de corte sobre a digestibilidade aparente dos nutrientes. In 'Proceedings of the XXI Congresso Brasileiro de Zootecnia' (The Brazilian Animal Science Association: Maceió)

Valente TNP, Detmann E, Valadares Filho SC, Cunha M, Queiroz AC, Sampaio CB (2011) *In situ* estimation of indigestible compounds contents in cattle feed and feces using bags made from different textiles. *Revista Brasileira de Zootecnia* **40**, 666–675. doi: 10.1590/S1516-35982011000300027

Van Soest PJ, Robertson JB (1985) 'Analysis of forages and fibrous foods.' (Cornell University: Ithaca, NY)

Van Soest PJ, Robertson JB, Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition.

Journal of Dairy Science **74**, 3583–3597. doi: 10.3168/jds.S0022-0302(91)78551-2

Weinberg ZG, Ashbell G, Hen Y, Azrieli A, Szakacs G, Filya I (2002) Ensiling whole-crop wheat and corn in large containers with *Lactobacillus plantarum* and *Lactobacillus buchneri*. *Journal of Indusexperiment Microbiology & Biotechnology* **28**, 7–11. doi: 10.1038/sj/jim/7000207

Weinberg ZG, Muck RE (1996) New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiology Reviews* **19**, 53–68. doi: 10.1111/j.1574-6976.1996.tb00253.x

Weinberg ZG, Muck RE, Weimer PJ (2003) The survival of silage inoculant lactic acid bacteria in rumen fluid. *Journal of Applied Microbiology* **94**, 1066–1071. doi: 10.1046/j.1365-2672.2003.01942.x

Weißbach F, Strubelt C (2008) Correcting the dry matter content of maize silages as a substrate for biogas production. *Landtechnik* **63**, 82–83.

Zuber P, Nakano MM, Marahiel MA (1993) Peptide Antibiotics. In '*Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics'. (Eds AL Sonenshein, JA Hoch, R Losick) pp. 897-916. (American Society for Microbiology: Washington DC)

IMPLICATIONS

"Implications were prepared reporting my experience with researches assessing bacterial inoculants as silage additives and their influence on fermentation and animal performance"

Bacterial inoculants are the most widely silage additives used in the world. Primarily, the utilization of these additives was recommended to reduce dry matter (DM) and energy losses of the ensiled crop, based on the efficient pathway played by homolactic lactic-acid bacteria (LAB) (1st generation of silage inoculants). Rapidly homolactic inoculants were adopted by farmers in Europe and North America. Owing the low aerobic stability of well-fermented silages when the silos are opened, a 2nd generation of silage inoculants (heterolactic LAB) was developed aiming to increase production of acetic acid. Acetic acid inhibits molds (i.e., particularly yeasts) overgrowth and preserves the nutritive value of silages during feed-out. Thereafter, several studies suggested combining homo and heterolactic LAB with the goal to preserve a greater amount of fermented forage in an anaerobic and aerobic environment. Nowadays, several commercial products based on combining homo and heterolactic LAB are available for farmers and also for researches conduction. Moreover, most recently other microorganisms have been studied regarding their potential to be used as a silage inoculant, such as *Bacillus subtilis* and beneficial yeasts.

Studies carried out around the world have showed that the effects of bacterial inoculants on silage quality are widely varied. For example, homolactic inoculants are claimed to reduce fermentative losses leading a suitable preservation of the ensiled crop, along with a better nutritive value. In addition, the reduction of DM losses during the fermentation phase caused by homolactic inoculation should reduce the costs regarding the silage produced compared to untreated silages. In the same way, heterolactic inoculants may increase aerobic stability of silages during feed-out, and there are some microorganisms useful for that. Heterolactic inoculants may be particularly important for silages produced under tropical climate due to the yeasts overgrowth. Moreover, combining homo and heterolactic inoculants should enhance preservation of silage by decreasing DM losses during fermentation and feed-out

phase, and ensuring a better nutritive value and sanitary aspects of silages. However, not always positive responses are reported. Combined results of studies carried out at UNESP/Jaboticabal revealed that responses on fermentation patterns and aerobic stability of silages are microorganisms- and strains-specific, along with dose dependent. Studies also indicated that an unsuitable silage making may compromise the silage quality, and in these cases there is often a lack of positive responses from bacterial inoculation.

Despite the animal performance, results are also largely varied and positive responses may occur with low frequency. In addition, data regarding the growth performance of animals fed inoculated silages under tropical climate are scarce and inconsistent. Enhanced animal performance may be closely linked with the microorganism(s) applied on silage and with the total mixed ration fed to the animals. In a first instance, feeding exclusively inoculated silage to the animals may confer a greater chance to find changes in animal performance caused by bacterial inoculants. However, positive responses can be encountered even in total mixed rations with elevated amount of concentrate (> 50%). Indeed, appears there is an interaction between bacterial inoculant used for silage and the forage:concentrate ratio used in total mixed rations. However, this interaction remains unknown and the relationships often can occur in different ways being pretty nebulous.

Owing the low frequency of positive responses on animal performance combined with low magnitude when they happen, the main reason to use bacterial inoculants as additives is still to reduce losses during fermentation or during feed-out, and thus preserving a greater amount of silage. However, these responses are deeply related with the silage quality, and when a suitable silage making and feed-out management are failed, thus, the action of bacterial inoculants likely will be negatively affected. In other words, the expected beneficial claimed by using bacterial inoculants on silage quality likely will not occur when silage making, and feed-out of silos are very poor, mainly under tropical climate. Nevertheless, further studies focusing on the effect of bacterial inoculants against aerobic spoilage microorganisms (i.e., molds) should be considered, because those microorganisms are able to produce micotoxins. Micotoxins are recognized to cause several damages

on animal health, but concerns about that are still few observed in the Brazilian literature, mainly in studies concerning bacterial inoculants.

Although the study of silage inoculants was the main topic in this dissertation and I wrote some things about my experience with it. I'd like to comment some points about methodology and analyses of interest in further studies. Overall, the majority of methodologies used in this dissertation are pretty good and desirable for silage studies. For example, the determination of DM content in a forced air oven is totally acceptable, and indeed is still the most common method used for silage. However, I think further studies that will be conducted at UNESP/Jaboticabal should focus on working with freeze-dried samples because there is DM loss by volatile compounds produced during fermentation, which can lead some error in determining DM content and mainly in predicting the nutritive value of silage.

About the variables, I recommend that further studies should analyze: insoluble detergent acid nitrogen (NIDA) because is a good indicative of Maillard reaction within the silo; protein fractionation because plant undergoes a great influence of ensiling, and fermentation process may reduce microbial protein synthesis by increasing protein solubilization; starch in cereal silages because it contribute a lot to understand animal performance; microbiology by real-time PCR because many microorganisms that can influence silage fermentation is still unknown, as well their interactions of known microorganisms within the silo and also rumen.

Finally, I'd like to say that surely I got a good experience about the topic of bacterial inoculants for silage during my Ph.D. course, but every day we can discovery more news about silage making because this topic is fascinating. Moreover, scientific maturity to understand a little bit more about silage making (or about any subject) is obtained with hard work and reading a lot.