DAVIANE MARTINELE COSTA

CORN SILAGES: DEVELOPMENT OF NOVEL INOCULANT AND PARTICLE SIZE ON REHYDRATED GRAIN

LAVRAS-MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Ruminantes, para a obtenção do título de Doutora.

Prof[a]. Dr[a]. Carla Luiza da Silva Ávila

Orientadora

LAVRAS – MG

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DAVIANE MARTINELE COSTA

CORN SILAGES: DEVELOPMENT OF NOVEL INOCULANT AND PARTICLE SIZE ON REHYDRATED GRAIN

SILAGENS DE MILHO: DESENVOLVIMENTO DE NOVO INOCULANTE E TAMANHO DE PARTÍCULAS EM GRÃO REIDRATADO.

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Produção e Nutrição de Ruminantes, para a obtenção do título de Doutora.

APROVADA em 28 de março de 2019.

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LAVRAS – MG
2019

Aos meus pais, Ilda e Tonico, meus alicerces, minha vida.

Ao meu amor, meu melhor amigo, Henrique.

À minha irmã, meu anjo de luz, Fernanda.

Dedico

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"Tudo posso Naquele que me fortalece."

Filipenses 4:13

RESUMO GERAL

Artigo 1. Cinquenta e três cepas de bactérias do ácido lático (BAL) isoladas de silagens de milho foram avaliadas como inoculantes quando o milho foi colhido em estágio avançado de maturidade. As cepas foram caracterizadas quanto ao crescimento e redução do pH no extrato de milho, crescimento em diferentes temperaturas e habilidade de inibir microrganismos deterioradores. As cepas: CCMA1362, 1363 e 1364 (*Lactobacillus farraginis*); CCMA1365 (*L. plantarum*); CCMA1366 (*L. buchneri*) e CCMA1367 (*Pediococcus acidilactici*) isoladas da silagem de milho e a cepa CCMA170 (*L. hilgardii*) de cana-de-açúcar foram avaliadas na silagem de milho colhido com alto teor de matéria seca (45,4%). Os silos experimentais foram abertos após 10, 32 e 100 dias de estocagem. Os teores de proteína bruta, cinzas e amido não foram afetados pela inoculação ou período de estocagem. Silagens controle e inoculadas com BAL homofermentativas mostraram maior perda de matéria seca e menor estabilidade aeróbia. Silagens com as cepas heterofermentativas obrigatórias, principalmente com a CCMA1362 (*L. farraginis*) e CCMA170 (*L. hilgardii*), mostraram menor população de leveduras (<2,00 log ufc g^{-1}) a partir de 10 dias de estocagem. Silagem inoculada com a cepa CCMA1362 mostrou menor perda de MS, menor população de microrganismos indesejáveis, estando entre as silagens com maior produção de ácido lático, acético e maior estabilidade aeróbia. A espécie *Lactobacillus farraginis* é citada pela primeira vez em estudos com silagem e a cepa CCMA1362 isolada da silagem de milho, mostrou ser promissora para o uso como inoculante em silagens de milho colhidos com alto teor de MS.

Artigo 2. O objetivo deste estudo foi avaliar o efeito do tamanho de partícula e do tempo de estocagem no perfil fermentativo, estabilidade aeróbia e na degradabilidade ruminal da silagem de grãos de milho reidratados. Os grãos de milho foram moídos para passar por uma tela de 3 mm (fina) ou 9 mm (grossa), reidratados para atingir cerca de 40% de umidade e ensilados em galões de 200 L. As amostras foram coletadas antes e depois da ensilagem aos 10, 30, 90 e 200 dias de estocagem para avaliar a contagem microbiana, os produtos finais da fermentação e a degradabilidade ruminal da matéria seca (MS). A degradabilidade ruminal *in situ* da MS foi avaliada com amostras sem prévias moagens para acessar o efeito do tamanho de partícula, com tempos de incubação de 0 (lavagem do saco), 3, 6 e 48 h em 3 vacas canuladas no rúmen. A degradação ruminal efetiva (DRE) foi calculada com base na fração solúvel (A), fração degradável (B) e taxa de passagem (kp) definida como 7,0% h^{-1}: A + B [kd / (kd + kp)]. A estabilidade aeróbia foi avaliada com 200 dias de estocagem. Aos 90 e 200 dias de estocagem, silagem de grãos de milho finamente moídos resultou em menor proteína bruta e maior concentração de NH_3-N em relação a silagem grossa. A silagem com grãos milho reidratados grosseiramente moídos apresentou menor temperatura no início dos tempos de estocagem e maior estabilidade aeróbica em relação a silagem com grãos finos (206 vs. 115 horas). A degradabilidade ruminal da MS aumentou ao longo dos tempos de armazenamento. O tamanho de partícula da silagem de grãos de milho reidratados não afetou os valores de Kd após 90 dias de estocagem, enquanto para a DRE foi necessário longo período de fermentação (200 dias). Silagem de milho reidratado grosseiramente moído (9mm), estocado por 200 dias alcança a degradabilidade ruminal do milho moído fino (3mm), com maior estabilidade aeróbia.

Palavras-chave: Amido. Degradabilidade. Heterofermentativa. *Lactobacillus farraginis*.

GENERAL ABSTRACT

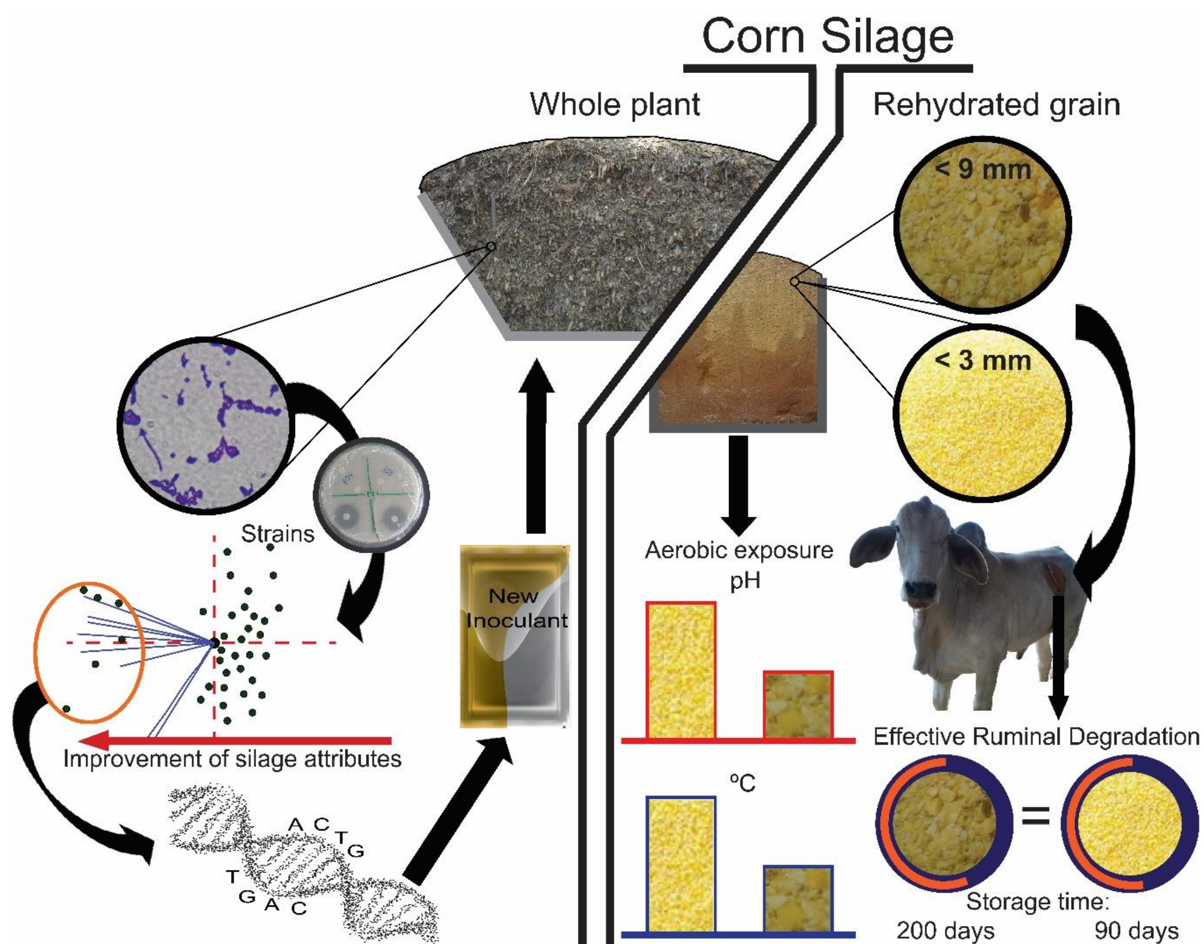
Paper1. Fifty-three strains of lactic acid bacteria (LAB) isolated from corn silages were evaluated for use as inoculants in corn silages harvested at late maturity. LAB strains were characterized for growth and pH reduction in corn extract, growth at different temperatures and the ability to inhibit silage-spoilage microorganism growth. Strains CCMA1362, 1363 and 1364 (*Lactobacillus farraginis*); CCMA1365 (*L. plantarum*); CCMA1366 (*L. buchneri*) and CCMA1367 (*Pediococcus acidilactici*) isolated from corn silage; and CCMA170 strain (*L. hilgardii*) isolated from sugarcane were evaluated in corn silages with 45.4% of dry matter (DM). The experimental silos were opened after 10, 32 and 100 days of storage. CP, ash and starch contents were not affected by the inoculation or storage time. Control and inoculated silages with homofermentative BAL showed higher DM loss and lower aerobic stability. Silages with obligate heterofermentative strains, especially with CCMA1362 (*L. farraginis*) and CCMA170 (*L. hilgardii*), showed the smallest yeast population (<2.00 log cfu g^{-1}) after 10 days of storage. Silage inoculated with strain CCMA1362 showed lower DM loss, showed a smaller population of undesirable microorganisms and was among the silages with higher lactic acid and acetic acid production and greater aerobic stability. The species *Lactobacillus farraginis* is reported for the first time in studies with silage, and strain CCMA1362 isolated from corn silage is shown to be promising for use as an inoculant in corn silages harvested at late maturity.

Paper 2. The objective of this study was to evaluate the effect of particle size and storage time on fermentative profile, aerobic stability, and ruminal degradability of rehydrated corn grain silage. Corn grains were ground to pass either a 3 mm (fine) or 9 mm (coarse) screen, rehydrated to achieve around 40% of moisture and ensiled in 200 L polyethylene silos. Samples were taken before and after ensiling at 10, 30, 90 and 200 days of storage to assess microbial counts, fermentation end products, and ruminal DM degradability. The DM degradation was evaluated with samples without ground to accessing the effect of original particle size, with incubation times of 0 (bag wash), 3, 6, and 48 h in 3 rumen cannulated cows. The effective ruminal degradation (ERD) was calculated based on soluble fraction (A), degradable fraction (B), and passage rate (kp) defined as 7.0% h-1: A + B [kd / (kd + kp)]. Aerobic stability was evaluated in silages with 200 days of storage. At 90 and 200 d of storage, fine rehydrated corn grain silage resulted in lower crude protein and greater NH3-N concentration than coarse grain. Coarsely ground rehydrated corn silage had lower temperature at the beginning of storage times and greater aerobic stability than finely ground corn (206 vs. 115 hour). Ruminal DM degradability increased over the storage times. Particle size of rehydrated corn grain silage did not affect kd values after 90 d of storage, while for the ERD was necessary a long time of fermentation (200 days). In summary, coarsely ground rehydrated corn silage storage by 200 days reaches the ruminal degradability of finely ground, with greater aerobic stability than finely ground corn.

Key words: Degradability. Heterofermentative. *Lactobacillus farraginis.* Starch.

RESUMO INTERPRETATIVO E RESUMO GRÁFICO

A presente tese teve como objeto central a silagem de milho, com dois propósitos distintos: (i) selecionar cepas de bactérias láticas para serem usadas como inoculantes em silagens de planta inteira colhidos com alto teor de matéria seca; e (ii) avaliar os efeitos do tamanho de partícula sobre a qualidade e estabilidade aeróbia da silagem de grão reidratado. No primeiro trabalho, foram avaliadas 52 cepas isoladas de silagens de milho de diferentes propriedades rurais. Essas cepas foram avaliadas quanto ao potencial de redução do pH, de crescimento em diferentes temperaturas e de inibir microrganismos indesejáveis na silagem. Foram selecionadas aquelas que apresentaram os melhores resultados e avaliado seu efeito como inoculantes na silagem de milho com 45% de matéria seca. As silagens inoculadas com *Lactobacillus farraginis*, bactéria lática heterofermentativa primeira vez avaliada em silagens, proporcionou melhor qualidade fermentativa, menor perda de matéria seca e elevada estabilidade aeróbia da silagem, sendo promissora para uso como inoculante. No segundo trabalho, grãos de milho maduros foram moídos e passados em peneiras de crivos de 3 ou 9 mm, reidratados e ensilados em tambores. Foi observado que a silagem com o grão moído mais grosso teve maior estabilidade aeróbia que os grãos finamente moídos. A moagem fina, proporcionou menores teores de proteína bruta, indicativo de maior disponibilização do amido para a degradação ruminal. De fato, moagem fina alcançou maior degradabilidade ruminal em menor tempo de estocagem, no entanto o efeito é anulado com longos períodos.



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PRIMEIRA PARTE

1 INTRODUÇÃO

Silagens de milho, em seus diferentes tipos e objetivos, são amplamente empregadas em sistemas intensivos de criação de bovinos de leite e corte. A silagem da planta inteira de milho, comparado a outras forragens, além de fonte de fibra, fornece energia dietética devido ao amido presentes nos grãos (FERRARETTO, SHAVER e LUCK, 2018). O milho grão é o principal cereal energético usado nas fazendas leiteiras e em confinamentos, e sua ensilagem após moagem e reidratação é uma técnica que permite ganhos no valor nutritivo (PEREIRA et al., 2013).

Já está amplamente difundido que corretas práticas de manejo durante a ensilagem da planta inteira de milho proporcionam silagens de ótima qualidade fermentativa. Os cuidados na ensilagem vão desde a colheita do milho com adequados teores de matéria seca (MS), compactação da massa, vedação, até o correto avanço do painel do silo (ALLEN; COORRS; ROTH, 2003). No entanto, imprevistos podem atrasar a colheita do milho, como clima desfavorável e indisponibilidade de maquinário no momento da ensilagem (WINDLE et al., 2014). Além disso, é preciso considerar que a colheita do milho em estágio mais avançado de maturidade para confecção de silagem pode ser uma estratégia para maior concentração de amido (BALL; COORS; SHAVER, 1997) conciliado com maior tempo de estocagem (HOFFMAN et al., 2011), bem como maior produção de matéria seca por hectare ou por kg de planta fresca (PEYRAT et al., 2016), reduzindo os custos operacionais com transporte e estocagem. Por outro lado, para o processo de conservação da forragem, elevados teores de matéria seca podem dificultar a compactação devido ao aumento do tamanho das partículas, principalmente quando o maquinário é deficiente, prejudicando o processo fermentativo e promovendo perdas da estabilidade aeróbia (KUNG Jr. et al., 2018; MUCK; HOLMES, 2000; RUPPEL et al., 1995). Assim como na silagem de planta inteira, no grão reidratado o tamanho de partícula pode influenciar o processo fermentativo e o valor nutricional.

Os grãos dos híbridos de milho cultivados no Brasil são predominantemente de textura dura, chamados "*flint*", devido à alta vitreosidade do endosperma (CRUZ et al., 2014). Essa característica é uma consequência do alto conteúdo de prolaminas que propicia maior resistência à quebra mecânica durante a colheita, entre outros atributos agronômicos desejáveis (KAMRA, 2005, p. 10). Porém, essa característica não favorece a nutrição animal, uma vez que essas proteínas que envolvem os grânulos de amido impedem a atuação microbiana e

enzimática no trato digestivo (FERRARETTO; CRUMP; SHAVER, 2013; HOFFMAN et al., 2011; McDONALD; HENDERSON; HERON, 1991). O processamento dos grãos, como a moagem e a ensilagem, melhora a digestibilidade do amido, além disso, os ganhos na digestibilidade do amido são positivamente correlacionados com maiores tempos de estocagem da silagem (FERRARETTO; SHAVER, 2012; FIRKINS et al., 2001). Contudo, nem sempre é possível armazenar a silagem por longos períodos na propriedade, longos tempos de estocagem tem sido relacionados com elevadas perdas de matéria seca e o maior grau de moagem dispende mais tempo operacional para a confecção da silagem (CARVALHO et al., 2016; CASTRO, 2017, p.71).

Na busca para solucionar esses problemas relacionados à conservação e valor nutritivo da silagem de planta inteira e de milho reidratado, uma das estratégias viáveis é o desenvolvimento de tecnologias. Nesse sentido, a seleção de novas cepas de bactérias láticas com potencial uso como inoculantes, objetivando atender os problemas que a forragem apresenta ao ser ensilada, pode contribuir de forma efetiva para sua conservação. A ensilagem dos grãos de milho reidratado é uma estratégia amplamente utilizada para aumentar a digestibilidade do amido. Entretanto, a influência do tamanho de partícula na conservação dessa silagem não foi elucidada, bem como na degradabilidade ruminal considerando curtos tempos de armazenamento e incubações de amostras sem previas moagens, representando o fator de estudo e um material mais próximo ao de fato ingerido pelo animal.

Essas estratégias e tecnologias foram os principais objetos de estudo desta tese, que teve como objetivos: (i) avaliar e selecionar cepas de bactérias do ácido lático isoladas de silagens de fazendas e avaliar seus efeitos sobre a o perfil fermentativo, perdas de matéria seca e estabilidade aeróbia de silagens de milho colhidos com alto de matéria seca; (ii) avaliar o efeito do tamanho de partícula e do tempo de estocagem sobre o perfil fermentativo, características químicas, estabilidade aeróbia e degradabilidade ruminal *in situ* da silagem de grãos de milho reidratados. Para alcançar esses objetivos, a tese foi compartimentalizada em dois artigos, ordenados na seguinte sequência:

a) Artigo 1 – New epiphytic strains of lactic acid bacteria improve the conservation of corn silage harvested at late maturity;

b) Artigo 2 - Particle size and storage time on conservation and ruminal degradability of rehydrated corn grain silage.

2 REFERENCIAL TEÓRICO

2.1 Silagens de milho

A planta de milho fornece características desejáveis para a ensilagem, que englobam desde a sua produtividade no campo, valor nutricional e substratos para a sua fermentação (ALLEN; COORS; ROTH, 2003). Além desses fatores, ainda existe a flexibilidade de colher milho para forragem ou para grãos, e recentemente fornece opções de fracionamento da planta para a produção de diferentes silagens. Diante disso, tem sido desenvolvido tecnologias, como os processadores e equipamentos especializados que permitem a colheita fracionada da planta de milho para a produção de diferentes tipos de silagens, como a *earlage* (grão e sabugo) e *snaplage* (grão, sabugo e palha) (FERRARETO; SHAVER; LUCK, 2018). Outras técnicas menos dependentes de maquinário especializado também proporcionam melhorias no valor nutritivo, como por exemplo, a opção por colher apenas a parte mais alta da planta onde estão inseridas as espigas (*toplage*) (COOK et al., 2016), dos grãos com 60 a 65% de umidade (grão úmido) ou dos grãos secos com posterior moagem e reidratação (reidratado) (HOFFMAN et al., 2011).

A silagem da planta inteira, entre as opções fornecedoras de fibra, ainda é a mais utilizada, principalmente devido ao seu alto valor nutritivo e rendimento de massa verde por hectare (BERNARDES; RÊGO, 2014; FERRARETTO; SHAVER; LUCK, 2018). Com práticas adequadas de manejo e com a colheita da planta no momento adequado para a ensilagem, as fases iniciais de fermentação da silagem acontecem de forma satisfatória, uma vez que contém quantidade de MS e carboidratos solúveis suficientes para fermentação (ALLEN; COORS; ROTH, 2003). O maior problema está na fase de abertura dos silos devido à maior instabilidade aeróbia que ocorre em virtude do acúmulo de substratos potencialmente oxidáveis por microrganismos oportunistas (SIQUEIRA; BERNARDES; REIS, 2005). Por outro lado, o milho colhido fora do limite recomendado para a concentração de MS, entre 30 e 35%, pode apresentar problemas com a fermentação. Diante dos inúmeros problemas que possam acontecer durante a ensilagem, o uso de inoculantes é uma ferramenta que visa melhorar o processo fermentativo da silagem e reduzir perdas durante a sua utilização (MUCK et al., 2018).

Além da silagem de planta inteira, é possível destacar a ensilagem dos grãos de milho, que vem se tornando cada vez mais comum. Essa técnica busca a melhoria da digestibilidade do amido, principalmente no Brasil onde os híbridos de milho possuem endosperma duro, de

alta vitreosidade e baixa digestibilidade (CORREA et al., 2002; CRUZ et al., 2014). A silagem de grãos de milho reidratados consiste na moagem dos grãos maduros secos e a adição de água para obter no mínimo 35% de umidade para ser ensilado (PEREIRA et al., 2013). O armazenamento do milho pela ensilagem do grão maduro reidratado é uma tecnologia de abordagem antiga (MCLAREN; MATSUSHIMA, 1968). As vantagens deste tipo de silagem englobam facilidade e economia na estocagem dos grãos, compra estratégica dos grãos na entressafra, menor dependência de maquinário, além de agregar valor nutricional ao produto (PEREIRA et al., 2013).

Durante a ensilagem dos grãos de milho ocorrem proteólises, o que resulta na degradação da matriz proteica que envolve os grânulos de amido. Esse efeito explica o incremento na degradabilidade ruminal desse carboidrato, uma vez que aumenta o acesso dos microrganismos do rúmen aos grânulos de amido (HOFFMAN et al., 2011). Além disso, outros fatores podem interferir no processo fermentativo e na extensão da degradabilidade ruminal da silagem de milho reidratado, como tipo do hibrido, o uso de aditivos na ensilagem, o tempo de estocagem e o grau de moagem dos grãos para a reidratação (FERRARETTO; SHAVER., 2015), sendo que a influência desse último fator pode ocorrer de forma mais destacada, em razão da maior flexibilidade no manejo com os grãos.

2.2 Principais inoculantes usados na silagem da planta inteira de milho

A ensilagem é um processo fermentativo no qual os microrganismos são os responsáveis pelas transformações bioquímicas que ocorrem na massa (PAHLOW, et al., 2003). A fermentação desejável da silagem, a fermentação lática, é baseada na combinação de anaerobiose e baixo valor de pH (KUNG Jr. et al., 2018). Como resultado, são reduzidas as atividades de respiração das células vegetais e de suas enzimas, bem como a de microrganismos indesejáveis. Fungos filamentosos, bactérias do gênero *Bacillus*, *Listeria*, Enterobactérias, bactérias acéticas e bactérias anaeróbias esporulantes do gênero *Clostridium*, são alguns dos microrganismos que podem estar presentes na massa ensilada e são inibidos pela acidez e anaerobiose (PAHLOW et al., 2003).

O ácido acético também pode ser produto da fermentação lática e por ser um ácido orgânico fraco, tem ação antimicrobiana pelo acesso ao espaço intracelular de microrganismo tolerantes a acidez, como as leveduras, interferindo no equilíbrio energético e osmótico desses microrganismos. Na silagem, onde valores de pH estão entre 3,5 e 4,8, o ácido lático por ter constante de dissociação (pka) menor em comparação ao ácido acético (pka = 3,86 *vs.* pka =

4,76, respectivamente), tem a maior parte das suas moléculas dissociadas (RAY; BHUNIA, 2013). Os íons H^+ presentes no meio ácido não têm livre acesso à célula microbiana, pois a membrana citoplasmática confere seletividade aos microrganismos, sendo ela semipermeável, e dessa forma não exercem efeito inibitório sobre os microrganismos ácido-tolerantes presentes na silagem (TORTORA et al., 2012). Por sua vez, a maioria das moléculas dos ácidos orgânicos fracos permanecem protonados e podem difundir através da membrana fosfolipídica de microrganismos tolerantes e não tolerantes a acidez (BREIDT et al., 2004; SCHNURER; MAGNUSSON, 2005). Assim, na silagem, pode se dizer que o efeito antimicrobiano do ácido acético, propiônico ou de qualquer outro ácido orgânico fraco é sempre dependente da redução do pH causado pelo ácido lático, no intuito de ter a maior parte das moléculas protonadas na massa ensilada. O ácido protonado no interior da célula microbiana se dissocia, pois encontra um ambiente neutro, com valores de pH maior do que o meio extracelular (RAY; BHUNIA, 2013). Uma vez dentro da célula, os prótons dos ácidos acidificam o citoplasma e a célula precisa trabalhar para expulsa-los, pois os microrganismos são dependentes da homeostase do pH intracelular para o perfeito funcionamento das enzimas e para manter as estruturas das proteínas, ácidos nucleicos e fosfolipídios (TORTORA et al., 2012). Como a membrana citoplasmática é impermeável a prótons, a eliminação de H^+ é realizado pela enzima ATPase, que gera um potencial de membrana, chamado de força próton motriz, com gasto de energia (DAVIDSON; TAYLOR, 2007). Este gasto de energia e exaustão metabólica é o principal efeito dos ácidos orgânicos fracos sobre a inibição do crescimento microbiano (NEAL; WEINSTOCK; OLIVER, et al., 1965). Em revisão, Dalié et al. (2010) citam que outra consequência dos prótons no citoplasma é o aumento da pressão osmótica celular, que desencadeia mecanismos de compensação de carga elétrica aumentando os níveis de sódio, potássio e a força iônica intracelular, provocando a ruptura da célula.

Quando em contato com o oxigênio, principalmente durante a fase de abertura dos silos para o fornecimento aos animais, a silagem é desafiada a manter sua estabilidade por mais tempo possível. Isso é possível quando a silagem apresenta baixos valores de pH e concentrações suficientes de principalmente os ácidos acético ou propiônico produzidos por bactérias do ácido lático (BAL) (AXELSSON, 2004, p. 63). Neste sentido, as bactérias láticas presentes na microbiota epífita são essenciais para a fermentação das silagens. Sua densidade pode variar, entre outros fatores, em função da espécie forrageira (PAHLOW et al., 2003) e do estágio de maturidade fisiológica da planta (LIN et al., 1992; COMINO et al., 2014). Entretanto, essas bactérias com atuação benéfica para a conservação da forragem, podem ser adicionadas durante a ensilagem. O princípio básico de atuação dos inoculantes é o incremento na população

inicial desses microrganismos, de forma que eles sejam capazes de competir com os microrganismos epifíticos da silagem e dominar o processo fermentativo (MUCK; KUNG, 1997; SAARISALO et al., 2007). As BAL correspondem ao principal grupo de microrganismos que atuam no processo fermentativo da silagem, sendo os gêneros mais comuns *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Enterococcus* e *Leuconostoc* (PAHLOW et al., 2003; MUCK et al., 2018). Além da produção de ácidos, as BAL podem produzir substâncias com potencial antimicrobiano como, dióxido de carbono, peróxido de hidrogênio e bacteriocinas (AXELSSON, 2004; CASTELLANO et al., 2008; SILVA et al., 2016). Esses compostos podem atuar na inibição de microrganismos indesejáveis na silagem (GOLLOP; ZAKIN; WEINBERG, 2005).

2.2.1 Bactérias do ácido lático

As duas vias principais de utilização de açúcares pelas BAL são a glicólise ou via de *Embden-Meyerhof Parnas* (EMP) e a via das pentoses fosfato. De acordo com as vias utilizadas, as BAL são classificadas em homofermentativas, heterofermentativas obrigatórias e heterofermentativas facultativas (MADIGAN et al., 2010).

As BAL homofermentativas, representadas pela espécie *Lactobacillus acidophillus, Lctococcus lactis* e algumas espécies de *Pediococcus* produzem quase que exclusivamente ácido lático na fermentação da glicose pela via EMP e não fermentam pentoses, pois têm apenas a enzima aldolase. As heterofermentativas facultativas, representadas pelas espécies *L. plantarum, L. rhamnosus, L. zeae e Enterococcus faecium* são semelhantes às anteriores. Contudo, também são capazes de fermentar pentoses em ácido lático e acético, pois além da enzima aldolase constitutiva, apresentam a fosfoquetolase, que são enzimas responsáveis por catalisar a reação de frutose 1,6 bifosfato a gliceraldeído-3-fosfato na rota de EMP (AXELSSON, 2004, p.63). As BAL heterofermentativas facultativas são os inoculantes mais antigos e comuns (MUCK et al., 2018), e entre as espécies desse grupo, *L. plantarum* esteve presente em 67% dos estudos sobre inoculantes homoláticos e heteroláticos facultativos (OLIVEIRA et al., 2016). No estudo da diversidade microbiana de silagens em 54 propriedades leiteiras de diferentes mesorregiões de Minas Gerais, Brasil, Santos (2016) detectou cepas de *L. plantarum* em todas as amostras analisadas.

Na maioria dos trabalhos, silagens sem inoculantes exibem um padrão de fermentação homolática. No entanto, fatores como temperatura ambiente e teor de MS da silagem podem alterar esse comportamento. Zhou, Drouin e Lafreniere (2016) observaram que em temperaturas

mais altas de ensilagem (25°C) há o predomínio de BAL heteroláticas, enquanto que em temperatura baixas houve maior população de heteroláticas. Embora ainda não evidenciado em silagens de milho, o padrão de fermentação de silagens mais secas parece ser homolática. Parvin e Nishino (2009) estudou as diferenças no Capim-Guiné ensilado com 28,6 e 44,3% MS. Aos 15 d, *Lactobacillus brevis* e *Lactococcus lactis*, foram as bactérias dominantes na silagem mais úmida, enquanto *L. plantarum* na silagem mais seca, com consequente maior relação ácido lático/ácido acético na silagem mais seca.

De modo geral, silagens tratadas com uma ou mais cepas de BAL homofermentativa ou heterofermentativa facultativa, apresentam maiores valores de ácido lático e recuperação de MS com menores valores de pH e concentração de ácido acético, comparado as silagens não inoculadas (MUCK; KUNG Jr, 1997). Na meta-análise desenvolvida por Oliveira et al. (2016), verificou-se que a inoculação com BAL heterofermentativas facultativa ou homofermentativas na silagem de milho não afetou a recuperação de MS, embora reduziu pH, ácido acético, ácido butírico e N-NH_3. No mesmo estudo, esses autores observaram que a inoculação reduziu a estabilidade dessas silagens e aumentou a contagem de leveduras em comparação com silagens não inoculadas. O início da deterioração das silagens é comumente relacionado ao crescimento de leveduras na massa (PAHLOW et al., 2003), sendo que seu aumento nas silagens inoculadas com bactérias homofermentativas pode ser devido ao maior aporte de ácido lático, uma vez que em aerobiose muitas espécies de leveduras usam esse ácido como substrato, e às menores concentrações de ácidos orgânicos fracos, como o ácido acético (OLIVEIRA et al., 2016).

As BAL heterofermentativas obrigatórias fermentam hexoses e pentoses, pela via fosfogluconato, em ácido lático, ácido acético, etanol e dióxido de carbono, podendo ser representadas pelas espécies *L. brevis*, *L. buchneri*, *L. diolivorans* e *L. hilgardii* (AXELSSON, 2004; HAMMES; HERTEL, 2003). Entre as espécies heteroláticas obrigatórias, *L. buchneri* é a mais usada nos trabalhos com silagem, com o objetivo de melhorar a estabilidade aeróbia devido a maior produção de ácido acético (MUCK et al., 2018). Posteriormente, Oude Elferink et al. (2001) observaram que o ácido acético pode ser produzido pelo *L. buchneri* e *L. parabuchneri* não só pela via da fosfoquetolase, mas também via conversão anaeróbia de ácido lático a ácido acético, 1,2-propanediol, etanol e CO_2, nas proporções de 1 mol de ácido lático para 0,48 mols de 1,2-propanediol, 0,48 mols de ácido acético, 0,04 mol de etanol e 0,52 mol de CO_2. Segundo Heinl et al. (2012), o metabolismo de *L. hilgardii* é semelhante a esse metabolismo do *L. buchneri*. Além da produção do ácido acético essa conversão anaeróbia do ácido lático é de interesse para aumentar a estabilidade aeróbia da silagem, uma vez que o 1,2 propanediol pode ser metabolizado a ácido propiônico por espécies de *L. diolivorans*

(KROONEMAN et al., 2002) e *L. reuteri* (SRIRAMULU et al., 2008), sendo *L. diolivorans* mais comum em silagem de milho (SANTOS, 2016, p. 137). Atualmente, em estudo *in vitro*, Zielinska et al. (2017) isolaram de silagens de milho cepas de *L. buchneri* capazes de metabolizar 1,2-propanediol em ácido propiônico, mas ainda é desconhecido os efeitos destas cepas na silagem.

A inoculação de silagens com bactérias heterofermentativas é relacionado com maiores perdas fermentativas de MS em relação as bactérias de metabolismo homolático, devido à produção de CO_2 (McDONALD et al., 1991). BAL heteroláticas fermentam hexoses e pentoses, com a relação de 1 mol de dióxido de carbono por 1 mol de glicose, podendo ocasionar até 24% de perda de MS (BORREANI et al., 2018). Porém, pequenos aumentos nas perdas fermentativas de MS podem ser prontamente aceitos se compensado por melhorias substanciais na estabilidade aeróbia da silagem durante a fase de utilização da silagem (MUCK et al., 2018), uma vez que as perdas pelo metabolismo de leveduras podem alcançar a ordem de 48% de MS (BORREANI et al., 2018). Além disso, durante a fase de abertura dos silos ou em momentos em que a silagem é exposta ao ar, microrganismos aeróbios oportunistas podem se desenvolver e produzir um vasto número de substâncias tóxicas que afetam a saúde animal e diminuem sua produtividade, como por exemplo as micotoxinas produzidas por fungos (MUCK; MOSER; PITT, 2003).

Alguns estudos indicam que a conversão anaeróbica de ácido lático em ácido acético pelo *L. buchneri* é relativamente demorado, sendo necessário de 30 a 60 dias de estocagem para detectar aumentos nas concentrações de ácido acético e 1,2-propanediol, e ter resultados positivos sobre a estabilidade aeróbia (MUCK et al., 2018). Sobre a relação entre tempo de armazenamento de silagens e o metabolismo tardio de bactérias heterofermentativas, Li e Nishino, (2011) observaram que silagens tratadas com *L. buchneri* na dose 1×10^6 ufc g^{-1} apresentaram deterioração aeróbia quando abertas aos 14 dias de fermentação. No estudo realizado por esses autores, silagens tratadas não deterioraram aos 56 dias e, silagens tratadas e controle não sofreram deterioração quando armazenadas por até 120 dias. Na silagem inoculada, o teor de ácido lático foi numericamente reduzido ao longo do tempo e ácido acético foi aumentado, de modo que aos 120 dias o valor observado era mais que o dobro (50,6 g kg^{-1} de MS) daquele encontrado aos 56 dias (20,0 g kg^{-1} de MS). Assis et al. (2014) adicionou 1×10^6 ufc de *L. hilgardii* por grama de forragem de milho, e isso resultou em melhoras na estabilidade aeróbica quando comparada com a silagem não tratada depois de 90 dias, mas não aos 30 d de estocagem. A estabilidade de silagens mantidas por mais tempo de estocagem é relacionada com maiores ganhos em concentrações de produtos da fermentação. Daniel, Junges

e Nussio (2014) demonstraram acréscimos de 0,4 hora por dia na estabilidade aeróbia até os 110 dias de armazenamento de silagens de milho, confirmando que tempos maiores de armazenamento são vantajosos para o alcance de silagens mais estáveis em aerobiose.

Novas espécies de *Lactobacillus*, especialmente as de metabolismo heterofermentativo obrigatório, têm sido avaliadas no intuito de antecipar a produção de ácido acético, bem como quanto ao seu potencial inibitório de microrganismos indesejáveis na silagem. Liu et al. (2014) isolaram uma cepa de *L. parafarraginis* (ZH1) da silagem de capim Sudangrass e avaliaram suas características metabólicas e o seu potencial uso como inoculante na silagem de milho doce (*Zea mays* L. var. rugosa Bonaf.). Uma cepa comercial de *L. buchneri* também foi avaliada. Os autores observaram que a 45 ºC a cepa ZH1 apresentou pouco crescimento e *L. buchneri* não cresceu; a cepa ZH1 diferiu da *L. buchneri* pela habilidade de fermentar D-Xylose, D-Manose, Esculin, D-Turanose e D-Arabitol, do contrário, ZH1 não utilizou ou apresentou menor habilidade para utilizar α-Methyl-D-Glucoside, Gluconato e 5-Keto-Gluconato. Nesse mesmo trabalho, a inoculação com a cepa de *L. parafarraginis* aumentou a concentração de ácido acético ($P<0.01$) e a estabilidade aeróbia ($P<0.01$) da silagem de milho doce estocada por 45 dias a 15 e 30 ºC, enquanto *L. buchneri* aumentou a estabilidade aeróbia apenas nas silagens estocadas a 30 °C. Na silagem de aveia pré-secada estocada por 45 dias e à 15 ºC , Liu e Zhang (2015) observaram que a inoculação com a cepa ZH1 proporcionou silagem com maior estabilidade aeróbia (144 h) em relação às silagens controle (32,9 h), tratada com *L. buchneri* (38,6 h) ou *L. plantarum* (29,8 h), e com inesperadas concentrações de ácido benzoico. Essas cepas também foram avaliadas no estudo de Liu, Lindow e Zhang (2018) para a produção de compostos antifúngicos no meio MRS, as quais apresentaram maior produção de ácido benzoico e ácido hexadecanoico, ambos compostos que exibiram inibição do crescimento em placa de *Candida krusei* e *Pichia membranefaciens.*

A espécie *L. parafarraginis* é descrita por Endo e Okada (2007). Nesse estudo as espécies *L. parafarraginis* e *L. farraginis* foram isoladas do Shochu, de uma bebida fermentada típica do Japão, e com base na sequência do gene 16S rRNA foram relacionadas ao grupo do *L. buchneri*. Segundo esses autores, *L. farraginis* e *L. parafarraginis* apresentam características metabólicas distintas, as quais permitem sua separação, sendo que *L. farraginis* apresenta crescimento a 45°C, não utiliza D-Xylose e não cresce em meio MRS caldo com 5% de NaCl. Até o momento, é desconhecido na literatura estudos com a avaliação de *L. farraginis* sobre perfil fermentativo e conservação de silagens.

2.2.2 Bactérias do ácido propiônico

As bactérias do ácido propiônico são anaeróbias facultativas, não formam esporos e adquirem energia por meio da fermentação de açúcares e ácido lático para produção de ácido propiônico, acético e dióxido de carbono (MADIGAN et al., 2010). Como a atividade antifúngica do ácido propiônico é maior do que a dos ácidos lático e acético, esse grupo de bactérias tem sido utilizado no intuito de reduzir as perdas associadas deterioração aeróbia (MUCK et al., 2018). No entanto, as silagens podem ser ambientes inóspitos para o crescimento ou sobrevivência dessas bactérias, uma vez que poucos trabalhos isolaram bactérias do ácido propiônico de silagens, e na planta de milho antes da ensilagem a população dessas bactérias é em média de 10 a 100 ufc g^{-1}, enquanto BAL variam de 10 a 1.000.000 de ufc g^{-1} (PAHLOW et al., 2003).

Muitos trabalhos têm avaliado o efeito da inoculação de bactérias do ácido propiônio, porém poucos mostraram resultados positivos. No estudo com a combinação de *Propionibacterium acidipropionici* com *L. plantarum*, Filya, Sucu e Karabulut (2004) observaram maior estabilidade aeróbia de silagens de milho quando a bactéria do ácido propiônico foi adicionada isoladamente, além disso, foi relatado por esses autores, concentrações consideráveis de ácido propiônico (4,9 g kg^{-1} de MS) apenas com 8 dias de estocagem. Com as mesmas espécies combinadas e adicionadas em silagens de milho e silagem de milho re-ensilada, Coelho et al. (2018) não encontraram efeitos na estabilidade aeróbia, com valores menores para silagens inoculadas e re-ensiladas (105h) em comparação ao controle re-ensilada (156h). Weinberg et al. (1995) avaliaram *Propionibacterium shermanii* na silagem de milho e não observaram efeitos sobre a estabilidade aeróbia. Rahman, et al. (2017) avaliaram silagem de milho inoculadas com *Propionibacterium freudenreichii* e observaram concentrações de 0,09 g kg^{-1} de MS, enquanto as silagens controle e a tratada com *L. plantarum* tiveram 0,29 e 0,54 g kg^{-1} de MS de ácido propriônico, respectivamente. Coral et al., (2008) citam que BAP podem ser inibidas pelos produtos da fermentação. Assim, a falta de respostas na inibição do crescimento de leveduras e fungos filamentosos ou melhorias na estabilidade aeróbia, é devido a essas bactérias não se desenvolverem bem quando as condições de ensilagem promovem uma rápida diminuição do pH (MERRY; DAVIES, 1999).

2.3 Inoculantes em função do teor de MS na ensilagem da planta inteira de milho

Muitas pesquisas já definiram que para uma melhor fermentação e conservação da silagem de milho, as plantas devem ser colhidas com teor de MS entre 32 e 35%, momento em

que os grãos atingem 2/3 da linha do leite (ALLEN et al., 2003; JOHNSON et al., 2002). Porém, na prática agrícola, o ponto de colheita é algo discutível. A opção por colher a planta em avançado estágio de maturidade para a ensilagem proporciona maior concentração de amido nos grãos (BAL; COORS; SHAVER, 1997), e quando conciliado com prolongados tempos de estocagem pode agregar ganhos na digestibilidade do amido (HOFFMAN et al., 2011). Além disso, é preciso considerar que a colheita do milho mais seco é uma estratégia quando o objetivo é a maior produção de MS/ ha ou por kg de planta fresca colhida (PEYRAT et al., 2016), o que consequentemente reduz custos com transporte e estocagem. Adicionalmente, muitos imprevistos podem atrasar a colheita do milho para a ensilagem, principalmente os eventos climáticos, problemas de manejo e logística, como a indisponibilidade ou quantidade insuficiente de colhedoras para acompanhar o avanço da maturidade das plantas (WINDLE et al., 2014). Erros no monitoramento da MS das plantas no campo também são factíveis de acontecer, principalmente em função dos diferentes ciclos dos híbridos disponíveis no mercado. Híbridos de ciclo superprecoce, por exemplo, apresentam janelas de corte mais estreitas, quando comparado aos híbridos de ciclo normal ou tardio.

O milho colhido com avançada maturidade fisiológica apresenta elevado conteúdo de MS, reduzida atividade de água e de concentração de carboidratos disponíveis para a fermentação (ALLEN et al., 2003; PEYRAT et al., 2016). Consequentemente, pode prejudicar a fermentação e a compactação da massa, com consequente formação de espaços porosos com oxigênio, que favorecerá o crescimento de microrganismos envolvidos com a deterioração aeróbia e perdas de MS (BUXTON e O'KIELY, 2003; XICCATO et al., 1994).

Ruppel et al. (1995) observaram que as perdas durante o armazenamento foram inversamente proporcionais à densidade. O modelo descrito por esses autores indicou que, ao longo de um período de armazenamento de seis meses, a perda de MS diminuiu de 20 para 10% quando a densidade aumentou de 160 para 320 kg MS m^{-3}. Muck e Holmes (2000) recomendaram 225 kg de MS m^{-3} como densidade mínima para a silagem de milho, no intuito de reduzir a quantidade de ar que penetra através da silagem de milho. Nesse sentido, no estudo conduzido por Harrison et al. (1998), foi demonstrado que a densidade diminuiu com avanços na maturidade fisiológica da planta de milho, em consequência dos maiores tamanhos de partícula. Johnson et al. (2002), em uma série de três experimentos com mini-silos, avaliaram, entre outros fatores, o efeito do estágio de maturidade do milho nas características fermentativas e na estabilidade aeróbia. Esses autores ensilaram híbridos de milho com 25 a 45% de MS, onde observaram uma tendência de redução na densidade da massa ensilada em relação ao avanço da maturidade e relataram que isso foi relacionado ao conteúdo mais grosseiro das partículas

em função do aumento da MS. Em condições de campo, as perdas ainda podem ser agravadas em áreas do silo de difícil compactação, como as próximas da superfície (BORREANI; BERNARDES; TABACCO, 2008).

Kung Jr. et al., (2018) compilaram dados de análises de diversas amostras de silagens de milho produzidas nos Estados Unidos, avaliaram o efeito do teor de MS da ensilagem nos valores de pH e nos produtos finais da fermentação, e observaram que os valores de pH mais baixos estavam relacionados a silagens com 30 a 35% de MS. No entanto, esses autores observaram que aumentos na concentração de MS, acima de 40-45%, os valores de pH também aumentaram, com redução na concentração de ácidos lático, acético e ácidos totais. Segundo esses autores, isso ocorre porque a atividade de água para o crescimento de bactérias do ácido láctico começa a ser limitante. Adicionalmente, esses efeitos durante a fermentação refletem sobre a estabilidade aeróbia dessas silagens, uma vez que reduz a concentrações de ácido acético.

Nesse sentido, a aplicação do inoculante no momento da ensilagem pode ser uma ferramenta disponível ao produtor para melhorar a fermentação e a estabilidade aeróbia de forragens ensiladas com elevada concentração de MS. Porém, poucos trabalhos avaliaram o uso de inoculantes em silagens de milho colhidos nessa condição, com poucos efeitos positivos ao uso.

Na avaliação dos efeitos de cepas comerciais de *Lactobacillus buchneri* 40788 (LB) e *L. plantarum* MTD-1 (LP), Hu et al., (2009) observaram que silagens confeccionadas com planta de milho apresentando 41% de MS, tiveram maiores valores de pH, concentrações de etanol e leveduras, em comparação aquelas ensiladas com 33% de MS. O tratamento com LP resultou em mais ácido lático somente na silagem com 33% de MS, enquanto LB aumentou a estabilidade aeróbia de ambas silagens. No estudo com avaliação de maturidade fisiológica, Johnson et al. (2002) não observaram efeitos da inoculação com LB e *Enterococcus faecium* na estabilidade aeróbia de silagens confeccionadas com plantas de milho com 44,7% de MS, que apresentaram em média 52 h estáveis. Comparando silagens de gramíneas úmidas e pré-secadas, Nishino e Touno (2005) não observaram efeito da inoculação com *L. buchneri* na silagem de pré-secado.

Comino et al. (2014) avaliaram o efeito do inoculante comercial, contendo *Lactobacillus casei* e *Lactobacillus buchneri*, em plantas de milho colhidas com 27, 32, 38 ou 44% de MS, com as respectivas avaliações visuais da linha do leite nos grãos: 5/6; 3/5; 1/4 e linha negra. O material antes da ensilagem apresentou reduções nos valores de atividade de água (0,991; 0,991; 0,982 e 0,973) e de carboidratos solúveis (155, 131, 75 e 35 g kg^{-1} de MS),

com aumentos na contagem de bactérias do ácido lático (6,44; 6,57; 7,76 e 7,29 log ufc g^{-1}) e fungos filamentosos (6,01; 6,09; 6,42 e 7,03 log ufc g^{-1}) em função do avanço da maturidade. Os autores observaram que o efeito da inoculação diminuiu com o aumento do teor de MS do milho, de forma que a estabilidade aeróbia de silagens com 32% de MS foi de 132 h, enquanto nas silagens com 44% de MS não houve efeito da inoculação (média 85 h). Esses autores reportam que a falta de efeitos do inoculante foi devido a elevada contagem de bactérias láticas epifíticas, baixa atividade de água e baixo teor de açúcares disponíveis para a fermentação no milho colhido com elevado teor de Ms.

Para o milho colhido com elevado teor de MS, as espécies ou cepas bacterianas avaliadas, podem não ser as mais indicadas. Um dos fatores de sucesso no uso de aditivos microbiológicos em silagens é a habilidade da bactéria em crescer rapidamente na massa ensilada e promover rápida e eficiente queda no pH (MUCK; MOSER; PITT, 2003; SAARISALO et al., 2007). Nesse sentido, incoulantes contendo cepas bacterianas mais eficientes no uso de substratos, hábeis no crescimento em condições menos favoráveis, compatível com as condições da planta ao ser ensilada, poderiam alcançar resultados positivos. É importante ressaltar que além da compatibilidade, muitos fatores podem afetar o desempenho do inoculante na silagem, entre eles, a taxa de inoculação (KLEINSCHMIT; KUNG Jr., 2006; MUCK et al., 2018), temperatura do tanque onde o inoculante fica armazenado durante a aplicação (WINDLE; KUNG, 2016) e espécie bacteriana utilizada (OLIVEIRA et al., 2016). Além disso, tem sido demonstrado que o efeito da inoculação também depende da estirpe. Saarisalo et al. (2007) e Santos, Ávila e Schwan, (2013) verificaram que a inoculação com diferentes estirpes, embora pertencentes à mesma espécie, resultou em silagens com características fermentativas diferentes.

2.4 Perfil fermentativo da silagem de grão de milho reidratado

Assim como na ensilagem da planta inteira, na ensilagem dos grãos de milho reidratado objetiva-se rápida redução dos valores pH e máxima anaerobiose. A velocidade com que esses processos ocorrem é dependente das características da planta, como capacidade tampão e carboidratos solúveis disponíveis, além da população de microrganismos epifíticos ou introduzidos com o uso de inoculantes (ÁVILA et al., 2009). Silagens de milho reidratado, bem como as de grão úmido, são reportadas como de difícil fermentação, uma vez que apresentam baixas concentração de carboidratos solúveis (CARVALHO et al., 2016). Durante o desenvolvimento da cultura do milho, carboidratos solúveis são polimerizados como amido no

endosperma do grão, resultando em pequenas quantidades de carboidratos prontamente disponíveis na colheita de grãos secos, os quais são os principais substratos para o crescimento de bactérias do ácido lático na silagem (McDONALD et al., 1991).

Embora as características fermentativas da silagem de grão úmido possam ser semelhantes às da silagem de milho reidratado (DA SILVA et al., 2018; KUNG Jr. et al., 2018), essas silagens possuem peculiaridades distintas. O estresse pelos quais os grãos são submetidos durante a secagem no campo e por aquecimento em secador comercial, podem modificar a população bacteriana do milho seco em comparação com o milho colhido na linha negra para a confecção de silagem de grão úmido. Carvalho (2014, p. 95) observou antes da ensilagem grande quantidade de bactérias do gênero *Clostridium* (19,5% do total de microrganismos) e menor população do gênero *Lactobacillus* (9,1% do total) no material ensilado de grãos de milho reidratados, resultado bem diferente do milho colhido para grão úmido, onde pode ser observado uma alta quantidade de *Lactobacillus* (33,5% do total) e a ausência de *Clostridium*. No estudo desenvolvido por Fernandes (2014, p. 97), silagens de grãos reidratados apresentaram menor teor de ácido lático (1,19 *vs* 2,07%, MS), maiores teores de etanol (1,1 *vs* 0,6%, MS) e ácido butírico (2,1 *vs* 5,8 mg kg^{-1} de MS), quando comparadas às silagens de grãos úmidos com 120 dias de fermentação.

Carvalho et al. (2016) avaliaram o perfil fermentativo de silagens de milho reidratado sem o uso de inoculantes e observaram baixa concentração de carboidratos solúveis no milho moído reidratado antes da ensilagem (20 g kg^{-1} de MS). Esses autores relataram um rápido consumo de carboidratos, onde aos 5 dias de ensilagem a concentração dos carboidratos solúveis atingiu 7 g kg^{-1} de MS. Porém, isso não foi observado para os valores de pH que foram reduzidos apenas após 30 dias de estocagem, bem como aumentou as concentrações de ácido lático (10,8 g kg^{-1} de MS). Nesse experimento, não foi realizado análise de estabilidade aeróbia, mas foi observado baixas concentrações de ácido acético ao longo dos tempos de estocagem, com máximo valor aos 150 dias de estocagem (2,7 g kg^{-1} de MS). Por outro lado, a concentração de ácido propiônio foi significativamente aumentada ao longo do tempo de estocagem (13 g kg^{-1} de MS), aproximadamente duas vezes maior do que aqueles observados em silagem preparada com planta inteira.

O objetivo da ensilagem dos grãos de milho é melhorar a disponibilidade do amido para os microrganismos ruminais, sendo uma forma de contrapor o efeito negativo da textura dura do endosperma sobre a digestibilidade do amido em grãos no estágio maduro de maturação. No Brasil, os híbridos de milho cultivados são caracterizados como duro (*flint*), com maior proporção de vitreosidade do endosperma (73%) (CORREA et al., 2002). No milho de

endosperma vítreo, os grânulos de amido são densos, possuindo fortes ligações entre os grânulos de amido e a matriz proteica de prolaminas, o que dificulta a penetração de água no grânulo, a hidrólise enzimática e a colonização por parte das bactérias do rúmen (HOFFMAN et al., 2011). Segundo esse mesmo autor, subunidades da zeína, a prolamina do milho, são altamente degradadas durante o processo fermentativo da silagem de grão úmido de milho. A proteólise das prolaminas que circundam os grânulos de amido pode ocorrer devido às enzimas da própria planta (SIMPSON, 2001, p.13), produtos finais da fermentação, principalmente ácidos (LAWTON, 2002, p. 18) e enzimas proteolíticas microbianas (BARON; STEVENSON; BUCHANAN-SMITH, 1986). As enzimas microbianas são consideradas como o principal contribuinte para a degradação da proteína (~ 60%) (JUNGES et al., 2017), sendo mais efetiva durante a ensilagem (FERRARETTO; CRUMP; SHAVER, 2013).

Lopes (2016, p. 114) observou aumento na digestibilidade *in vitro* da MS do milho reidratado e dos valores de kd dos 30 para 90 dias de fermentação, sem diferença entre 90 e 120 dias, quando as amostras foram incubadas por 7 e 18 h. No mesmo trabalho, o volume acumulado de gás durante as incubações foi aumentado em silagens estocadas por 120 dias. Carvalho et al. (2016) observaram aumento na digestibilidade *in vitro* da MS do milho reidratado, com 7 h de incubação, a partir de 30 até 180 dias de ensilagem, e com 3 h de incubação após 90 dias de ensilagem. Castro (2017, p. 71) observou aumento de 39% na fração A (rapidamente degradável) de silagem de milho reidratados estocadas por 247 dias em comparação ao milho seco moído. Nesse mesmo trabalho, a degradabilidade ruminal da MS foi aumentada em 60,5; 57,7; 44,2 e 19,5% nos tempos 3, 6, 18 e 48 h de incubação ruminal, em função da ensilagem.

O perfil fermentativo foi estudado por Arcari et al. (2016) em silos laboratoriais, com milho moído a 2mm e avaliações foram feitas nos tempos 3, 30, 90 e 330 dias de estocagem. A queda do pH ocorreu aos 30 d de estocagem quando o valor reduziu de 5,2 (3 dias) para 3,8, e permaneceu constante até os 330 dias. Os autores também observaram que a ensilagem não alterou a MS ou a PB, mas aumentou nitrogênio amoniacal 8 vezes durante a ensilagem e reduziu o teor de amido gradativamente com variação final de 2,4% comparando valores de 3 e 330 dias. De maneira geral, a produção de ácidos foi crescente até 60 dias, bem como de etanol que teve máxima de 0,7% da MS. Ainda no mesmo trabalho, o aumento da degradação do amido *in vitro,* com12 h de incubação ruminal, foi linear aumentando com o tempo de ensilagem, sugerindo que aumentos na digestibilidade é proporcional ao tempo de estocagem, embora a fermentação parece estabilizar com 60 dias após a ensilagem.

Dessa forma, tem sido verificado que a ensilagem dos grãos de milho resulta em melhorias na digestibilidade, e maiores ganhos são obtidos desde que haja longos períodos de estocagem, porém esse efeito é acompanhado de progressiva perda de MS (BENTON; KLOPFENSTEIN; ERICKSON, 2005; CARVALHO et al., 2016).

2.4.1 Tamanho de partícula na silagem de milho reidratado

Na ensilagem do milho reidratado, o primeiro passo é a moagem dos grãos. A decisão sobre o grau de moagem tem sido direcionada para a obtenção de melhores digestibilidades do amido, ou seja, mais fino possível (HOFFMAN; SHAVER, 2019). A quebra do grão em tamanhos menores aumenta o contato da partícula com a água, a área de superfície para a adesão de microrganismos e atuação de enzimas, proporcionando melhorias na digestibilidade do amido (McALLISTER et al., 1990). Baron, Stevenson e Buchanan-Smith (1986) observaram que grãos de milho que passavam por uma tela com malha de 8 mm apresentavam maior teor de N solúvel e não-proteico como proporções de N total, sugerindo que o tamanho das partículas de grãos pode determinar a proteólise no silo. Por outro lado, uma moagem mais fina dispende mais tempo, ou seja, torna-se mais caro o custo da tonelada de milho processado para a ensilagem (HEADLEY; PFOST, 1968). Castro (2017, p.71), observou um aumento expressivo na taxa de moagem de 3,9 ton h^{-1} com moagem fina para 11,7 ton h^{-1} com moagem grossa.

Poucos trabalhos avaliaram o efeito do tamanho de partícula de silagens de milho reidratado sobre o perfil fermentativo e estabilidade aeróbia. Em silagens de grão úmido sem uso de inoculantes, a estabilidade aeróbia tem alcançado valores entre 50 e 84 horas (KUNG Jr. et al., 2007; BASSO et al., 2012; Da SILVA et al., 2015). Da Silva et al. (2018) observaram que silagem de grão de milho reidratado finamente moído (peneira com crivos de 2 mm), sem inoculante e estocadas por 124 dias permaneceram estáveis durante a exposição aeróbia por até 71 horas. No trabalho de Morais (2016), com o mesmo tamanho de partícula citado anteriormente, foi observado 120 horas de estabilidade aeróbia para a silagem. A variação desses valores faz questionar a extrapolação dos dados para silos em condição de fazendas, onde muitas variáveis podem afetar a estabilidade aeróbia.

Na silagem de milho maduro reidratado sem o uso de inoculante o tempo para redução dos carboidratos solúveis pode variar, sendo de 5 dias para milho moído em crivo de 3 mm (CARVALHO et al., 2016) e 21 dias para milho moído em crivo 12 mm (FERNANDES, 2014, p.97). Já a redução no pH ocorre de forma mais lenta em ambos os graus de moagens, onde Carvalho et al. (2016) observaram a primeira redução aos 30 dias de estocagem (pH = 4,68) e

valores finais de 4,2 após 210 dias de ensilagem. Para o milho moído com 12 mm e estocado por 21 dias, os valores de pH foram próximos a 5, permanecendo com valores acima de 4,5 até 120 dias (FERNANDES, 2014, p.97).

O processo de ensilagem e a moagem dos grãos são fatores que auxiliam na degradação das prolaminas do milho, principalmente devido à ação das enzimas microbianas e da planta presentes na silagem (HOFFMAN et al., 2011; FERRARETTO; CRUMP; SHAVER, 2013; JUNGES et al., 2017) e o ganho na digestibilidade pode ser relacionado o período de estocagem (LOPES, 2016, p.105). Dessa forma, a interação desse fatores tem sido estudado, mas ainda não está evidente qual o tempo mínimo de estocagem necessário para anular o efeito do grau de moagem sobre a degradabilidade ruminal da MS. Ainda, a maior parte dos trabalhos publicados sobre o estudo de cinética ruminal de silagens de grãos de milho utilizaram amostras de silagem moídas novamente, para serem incubadas em sacos convencionais *in situ*, eliminando possíveis efeitos de fatores de estudo, como o grau de processamento (JOHNSON et al., 2002).

Lopes (2016, p.105) avaliou o efeito da ensilagem de milho maduro reidratado, processados para passar em peneiras de crivos de 3 e 8 mm, sobre a digestibilidade ruminal da MS *in vitro*. A degradação da MS não foi afetada pelo tamanho de partículas, mas houve tendências de menor degradação ruminal da MS em 7h (P = 0,10) e 18 h (P = 0,12) de incubação, com menor kd 3 -7 h (P = 0,09) para milho grosso comparado ao milho finamente moído. A produção acumulada de gás aumentou de 169,0 para 198,1 mL quando a silagem foi processada para o menor tamanho de partículas. Na avaliação sobre efeitos de tamanho de partícula e tempo estocagem (30, 90 e 120 dias), não houve evidências que sugerissem que o tamanho das partículas de milho fosse um fator no efeito de ensilagem, uma vez que as concentrações de prolamina não foi afetada, mas o milho moído grosseiramente teve tendência a ser menos digerível *in vitro* do que o milho finamente moído.

No trabalho de Castro (2017, p. 71), a avaliação dos efeitos do grau de moagem e ensilagem (247 dias) sobre a cinética de degradação ruminal foram analisadas com incubações das amostras no seu estado integro inicial, ou seja, sem posteriores moagens. Antes da ensilagem, a fração solúvel do milho moído grosso (9 mm) não diferiu do milho moído fino (3 mm); a degradabilidade ruminal da MS do grão moído fino foi maior nos vários tempos de incubação (3, 6, 18 e 48 h), mas não afetou o kd e a degradabilidade ruminal efetiva da MS. Com a ensilagem, o autor observou uma tendência (P = 0.08) na redução do kd da fração B (2.03 vs. 2.15% h^{-1}), mas o tamanho da partícula não afetou a degradabilidade ruminal em nenhum dos tempos de incubações avaliados, o kd e a degradabilidade ruminal efetiva, o que

sugere que o tempo de estocagem avaliado foi suficiente para anular os efeitos do tamanho de partícula sobre a degradabilidade da MS.

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SEGUNDA PARTE

3 ARTIGO 1: New strains of epiphytic lactic acid bacteria improve the conservation of corn silage harvested at late maturity

Artigo formatado de acordo com as normas do periódico científico Journal of Dairy Science

1 RUNNING HEAD: INOCULANT FOR CORN SILAGE HARVESTED AT LATE
2 MATURITY

3

4 **New epiphytic strains of lactic acid bacteria improve the conservation of corn silage**
5 **harvested at late maturity**

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ABSTRACT

Fifty-three strains of lactic acid bacteria (LAB) isolated from corn silages from different farms were evaluated for use as inoculants in corn silages. LAB strains were characterized for growth and pH reduction in corn extract, growth at different temperatures and the ability to inhibit silage-spoilage microorganism growth. Strains CCMA1362, 1363 and 1364 (*Lactobacillus farraginis*); CCMA1365 (*L. plantarum*); CCMA1366 (*L. buchneri*) and CCMA1367 (*Pediococcus acidilactici*) isolated from corn silage; and CCMA170 strain (*L. hilgardii*) isolated from sugarcane were evaluated in corn silages harvested at an advanced stage of physiological maturity. The inoculants were applied at a rate of 6 log cfu g^{-1} of whole-corn crop and harvested with 454 g kg^{-1} of DM. The experimental silos were opened after 10, 32 and 100 days of storage. CP, ash and starch contents were not affected by the inoculation or storage time. Inoculation with obligate homofermentative LAB resulted in silages with higher DM loss and lower aerobic stability. Silages with obligate heterofermentative strains, especially with CCMA1362 (*L. farraginis*) and CCMA170 (*L. hilgardii*), showed the smallest yeast population (<2.00 log cfu g^{-1}) after 10 days of storage. Silage inoculated with strain CCMA1362 showed lower DM loss, showed a smaller population of undesirable microorganisms and was among the silages with higher lactic acid and acetic acid production and greater aerobic stability. This strain also provided good results in the laboratory tests. The species *Lactobacillus farraginis* is reported for the first time in studies with silage, and strain CCMA1362 isolated from corn silage is shown to be promising for use as an inoculant in corn silages harvested at an advanced stage of physiological maturity.

Key words: heterofermentative, high dry matter, inoculant, *Lactobacillus farraginis*

INTRODUCTION

The use of microbial inoculants in forage preservation is a technique that has been used for many years. The metabolic pathway of lactic acid bacterial (LAB), the variations between the species and, more recently, the variations between strains of the same species are factors related to microorganisms that will influence in inoculant response (Santos et al., 2013). In addition to these features, inoculation effectiveness is highly dependent on forage physicochemical conditions at the time of silage (Muck, 2010; Comino et al., 2014; Romero et al., 2018), mainly dry matter (DM) content, which strongly alterations in fermentation profile of silage (Kung et al., 2018).

According to Muck et al. (2018), despite of *L. buchneri* group includes 25 different species, most studies about the use of inoculants in silage fermentation have been used *L. buchneri,* and only a few another species were evaluated, such as *L. brevis*, *L. hilgardii*, *L. diolivorans* and more recently *L. parafarraginis*. Corn plants and corn silage itself have wide range of lactic acid bacterial species (Santos, 2016b; Zhou et al., 2016), which may indicate an opportunity to knowledge of new species and strains to be used as starter cultures in silage conservation process. However, few studies have evaluated the potential of LAB strains isolated from the corn silage, as well as few are studies that begin with a larger range of isolates, laboratory tests to assess their metabolism and potential to inhibit growth of undesirable microorganisms in silage, with the aim of select those strains with potential for use as inoculants.

Many studies have already defined the ideal harvest window of corn plants for silage as the time that the grains reach between 50 and 60% of the milk line, 2/3 milk line, at which time the forage has a DM content between 32 and 35% (Bal et al., 1997; Allen et al., 2003). However, in agricultural practice, many unforeseen factors can delay harvest of the corn, mainly climatic events, problems with hybrid DM monitoring and harvester logistics, which results in harvest

the corn outside desirable silage standards (Windle et al., 2014). Corn harvest stage is crucial as it influence the quality and quantity of the silage material (Ferraretto and Shaver, 2012). In this sense, it is necessary to consider that corn harvested at an advanced stage of maturity for silage production may be a strategy to obtaining higher starch content (Bal et al., 1997), mainly when silage will be stored for a longer time, which provides gains on digestibility (Hoffman et al., 2011). Besides the gains in nutritive value, higher dry matter yield per hectare or higher dry matter of fresh plant (Peyrat et al., 2016), with consequent reduction in operational costs with transportation and storage, can also be achieved when corn is harvested with high dry matter content.

Corn harvested at advanced stage of maturity has a high DM content, with a reduced water activity (a_w) and water-soluble carbohydrate concentration, which negatively affect packing, reduce fermentation and accelerate the aerobic deterioration of the silage (Buxton and O'Kiely, 2003; Kung et al., 2018). The inoculant can be an additional tool, especially when using corn harvested outside the recommended standard for good fermentation. However, the choice to use inoculant should be determined based on the challenges faced by the forage when ensiled (Kung et al., 2003), with efficient microorganisms to the use of substrates and ability of growth in less favorable conditions.

Thus, we hypothesized that epiphytic LAB from corn silage are more efficient for the preservation of corn ensiled at advanced physiological maturity. The objective of this study was to select LAB strains isolated from silages from different farms and to evaluate their effects on the fermentation profile, DM losses and aerobic stability of corn silages harvested at late maturity.

MATERIALS AND METHODS

Screening of Strains for Silage

Eighty-eight strains of LAB isolated from corn silage produced in different regions of Minas Gerais State, Brazil, were grouped based on metabolic and genotypic characterization (Santos, 2016a). Fifty-three strains were pre-selected considering specimens of each identified species, excluding clones, and with higher lactic or acetic acid production and lower ethanol production. The isolated species and their respective number of strains were: *Lactobacillus acidophilus* (1) e *Pediococcus acidilactici* (5), *L. casei* (3), *L. paracasei* (8), *L. plantarum* (3), *L. rhamnosus* (9), *L. zeae* (2), *L. hilgardii* (7), *L. diolivorans* (5), *L. farraginis* (3) e *L. buchneri* (7). The 53 strains were evaluated for their growth in corn plant extract, ability to reduce the pH of the extract and growth at different temperatures. The extract was produced with corn plants, according to Saarisalo et al. (2007). One-millimetre aliquots of standardized inoculum (OD, 600 nm) were added to 100 mL of extract and incubated at 30°C for 36 hours to evaluate the growth (OD, 600 nm) and pH. Growth of LAB strains was also evaluated at temperatures of 35, 40 and 45°C, in MRS broth (OD, 600 nm) after 48 hours of incubation.

For the next stage of evaluation, strains were ranked according to, first, better growth in the extract, followed by greater efficiency in reducing the pH, combined with higher growth at different temperatures (Table S1). In total, 37 strains were evaluated for their potential to inhibit the growth of corn silage pathogenic and spoilage microorganisms. The antimicrobial activity was evaluated using the ‘spot on the lawn’ antagonism test according to Harris et al. (1989), with modifications. The bacteria *Bacillus cereus* (CCT 1436) and *Escherichia coli* (ATCC 25922) and the yeasts *Issatchenkia orientalis* (CCMA 902) and *Pichia manshurica* (CCMA 48) were used as indicators. Antagonism experiments were conducted by spotting 25 µL of an overnight lactic acid bacterial culture onto the surface of an MRS agar plate and incubation at 37°C for 48 hours. Subsequently, 20 mL of culture of the indicator microorganism grown

overnight was transferred to Erlenmeyer flasks containing 200 mL of yeast extract peptone glucose (YEPG) for yeast and Brain-Heart Infusion (BHI) for bacteria, both with soft agar (0.75% agar). These solutions were poured onto the plates containing the LAB cultures and incubated for 24 hours at 28°C for evaluation of the yeast and 30°C for bacteria. The inhibition potential was evaluated by the halo size, which was measured in millimetres using a calliper. To test the inhibition of filamentous fungi, the species *Aspergillus flavus* (CCDCA 1054) and *Aspergillus parasiticus* (CCDCA 10606) were used. A fungal spore suspension was making with soft agar (0.75% agar). A standardization of LAB inoculum was performed using the number 1 standard of the McFarland scale. Then, on 1/3 of the plate with De Man, Rogosa, Sharpe (MRS) agar (M641I, Himedia; Mumbai, India) 20 µL of BAL inoculum was spread and incubated at 37 °C for 48h. Afterwards, each filamentous fungi was inoculated in the same plate with LAB, using a platinum handle embedded in spore suspension. The plates were incubated at 30 ° C for 7 days. The inhibition potential was evaluated by distances between LAB and mold colony.

Evaluation of Strains in Experimental Silos

The strains CCMA 1362, CCMA 1363 and CCMA 1364 (*Lactobacillus farraginis*), CCMA 1365 (*L. plantarum*), CCMA 1366 (*L. buchneri*) and CCMA 1367 (*Pediococcus acidilactici*) were selected and evaluated in experimental silos. The strain *L. hilgardii* (*A. cepa L. hilgardii* 170) isolated from sugarcane silage was included as a treatment because it showed favourable results in previous studies (Ávila et al., 2014; Carvalho et al., 2015).

Corn (Biomatrix 3063PRO2) was harvested in the second-season, at late maturity, approximately 140 days after sowing, when the sample showed 45% of DM, analyzed via the microwave (Donnelly et al., 2018). The inoculants were previously prepared according to Ávila et al. (2009), mixed with 80 mL of distilled water and homogenized on 3 kg of fresh corn (FC),

reaching a concentration of 6 log cfu g^{-1} ± 0.16. For the control treatment, only 80 mL of distilled water was added.

Experimental polyvinyl chloride (PVC) mini-silos, 10 cm in diameter and 60 cm in length, were used. The experimental silos were sealed with tight lids containing Bunsen valves for gas release. Each silo was packed to achieve a packing density of 535 ± 21 kg m^{-3} of FC. The silos were closed, weighed and stored in a covered place. After 10, 32 and 100 days of storage, the silos were weighed and opened. The dry matter loss was calculated using the weights and the DM concentration of the FC and of the silage.

The water extracts for sequential 10-fold dilutions and the lactic acid bacteria, anaerobic spore-forming bacteria, yeast and mould colonies were prepared according to Santos et al. (2013). Total aerobic bacteria were enumerated on nutrient agar (Himedia Laboratories), and the plates were incubated at 30°C for 24 hours. The pH of the extract was determined using a pH metre (DIGIMED DM 20 Potentiometer, Digicrom Instruments, SP, Brazil). The acid and alcohol contents were analyzed by high-performance liquid chromatography according to the method described by Carvalho et al. (2017).

To determine the DM contents, the samples were initially prepared using a forced draft oven at 60°C for 72 hours. The dried samples were then ground in a Wiley type grinder through a 1-mm screen, and then 1 g samples were dried at 105°C for 24 hours. Crude protein (CP) was determined according to AOAC method 990.03, and the mineral portion (ash) was obtained by burning the sample in a muffle furnace at 550°C for 6 hours (AOAC, 1990). Water-soluble carbohydrates (WSC) were determined by the phenol method using a standard glucose curve (Dubois et al., 1956). To determine the concentration of neutral detergent fibre (NDF), the samples were treated with thermostable a-amylase and addition of sodium sulphite, according to Van Soest et al. (1991). Starch was analyzed enzymatically according to Hall (2009).

Aerobic Stability

After 100 days of storage, 2 kg of silage was placed in pails and kept in a closed space at room temperature. Data Logger's (Impac, model MI-IN-D-2-L; São Paulo, Brazil) were placed in the centre of the silage mass. Ambient and silage temperatures were measured every 30 minutes. The loss of aerobic stability was calculated as the time required for the silage mass to raise the temperature by 2 ºC above the ambient temperature. Aerobic deterioration was defined as the sum of the daily temperature increases (°C) above the reference temperature during 10 days (Conaghan et al., 2010).

Statistical Analysis

The data for growth microbial and pH reduction, both in corn extract, the inhibition of undesirable microorganisms and growth microbial at different temperatures for the 37 LAB strains were analyzed by principal component analysis (PCA) using Statistica software (2009).

Evaluation of the strains in experimental silos was carried out in a completely randomized design. The treatments were constituted in a factorial arrangement with 24 combinations of 8 inoculants (7 LAB strains and control) and 3 storage times (10, 32 and 100 days), with 3 replicates, except the data of dry matter loss, which had 4 replicates. The data were analyzed with SISVAR software (Lavras, Brazil) version 4.5, using a model that included the inoculant and storage time as fixed effects, as well as inoculant-time interaction. Data from aerobic stability was analyzed using the model: $Yi = \mu + Pi + \varepsilon i$, where: μ = overall mean; Pi = inoculant effect (i = 7 LAB strains and control) and, εi= experimental error. The means were compared using the Scott-Knott test at 5% probability.

RESULTS

Screening of Strains for Silage

Bacterial growth and pH reduction in the corn extract varied among the 53 LAB strains, some of which displayed higher growth and a greater pH reduction (Table S1). The growth of LAB in the aqueous corn extract was positively correlated with the pH reduction capacity (0.66, P<0.01). At 35°C, *L. buchneri* had the largest number of strains with an OD above average, and at 40°C, *L. acidophilus*, *L. paracasei* and *L. farraginis* showed better growth. At 45°C, few strains exhibited good growth, namely, *L. farraginis* strains, a *L. plantarum* strain and a *P. acidilactici* strain (Table S1). In general, *L. buchneri* and *L. hilgardii* strains did not grow at 45°C. According to these results, 37 strains were selected to evaluate the inhibitory potential of undesirable microorganisms in silage.

None of the strains was able to inhibit *I. orientalis* growth. In the principal component analysis (Figure 1), the CCMA1362, CCMA1363 and CCMA1364 (*L. farraginis*) strains, located in the upper left quadrant, were efficient in inhibiting the growth of *A. flavus* and *P. manshurica* and showed vigorous growth in corn extract. In the same quadrant, strain CCMA 1367 (*P. acidilactici*) was associated with an efficient inhibition of *E. coli* and *A. parasiticus* and good growth at 45°C. Strains CCMA 1365 (*L. plantarum*) and CCMA1366 (*L. buchneri*) were also associated with the inhibition of *P. manshurica*. Strain CCMA 1366 showed a stronger relationship with the pH reduction in the extract and with the inhibition of *A. parasiticus* and *B. cereus*. According to the PCA results, strains CCMA1362, CCMA1363, CCMA1364, CCMA165, CCMA1366 and CCMA1367 were chosen to be evaluated as corn silage inoculants in experimental PVC silos.

Evaluation of Strains in Experimental Silos

The corn was ensiled with a high DM concentration (454 g kg^{-1}), and the other chemical composition parameters are related to such high DM concentration (Table 1). The population of LAB, yeast, filamentous fungi, total aerobic bacteria and anaerobic spore-forming bacteria of corn plants was, on average, 8.04, 6.80, 6.0, 8.50 and 7.90 log cfu g^{-1} fresh whole plant corn, respectively.

The DM concentration decreased and NDF increased over the storage time (Table 2). The silages inoculated with strains CCMA1362, CCMA1365 and CCMA1366 showed the highest levels of DM. CP, ash and starch contents were not affected by the inoculation or storage time (P>0.05), with mean values of 57, 27 and 400 g kg^{-1} of DM, respectively (Table 2).

There was interaction between the inoculants and storage time (P<0.01) for the variables DM losses, WSC and pH (Figure 2). DM loss increased over time in all treatments, except for the treatment with strain CCMA1362 (*L. farraginis*). At the end of 100 days of storage, the silage treated with this strain showed the lowest DM loss (4.0% of DM), while the highest DM losses were observed for the control silage and the silage treated with strain CCMA1367 (*P. acidilactici*) (Figure 2a). After 10 days of storage, a greater drop in WSC levels was observed in the silages with strains CCMA1362, CCMA1366 and CCMA1367. At 10 days, there was no difference in pH values (P>0.05) between the silages (Figure 2b). At the end of the storage time, the pH increased in all silages, demonstrating the highest values in the treatment with strain CCMA1366 (*L. buchneri*) (Figure 2c); this silage also showed the lowest lactic acid concentration at 27.13 g kg^{-1} of DM (P<0.01) (Figure 3a). Silages inoculated with CCMA1365 (facultative heterofermentative) and CCMA1367 (obligate homofermentative) showed the lowest pH values and highest lactic acid concentrations at the end of the evaluation time.

After 10 days of storage, the highest lactic acid concentration (P<0.01) was observed in silage treated with strain CCMA1364 (*L. farraginis*), followed by silage containing

CCMA1365 (*L. plantarum*) (P<0.01), with concentrations of 67.95 and 59.55 g kg-1 of DM, respectively (Figure 3a). However, for the former, an intense reduction of the lactic acid concentration was observed over the storage time. The control silage (without inoculant) had the lowest acetic acid concentration relative to those inoculated with the obligate heterofermentative strains (CCMA1362, 1363, 1364 and 1366), starting at 32 days of storage (Figure 3b). With the exception of silage treated with strain CCMA1367, the acetic acid concentration increased in all silages as a function of the storage time. The highest acetic acid concentration (24.5 g kg^{-1} DM) at 100 days storage was observed in the silage treated with strain CCMA1366 (*L. buchneri*), followed by the silages CCMA1364, CCMA1363 and CCMA1362 (all three *L. farraginis*) and CCMA170 (*L. hilgardii*), both obligate heterofermentative.

Tartaric, malic, oxalic, citric, succinic, propionic, isobutyric, butyric and isovaleric acids were not detected in any silage. After 10 days of storage, the ethanol concentration was higher in the control silage and those inoculated with the strains CCMA1364 (*L. farraginis*) and CCMA1367 (*P. acidilactici)* (Figure 3c). With the increase in storage time, the ethanol concentration increased exclusively in the CCMA1366 treatment, with a mean of 7 g kg^{-1} of DM. The highest 1,2-propanediol concentration was also observed in this silage after 100 days of storage, and this metabolite was detected only in silages treated with CCMA1366 (*L. buchneri*) and CCMA170 (*L. hilgardii*) (Figure 3d). There was no difference in the ethanol concentration at 32 days of storage, while at 100 days, the silages inoculated with the *L. farraginis* strains presented the lowest concentrations. With the exception of strains 1365 and 1367, after 32 days, inoculation reduced the yeast count at all fermentation times (Table 3). In the silages with strains CCMA1362 *(L. farraginis*) and CCMA170 *(L. hilgardii*), the count was below 2.0 log CFU g^{-1} throughout the evaluated period. For silages treated with other strains, inhibition of yeast growth occurred after 32 days of storage. The control silage and those treated

with strains CCMA1367 (*P. acidilactici*) and CCMA1365 (*L. plantarum*) had the highest yeast population at 32 and 100 days of storage ($P<0.01$).

The LAB population size decreased in all treatments over the storage time, except in the silage treated with strain CCMA1366 (*L. buchneri*) (Table 3). The silage with strain CCMA1367 (*P. acidilactici)* showed a greater reduction in the LAB count over the storage time. The control silage had the smallest LAB population at 100 days, with a mean of 5.64 log cfu g^{-1}. The total mesophilic aerobic bacteria decreased over time in all silages ($P<0.01$), and the largest population of these bacteria was observed in the treatment with strain CCMA1365 (*L. plantarum*).

The growth of filamentous fungi was detected only in the control silage and those inoculated with CCMA1367 (*P. acidilactici*) and CCMA170 (*L. hilgardii*) (data not shown). At 10 days, the control silage had the largest filamentous fungus population (2.90 log cfu g^{-1}) ($P<0.01$). At 32 days of storage, filamentous fungi count was reduced ($P<0.01$) to values below the detection level (<2.00 log cfu g^{-1}) in the inoculated silages and to 2.47 log cfu g^{-1} in the control silage.

At 10 days of storage, the population of anaerobic spore-forming bacteria was the smallest ($P<0.01$) in the treatments with strains CCMA1362 (*L. farraginis*) and CCMA1366 (*L. buchneri*) (Table 3). During the evaluation period, the population of these bacteria remained constant in the silages inoculated with strains CCMA1362, 1363 and 1366, while there was a reduction in population size in the other silages ($P<0.05$). At 32 and 100 days, there was no difference in the population of anaerobic spore-forming bacteria in the silages treated with the different strains ($P>0.05$).

Silages inoculated with strains CCMA1362, CCMA1363, CCMA1364, CCMA1366 and CCMA170 (obligate heterofermentative) were the most stable upon aerobic exposure ($P<0.01$), with an average of 116 hours (Table 4). In these silages, there were no sharp changes

in temperatures, which remained close to ambient temperature throughout the evaluation period (Figure 4). Control silage and silages inoculated with CCMA1365 (*L. plantarum*) and CCMA1367 (*P. acidilactici*) lost aerobic stability on average after 63 hours of exposure to air. These silages had the highest maximum temperature and aerobic deterioration (Table 4 and Figure 4).

DISCUSSION

During the process of strain selection, many parameters can be analyzed to improve the screening of the best strains for evaluation. In the present experiment, the variables studied in the selection of LAB strains made it possible to distinguish a group of microorganisms that were correlated with desirable characteristics for their use as inoculants in corn silage. Microbial growth was accompanied by reductions in pH values. The same findings were reported by Santos et al. (2013) and Dogi et al. (2013). This result is characteristic of LAB, since this group of bacteria is known for its acidogenicity (Quivey et al., 2000).

Temperatures of 40 and 45°C were limiting for the growth of most of the LAB strains tested, but strains CCMA1363 and CCMA1364 (*L. farraginis*), CCMA1367 (*P. acidilactici*) and CCMA1365 (*L. plantarum*) showed favourable growth at 45°C. It is important to evaluate the thermotolerance of strains that can be used as inoculants, since one of the factors that affect their efficiency is the temperature (Weinberg and Muck, 1996). High temperatures may occur at the beginning of the fermentation process, due to the presence of residual oxygen and aerobic microbial activity (Borreani et al., 2018), and in the inoculant storage tanks during field applications (Windle and Kung, 2016). Although some species appeared to be more resistant to high temperatures, this phenomenon differed among strains of the same species. In laboratory tests, Mulrooney and Kung (2008) observed that a *L. plantarum* strain was most thermotolerant at 45°C compared with *Pediococcus pentosaceus, L. buchneri* and *Enterococcus faecium.* For

some species, such as *L. plantarum*, studies examining adaptation mechanisms to different temperatures have shown that the main change occurs in the fatty acid composition of the cell membrane to maintain fluidity (Russel et al., 1995).

In the present study, the reduction in pH of the corn extract was correlated ($P<0.01$) with the inhibition of *B. cerus, E. coli, P. manshurica, A. flavus* and *A. parasiticus*. The antimicrobial activity of LAB is mainly associated with the production of organic acids. The production of strong organic acids has an effect on non-acid-tolerant microorganisms, whereas weak organic acids act on acid-tolerant microorganisms, mainly by disrupting the osmotic balance (Dalié et al., 2010).

The yeasts species *P. manshurica* and *I. orientalis* are lactate users and commonly found in corn silages (Carvalho et al., 2016; Santos et al., 2016). In the present study, only 21% of the evaluated strains were able to inhibit the growth of *P. manshurica*, including strains CCMA1362, CCMA1363 and CCMA1364 (all three *L. farraginis*). These strains were also efficient in inhibiting the growth of *A. flavus* and *A. parasiticus*, which are aflatoxin-producing fungi.

The physiological maturity stage of corn at the time of harvesting influences its chemical, nutritional and microbiological quality (Johnson et al., 2002; Opsi et al., 2013). As a consequence, this will affect the activity of the inoculated microorganism in the silage (Hu et al., 2009; Comino et al., 2015). The evaluated LAB found a challenging environment for microbial activity, such as a high DM content (450 g kg^{-1} of DM) and low soluble carbohydrate concentration (31.8 g kg^{-1} of DM), since in corn silages harvested with DM between 30 and 35%, the WSC concentration is, on average, 10% of DM (Santos et al., 2013, Comino et al., 2014).

Although the DM content of corn was higher than normally recommended, intense fermentation seemed to occur, as observed by the high counts of the different groups of

microorganisms evaluated, mainly the LAB, and by the high concentrations of the different metabolites produced. This result was also observed by Hu et al. (2009), which corn silages with moderately high (40.6%) and normal (33.1%) DM showed no differences in lactic and acetic acid concentrations, with higher count of yeasts in silages with high DM.

It was possible to observe a reduction in DM content and an increase in NDF, which are indicative of sugar metabolism during fermentation (Pahlow et al., 2003). The highest DM concentrations were observed in silages inoculated with strains CCMA1362 (*L. farraginis*) and CCMA1365 (*L. plantarum*), which also showed the lowest DM losses during the evaluated times. The first has an obligate heterofermentative metabolism, and in the studied silages it led to high acetic acid concentrations throughout the fermentation, as well as high lactate production in the initial storage times. The second has a facultative heterofermentative metabolism, and in this experiment, despite the low acetic acid concentration, the silages contained a high lactic acid concentration throughout the process. In this phase of the fermentation process, while the silos are closed, both homo and heterofermentative metabolism can reduce DM losses by inhibiting different spoilage microorganisms due to the dominance of the inoculated strains over the epiphytic population (Ávila et al., 2014). The CCMA1362 strain was the most efficient in inhibiting yeasts and, therefore, was more efficient in reducing losses during the final fermentation times. The control silage and the silage inoculated with a *P. acidilactici* homofermentative strain had the largest yeast population and the highest DM losses. That result was also observed by Arriola et al. (2011) when comparing *P. pentosaceus* and heterofermentative species in corn silages.

Regardless of the metabolic pathway for the use of sugars, all strains evaluated significantly affected the fermentation profile of the silages, which may indicate the dominance of the strain, a criterion for the inoculant efficiency (Kung et al., 2003). In contrast to the results of the present study, Comino et al. (2015), when evaluating the effect of *L. casei* and *L. buchneri*

inoculation on corn silage harvested with 44% of DM, did not observe any effects on the fermentative characteristics and DM loss. According to these authors, the strains used were unable to dominate the fermentation process given the large epiphytic population of LAB in the corn harvested at an advanced stage of physiological maturity.

The intensity of reactions inside the silo is indicative of the quality of fermentation and of the efficiency of the microorganisms in the use of available carbohydrates (Pahlow et al., 2003). At 10 days of storage, the lowest WSC concentration combined with the lowest lactic acid production in the silage treated with strain CCMA1367 (*P. acidilactici*) suggested that this strain was the least efficient in using the available sugars. An opposite behaviour was observed in the silage inoculated with strain CCMA1365 (*L. plantarum*), which exhibited high residual soluble carbohydrate and lactic acid contents at all evaluation times. However, despite differences in the use of WSC and in lactic acid contents at 10 days of storage, the reduction in pH values did not vary among silages.

In all silages, the pH increased over the storage time but remained below 4.2, which is characteristic of well-preserved silages and a good fermentation quality. The silage inoculated with *L. buchneri* strain CCMA1366 showed the highest increase in pH, reaching 4.17 at 100 days, with the highest ethanol concentration. Since the yeast population in this silage decreased at 32 days of storage, these results are more related to the metabolism of *L. bucnheri,* because of the moderate conversion of lactic acid to acetic acid, 1,2-propanediol and ethanol (Oude Elferink et al., 2001). Thus, it is possible that these metabolic pathways were active in the silage inoculated with the *L. buchneri* strain. According to the same authors, the degradation of lactic acid is a self-protective mechanism against the decrease in pH. It is one of the mechanisms that may provide an explanation for the lack of variation in the LAB population ($P>0.05$) between storage times in the silage inoculated with CCMA1366 (*L. buchneri*).

Before ensiling, the corn presented high filamentous fungi (6.0 log cfu g-1) and yeast population (6.8 log cfu g-1), which is consistent with studies reporting a larger population of these epiphytic microorganisms when forage is harvested at advanced physiological stages (Muller et al., 2009; Comino et al., 2014). However, with the ensiling, the rapid establishment of anaerobiosis and the pH decrease, the growth of filamentous fungi was inhibited. However, this inhibition was less intense in the silages inoculated with strains CCMA1367, CCMA170 and control silage, which had the highest filamentous fungi counts at 10 days of storage. Inoculation with the obligate heterofermentative strains, mainly CCMA1362 and CCMA170, reduced the yeast population. The inhibitory action on yeasts is commonly associated with the presence of weak organic acids (Dalié et al., 2010; Hassan et al., 2015); in the present work, this inhibition may be associated with the increase in acetic acid concentrations. Silage treated with strain 1362 also produced a large amount of lactic acid (68 g kg^{-1} of DM) at 10 days of storage. This rapid production of lactic acid may have inhibited the population of total aerobic bacteria and anaerobic spore-forming bacteria, which was, on average, lower in the silage treated with strain CCMA 1362 (*L. farraginis*), reflecting greater aerobic stability and lower DM loss.

Inoculation with *L. farraginis, L. buchneri* and *L. hilgardii* species significantly increased the aerobic stability of silage. The silages inoculated with these strains had the highest acetic acid concentrations, indicating their action on yeasts. These strains also showed efficiency in laboratory tests in inhibiting the evaluated undesirable microorganisms (Table S1). The spoilage of corn silage during the feed-out phase is a problem, as evidenced by many scientific studies and field observations (Berger and Bolsen, 2006; Borreani and Tabacco, 2010). In cases where silage is produced with corn harvested at an advanced stage of physiological maturity, these silages tend to be more porous in the silo, especially in field condition (Borreani et al., 2018), and sufficient amounts of organic acids, as observed in this

work, can suppress the growth of undesirable microorganisms during fermentation process, as well as during the feed-out phase.

CONCLUSIONS

The inoculation with obligate homofermentative LAB isolated from corn silages resulted in silages with higher loss of DM and lower aerobic stability. The strain CCMA1362 (*Lactobacillus farraginis*) presented the best results promising for use as an inoculant in corn silage harvested at the advanced stage of physiological maturity. The silages inoculated with this strain showed lowest DM loss, lowest population of undesirable microorganisms, good production of lactic and acetic acid and greater aerobic stability. More studies are need to evaluate the efficiency of these strains under field conditions.

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Captions for figures

Fig.1. Principal component (PC) analysis of the growth in corn extract (GE), pH reduction (pH), growth in different temperatures (35, 40 and 45°C) and ability to inhibit the growth of spoilage microorganisms (*Aspergillus flavus, Aspergillus parasiticus, Bacillus cerus, Escherichia coli* and *Pichia manshurica*) of strains of lactic acid bacteria.

Fig.2. Dry matter loss (a), water-soluble carbohydrates (b) and pH (c) contents in corn silages as a function of the microbial inoculant within each storage time. Means fallowed by the same letter (lowercase for LAB strains and uppercase for storage time) are not significantly different by the Scott-Knott test (P>0.05). CCMA1362, 1363 and 1364 (*L. farraginis*); CCMA1365 (*L. plantarum*); CCMA1366 (*L. buchneri*); CCMA1367 (*P. acidilactici*) and CCMA170 (*L. hilgardii*). (a): SEM = 2.45; inoculant effect (I), P < 0.01; storage time effect (T), P < 0.01; inoculant-storage time effect (I * T), P < 0.01. (b): SEM = 0.67; I, P < 0.01; T, P = 0.38; I*T, P < 0.01. (c): SEM = 0.01; I, P < 0.01; T, P < 0.01; I*T, P < 0.01.

Fig.3. Concentrations of lactic acid (a), acetic acid (b), ethanol (c) and 1,2-propanediol (d) in corn silages as a function of the microbial inoculant within each storage time. Means followed by the same letter (lowercase for LAB strains and uppercase for storage time) are not significantly different by the Scott-Knott test (P>0.05). CCMA1362, 1363 and 1364 (*L. farraginis*); CCMA1365 (*L. plantarum*); CCMA1366 (*L. buchneri*); CCMA1367 (*P. acidilactici*) and CCMA170 (*L. hilgardii*). (a): SEM = 1.27; inoculant effect (I), P < 0.01; storage time effect (T), P < 0.01; inoculant-storage time effect (I * T), P < 0.01. (b): SEM = 0.74; I, P < 0.01; T, P < 0.01; I*T, P < 0.01. (c): SEM = 0.29; I, P < 0.01; T, P < 0.01; I*T, P < 0.01. (d): SEM = 0.44; I, P < 0.01; T, P < 0.01; I*T, P < 0.01.

Fig.4 Variation in temperature during aerobic exposure of control silages and silages inoculated with strains of lactic acid bacteria.

Figure 1

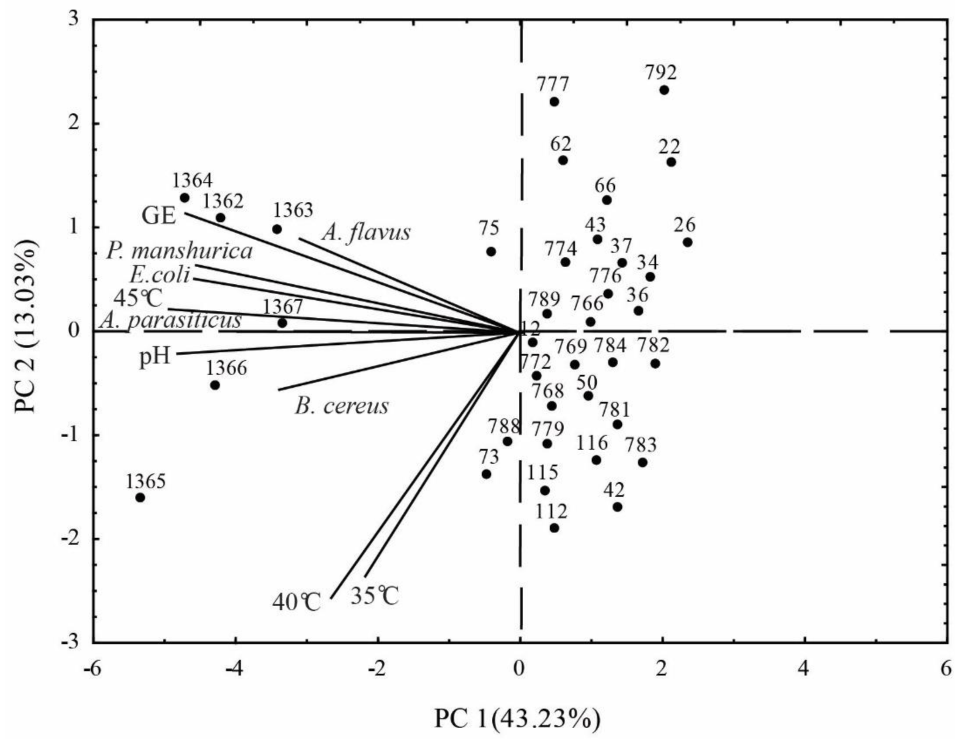

Figure 2

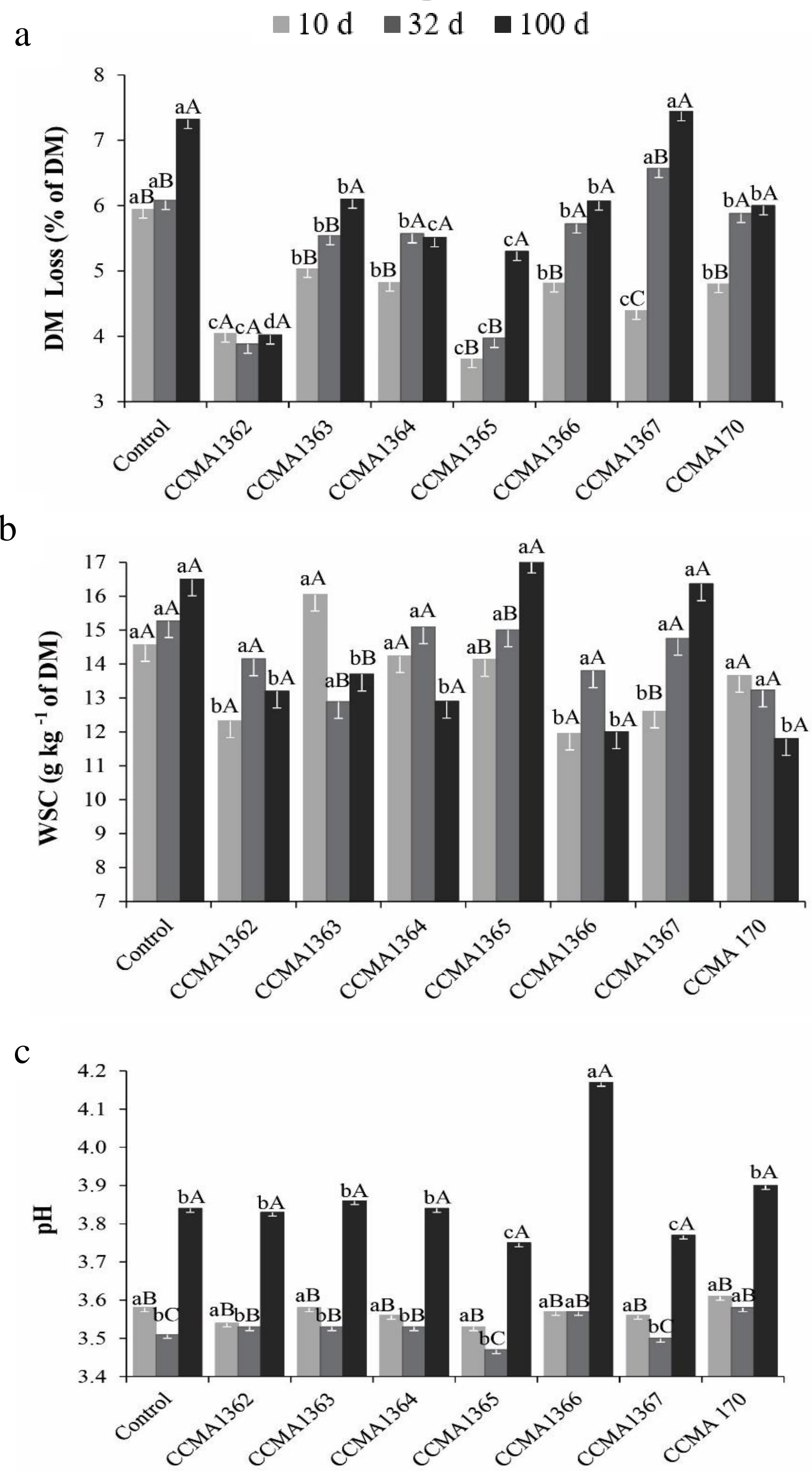

Figure 3

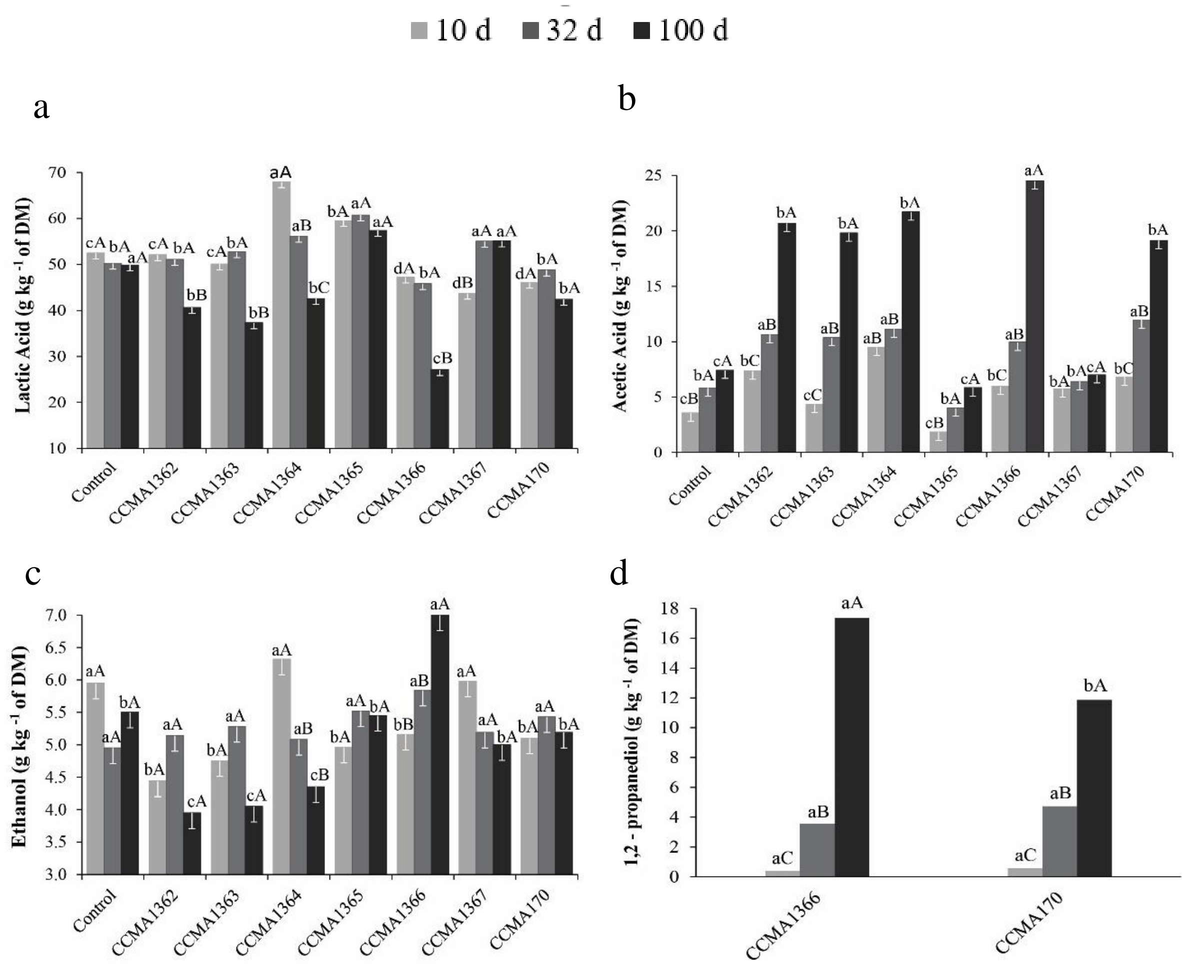

Figure 4

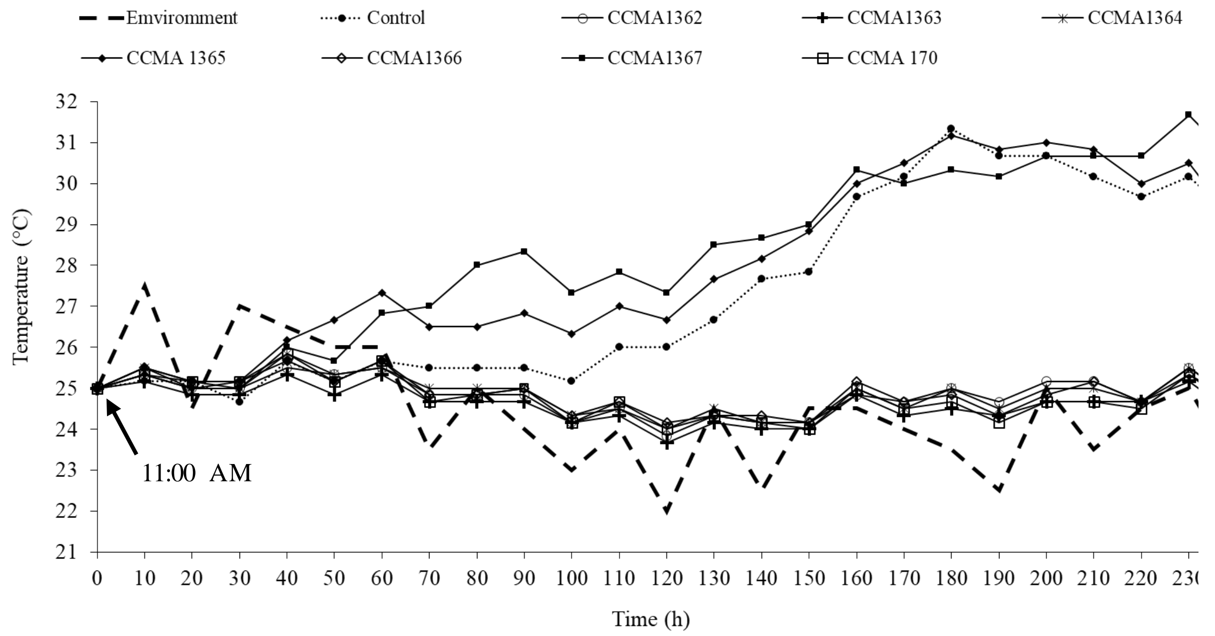

Table 1. Chemical composition and microbial population in whole plant corn before ensiling

Item	
Dry Matter (g kg^{-1} as fed)	454.0
Crude Protein (g kg^{-1} of DM)	58.0
Ash (g kg^{-1} of DM)	25.0
Neutral Detergent Fiber (g kg^{-1} of DM)	400.4
Water-soluble carbohydrates (g kg^{-1} of DM)	31.8
Starch (g kg^{-1} of DM)	404.0
Lactic acid bacteria (log cfu g^{-1})	8.04
Total aerobic bactéria (log cfu g^{-1})	8.50
Filamentous fungi (log cfu g^{-1})	6.00
Yeasts (log cfu g^{-1})	6.80
Anaerobic spore-forming bacteria (log cfu g^{-1})	7.90
pH	6.44

Table 2. Concentrations of dry matter (DM), neutral detergent fiber (NDF), crude protein (CP), ash and starch in silages inoculated with different strains of lactic acid bacteria and at different storage times (10, 32 and 100 days)

	Silages									Storage (days)				*P-value*		
	Control	CCMA 1362	CCMA 1363	CCMA 1364	CCMA 1365	CCMA 1366	CCMA 1367	CCMA 170	SEM	10	32	100	SEM	I	Time	I*T
DM g kg^{-1} as fed	406.0 b	422.2 a	409.2 b	410.9 b	428.8 a	420.3 a	410.6 b	412.2 b	3.4	421.2 a	413.5 b	410.3 b	2.1	<0.01	<0.01	0.54
				g kg^{-1} of DM												
NDF	423.8	409.1	413.3	415.0	416.7	417.3	415.6	420.6	11.3	401.6 b	409.8 b	437.8 a	7.1	0.99	<0.01	0.87
CP	57.2	58.1	57.1	56.9	56.9	58.5	58.3	58.0	7.0	59.0	57.5	57.0	4.3	0.45	0.61	0.97
Ash	27.5	26.7	27.3	27.1	26.3	26.9	27.4	27.2	0.4	26.7	27.4	27.3	0.2	0.26	0.95	0.31
Starch	389.4	410.2	408.6	408.5	406.3	407.3	382.6	378.7	10.4	403.0	396.6	397.3	6.4	0.16	0.74	0.50

I: inoculant effect. I*T: interaction inoculant-storage time effect. Means followed by different letter are statistically different by Scott-Knott test (P<0.05).
CCMA1362, 1363 and 1364 (*L. farraginis*); CCMA1365 (*L. plantarum*); CCMA1366 (*L. buchneri*); CCMA1367 (*P. acidilactici*) and CCMA170 (*L. hilgardii*).

Table 3. Means of microbial populations (fresh weight basis) in corn silage after 10, 32 and 100 days of storage

Storage (days)	Control	CCMA 1362	CCMA 1363	CCMA 1364	CCMA 1365	CCMA 1366	CCMA 1367	CCMA 170
Lactic acid bacteria (log cfu g^{-1})								
10	8.53 bA	8.36 cA	8.72 aA	8.72 aA	8.56 bA	8.79 aA	7.89 dA	8.35 cA
32	7.55 dB	8.41 bA	8.36 bB	8.51 bB	7.77 cB	8.76 aA	7.62 dB	7.50 dC
100	5.64 eC	8.06 bB	8.11 bC	7.97 bC	6.92 cC	8.82 aA	5.97 dC	8.00 bB
P-value	I: <0.01		Time: <0.01		I*T: <0.01		SEM: 0.06	
Total aerobic bacteria (log cfu g^{-1})								
10	8.47 aA	7.63 cA	8.39 aA	8.34 aA	8.47 aA	8.06 bA	7.90 bA	8.33 aA
32	7.40 bB	7.30 bB	7.18 bB	7.20 bB	7.72 aB	7.26 bB	7.31 bB	7.30 bB
100	5.73 cC	5.61 cC	5.64 cC	5.82 cC	6.56 aC	6.04 bC	5.98 bC	5.80 cC
P-value	I: <0.01		Time: <0.01		I*T: <0.01		SEM: 0.07	
Anaerobic spore-forming bacteria (log cfu g^{-1})								
10	4.20 aA	3.50 cA	3.77 bA	4.33 aA	3.70 bA	3.57 cA	3.72 bA	3.71 bA
32	3.89 aB	3.55 aA	3.75 aA	3.57 aB	3.75 aB	3.67 aA	3.63 aA	3.70 aA
100	3.49 aC	3.56 aA	3.62 aA	3.35 aB	3.50 aB	3.55 aA	3.44 aB	3.40 aB
P-value	I: <0.01		Time: <0.01		I*T: <0.01		SEM: 0.09	
Yeasts (log cfu g^{-1})								
10	3.95 aA	<2.00 dA	2.61 bA	2.90 bA	2.36 cB	3.14 bA	3.20 bA	<2.00 dA
32	3.48 aA	<2.00 bA	<2.00 bB	<2.00 bB	3.51 aA	<2.00 bB	3.28 aA	<2.00 bA
100	3.35 aA	<2.00 bA	<2.00 bB	<2.00 bB	3.09 aA	<2.00 bB	2.89 aA	<2.00 bA
P-value	I: <0.01		Time: <0.01		I*T: <0.01		SEM: 0.16	

I: inoculant effect. I*T: interaction inoculant-storage time effect. ND, not detected by the culture-dependent technique. Means followed by the same letter (lowercase for LAB strains and uppercase for storage time) are not significantly different by the Scott-Knott test (P<0.05). CCMA1362, 1363 and 1364 (*L. farraginis*); CCMA1365 (*L. plantarum*); CCMA1366 (*L. buchneri*); CCMA1367 (*P. acidilactici*) and CCMA170 (*L. hilgardii*).

Table 4. Aerobic stability of corn silages inoculated with strains of latic acid bacteria after 100 days of storage.

	Control	CCMA 1362	CCMA 1363	CCMA 1364	CCMA 1365	CCMA 1366	CCMA 1367	CCMA 170	SEM	*P-value*
Aerobic stability, h	68.7b	125.0a	117.5a	115.0a	58.2b	107.6a	62.3b	107.5a	5.47	<0.01
Maximum temperature, °C	31.3a	25.6b	25.8b	25.3b	32.2a	25.6b	32.0a	25.8b	0.38	<0.01
Time to reach maximum temperature, h	177.0a	55.1b	64.6b	49.6b	185.9a	64.9b	193.6a	57.0b	13.1	<0.01
Aerobic deterioration, °C	5.83a	1.66b	1.30b	1.30b	5.5a	1.0b	4.8a	1.16b	0.43	<0.01

Means followed by different letter are statistically different by Scott-Knott test ($P<0.05$).
CCMA1362, 1363 and 1364 (*L. farraginis*); CCMA1365 (*L. plantarum*); CCMA1366 (*L. buchneri*); CCMA1367 (*P. acidilactici*) and CCMA170 (*L. hilgardii*).

Supporting information

Table S1. Test results during screening of strains of lactic acid bacteria

		Corn extract		Growth in temperatures			Antimicrobial activity (cm)				
LAB species	**Strain code**	**Growth[1]**	**pH[1]**	**35°C**	**40°C**	**45°C**	***E. coli***	***B. cereus***	***P. manshurica***	***A. flavus***	***A. parasiticus***
L. acidophilus	CCMA779	0.24	1.02	0.65	1.82	0.00	0.90	1.50	0.00	1.30	1.50
L. buchneri	CCMA768	0.21	1.40	1.20	1.08	0.00	0.90	1.25	0.00	1.70	1.20
L. buchneri	CCMA769	0.21	1.50	1.13	0.90	0.00	1.10	1.00	0.00	1.50	1.40
L. buchneri	UFLASLM12	0.19	1.39	1.19	0.78	0.00	1.30	1.30	0.00	1.70	1.20
L. buchneri	CCMA772	0.17	1.33	1.19	0.75	0.00	1.30	1.00	0.00	1.40	1.45
L. buchneri	UFLASLM50	0.18	1.40	1.28	0.65	0.00	0.60	1.50	0.00	1.60	1.10
L. buchneri	CCMA777	0.16	1.38	0.55	0.00	0.00	1.30	1.00	0.00	1.80	1.50
L. buchneri	CCMA1366	0.30	1.62	1.12	1.52	0.50	1.50	2.00	0.35	1.50	2.00
L. casei	CCMA783	0.09	1.12	1.35	0.75	0.00	0.90	1.50	0.00	1.30	1.10
L. casei	CCMA784	0.11	1.03	0.70	1.10	0.00	1.00	1.50	0.00	1.50	1.30
L. casei	CCMA 0785	0.02	0.45	0.14	0.00	0.00	-	-	-	-	-
L. diolivorans	CCMA775	0.11	0.99	0.43	1.01	0.12	-	-	-	-	-
L. diolivorans	UFLASLM62	0.13	0.98	0.40	0.50	0.34	1.21	1.50	0.15	1.50	1.10
L. diolivorans	CCMA776	0.13	1.15	0.32	0.89	0.50	0.50	1.60	0.00	1.20	1.50
L. diolivorans	UFLASLM73	0.19	1.50	1.05	1.88	0.23	0.90	1.00	0.00	1.80	1.50
L. diolivorans	UFLASLM75	0.14	1.22	0.73	0.73	0.24	1.10	1.50	0.25	1.50	1.30
L. farraginis	CCMA1362	0.64	1.94	0.86	1.08	0.50	1.50	2.00	0.15	1.75	1.70
L. farraginis	CCMA1363	0.60	1.88	0.67	1.11	1.10	1.30	1.50	0.20	1.80	1.50
L. farraginis	CCMA1364	0.67	1.87	0.80	1.26	1.11	1.35	1.70	0.20	1.70	1.50
L. hilgardii	UFLASLM14	0.04	0.43	0.22	0.06	0.00	-	-	-	-	-
L. hilgardii	UFLASIL22	0.26	0.63	0.39	0.54	0.00	1.00	1.00	0.00	1.40	1.30
L. hilgardii	CCMA770	0.05	0.57	0.19	0.06	0.00	-	-	-	-	-
L. hilgardii	CCMA771	0.18	1.37	1.22	1.46	0.00	-	-	-	-	-
L. hilgardii	UFLASLM26	0.23	1.24	0.71	0.45	0.00	1.00	0.40	0.00	1.10	1.00
L. hilgardii	UFLASLM66	0.14	1.13	0.29	0.70	0.00	1.25	1.50	0.10	1.30	1.00
L. hilgardii	UFLASLM67	0.02	0.61	0.14	0.00	0.00	-	-	-	-	-
L. paracasei	CCMA773	0.19	1.50	1.19	1.30	0.23	-	-	-	-	-
L. paracasei	UFLASLM42	0.15	1.11	1.13	1.50	0.21	0.75	1.00	0.00	1.20	1.20
L. paracasei	CCMA781	0.17	0.95	0.88	1.45	0.21	1.00	1.50	0.00	1.30	1.40
L. paracasei	UFLASLM112	0.15	1.35	0.95	1.71	0.35	1.00	1.50	0.00	1.00	1.20
L. paracasei	UFLASLM115	0.17	1.40	0.80	1.78	0.34	0.80	1.50	0.00	1.30	1.20
L. paracasei	UFLASLM116	0.18	1.52	0.64	1.42	0.00	0.80	1.80	0.00	0.90	1.10
L. paracasei	CCMA788	0.16	1.52	0.98	1.24	0.32	1.00	2.00	0.00	1.50	1.00
L. paracasei	CCMA789	0.18	1.25	0.72	1.41	0.26	1.00	0.65	0.00	1.80	1.50
L. plantarum	CCMA780	0.02	0.46	0.14	0.10	0.00	-	-	-	-	-
L. plantarum	CCMA792	0.26	1.27	0.10	0.00	0.00	0.90	1.50	0.00	1.20	1.00
L.plantarum	CCMA1365	0.36	1.96	1.70	1.64	0.90	2.00	2.00	0.25	1.50	1.50
L. rhamnosus	CCMA767	0.07	0.62	0.17	0.00	0.00	-	-	-	-	-
L. rhamnosus	UFLASLM34	0.17	1.21	0.70	0.70	0.27	1.10	0.90	0.00	1.10	1.35
L. rhamnosus	UFLASLM35	0.18	1.53	0.22	0.00	0.10	-	-	-	-	-
L. rhamnosus	UFLASLM36	0.22	1.50	0.70	0.70	0.00	0.60	1.50	0.00	1.30	0.80
L. rhamnosus	UFLASLM37	0.20	1.52	0.65	0.75	0.27	0.90	0.75	0.00	1.25	1.30
L. rhamnosus	CCMA786	0.04	0.46	0.18	0.00	0.00	-	-	-	-	-
L. rhamnosus	CCMA787	0.02	0.60	0.18	0.00	0.00	-	-	-	-	-
L. rhamnosus	UFLASLM110	0.10	0.40	0.17	0.00	0.00	-	-	-	-	-
L. rhamnosus	CCMA790	0.20	1.13	0.16	0.00	0.00	-	-	-	-	-
L. zeae	CCMA774	0.21	1.23	0.63	0.74	0.00	0.75	1.50	0.00	1.60	1.40
L. zeae	UFLASLM43	0.27	1.09	0.68	0.70	0.00	1.00	1.30	0.00	1.50	1.20
P. acidilactici	CCMA766	0.23	1.25	0.77	0.89	0.50	1.00	1.00	0.00	1.30	0.80
P. acidilactici	CCMA782	0.26	1.02	0.86	0.89	0.26	1.00	1.50	0.00	1.20	0.90
P. acidilactici	CCMA1367	0.40	1.62	0.70	1.53	1.02	1.80	2.00	0.00	1.50	1.50
P. acidilactici	UFLASLM128	0.03	0.86	0.12	0.00	0.00	-	-	-	-	-
P. acidilactici	UFLASLM223	0.01	0.04	0.15	0.00	0.00	-	-	-	-	-

[1] Crescimento microbiano (OD_{600}) e valores de pH correspondem a diferença entre tempo inicial e final de avaliação.

4 ARTIGO 2: Particle size and storage time on conservation and ruminal degradability of rehydrated corn grain silage

Artigo formatado de acordo com as normas do periódico científico Grass and Forage Science

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Particle size and storage time on conservation and digestibility of rehydrated corn grain silage

Particle size and storage of rehydrated corn silage

Abstract

The objective of this study was to evaluate the effect of particle size and storage time on chemical and microbiological characteristics, aerobic stability, and ruminal degradability of rehydrated corn grain silage. Corn grains were ground to pass either a 3 mm (fine) or 9 mm (coarse) screen, rehydrated to achieve around 40% of moisture and ensiled in 200 L polyethylene gallons. Samples were taken before and after ensiling at 10, 30, 90 and 200 days of storage to assess microbial counts, fermentation end products, and DM ruminal degradability. The DM degradation was evaluated with incubation times of 0 (bag wash), 3, 6, and 48 h in 3 rumen cannulated cows. The effective ruminal degradation (ERD) was calculated based on soluble fraction (A), degradable fraction (B), and passage rate (kp) defined as 7.0% h-1: A + B [kd / (kd + kp)]. Aerobic stability was evaluated in silages with 200 days of storage, pH and temperature were analyzed up to 240 hours of aerobic exposure. At 90 and 200 d of storage, fine rehydrated corn grain silage resulted in lower crude protein and greater NH3-N concentration than coarse grain. Coarsely ground rehydrated corn silage had lower temperature at the beginning of storage times and greater aerobic stability than finely ground corn (206 *vs.* 115 hour). DM ruminal degradability increased over the storage times. Particle size of rehydrated corn grain silage did not affect kd values after 90 d of storage, while for the ERD was necessary a long time of fermentation (200 d).

Key words: *conservation. ensiling, grinding, starch.*

Introduction

In corn grains, the starch is involved by hydrophobic starch-protein matrix, that prevents microbial attachment and reduces ruminal degradability of starch (San Emeterio et al., 2000; Rémond et al., 2004), as well as reduces enzymatic digestion in the abomasum and small intestine (Giuberti et al., 2014). In corn grains with a high proportion of vitreous endosperm, such as flint hybrids, which are predominantly used in Brazil, there is a higher proportion of prolamin proteins (Correa et al., 2002; Cruz et al., 2014). Rehydrated grain silage consists of ground mature grain rehydration, which provides adequate conditions for storage and fermentation as silage (Andrade et al., 2010; Carvalho et al., 2016). During the ensiling process, proteolysis of the prolamins involving the starch granules occurs meanly by the action of microbial proteases (Junges et al., 2017), improving DM ruminal degradability (Hoffman et al., 2011; Ferraretto et al., 2015). The effect of this technique would be even greater in corn grain with a high proportion of vitreous endosperm (Correa et al., 2002).

Improvements in starch digestibility are related to prolonged storage period of silage, but at the expense of increased DM loss (Carvalho et al., 2016). Grinding corn grain is another technique that improves ruminal degradability of starch, because it increases the surface area to ruminal microbial attachment (McAllister et al., 1990). Therefore, the effectiveness of ensiling as a strategy for manipulating corn grain digestibility can also be dependent on particle size. Many studies have evaluated the effect of storage time on DM digestibility of rehydrated ground corn silage; however, the variation of this response influenced by particle size and short storage time is not well known. Additionally, the majority of the ruminal disappearance and degradation rate data for silage that has been published used finely ground samples, which eliminates physical differences mechanical processing (Johnson et al., 2002).

Ensiling of rehydrated ground corn is a fermentative process and many factors can change fermentation patterns. Effects of particle size on fermentative characteristics and aerobic

stability of whole-plant corn silage were well elucidated (Muck & Holmes, 2000). Yet, in spite of not being a new ensiling process, the literature on rehydrated corn grain silage is scarce of studies detailing the fermentation profile and aerobic stability, as affected by particle size and storage time. We hypothesized that fine grains might improve the fermentation profile of rehydrated corn grain silage by fast action of bacteria of silage. Additionally, ruminal degradability of coarse grains might reach fine grains with different storage time of silage. The aim of this study was to evaluate the effect of particle size and storage time on chemical and microbiological characteristics, aerobic stability, and ruminal degradability of rehydrated corn grain silage.

Materials and Methods

Rehydrated Corn Grain Silage

Grains from a mature corn hybrid (Dow 2B707, Dow AgroSciences Industrial 76 Ltda, São 77 Paulo, Brazil) were ground with a hammer mill (Nogueira TN-8, Nogueira Máquinas 79 Agrícolas, São João da Boa Vista, Brazil) to pass either a 3 mm (fine) or 9 mm (coarse) screen. The corn hybrid showed proportion of vitreous endosperm (84 ± 3 % of endosperm); Geometric Mean Particle Size (GMPS) and surface area of finely or coarsely ground corn before ensiling was 1,591 vs. 2,185 µ and 24.7 vs. 20.9 cm^2 g^{-1}, respectively (Castro, 2017).

Ground corn was mixed with water in a TMR mixer to achieve at least 35 % moisture for ensiling in 10 silos of polyethylene of 200 L, sized 57 cm in diameter and 95 cm in length. Packing was performed manually, with a mean density of 985 ± 44 kg m^{-3}. All silages were inoculated with KeraSIL grão úmido® (Kera Nutrição Animal, Bento Gonçalves, Brazil) added at 4 g t^{-1} of hydrated ground corn. KeraSIL is composed by *Lactobacillus plantarum* (4.0×10^{10} ufc g^{-1}) and *Propionibacterium acidipropionici* (2.6×10^{10} ufc g^{-1}). At the time of filling the silos, one data logger (model MI-IN-D-2-L; Impac, São Paulo, Brazil) was buried into of each silo

(60 cm deep, approximately) to measure temperatures during storage times. Samples were taken before and after ensiling at 10, 30, 90 and 200 days of storage through acylindrical sampler (5 cm diameter and 70 cm length) inserted in the center of the silo. The experiment was carried out in completely randomized design with two treatments (particle size) and five replicates (silos) with repeated measures (storage times).

Analytical procedures

To obtain the aqueous extract, a 25 g sample of pre-ensiled material and silages was blended in 225 ml of 0.1% sterile peptone water and homogenized in an orbital shaker for 20 min. The pH of each sample was then determined (DigimedDM 20 Potentiometer; Digicrom Instrumentos, SP, Brazil). Aliquots of 2 ml of aqueous extracts were acidified with 10 µl of 50% (v/v) H_2SO_4 and frozen prior to analysis to determine malic, succinic, lactic, acetic, propionic, isobutyric, butyric and isovaleric acids and 1,2-propanediol and ethanol content by high-performance liquid chromatography according to the method described by Santos et al. (2014).

For ammonia nitrogen (NH_3-N) analysis, a 50 g of sample was blended in 450 ml of deionized water and homogenized in an orbital shaker for 10 min. Aliquots of 50 ml of aqueous extracts were utilized, and reading of the ammonia concentration in mol l^{-1} was determined using an ion selective electrode coupled to a multiparameter (High Performance Ammonia Ion Selective; Thermo Fisher Scientific Inc., Waltham, MA).

The pre-ensiled material and silages were dehydrated in a forced ventilation oven at 55°C for 72 h and ground through a 1-mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA). To determine dry mater (DM) contents the samples were dried at 105°C for 24 h. Crude protein (CP) was determined according to the AOAC method 2001.11 (AOAC International, 2012). Starch was analyzed enzymatically according to Hall (2009). Water-

soluble carbohydrates (WSC) were analyzed using the phenol method with a glucose standard curve (Dubois et al. 1956).

Microbiological analysis

Another extract was used for counting LAB, yeasts and filamentous fungi. A 25g sample of pre-ensiled material and silages was blended in 225 mL of 0.1% sterile peptone water and homogenized in an orbital mixer for 20 min. Sequential 10-fold dilutions were prepared to quantify the microbial groups using spread-plat method. Yeasts and filamentous fungi were enumerated on dichloran rose bengal chloramphenicol medium (DRBC, Difco; Becton Dickinson, Sparks, MD, USA). The plates were incubated at 28°C for 72 h. Yeasts were distinguished from filamentous fungi by colony appearance and cell morphology. For enumeration of LAB, the medium Man, Rogosa, Sharpe agar (M641I, Himedia) plus nystatin (4 mL L^{-1}) was used. The plates were incubated at 30°C for 72 h.

Aerobic Stability

To evaluate aerobic stability, samples of approximately 3 kg were removed from each silo with 200 days of storage and placed in plastic buckets. Two sets of buckets were made. In the first set, the temperatures were measured each 15 min using data loggers (model MI-IN-D-2-L; Impac, São Paulo, Brazil) inserted into the silage mass at a depth of 10 cm. The aerobic stability was defined as the number of hours the silage remained stable before rising more than 2°C above the ambient temperature (Moran et al., 1996). An additional set buckets was subjected to exposure aerobic and silage samples were collected at 0, 24, 72, 144, 192 and 240 h for pH analysis.

Ruminal *in situ* degradability

Measurement of in situ ruminal DM disappearance were performed with four incubation time-points 0, 3, 6 and 48 h. Two empty bags were put together to correct for DM adhesion at each incubation time. Dry samples, without ground to accessing the effect of original particle size, were weighted (5.15 ± 0.5 g) in 10 x 20 cm non-woven textile bags (NWT – 100 g/m2). Three rumen cannulated cows were used (Ethics Committee of Federal University of Lavras, code 085/2018). Cows were fed with corn silage ad libitum and 1 kg of DM from a concentrate, approximately. After removal, bags were immersed in cold water for 15 min to stop fermentation. The 0 h and the incubated bags were washed with cold tap water until rinse water was clear.

The model to estimate the fractional degradation rate (kd) was a two-pool model comprised of a fast degrading fraction (A) and a slow degrading fraction (B). An indigestible residue was not included in the model. Fraction A was the bags at 0 h, and a fractional degradation rate was determined using the other incubation times. The kd was obtained by the slope of the natural logarithm of each time point residue as a percentage of the incubated, which resulted in a linear regression. Effective rumen degradability (ERD) was calculated as: A + B [kd/(kd + kp)], where: A = fraction A, B = fraction B (100 – fraction A), kd = fractional degradation rate, kp = fractional passage rate. The fractional passage rate (kp) used was calculated using the average experimental cow being fed experimental diet and using the CNCPS formula (Tylutki et al., 2008). The kp obtained was equal to 7.0 % h^{-1}.

Statistical analysis

Microbial, chemical, ruminal degradability data during storage times, pH, and temperature during aerobic stability were analyzed with MIXED procedure of SAS, version 9.3 (Statistical Analysis System, version 9.4), as repeated measures. Covariance structures utilized

were chosen based on the lowest Akaike information criterion value. Microbial, chemical and ruminal degradability data were analyzed according to model: $Y_{ij} = \mu + P_i + \varepsilon_i + T_j + P_i{*}T_j + \varepsilon_{ij}$, where: μ = overall mean; P_i = fixed effect associated with the particle size (i = 3 or 9 mm), ε_i = experimental error used to test the particle size effect, T_j = fixed effect associated with storage time (j = 0, 10, 30, 90 or 200 d), $(P{*}T)_{ij}$ = interaction between particle size and time of storage effect; ε_{ij} = experimental error. For temperature and pH data during aerobic stability were used the same model considering times (j = 0, 24, 72, 144, 192 and 240 h). Aerobic stability (h) was analyzed using the model: $Y_i = \mu + P_i + \varepsilon_i$, where: μ = overall mean; Pi = particle size effect (i = 3 or 9 mm) and, ε_i= experimental error. The means were compared using the Tukey test at 5% probability.

Results

Chemical and microbiological characteristics

The DM concentration was lower at finely (3-mm) than coarsely ground (9-mm) rehydrated corn grain silage (RCGS) (Table 1, P<0.01). Regardless of particle size, DM concentration decreased from 588.5 to 537.7 g kg $^{-1}$ of DM after 90 d of storage, and then remained constant up to 200 d. The total reduction in DM concentration was 9.2%, from day 0 to day 200 of ensiling. A treatment by time interaction was observed for CP (P<0.01) and NH_3-N (P<0.01) concentration (Figure 1A). In fine RCGS, there was reduction of CP content at 90 and 200 d of storage, while in coarse RCGS CP content decreased only at 200 d. The NH_3-N concentration increased throughout the storage time in both silages; yet, at 90 and 200 d of storage, the amount of NH_3-N was greater in fine RCGS.

There was no interaction between the particle size and storage time on starch and WSC concentration (Table 1). Starch concentration was lower in fine than coarse silage (P=0.01) and reduced, on average, 5.7% at 30 d of storage (P=0.02), and then remained constant up to day

200. The WSC concentration was reduced from 22.5 g kg^{-1} of DM before ensiling to 14.2 g kg^{-1} of DM after 10 d of storage, and remained constant until day 90. Moreover, there was a reduction of WSC at 200 d.

There was an expressive reduction in pH value at 10 d of fermentation in both silages. Nevertheless, finely ground RCGS showed greater reduction at 10 and 30 d of storage, as well as greater lactic acid concentration than coarsely ground (Figure 2A and 2B). At 90 d, pH values were similar and maintained up to 200 d. Lactic acid concentration was greater in fine RCGS than coarse RCGS at 10 and 30 d of storage ($P<0.01$); at 90 and 200 d there was no difference. The concentrations of 1,2-propanediol, propionic acid, butyric acid, isobutyric and isovaleric acids were below the detection limit of the technique employed. Particle size did not change acetic acid concentration ($P>0.05$); there was increased at 30 d and at 200 d of storage ($P<0.01$) (Table 1).

Greater ethanol concentration was observed in finely ground RCGS than coarsely ground at all evaluation times ($P<0.01$); which at 30 and 200 d were obtained the greatest concentration (Figure 2C). Before ensiling, yeast count was, on average, 3.7 log CFU g^{-1}; solely at 90 d, there was reduction on population of yeasts in both silages ($P<0.01$) (Figure 2D). Coarsely ground RCGS showed lower population of yeasts at 30, 90 and 200 d than fine grinding ($P<0.01$), and at 200 d the population was <2.00 log CFU g^{-1} in coarsely ground RCGS. Differences in particle size did not change the population of LAB ($P>0.05$); with 10 d of storage, the amount of LAB increased in 3.08 log CFU g^{-1} of silage and then was constantly reduced over storage times ($P<0.01$) (Table 1). The filamentous fungi growth occurred only before ensilage, mean 3 log CFU g^{-1} silage. After ensilage, the population was below the minimum detection limit in both silages and all times evaluated (<2.00 log CFU g^{-1} silage). During storage time, there was intense variation in temperature of the silages with magnitude of 10 °C, from 17 to 27 °C, the highest temperature peaks were observed at the beginning, with 12 hours after

ensilage and at 120 days (Figure 3). Between fine and coarse RCGS, the greatest temperature difference was observed on the first day of storage, when finely ground corn reached temperature around 26 °C and coarsely ground corn approximately 23 °C, both with 12 hours after ensilage.

Aerobic Stability

Silage particle size affected the aerobic stability (P<0.01), in which coarse grinding had greater aerobic stability than the fine one (206 vs. 115 h, respectively) (Figure 4A). Temperature and pH values during the aerobic exposure are shown in Figure 4B. From opening time (0h) to 72h of aerobic exposure, the temperature and pH value was similar between silages. At 144 h of aerobic exposure, there were marked differences on pH and temperatures values of silages. Finely ground had greater temperature (30.7 vs. 25.7 °C, P<0.01) and pH (6.07 vs. 3.67, P<0.01) than coarsely ground RCGS, respectively.

Ruminal degradability

Interactions between particle size and storage time were detected for all times of ruminal degradations, kd and ERD (Figure 5). The storage times increased ruminal DM degradability in both silages. Before ensilage there was no difference of A fraction (P>0.05) (Figure 5A). At 10 d of storage, fine grinding showed greater size of the A fraction than coarse grinding (19.45 vs. 12.32 % of DM) (P<0.01). There was no difference of A fraction between fine and coarse RCGS at 30, 90 or 200 d of storage (P>0.05). Higher values of A fraction for fine and coarse RCGS (25.83 and 26.03 % of DM) were achieved with 30 and 90 d, respectively. Ruminal DM degradability during 3 and 6 hours of incubation was greater in fine than coarse RCGS up to 10 d of storage (P<0.01); however, with 3 h of incubation ruminal, DM degradability of fine and coarse silage was not different after 30 d of storage (Figure 5B and 5C). With 6 h of incubation

was observed greater values for fine RCGS than coarse RCGS at 90 d of storage ($P<0.01$), however there was no differences at 200 d of storage ($P>0.05$). Finely ground corn before ensiling showed greater DM degradability than coarsely ground with 48 h of ruminal incubation ($P=0.03$); at 90 and 200 d of storage there was no difference between silages ($P>0.05$) (Figure 5D). Up to 10 d of storage kd values did not differ between silages (1.95 % h^{-1}, $P>0.05$) (Figure 5E). At 30 d of storage, kd of fine RCGS was greater than coarse (3.80 vs. 2.82 % h^{-1}, $P<0.01$), and at 90 and 200 d finely and coarsely ground RCGS showed similar kd (mean of 3.70% h^{-1}; $P>0.05$). Before ensiling and up to day 30, fine grinding provided greater ERD than coarse grinding ($P<0.01$) (Figure 5F). Only at 200 d of storage, there was no difference in ERD between silages ($P>0.05$, mean of 55.5 % of DM). ERD of finely ground RCGS stored by 30 d did not differ to ERD of coarsely ground stored by 90 d ($P>0.05$), as well as fine at 90 d did not differ to coarse with 200 d of storage ($P>0.05$).

Discussion

The studied factors, particle size and storage time, affected the fermentative profile of RCGS. In the present experiment, longer storage time reduced CP content and increased NH_3-N concentrations, occurring more quickly in fine RCGS. This effect is related to the breakdown of the protein matrix and the reductions in prolamin concentrations (Hoffman et al., 2011). The proteolysis of prolamin proteins surrounding the starch granules can occur due to corn kernel enzymes (Simpson, 2001), fermentation end-products, mean acids (Lawton, 2002), and microbial proteolysis (Baron etal., 1986). All silages were inoculated with lactic acid bacteria and propionic bacteria. This way, although substantial changes in acid load are not the main mechanism to break down zein proteins in corn grain silage (Junges et al., 2017), the greater proteolysis in fine grinding can be due to additive effect of greater lactic acid concentration and lower pH value in this silage, at the beginning of storage times.

Increase in NH_3-N during storage times has been associated with decrease in prolamin concentration and consequent improvement of ruminal DM degradability of corn grain silages (Kung Jr. et al., 2014; Da Silva et al., 2018). Regardless of the particle size of RCGS, ensiling and storage time increased DM degradability. However, as well as the reduction in crude protein and increase in NH3-N, the maximum degradability, kd and ERD values occurred faster in fine RCGS. Storage for 10 d increased DM degradability in relation to corn rehydrated before ensiling in all times of ruminal incubation, except fine RCGS during 48 h of incubation. Under different conditions, Carvalho et al. (2016) observed increase *in vitro* DM digestibility, with samples ground at 2 mm with 3 h of incubation, only after 90 d of storage. This variation may be due to the fact that samples of the present study had been incubated into rumen without previous grinding, since grinding size affects tall fractions, disappearance rate, and effective ruminal disappearance (Fernandes et al., 2018).

The storage for 30 d eliminated the effect of particle size on soluble or rapidly degradable fraction (fraction A), and DM degradation with 3 h of ruminal incubation. Castro, (2017) also observed no differences in size of fraction A and DM degradation during incubations of 3, 6 and 48 h, when fine and coarse rehydrated corn silages were stored for 247 d. The current experiment showed that with ruminal incubations up to 6 h, finely ground RCGS reached maximum DM degradability with 90 d of storage, whereas coarse delayed 200 d for maximum degradability. This behavior occurred with 48 h of ruminal incubation, even though both silages reach maximum degradability with shorter storage time compared to 6h. As a consequence of reductions of bag residues over time, kd and ERD increased in function of storage time. Thirty days of storage was enough to increase kd values of coarse and fine RCGS, which finely ground had greater kd (3.79 % of DM), value close to that observed by Fernandes et al. (2016), with rehydrated corn silage ground at 3 mm and stored up to 30d. At 90 and 200

d of storage, the range of corn particle size evaluated did not change kd; similarly, ERD values were the same at 200 d of storage.

Rehydrated corn grain may have an impaired fermentation, due to little soluble carbohydrates content, the main substrates for the growth of LAB in silage (McDonald et al., 1991). Nevertheless, we observed that WSC concentrations before ensiling were satisfactory to promote growth of LAB. In contrast with the findings of Carvalho et al. (2016), in this experiment the reduction of values of pH occurred quickly, at 10 d of storage, which was possibly a consequence from the use of inoculant containing homofermentative bacteria. Regardless of particle size, there was a first reduction of WSC concentration at the beginning of storage time and another at 200 d of storage. Carvalho et al. (2016) observed minimum values of WSC at 5 d of ensiling with corn ground at 2 mm. In addition, in both silages, there was 5.70% reduction in starch concentration with 30 d of storage. This hydrolysis of starch to glucose monomers during fermentation of RCGS can be considered one way to provide soluble carbohydrate for microbial growth in silage. It is possible that, during the fermentative process of silage, there are amylolytic enzyme producing microorganisms. Giraud et al. (1994) found a strain of *Lactobacillus plantarum*, isolated from fermented cassava, which can break down cassava raw starch.

Even though the population of LAB was not affected by particle size, finely ground RCGS showed greater lactic acid concentration and lower pH values than coarsely ground at the start of storage times. On the other hand, fine grinding was also favorable to yeast metabolism with consequent increase in ethanol concentration. This behavior can also be explained by the increase in the specific area, which in turn provides a more favorable environment for the development of the present microorganisms. Additionally, fine RCGS was warmer at the beginning of storage times, which can be explained by greater aerobic microbial metabolism in this silage when compared with coarse RCGS (McDonald et al., 1991). The

temperature of both silages rose up to 12 h after ensilage, and the higher temperature in fine RCGS was evident. At the start of ensilage, vegetal cells' breathing and aerobic microorganisms' metabolism can elevate silage temperature (Muck et al., 2003; Borreani et al., 2018). Although no significant difference in the yeast population was observed at the beginning of storage (10 d), this population was numerically bigger in fine RCGS. Furthermore, this silage showed higher concentration of ethanol in all storage times ($P<0,01$).

Particle size did not affect acetic acid concentration, and the observed value at 200 d of storage is in agreement with that observed by Carvalho et al. (2016), without use of inoculants, and with Da Silva et al. (2018), with inoculation of homofermentative bacteria. Acetic acid and propionic acid possess high antifungal activity (Kleinschmit et al., 2005; Tabacco et al., 2011). The inoculant used in this experiment contained *Propionibacterium specie*, but propionic acid was not detected in these silages. According to Muck et al. (2018), *Pr. acidipropionici*, when applied alone, has been most successful in keeping yeast counts low. Merry and Davies (1999) indicated that these bacteria do not grow well when ensiling conditions lead to a rapid decrease in pH value.

Finely ground RCGS exhibited the lowest aerobic stability. We observed other parameters that indirectly demonstrated the highest aerobic stability of coarsely ground RCGS, such as stable pH and low peak of temperature during aerobic exposure. This variation of aerobic stability in relation to particle size, as well as in some attributes during silage storage times, can also be due to change in the microbial community composition. Vermeulen et al., (2018) observed that reduced particle size wheat bran is efficiently colonized by lactic acid producing community in the cecal microbiota of broilers. These findings suggest that particle size was a determinant factor to aerobic stability of RCGS. Besides enlarging the contact surface area, particle size affects hydration properties, where the smallest particle size is quickly

hydrated in comparison with thick particles, making the material more susceptible to microbial attack (Jacobs et al., 2016; Vermeulen et al., 2017).

Conclusion

Particle size affected the fermentative profile, chemical characteristics and aerobic stability of RCGS. Fine grinding (3 mm) was more favorable to microbial metabolism at the beginning of storage times, which in turn showed greater lactic acid and pH value. However, finely ground RCGS had greater yeast counts and ethanol concentration than coarsely ground RCGS, during storage time. Fine RCGS was more susceptible to aerobic deterioration, reaching maximum temperature and pH value faster than coarse RCGS. Ruminal DM degradability increased over the duration of storage. Particle size of rehydrated corn grain silage did not affect kd values after 90 d of storage, while for the ERD was necessary a long time of fermentation (200 days).

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Table 1. Concentration of dry matter (DM), water soluble carbohydrates (WSC), starch, acetic acid and lactic acid bacteria (LAB) of rehydrated corn grain silage ground with 3 mm or 9 mm and means in storages times

	Particle size (PS)		SEM	Storage time (days)					SEM	*P-value*		
	3-mm	9-mm		0	10	30	90	200		PS	Time	PS*T
Dry Matter, g kg^{-1} as fed	552.2	561.9	1.47	588.5a	568.0b	556.5c	537.7d	534.4d	2.32	<0.01	<0.01	0.75
WSC, g kg^{-1} of DM	14.2	15.2	0.03	22.5a	14.2b	14.5b	13.4b	8.8c	0.07	0.05	<0.01	0.16
Starch, g kg^{-1} of DM	664.9	685.5	3.7	700.6a	682.0a	660.6b	661.3b	660.0b	8.70	0.01	0.02	0.21
Acetic acid, g kg^{-1} DM	1.66	1.60	0.06	ND	1.49c	2.11b	1.88b	2.25a	0.09	0.36	<0.01	0.66
LAB, log CFU g^{-1} of silage	4.64	4.67	0.03	4.10c	7.03a	5.31b	3.93c	2.88d	0.08	0.68	<0.01	0.97

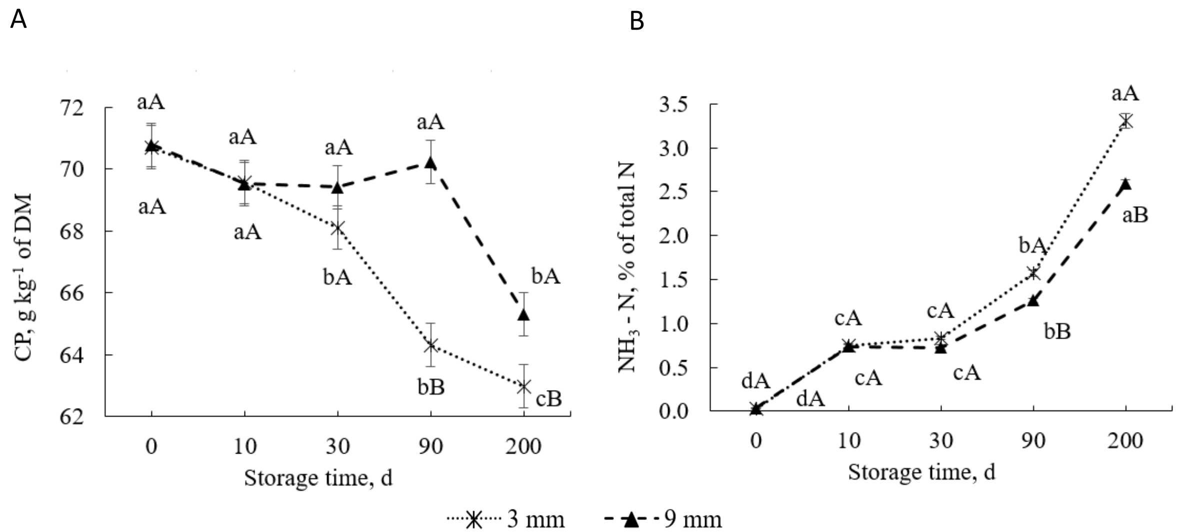

Figure 1. Crude protein (CP) (A) and ammonia N (NH_3-N) (B) of rehydrated corn grain silage ground at 3-mm or 9-mm stored for 0, 10, 30, 90 and 200 days. (A): SEM = 0.90; Effect of particle size (PS), P < 0.01; effect of storage time (T), P < 0.01; interaction between PS and T (PS x T), P < 0.01. (B): SEM = 0.06.; Effect of particle size (PS), P < 0.01; effect of storage time (T), P < 0.01; interaction between PS and T (PS x T), P < 0.01. Means with different letters differ statistically by Tukey test (P<0.05). Lowercase letters represent time and capital letters particle size. Error bars indicate SEM.

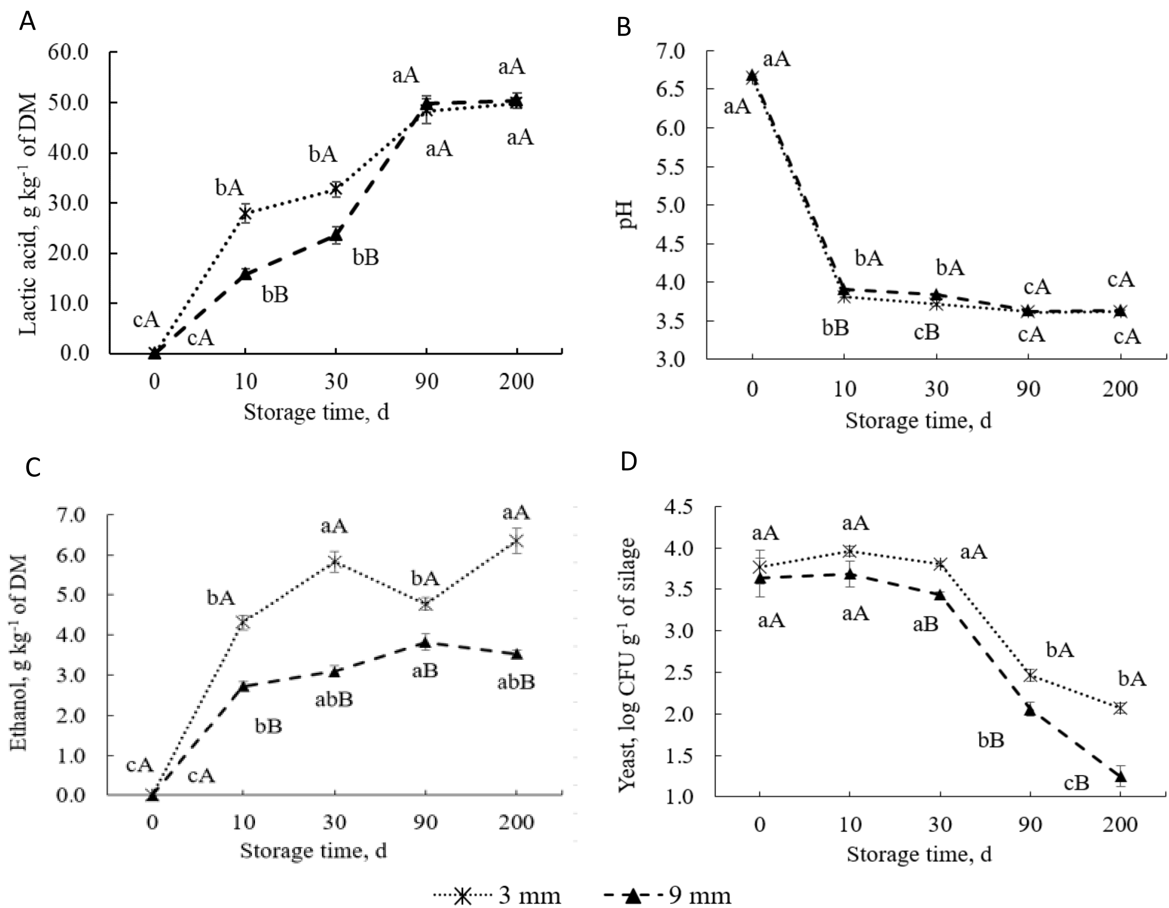

Figure 2. Lactic acid (A), pH (B), ethanol (C), and yeast (D) of rehydrated corn grain silage ground at 3-mm or 9-mm stored for 0, 10, 30, 90 and 200 days. (A): SEM = 2.10; Effect of particle size (PS), $P = 0.37$; effect of storage time (T), $P < 0.01$; interaction between PS and T (PS x T), $P < 0.01$.(B): SEM = 0.004; PS, $P <0.01$; T, $P < 0.01$; PS x T, $P < 0.01$. (C): SEM = 0.22; PS, $P < 0.01$; T, $P < 0.01$; PS x T, $P < 0.01$. (D): SEM = 0.19; PS, $P < 0.01$; T, $P < 0.01$; PS x T, $P = 0.04$. Means with different letters differ statistically by Tukey test ($P<0.05$). Lowercase letters represent time and capital letters particle size. Error bars indicate SEM.

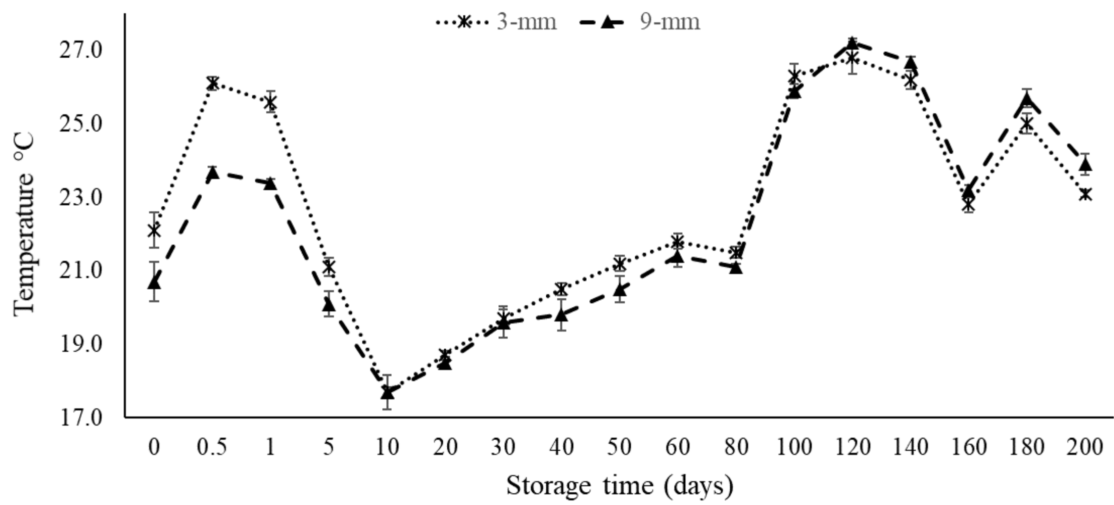

Figure 3. Temperature during storage times of finely (3-mm) and coarsely (9-mm) ground rehydrated corn gran silage. Error bars indicate SEM.

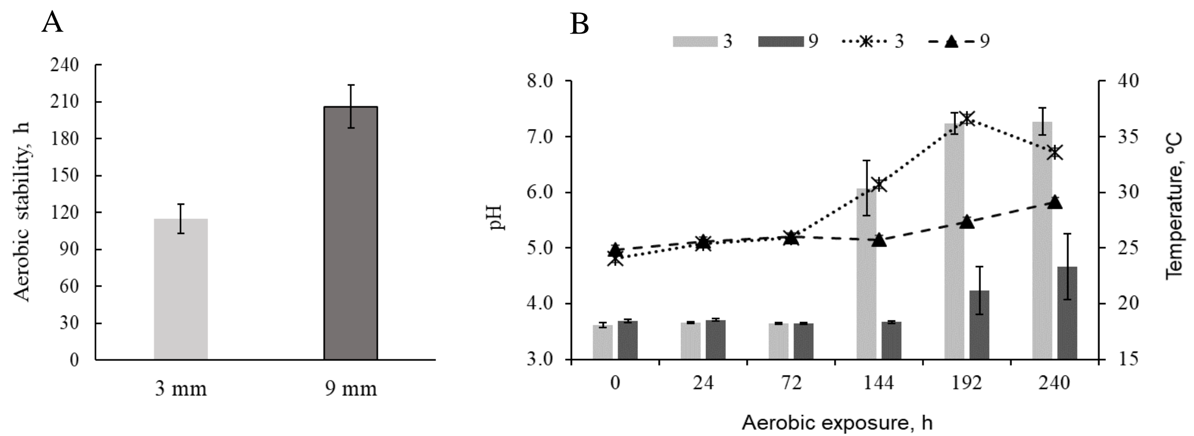

Figure 4. Aerobic stability (A), pH and temperature (B) (bar and lines, respectively) during aerobic exposure of rehydrated corn grain silage ground at 3-mm or 9-mm stored for 200 days. (A): SEM = 11.8, Effect of particle size (PS), P<0.01. (B pH): (SEM = 0.24; PS, P < 0.01; Effect of aerobic exposure (T), P < 0.01; interaction between PS and T (PS x T), P < 0.01. (B temperature): SEM = 0.40; PS, P < 0.01; T, P < 0.01; PS x T, P < 0.01. Error bars indicate SEM.

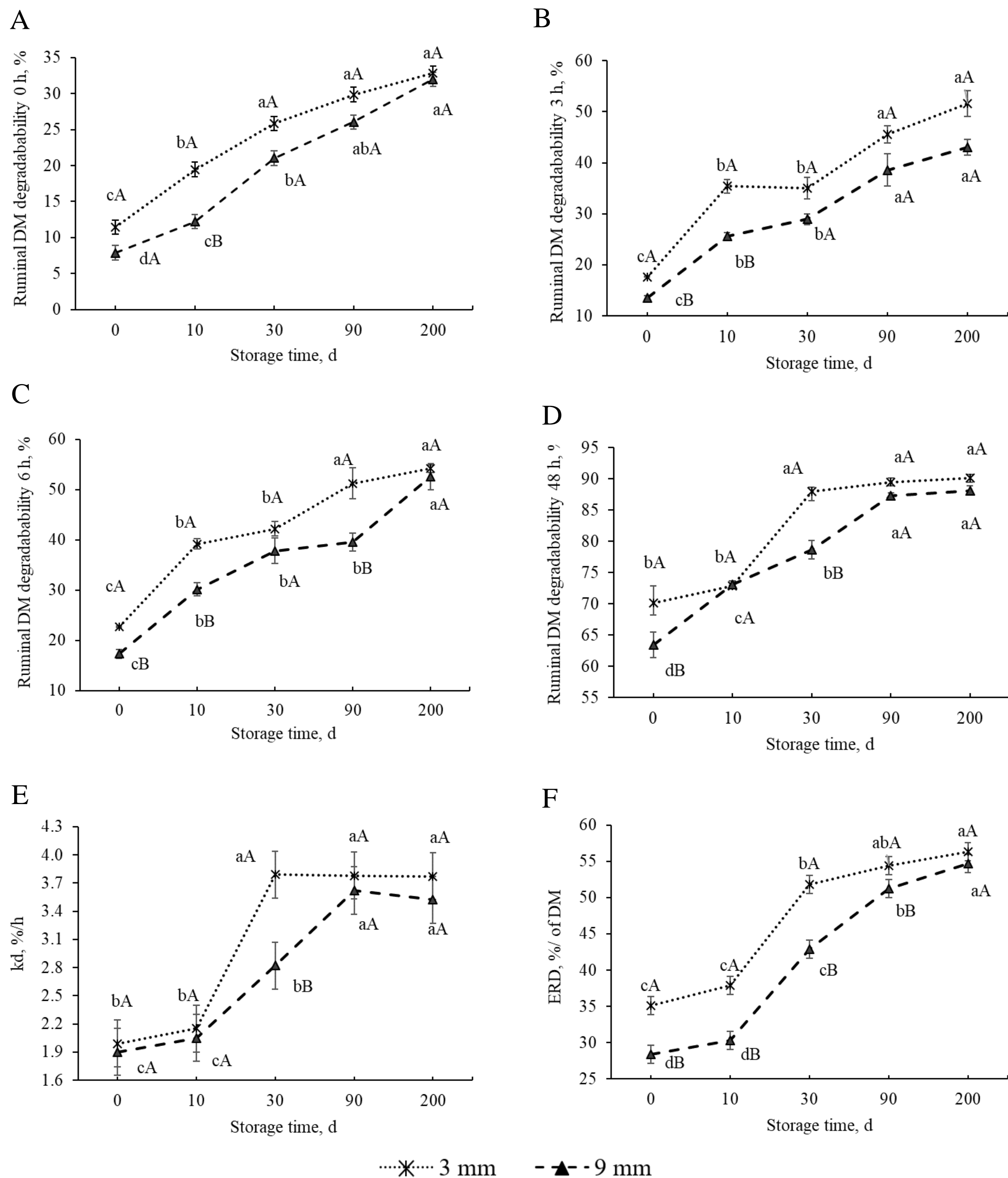

Figure 5. Kinetics of ruminal DM degradation of rehydrated corn grain silage ground at 3-mm or 9-mm during storage times. (A): SEM = 2.32; Effect of particle size (PS), $P < 0.01$; effect of storage time (T), $P < 0.01$; interaction between PS and T (PS x T), $P < 0.01$.(B): SEM = 2.55; PS, $P <0.01$; T, $P < 0.01$; PS x T, $P = 0.05$. (C): SEM = 2.48; PS, $P < 0.01$; T, $P < 0.01$; PS x T, $P = 0.03$. (D): SEM = 2.40; PS, $P < 0.01$; T, $P < 0.01$; PS x T, $P = 0.04$. (E): SEM = 0.14; PS, $P = 0.06$; T, $P < 0.01$; PS x T, $P < 0.01$. (F): SEM = 0.80; PS, $P < 0.01$; T, $P < 0.01$; PS x T, $P < 0.01$. Means with different letters differ statistically by Tukey test ($P<0.05$). Lowercase letters represent time and capital letters particle size.