UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

IMPACTO DO ESTÁDIO DE MATURIDADE, DO USO DE INOCULANTE BACTERIANO E DO TEMPO DE ARMAZENAGEM SOBRE A QUALIDADE DA SILAGEM DE MILHO

Luis Gustavo Rossi

Zootecnista

2020

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Orientador: Prof. Dr. Ricardo Andrade Reis

Coorientador: Prof. Dr. Carlos Henrique Silveira Rabelo

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Campus de Jaboticabal, como parte das exigências para a obtenção do título de doutor em Zootecnia

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: IMPACTO DO ESTÁDIO DE MATURIDADE, DO USO DE INOCULANTE BACTERIANO E DO TEMPO DE ARMAZENAGEM SOBRE A QUALIDADE DA SILAGEM DE MILHO

AUTOR: LUÍS GUSTAVO ROSSI
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Jaboticabal, 21 de setembro de 2020

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LUIS GUSTAVO ROSSI – nascido no dia 6 de maio de 1987, na cidade de Jardinópolis - São Paulo, filho de José Luis Rossi e Aparecida de Jesus Rodighero Rossi. No mês de março do ano de 2007, iniciou o curso de Zootecnia na Faculdade de Ciências Agrárias e Veterinárias – Unesp Jaboticabal, e obteve o título de Zootecnista em março de 2012. Durante a graduação foi bolsista de Iniciação Científica pela agência de fomento Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) e participou do Grupo PET Zootecnia nesta instituição de ensino. No mês de março de 2012, obteve bolsa de treinamento técnico nível III, pela FAPESP, auxiliando em projetos de pesquisa no setor de Digestibilidade da Faculdade de Ciências Agrárias e Veterinárias – Unesp Jaboticabal, sob orientação da Profa. Dra. Telma Teresinha Berchielli. No mês de março de 2013 ingressou no curso de Mestrado do Programa de Pós-Graduação em Zootecnia da Faculdade de Ciências Agrárias e Veterinárias – Unesp Jaboticabal, sob orientação da Profa. Dra. Telma Teresinha Berchielli. Em agosto de 2016 ingressou no curso de Doutorado do Programa de Pós-Graduação em Zootecnia da Faculdade de Ciências Agrárias e Veterinárias – Unesp Jaboticabal, sob orientação do Prof. Dr. Ricardo Andrade Reis, onde foi bolsista pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

“Apressa-te a viver bem e pensa que cada dia é, por si só, uma vida”

Lucius Annaeus Seneca

DEDICO

AOS MEUS PAIS,

JOSÉ LUIS ROSSI
APARECIDA DE JESUS RODIGHERO ROSSI
Pelos conselhos e ensinamentos.

ÀS MINHAS IRMÃS,

GEISA APARECIDA ROSSI (*In memorian*)
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"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto intitulado **"Impacto do estádio de maturidade, inoculantes bacterianos e tempos de armazenagem sobre a qualidade da silagem de milho"**, protocolo nº 006764/17, sob a responsabilidade do Prof. Dr. Ricardo Andrade Reis, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de junho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 04 de maio de 2017.

Vigência do Projeto	15/06/2017 a 31/12/2018
Espécie / Linhagem	Ovina - Dorper / Santa Inês
Nº de animais	108
Peso / Idade	20 kg
Sexo	Fêmea
Origem	Setor de Forragicultura – FCAV – UNESP

Jaboticabal, 04 de maio de 2017.

Profª Drª Lizandra Amoroso
Coordenadora – CEUA

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IMPACT OF MATURITY STAGE, USE OF BACTERIAL INOCULANT AND STORAGE TIME ON THE QUALITY OF CORN SILAGE

ABSTRACT - This study aimed to evaluate the impact of the maturity stage, bacterial inoculation and storage time in fermentation, aerobic stability and nutritional characteristics of flint corn silage, in addition to evaluate the impact of prolonged storage and inoculation of flint corn silage with *Lactobacillus buchneri* in the metabolic and performance responses of lambs. For this purpose, two studies were carried out. The first consisted of harvesting a flint corn hybrid very early, early and medium (at 250, 300 and 350 g dry matter (DM)/kg as fed, respectively) and ensiled in mini-silos without (control) or with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage for 120, 240 and 360 days to investigate how these factors interact with each other. The second study, was harvested with 315 g dry matter (DM)/kg and ensiled in concrete pipe silos (n = 2) without (control) or with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage for 120, 240 and 360 d. Such silages were used to formulate six different diets. Sixty non-castrated male Dorper × Santa Inês lambs were used in the feeding program. Additionally, eight ruminally cannulated Dorper × Santa Inês crossbred lambs were used to measure the ruminal fermentation. In spite of reducing silage digestibility, the harvest of whole-crop flint corn with 300 to 350 g/kg DM is desirable to have higher DM yield and starch accumulation. Inoculation with *Lactobacillus buchneri* preserved the silage against aerobic deterioration. The productivity responses of lambs were enhanced by increasing the storage length of flint corn silage, but inoculation with *L. buchneri* did not contribute to improve animal performance.

Keywords: animal performance, flint corn silage, *Lactobacillus buchneri*, maturity, storage length, starch digestion

IMPACTO DO ESTÁDIO DE MATURIDADE, DO USO DE INOCULANTE BACTERIANO E DO TEMPO DE ARMAZENAGEM SOBRE A QUALIDADE DA SILAGEM DE MILHO

RESUMO - Objetivou-se avaliar o impacto do estágio de maturidade, inoculação bacteriana e tempo de armazenamento na fermentação, estabilidade aeróbia e características nutricionais da silagem de milho flint, além disso, avaliar o impacto do armazenamento prolongado e inoculação de silagem de milho flint com *Lactobacillus buchneri* nas respostas metabólicas e de desempenho de cordeiros. Para tanto foram realizados dois estudos. O primeiro, consistiu em colher um híbrido de milho flint muito cedo, cedo e mediano (250, 300 e 350 g de matéria seca/kg, respectivamente) e ensilado em mini-silos sem (controle) ou com *Lactobacillus buchneri* CNCM I-4323 a 1×10^5 ufc/g de forragem fresca por 120, 240 e 360 dias para investigar como esses fatores interagem entre si. O segundo estudo, um híbrido de milho flint foi colhido com 315 g de matéria seca/kg e ensilado em silos de tubos de concreto sem (controle) ou com *Lactobacillus buchneri* CNCM I-4323 a 1×10^5 ufc/g de forragem fresca por 120, 240 e 360 dias. Essas silagens foram utilizadas para formular seis dietas diferentes. Sessenta cordeiros machos não castrados Dorper × Santa Inês foram utilizados no programa de alimentação. Adicionalmente, oito cordeiros mestiços Dorper × Santa Inês canulados no rúmen foram usados para medir a fermentação ruminal. Apesar de reduzir a digestibilidade da silagem, a colheita de milho flint integral com 300 a 350 g/kg de matéria seca é desejável para maior produtividade de matéria seca e acúmulo de amido. A inoculação com *Lactobacillus buchneri* é recomendada para preservar a silagem contra a deterioração aeróbia. As respostas de produtividade dos cordeiros aumentaram com o aumento do tempo de armazenamento da silagem de milho flint, mas a inoculação não contribuiu para melhorar o desempenho animal.

Palavras chave: desempenho animal, digestão de amido, estádio de maturidade, *Lactobacillus buchneri*, silagem de milho flint, tempo de armazenamento

CAPITULO 1 - REVISÃO DE LITERATURA

1. Introdução

No Brasil, o milho na forma de silagem é a principal opção para a alimentação animal. A cultura se destaca para essa finalidade por ser uma alternativa economicamente viável e apresentar um bom rendimento de massa verde, excelente qualidade de fermentação e manutenção do valor nutritivo da massa ensilada (de Melo et al., 2019).

Os híbridos de milho cultivados no Brasil têm predominantemente grãos flint e maior proporção associada ao endosperma vítreo (Bernardes et al., 2018). São largamente utilizados por apresentarem maior resistência ao ataque de pragas e doenças, porém, o grão de milho destes híbridos possui maior resistência ao ataque dos microrganismos ruminais quando comparado com os híbridos dentados (Philippeau et al., 1999; Menezes et al., 2017), reduzindo a digestibilidade do amido (Correa et al., 2002). Desta maneira, a busca por estratégias que melhorem a eficiência de utilização do amido e da silagem de milho como um todo visando otimizar a produção animal se faz necessária.

Estudos têm apontado que o estádio de maturidade (Der Bedrosian et al., 2012; Windle et al., 2014; Horst et al., 2020), utilização de inoculantes bacterianos (Hu et al., 2009; Saylor et al., 2020) e o tempo de armazenagem da silagem (Daniel et al., 2015; Carvalho et al., 2017; Hristov et al., 2020) alteram o processo fermentativo, podendo também ter efeito direto sobre a disponibilidade do amido do grão. De maneira geral e considerando-se cada um destes pontos, sabe-se que com o avanço do estádio de maturidade da planta de milho, há uma redução na digestibilidade do amido devido à maior vitreosidade do grão (Correa et al., 2002; Szasc et al., 2007; Windle et al., 2014). Além disso, observa-se maior complexidade da interação entre a matriz proteica (zeínas hidrofóbicas classificadas como prolamina) e os grânulos de amido em plantas mais maduras comparativamente àquelas colhidas com maior umidade (Hoffman et al., 2011).

Visando maximizar a digestibilidade do amido, Daniel et al. (2015) recomendaram que o tempo mínimo de armazenagem da silagem de milho deve ser de 120 dias. Aliado ainda ao tempo de armazenagem, Hallada et al. (2008) apontaram

que a digestibilidade da fibra em detergente neutro (FDN) da silagem de milho aumentou conforme o tempo de armazenagem aumentou. Sabe-se que durante o processo fermentativo, alguma degradação da hemicelulose é esperada devido à hidrólise ácida e/ou ação enzimática de alguns microrganismos (McDonald et al., 1991).

Do mesmo modo, inoculantes bacterianos parecem influenciar o processo fermentativo e a digestibilidade da silagem de maneira diferente quando testados em diferentes estádios de maturidade (Hu et al., 2009; Rabelo et al., 2014). Além disso, a interação entre inoculação bacteriana, estádio de maturidade e tempos de armazenagem não tem sido objeto de investigação em silagens produzidas em condições tropicais.

Diante deste cenário, evidencia-se a necessidade de novas pesquisas que vislumbrem maximizar a utilização de silagens de milho por meio da avaliação da interação entre estádio de maturidade da planta no momento da colheita para ensilagem e inoculação bacteriana, bem como estabelecer o tempo ideal de armazenagem para fornecimento aos animais.

2. Revisão de literatura

2.1. Estádio de maturidade da planta

A silagem de milho apresenta vantagens, como boa palatabilidade e alto valor nutritivo. No entanto, a maturidade na colheita pode afetar a qualidade da fermentação e a composição dos nutrientes (Wang et al., 2015).

Um material colhido com baixos teores de matéria seca (MS) favorece o crescimento de bactérias do gênero *Clostridium*, as quais promovem a proteólise e, consequentemente, produção de nitrogênio amoniacal. O crescimento destas bactérias ocorre em teores de umidade acima de 72% e pH em torno de 5,5 (Mcdonald, 1991). Por outro lado, a ensilagem de milho com alto teor de matéria seca pode trazer problemas decorrentes da dificuldade de compactação, aumento da porosidade da silagem, diminuição da densidade, retenção de oxigênio e desenvolvimento de fungos (Nussio et al., 2001).

O teor de MS da cultura ensilada, assim como tipo de silo, grau de compactação e processamento físico da forragem, também influenciam na produção de efluente

(Bal et al., 2000). Perdas por efluente são maiores quando a forragem apresenta alto teor de umidade. No corte precoce (menor que 30% MS), as plantas não possuem os grãos devidamente formados ou cheios, porque a porcentagem de água ainda é alta, resultando em perdas por efluente (Factori et al., 2008). Em forragens ensiladas com aproximadamente 30% de MS, a produção de efluente pode ser pouco significativa (Haigh, 1999). Já em estágios mais tardios (42% MS) a silagem tem menor valor nutritivo, prejudica o processamento dos grãos e prejudica a compactação havendo maiores perdas de MS (Factori et al., 2008).

A literatura sugere teores entre 30 a 35% de matéria seca da planta no momento da ensilagem (Nussio et al., 1999), no entanto, as recomendações do estádio ideal da colheita da lavoura são frequentemente contestáveis e, nos dias atuais, atenções estão voltados ao maior teor enérgico no alimento, o que caracteriza a colheita da lavoura em estádio em que a deposição de amido esteja em fase final ou já tenha ocorrido em sua totalidade (Marafon et al., 2015).

Durante a maturação da planta de milho, os açúcares nos grãos são convertidos em amido e o conteúdo de MS nos núcleos aumentam (Allen et al., 2003). Portanto, a maturação tardia na colheita foi sugerida como uma ferramenta de gestão para aumentar os rendimentos de MS e amido por hectare (Owens e Basalan, 2012).

O atraso na colheita, com maturação avançada pode prejudicar a digestibilidade da silagem de planta inteira de milho (Allen et al., 2003; Ferraretto e Shaver, 2012), principalmente a digestibilidade da FDN (Row et al., 2016; Arriola et al., 2017). Logo, nem sempre a maior proporção de grãos na forragem conferirá qualidade à silagem, indicando que o valor nutritivo também está associado à qualidade da fração vegetativa, como o colmo e folhas (Horst et al., 2015).

O atraso na colheita também pode prejudicar a utilização da fração de amido do grão pelo animal, que está relacionado com o aumento na proporção de endosperma vítreo no núcleo (Correa et al., 2002) e, correspondentemente, pode limitar a porcentagem de grãos fraturados durante o processamento de silagem de milho de planta inteira (Ferraretto et al., 2018).

Dois polímeros de glicose formam o amido, amilose (22% a 28%) e amilopectina (72% a 78%). Esses dois polímeros são diferentes em estrutura química, tamanho da molécula e propriedades químicas (Menezes et al., 2017). A amilopectina

se constitui na parte mais organizada e cristalina dos grânulos, mais densa e que oferece maior resistência à penetração de água ou ação enzimática. A amilose se constitui na parte menos organizada ou amorfa e menos densa que a cristalina, podendo a água mover-se livremente através dela.

Os grânulos de amido são interligados e envoltos por uma camada ou matriz proteica. A digestibilidade do amido é inversamente proporcional ao seu teor de amilose, em virtude de interações desta com a matriz proteica do grânulo de amido (Pflugfelder e Rooney, 1986).

A matriz proteica do amido no milho foi definida anteriormente como um impedimento fisico-químico para a digestão do amido em ruminantes (Owens, Zinn e Kim, 1986). No milho, as proteínas zeínas hidrofóbicas são as proteínas primárias na matriz amido-proteína, e compreendem 50 a 60% da proteína total no milho integral. Essas proteínas são classificadas como prolaminas, consistindo de 4 subclasses (α, β, γ, δ). As zeinas não são intrínsecas dentro do grânulo de amido, mas são principalmente localizadas na superfície no exterior dos grânulos de amido. À medida que se desenvolvem e se distendem com o avanço da maturidade, as ligações cruzadas de β e γ-zeínas e as α e δ-zeínas penetram em sua rede, encapsulando assim o amido numa matriz proteica de amido hidrofóbica (Hoffman et al., 2011).

Ngonyamo-Majee et al. (2009) relataram baixa digestibilidade ruminal in vitro do amido quando o milho foi colhido em intervalos entre a metade da linha do leite e a linha negra e isto foi relacionado ao aumento da proporção de endosperma vítreo em grãos de milho com maturidade avançada.

2.2. Inoculantes bacterianos

A correta preservação da silagem depende da ausência de oxigênio e a acidificação da forragem (Mcdonald et al., 1991). Uma vez aberto, é exposto ao oxigênio iniciando um processo de deterioração devido à atividade microbiana aeróbia, que pode influenciar negativamente a qualidade da silagem e a lucratividade dos sistemas de produção (Tabacco et al., 2011).

Como estratégia no combate a deterioração das silagens faz-se o uso de inoculantes microbianos, estes, contêm principalmente as bactérias ácido-láticas (BAL), que são utilizadas para melhorar a ação da população já existente na massa

que está sendo ensilada, potencializando assim, o processo fermentativo (Muck et al., 2018).

Primordialmente, os inoculantes objetivam propiciar a produção eficiente de ácidos orgânicos, acelerar o abaixamento de pH, preservar os nutrientes da forragem conservada e inibir o desenvolvimento de microrganismos indesejáveis, contribuindo dessa forma, na melhora da recuperação da MS. Também devem possuir a capacidade de inibição de atividades relacionadas as proteases e deaminases, bem como, adicionar de forma predominante os microrganismos benéficos para o processo de fermentação (Kung Jr et al., 2003).

Inoculantes contendo bactérias homofermentativas foram desenvolvidos com o objetivo de produzir ácido láctico e reduzir o pH rapidamente (Wilkinson e Davies, 2013). No entanto, as bactérias homofermentativas também foram responsáveis por reduzir estabilidade aeróbia na silagem de milho devido à baixa produção de ácidos graxos voláteis que inibem atividade fúngica e também à alta produção de ácido lático, substrato para microrganismos indesejáveis (Muck e Kung, 1997). Para esses fins, inoculantes bacterianos contendo bactérias heterofermentativas foram desenvolvidas, como o *Lactobacillus buchneri*, melhorando a estabilidade aeróbia da silagem (Wilkinson e Davies, 2012; Basso et al., 2012; Santos et al., 2016; Muck et al., 2018).

O grupo das bactérias heterofermentativas além de produzirem o ácido lático, produzem o ácido acético, etanol e CO_2. Os ácidos orgânicos, quando se trata da inibição de microrganismos, agem por meio da redução do pH ou na forma dissociada, pois dessa forma conseguem entrar na célula e causar desequilíbrio energético do microrganismo indesejável (como a levedura) presente na silagem, como exemplo ácido acético e propiônico (Silva, 2019), garantindo assim o aumento da estabilidade aeróbia da silagem. Além desta vantagem, Hoffman et al. (2011) sugeriram que a atividade proteolítica foi responsável por melhorias na digestão do amido do grão de milho com alto teor de umidade durante longos períodos de ensilagem e Kleinschmit e Kung (2006) relataram que *Lactobacillus buchneri* permaneceu razoavelmente ativo por períodos prolongados de tempo (até um ano) em silagem, mesmo em condições anaeróbias e com baixo pH.

Os inoculantes atuais requerem um mínimo de 45 a 60 dias de armazenamento antes dos benefícios substanciais para a estabilidade aeróbia, tornando-os uma

escolha ruim nessas circunstâncias onde uma silagem é fornecida após um curto armazenamento período (Borreani et al., 2018).

2.3. Tempo de armazenamento

No processo de ensilagem ocorrem várias reações bioquímicas, mediadas por enzimas e produtos metabólicos dos microrganismos, que visam manter a qualidade no processo de fermentação (Zanette et al., 2012). A estabilidade fermentativa da silagem acontece quando a massa ensilada atinge o pH necessário para que ocorra a diminuição das atividades microbianas (Anjos et al., 2018).

Embora o pH da silagem estabilize em uma semana, vários estudos relataram aumento da digestibilidade do amido em períodos prolongados de armazenamento (Newbold et al., 2006; Kung, 2013; Carvalho et al., 2017). Quando comparados os tipos de híbridos na ensilagem, Fernandes (2014) não observou diferenças na digestibilidade do amido após 60 dias de armazenamento entre híbridos flint ou dentado. Assim, a vitreosidade do grão continua sendo importante para a seleção dos tipos de grãos a serem laminados, moídos e alimentados como grão de milho seco, mas não para silagens (Owens, 2008).

O armazenamento por períodos mais longos antes da abertura do silo, é uma estratégia para melhorar a digestibilidade da silagem de milho, especialmente se o milho duro é colhido com maior teor de MS (Daniel et al., 2019). Como o amido e a fibra em detergente neutro (FDN) são os principais nutrientes na silagem de milho, e considerando que a digestibilidade do amido normalmente aumenta durante a ensilagem (Daniel, Junges e Nussio, 2015), o teor de amido e a digestibilidade da FDN parecem ser mais relevantes do que a vitreosidade do grão entre as características nutricionais para a seleção de híbridos de milho para produção de silagem (Carvalho e Carbonare, 2017).

A matriz proteica que envolve os grânulos de amido em grãos de milho é composta primariamente de prolaminas, no milho essa prolamina é definida como zeína e representa fator inibitório à digestão de amido (Hoffman et al., 2011). Evidências apontam que a atividade proteolítica bacteriana pode explicar os aumentos de digestibilidade da fração amido da silagem. Em revisão realizada por Hoffman et al. (2011) é frequente a hipótese de que as proteínas hidrofóbicas (zeínas) são

degradadas no processo de ensilagem, e assim, maior acesso das bactérias do rúmen aos grânulos de amido. Proteínas como a zeína nas ligações proteína-amido são potencialmente degradas no processo de ensilagem por solubilização ou pela atividade proteolítica (Junges, 2014).

Segundo Silva (2019) o teor de MS das silagens diminuiu com o tempo da estocagem, o que pode estar relacionado com os percentuais de perdas de MS durante o período fermentativo das silagens (Oliveira et al., 2017). As perdas de MS durante a estocagem podem estar associadas ao intenso metabolismo das BAL heterofermentativas obrigatórias sobre o consumo de matéria orgânica do material ensilado, resultando em aumento de componentes não degradáveis como por exemplo, as concentrações de cinzas (Ferrero et al., 2019), assim como pelos processos proteolíticos vegetais e microbianos que acarretam em alterações dos compostos nitrogenados em silagens (Kung Jr et al., 2018).

Neste contexto, o teor de nitrogênio amoniacal durante o tempo de estocagem pode aumentar, permanecendo com teores semelhantes a partir dos 103 dias de estocagem e comumente isso ocorre em silagens mais úmidas (Kung Jr et al., 2018). Outro fator para tal resultado está associado com as quebras de subunidades de zeínas que ocorre com o tempo da estocagem, aumentando as concentrações de nitrogênio amoniacal e proteína total, como observados em outros experimentos de silagem de planta inteira de milho (Windle et al., 2014; Ferrareto et al., 2015; Ferrareto et al., 2016).

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CHAPTER 2

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

CHAPTER 2 - FLINT CORN SILAGE MANAGEMENT: INFLUENCE OF MATURITY STAGE, INOCULATION WITH *Lactobacillus buchneri*, AND STORAGE TIME ON FERMENTATION PATTERN, AEROBIC STABILITY AND NUTRITIONAL CHARACTERISTICS

Abstract

A flint corn hybrid was harvested very early, early and medium (at 250, 300 and 350 g dry matter (DM)/kg as fed, respectively) and ensiled in mini-silos without (control) or with *Lactobacillus buchneri* CNCM I-4323 at 1 × 10^5 cfu/g of fresh forage for 120, 240 and 360 d to investigate how these factors interact with each other. There was only a small increase (1.1%; P = 0.003) in starch digestibility (starch-D) in the silages stored for 360 d when compared to that stored for 240 d, but with no difference for 120 d. Despite the reduced starch-D (526 vs. 694 g/kg starch; P < 0.001), silages produced from medium harvest had higher (P < 0.001) starch content (317 vs. 137 g/kg DM) and higher amount of digestible starch (169 vs. 98.5 g/kg DM; P < 0.001) compared to very early harvest. Overall, lactic acid of corn silage decreased by harvesting more mature crops (P = 0.002), and acetic acid was higher for corn silage inoculated with *L. buchneri*, mainly in that stored for 360 d (P = 0.003). Despite changes in fermentation end-products, a three-way interaction (P = 0.009) for DM recovery did not show clear responses to the factors examined. However, the recovery of digestible DM decreased (P = 0.035) by 2.6% in corn silages inoculated with *L. buchneri*. The 2-way interactions (inoculation × storage time and maturity × storage time) showed, in general, that inoculation of corn silage with *L. buchneri* persistently increased (P < 0.001) the aerobic stability (+123 h on average compared to the control), and that more mature crop silage had higher aerobic stability (140 h; P = 0.036) than the others (118 and 48.5 h for those silages from very early and early harvest). The estimated milk yield increased (P < 0.001) as the maturity stage advanced (on average 875; 1,288 and 1,319 L/t of DM for silages produced at very early, early and medium harvest, respectively), which can be attributed to the higher starch accumulation in the corn grains (P < 0.001). In conclusion, storage for a longer time (i.e., >120 d) with the goal of increasing silage digestibility did not occur. In spite of reducing silage digestibility, the harvest of whole-crop flint corn with 300 to 350 g/kg DM is desirable to have higher

DM yield and starch accumulation, which lead to increased milk yield. Inoculation with *L. buchneri* is recommended to preserve the silage against aerobic deterioration.

Keywords: corn silage, flint hybrid, *Lactobacillus buchneri*, maturity, starch digestion, storage length

1. Introduction

Most of the corn utilized for silage production in Brazil is flint type, which is recognized to have a high vitreousness that compromises starch digestion (starch-D; Correa et al., 2002; Bueno et al., 2020) because the protein matrix surrounding the starch granules impairs ruminal microbial attachment. Ensiling has been proposed to increase starch-D (Hoffman et al., 2011), and prolonging the storage time of silage was found to effectively improve starch-D (Der Bedrosian et al., 2012; Da Silva et al., 2019). Such benefits are attributed to protein breakdown caused by the proteolytic activity of bacteria and plant enzymes (Junges et al., 2017). In this regard, a meta-analysis suggested that corn silage should be stored for at least 120 d to maximize starch utilization (Daniel et al., 2015).

As enhanced starch utilization is achieved by increasing storage time, this management is probably a feasible strategy to minimize the negative effects of the advances in plant maturity on starch-D. Indeed, starch-D is depressed by harvesting whole-crop corn with higher dry matter (DM) content (Der Bedrosian et al., 2012; Bueno et al., 2020), a physiological response attributed to the increased vitreousness of the corn grain (Philippeau and Michalet-Doreau, 1997). Conversely, despite the lowered herbage production and starch accumulation, it is known that harvesting whole-crop corn earlier than desirable (< 30% DM content) results in enhanced starch-D because the starch granules are more accessible to ruminal bacteria (Kotarski et al., 1992; Philippeau and Michalet-Doreau, 1997). Even though not desirable, livestock producers are forced to anticipate the harvest of whole-crop corn for silage production in some cases because of the lack of feed on the farm as a consequence of adverse climatic conditions that compromise feed production, lack of a feeding plan, and so on. Notably, many surveys carried out in Brazil have reported corn silages produced with DM contents lower than 30% (Oliveira et al., 2017; Giombelli et al., 2019; Santos et al.,

2020). In this case, where the harvest is anticipated, lower storage times of corn silage should be enough to ensure high starch-D once the starch granules are more susceptible to ruminal fermentation.

Moreover, silage inoculation has become increasingly common on farms, and in many cases, *Lactobacillus buchneri* is the bacterium used for improving the aerobic stability of silages. In addition to the reduction of silage spoilage in the presence of air, the inoculation of high-moisture corn (HMC) silage and rehydrated corn grain silage with *L. buchneri* was found to decrease the concentration of prolamin (Da Silva et al., 2018, 2019), and this effect was more evident in HMCs after 120 d of storage (Da Silva et al., 2019). The increased prolamin breakdown was suggested to be related to changes in the microbial community within the silo (Da Silva et al., 2019), probably directing towards those bacteria having higher proteolytic activity (Junges et al., 2017). Thus, inoculation with *L. buchneri* might be useful to increase starch-D in whole-crop flint corn silage as well.

We aimed to investigate the impact of maturity stage, bacterial inoculation, and storage time on fermentation, aerobic stability, and nutritional characteristics of flint corn silage and their implications for corn silage management. It was hypothesized that increasing storage time and using *L. buchneri* are feasible strategies to enhance starch-D of silages produced with 30–35% DM content at similar levels of that harvested very early and stored for less time.

2. Material and methods

2.1. Ethics statement

All procedures adopted in this study were performed according to Ethical Principles in Animal Experimentation from the National Council for Animal Experiment Control (CONCEA) and were approved by the Ethics Committee on the Use of Animals (CEUA) from São Paulo State University (UNESP) at a regular meeting (Protocol No. 006764/17).

2.2. Crop harvest and ensiling procedure

A flint corn hybrid (2B 710 PW, Dow AgroSciences, São Paulo, SP, Brazil) was planted at a sowing density equivalent to 54,000 seeds/ha in 0.90-m rows in fields at São Paulo State University (at Jaboticabal, SP, Brazil: 21°150S, 48°180W; altitude 615 m). One week prior to planting, herbicides (4 L Zapp®/ha and 0.5 L Select®/ha; Syngenta, Matão, SP, Brazil) and mineral oil (0.5 L/ha) were applied to the field. The sowing date was 10 November 2016, and the soil was classified as Haplustox. The fields were fertilized with 350 kg/ha of 8-28-16 (N–P–K) at planting. Thereafter, on 17 November 2016, an additional fertilizer, 300 kg/ha of 30-0-10 (N–P–K), was applied after a week of corn growth, and a further 350 kg/ha of urea was applied after 4 weeks of corn growth on 7 December 2016. Herbicides (3 L Zapp®/ha and 2 L Atrazine®/ha; Syngenta, Matão, SP, Brazil) and insecticide (0.25 L Engeo Pleno S®/ha; Syngenta, Matão, SP, Brazil) were applied after 4 weeks of corn growth on 5 December 2016. Fungicide (0.5 L Priori Xtra®/ha; Syngenta, Matão, SP, Brazil), insecticide (0.15 L Ampligo®/ha; Syngenta, Matão, SP, Brazil) and mineral oil (0.5 L/ha) were also applied after 5 weeks of corn growth on 12 December 2016. The climate where the corn was cultivated is classified as ‘Aw’ (Rolim et al., 2007) and characterized as tropical with a wet summer season and dry winter season.

On 31 January 2017, whole-crop corn (83-days growth) was randomly harvested in different locations in the field at 246 g of whole-plant DM/kg as fed (called ‘very early harvest at 250 g/kg DM’) at a stubble height of 20 cm using a pull-type New Pecus forage harvester (Nogueira, São João da Boa Vista, SP, Brazil). Forage was cut to 10 mm, and kernels were processed. The same process was repeated on 9 February 2017 (92-days growth) and 16 February 2017 (99-days growth) when the corn forage had 306 g/kg DM (called ‘early harvest at 300 g/kg DM’) and 353 g/kg DM (called ‘medium harvest at 350 g/kg DM’), respectively. To determine the DM yield and percentage of corn grains in each maturity stage, five points in the field were sampled for number of plants per linear meter, weight of plants and grains, and DM content. Thereafter, for each maturity stage in which corn plant was harvested, one pile of corn forage for each silo was randomly treated either with water (5 L/t; control) or with *Lactobacillus buchneri* (CNCM I-4323) at 1×10^5 cfu/g of fresh forage (inoculated;

Lallemand Animal Nutrition, Goiânia, GO, Brazil). The inoculant was dissolved in distilled water (5 L/t) and sprayed onto fresh forage during the filling of the silos.

Treated forages (3.21 ± 0.103 kg) were then placed into each mini-silo (n = 4). Forage packing was achieved by using wood sockets, and a final bulk density of 743 ± 23.9 kg fresh forage/m3 was obtained. PVC tubes (4.3 L) were used as mini-silos, and they were closed with plastic lids and sealed with adhesive tape. Mini-silos were stored in a barn at ambient temperature for 120, 240 and 360 d. One fresh sample of corn forage was collected from each silo during filling for chemical analysis. The same procedure was repeated when the silos were opened; both forage and silage samples were stored at −20°C. The DM recovery for each silo was calculated based on the initial and final weight and DM contents of the fresh forages and silages (Jobim et al., 2007). Moreover, the recovery of digestible DM was calculated taking into account the initial and final weight of digestible DM, which was obtained by multiplying the dry weight of forage and silage placed inside each mini-silo by its respective in vitro DM digestibility (IVDMD).

2.3. Aerobic stability

Aerobic stability was determined by placing a silage sample (1.34 ± 0.133 kg) from each mini-silo in a plastic bucket of 5 L capacity and kept in a room at ambient temperature. The silage temperature was measured every half hour by using a datalogger (ESCORT Intelligent MINI; Escort Console, Buchanan, VA, USA) placed in the center of the mass for 10 d. The ambient temperature was also measured every half hour by two dataloggers placed near the buckets. Aerobic stability was defined as the number of hours that the silage temperature remained stable before increasing more than 2°C above the ambient temperature (Moran et al., 1996). Aerobic deterioration (°C) was defined as the sum of the daily temperature increases above the ambient temperature during the first 5 d of aerobic exposure (Conaghan et al., 2010).

2.4. Sample preparation and chemical analyses

Twenty-five grams of each sample of forage or silage was mixed with 225 mL of distilled water and blended in a Phillips Walita blender (Walita, Varginha, MG, Brazil)

for 1 min at the highest setting and filtered through two layers of cheesecloth. The pH of the filtrate was measured immediately by using a pH meter (model MA522, Marconi Laboratory Equipment, Piracicaba, SP, Brazil). After the pH was measured, the filtrate was stored at −20°C for subsequent analysis of lactic acid and volatile fatty acids (acetic, propionic and butyric acids) using a high-performance liquid chromatograph (HPLC; Shimadzu model Prominence, Shimadzu Corp., Kyoto, Japan) equipped with a UV/VIS detection system and a refractive index detector (SPD-20). An apolar column (C-18 model Shimpack VP-ODS; 4.6 mm × 250 mm) was used at 35°C for chromatographic separation. The polar mobile phase consisted of a 20 mM buffer phosphate solution at pH 2.5, and acetonitrile was used as the apolar solvent. The presence of the acids was detected by UV absorbance, at a wavelength of 210 nm. Ammonia N was measured by distillation according to the AOAC (1996; method no. 941.04).

Samples of forage and silage were oven-dried (at 55°C for 72 h) and processed in a knife mill (Willey mill model 4; Arthur H. Thomas Company, Philadelphia, PA) before being ground through a 1 mm screen and analyzed for DM (105°C for 12 h) and ash (500°C for 5 h) according to the AOAC (1990; methods no., 943.01 and 924.05, respectively). Silage DM content was corrected for volatile compounds according to Weissbach (2009). Ether extract (EE) was determined according to the procedures described by the AOAC (1996; method no. 920.39). The total nitrogen (TN) was measured by rapid combustion by using a LECO Analyzer (model F528 N; LECO Corp., St. Joseph, MI, USA); crude protein (CP) was calculated as TN × 6.25. Soluble protein was determined following the procedures described by Licitra et al. (1996). The neutral detergent fiber (aNDF) and acid detergent fiber (ADF) were sequentially determined in an ANKOM200 Fiber Analyzer (ANKOM Technology Corporation, Fairport, NY, USA) following the procedures described by Mertens (2002) and AOAC (1990; method no. 973.18), respectively. For aNDF analysis, a heat-stable α-amylase was used without sodium sulfite. The aNDF and ADF were expressed inclusive of residual ash. Hemicellulose was calculated as aNDF minus ADF. Neutral detergent insoluble nitrogen (NDIN) and acid detergent insoluble nitrogen (ADIN) were determined in the residual samples of aNDF and ADF, respectively, using the LECO Analyzer. Starch was determined by an enzymatic-colorimetric assay using acetate

buffer in gelatinization solution (Hall, 2009). The 30-h IVDMD, 7-h in vitro starch digestibility (starch-D), and 30-h in vitro aNDF digestibility (aNDF-D) were determined using the approach proposed by Goering and Van Soest (1970). The milk yield per tonne of DM was estimated using the Milk2006 spreadsheet (Shaver et al., 2006).

2.5. Statistical analyses

All silage variables were analyzed as a completely randomized design with four replicates using the MIXED procedure of SAS (v 9.2; SAS Inst. Inc., Cary, NC). The maturity stage (2 degrees of freedom (DF)), bacterial inoculation (1 DF), storage time (2 DF), and their interactions were considered fixed effects, while the residual error was considered a random effect. Differences between silage means were compared using the PDIFF option of the LSMEANS command, which is based on Fisher's F-protected least significant difference test (multiple t-test comparisons). Similarly, when significant interactions between the factors examined occurred, means were also compared using the PDIFF option as described above. Outliers were identified and deleted if absolute values of studentized residuals exceeded ± 3. Significant differences were declared at $P \leq 0.05$. Significant differences for the main effects assessed in this study (i.e., maturity stage, bacterial inoculation, and storage time) are shown in figures only whether significant interactions between them did not occur (i.e., maturity stage × bacterial inoculation, maturity stage × storage time, bacterial inoculation × storage time, and maturity stage × bacterial inoculation × storage time); variables unaffected by treatments were only described in the text.

3. Results

3.1. Agronomic characteristics and chemical composition of whole-crop corn prior to ensiling

Whole-crop corn had a higher DM yield and percentage of grains according to the advanced maturity stage (Table 1). Corn forage harvested very early, early and medium had 246, 306 and 353 g/kg DM, respectively. Overall, starch content increased with advances in corn plant maturity, while soluble CP, IVDMD, starch-D, and aNDF-D decreased.

Table 1. Characteristics of whole-corn crop prior to ensiling (data are given in g/kg DM, unless otherwise stated; mean ± SD) as influenced by maturity and bacterial inoculation[1].

Item	Whole-crop corn at ensiling					
	Very early (250 g/kg DM)		Early (300 g/kg DM)		Medium (350 g/kg DM)	
	Control	*L. buchneri*	Control	*L. buchneri*	Control	*L. buchneri*
Forage characteristics						
DM yield, t MS/ha	13.2 ± 0.896		15.7 ± 1.23		16.9 ± 0.945	
Grains, g/kg DM	272 ± 30.6		380 ± 44.4		449 ± 56.8	
Bulk density, kg/m^3						
Wet	750 ± 12.1	769 ± 21.6	745 ± 17.9	754 ± 14.3	709 ± 26.4	729 ± 17.2
DM	184 ± 4.82	189 ± 4.49	227 ± 5.72	232 ± 5.35	248 ± 9.07	258 ± 10.6
Nutritional value index						
pH	5.69 ± 0.080	5.66 ± 0.050	5.91 ± 0.053	6.04 ± 0.330	6.06 ± 0.105	6.22 ± 0.343
Ammonia-N, g/kg TN	46.5 ± 0.580	46.5 ± 0.420	34.1 ± 0.930	33.9 ± 0.350	28.1 ± 0.930	27.0 ± 0.570
DM, g/kg as fed	246 ± 4.54	246 ± 3.91	305 ± 4.56	307 ± 4.29	351 ± 6.79	354 ± 7.81
Ash	38.0 ± 0.900	37.5 ± 0.810	36.6 ± 1.85	36.1 ± 1.69	30.4 ± 1.91	29.7 ± 1.96
CP	109 ± 7.87	111 ± 5.49	93.6 ± 7.11	96.1 ± 14.6	83.3 ± 12.2	91.0 ± 6.44
Soluble protein, g/kg CP	403 ± 12.3	405 ± 10.1	395 ± 14.9	390 ± 9.94	355 ± 5.06	358 ± 12.6
EE	32.6 ± 0.810	33.6 ± 0.560	36.2 ± 0.320	35.6 ± 0.480	37.3 ± 0.300	37.1 ± 0.450
aNDF	483 ± 7.20	479 ± 3.40	497 ± 10.3	486 ± 10.2	516 ± 10.2	516 ± 4.77
aNDF-D, g/kg	595 ± 27.3	607 ± 22.7	541 ± 14.4	571 ± 13.2	524 ± 15.7	535 ± 19.0
ADF	247 ± 4.82	247 ± 5.59	256 ± 5.64	256 ± 5.25	271 ± 5.32	271 ± 5.44
Hemicellulose	237 ± 8.25	232 ± 8.14	241 ± 11.0	230 ± 9.91	246 ± 10.8	245 ± 5.49
Starch	158 ± 7.41	165 ± 4.96	288 ± 12.1	293 ± 7.36	353 ± 7.21	356 ± 10.0
Starch-D, g/kg	664 ± 21.2	649 ± 15.6	555 ± 6.63	588 ± 5.83	474 ± 18.2	476 ± 15.3
Digestible starch	105 ± 8.30	107 ± 5.19	164 ± 3.55	171 ± 3.89	167 ± 5.96	169 ± 5.22
IVDMD, g/kg	708 ± 12.0	724 ± 6.10	650 ± 25.5	658 ± 12.5	534 ± 12.6	552 ± 19.6
TDN_{-1x}, g/kg	662 ± 16.9	677 ± 17.1	679 ± 11.2	698 ± 8.01	669 ± 9.19	674 ± 12.2
NE_{L-3x}, Mcal/kg DM	1.40 ± 0.038	1.44 ± 0.033	1.48 ± 0.023	1.52 ± 0.017	1.47 ± 0.018	1.48 ± 0.021

Milk yield, kg/t DM	1,307 ± 54.2	1,354 ± 50.7	1,398 ± 33.8	1,450 ± 25.1	1,376 ± 26.9	1,386 ± 33.7

[1]Corn forage was treated at ensiling either without (control) or with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g fresh forage.
*DM = dry matter; TN = total nitrogen; CP = crude protein; EE = ether extract; aNDF = neutral detergent fiber; aNDF-D = NDF digestibility; ADF = acid detergent fiber; Starch-D = starch digestibility; IVDMD = in vitro dry matter digestibility; $TDN_{-1\times}$ = total digestible nutrients at 1× maintenance; $NE_{L-3\times}$ = net energy for lactation at 3× maintenance.

3.2. Fermentation, chemical composition, and aerobic stability of corn silages

The DM content of corn silage was affected by the interaction between all factors assessed (Fig. 1). As expected, DM content increased (P < 0.001) according the maturity stage advanced. With few exceptions, silages stored for 240 and 360 d had higher (P < 0.001) DM content than that stored for 120 d, and in general, inoculation did not increase the DM content of silages.

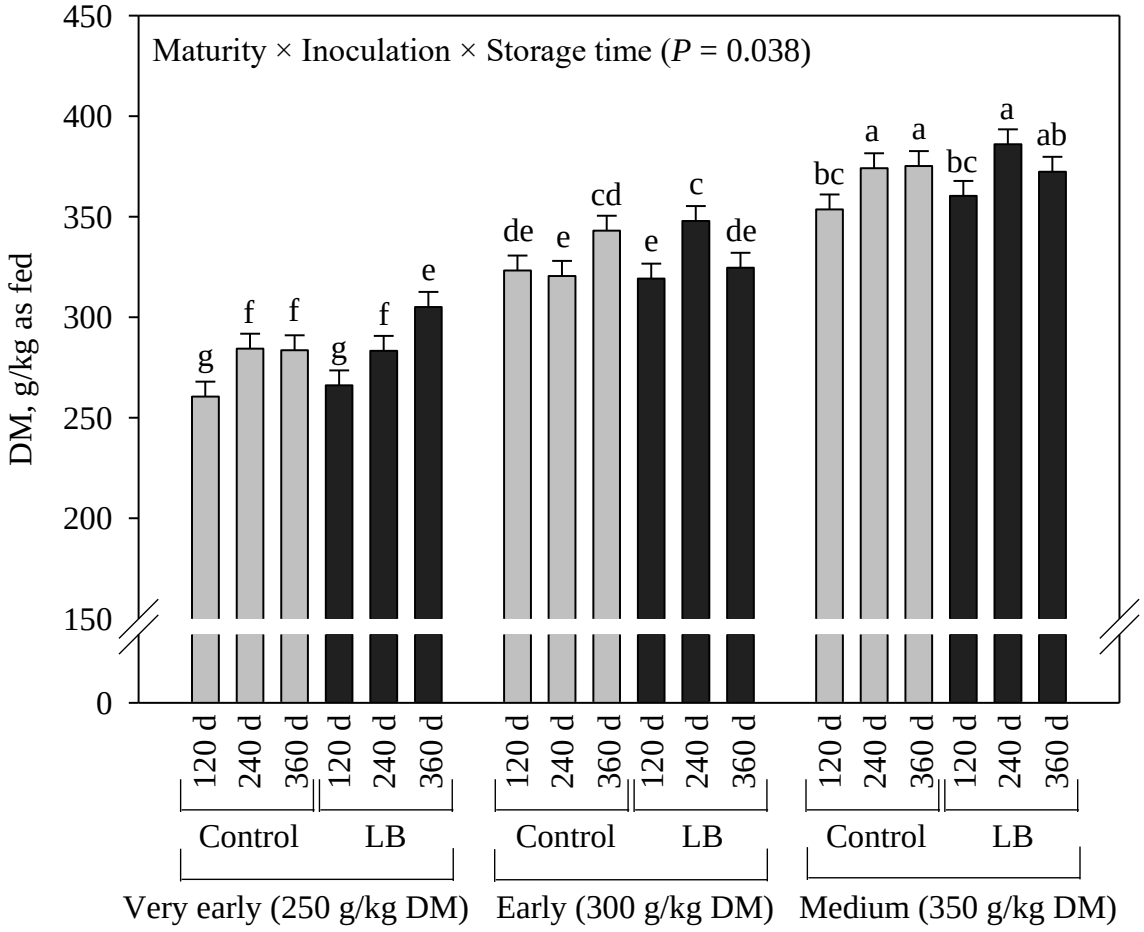

Fig. 1. Dry matter content of corn silage as influenced by the interaction among maturity, inoculation and storage time (LB = *Lactobacillus buchneri*).

Inoculation of corn silage with *L. buchneri* resulted in higher CP preservation (+5.9%; P = 0.018) compared to the control silage, and the CP content decreased as the storage time increased (P < 0.001; Fig. 2A). Compared with the corn silage

produced very early, the soluble CP decreased ($P < 0.001$) by –8.9% and –12.8% for those harvested early and at medium, respectively (Fig. 2B). The soluble CP increased by 3.3% ($P < 0.001$) following silage inoculation and increased ($P < 0.001$) by 7.7% and 9.3% after 240 and 360 d of storage compared to the silage stored for 120 d (Fig. 2B). With the exception of corn silages produced from plants harvested very early, increasing the storage time led to increased ammonia-N ($P = 0.009$) in the other silages; also, silage inoculation increased ammonia-N by 13.2% ($P < 0.001$; Fig. 2C).

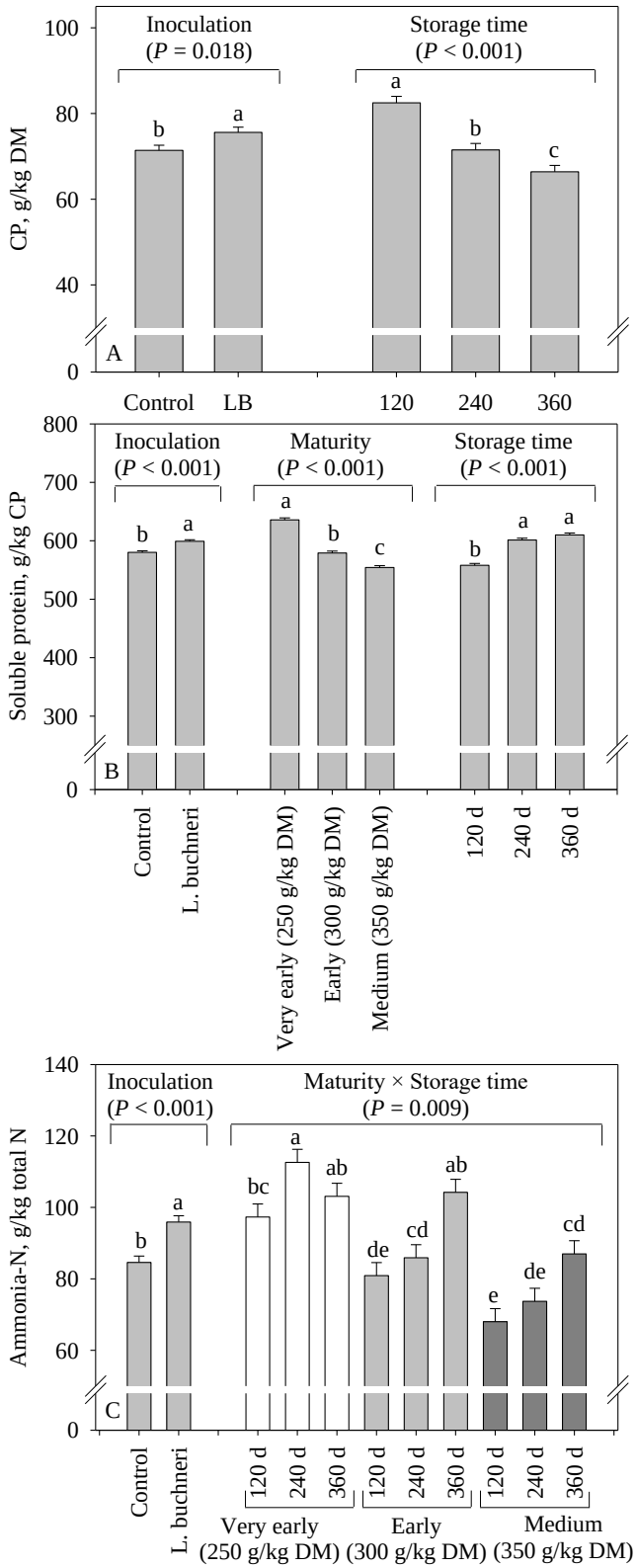

Fig. 2. Crude protein content (A), soluble CP (B) and ammonia-N (C) of corn silage as influenced by inoculation, maturity, storage time (days), and its interaction (LB = *Lactobacillus buchneri*).

There was a small (1.3%) but significant ($P = 0.017$) increase in aNDF content following silage inoculation; overall, aNDF of corn silage increased by advancing plant maturity and decreased as the silages remained more time stored ($P = 0.011$; Fig. 3A). Inoculation resulted in higher ($P = 0.002$) aNDF-D of corn silages produced early (+3.9%) and at medium (+6.0%), a response not observed for very early harvest; overall, aNDF-D lowered owing advances in maturity stage and storage time ($P = 0.024$; Fig. 3B). Inoculation decreased the ADF content by 2.7% ($P = 0.034$), while advances in maturity and increasing storage time resulted in higher ADF content ($P < 0.001$; Fig. 3C). Hemicellulose increased ($P < 0.001$) by 8.4% due to silage inoculation and decreased consistently with storage length ($P < 0.001$; Fig. 3D).

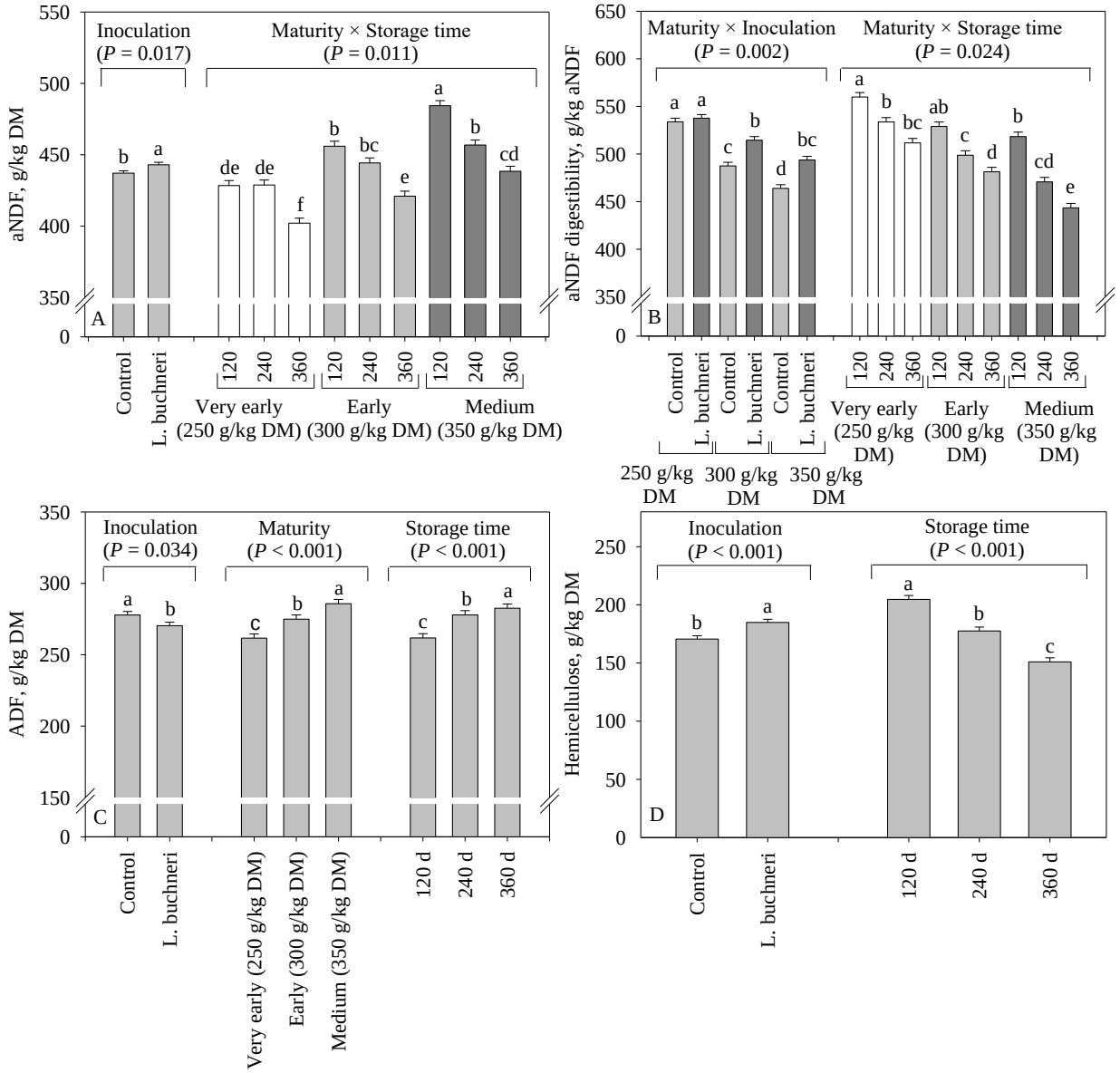

Fig. 3. Neutral detergent fiber (A), NDF digestibility (B), acid detergent fiber (C), and hemicellulose (D) content of corn silage as influenced by inoculation and interaction between maturity and storage time (days).

Starch content increased ($P < 0.001$) from 137 g/kg DM in the silage produced with plants harvested very early to 317 g/kg DM in that harvested at medium (Fig. 4A). Moreover, in comparison with 120 d of storage, the starch content decreased by 2.7% in corn silages stored for 360 d ($P < 0.001$). As the maturity stage advanced, starch-D decreased ($P < 0.001$) from 694 to 526 g/kg starch; there was a small increase (1.1%; $P = 0.003$) in starch-D in the silages stored for 360 d when compared to those stored for 240 d, but with no difference to 120 d (Fig. 4B). The digestible starch of corn silage increased ($P < 0.001$) from 98.5 g/kg DM at the very early harvest to 169 g/kg DM at medium harvest (Fig. 4C). Furthermore, the digestible starch was reduced ($P = 0.005$) by inoculation of corn silage stored for 360 d, a response not observed for the other storage times. Contents of ash (on average 41.9 g/kg DM; $P \geq 0.75$), NIDN (on average 139 g/kg total N; $P \geq 0.07$) and ADIN (on average 46.4 g/kg total N; $P \geq 0.20$) were not affected by maturity stage, inoculation, or storage time.

There was an interaction ($P = 0.009$) between all the factors investigated in this study for DM recovery (Fig. 5). However, the results were very variable depending on the maturity stage, bacterial inoculation and storage time, with no clear tendency being observed. The IVDMD slightly increased (1.0%; $P = 0.027$) due to silage inoculation and increased by 1.4% in the corn silage stored for 360 d in comparison with that stored for 120 d ($P = 0.033$); moreover, IVDMD decreased from 705 g/kg DM in the silage produced at very early harvest to 510 g/kg DM at medium harvest ($P < 0.001$; Fig. 6A). The recovery of digestible DM decreased ($P = 0.035$) by 2.6% in corn silages inoculated with *L. buchneri* (Fig. 6B).

Silage pH was only affected by maturity ($P < 0.001$), and values ranged from 3.17 (early harvest) to 3.45 (medium harvest; Fig. 7A). Overall, lactic acid of corn silage decreased by harvesting more mature plants, and inoculation resulted in a higher lactic acid concentration in comparison to the control ($P = 0.002$; Fig. 7B). The 2-way interaction showed that acetic acid was higher for corn silage stored for 360 d and inoculated with *L. buchneri* ($P = 0.003$); moreover, as the storage time increased, the acetic acid increased in the silages produced with more mature crops, but the silages stored for 120 d showed lower acetic acid as the maturity advanced ($P < 0.001$; Fig. 7C). The concentration of propionic acid was higher ($P < 0.001$) in corn silages stored for 240 and 360 d (13.3 g/kg DM on average) in comparison with that stored for 120 d

(9.30 g/kg DM); also, there was an interaction between inoculation and maturity stage, in which the inoculated silage produced at very early harvest had a higher ($P = 0.008$) concentration of propionic acid (1.51% DM) than the others (Fig. 7D).

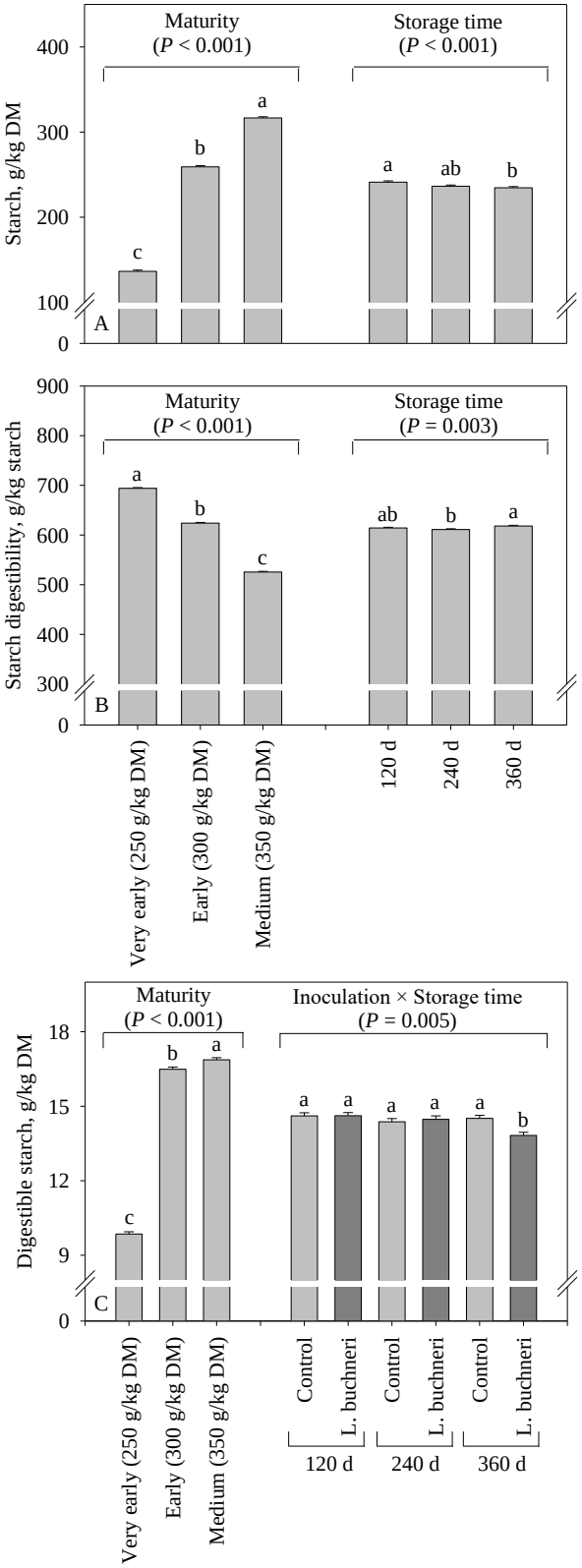

Fig. 4. Starch content (A), starch digestibility (B), and digestible starch (C; starch content × starch digestibility) of corn silage as influenced by inoculation, maturity, storage time and its interaction.

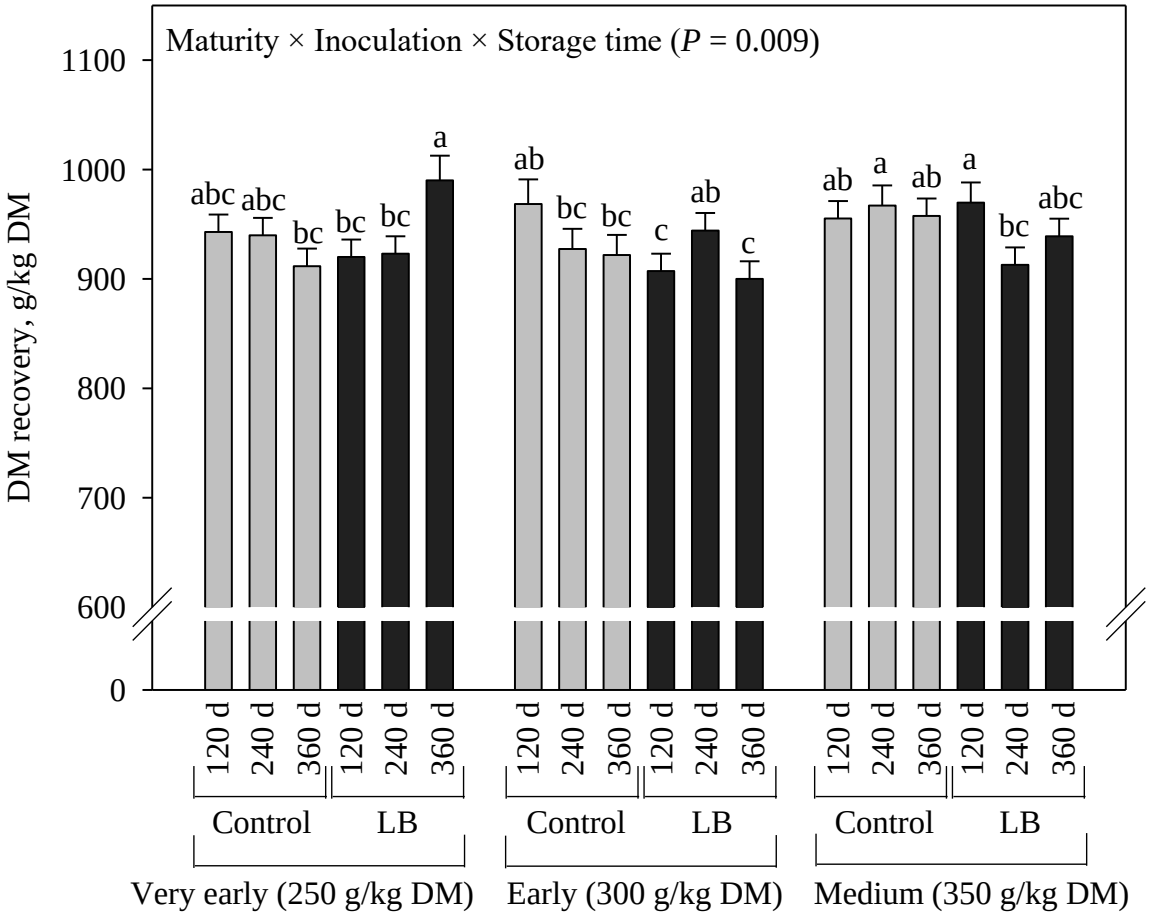

Fig. 5. Dry matter recovery of corn silage as influenced by inoculation and the interaction between maturity and storage time (LB = *Lactobacillus buchneri*).

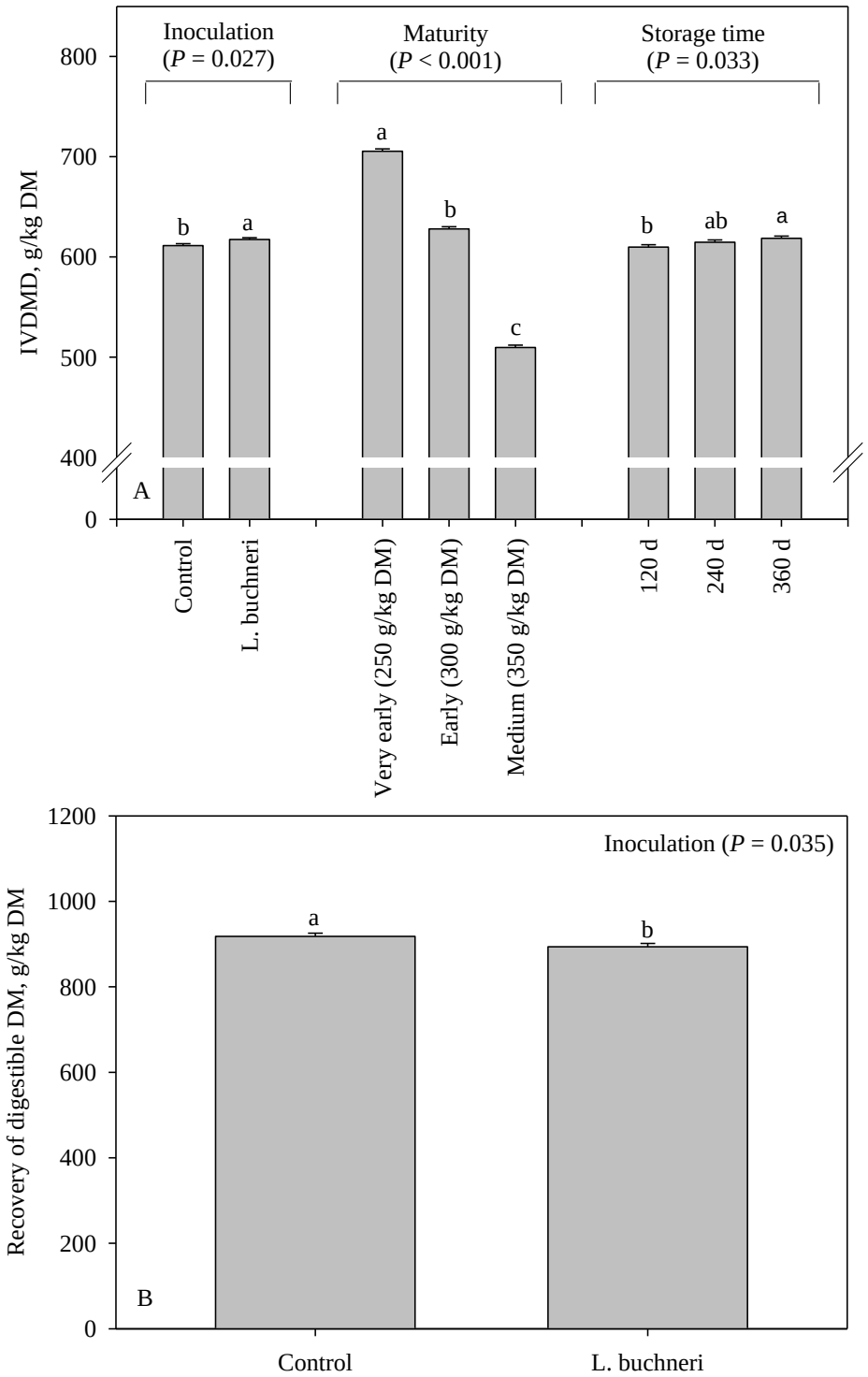

Fig. 6. In vitro dry matter digestibility (A) and recovery of digestible DM (B) of corn silage as influenced by inoculation, maturity, and storage time.

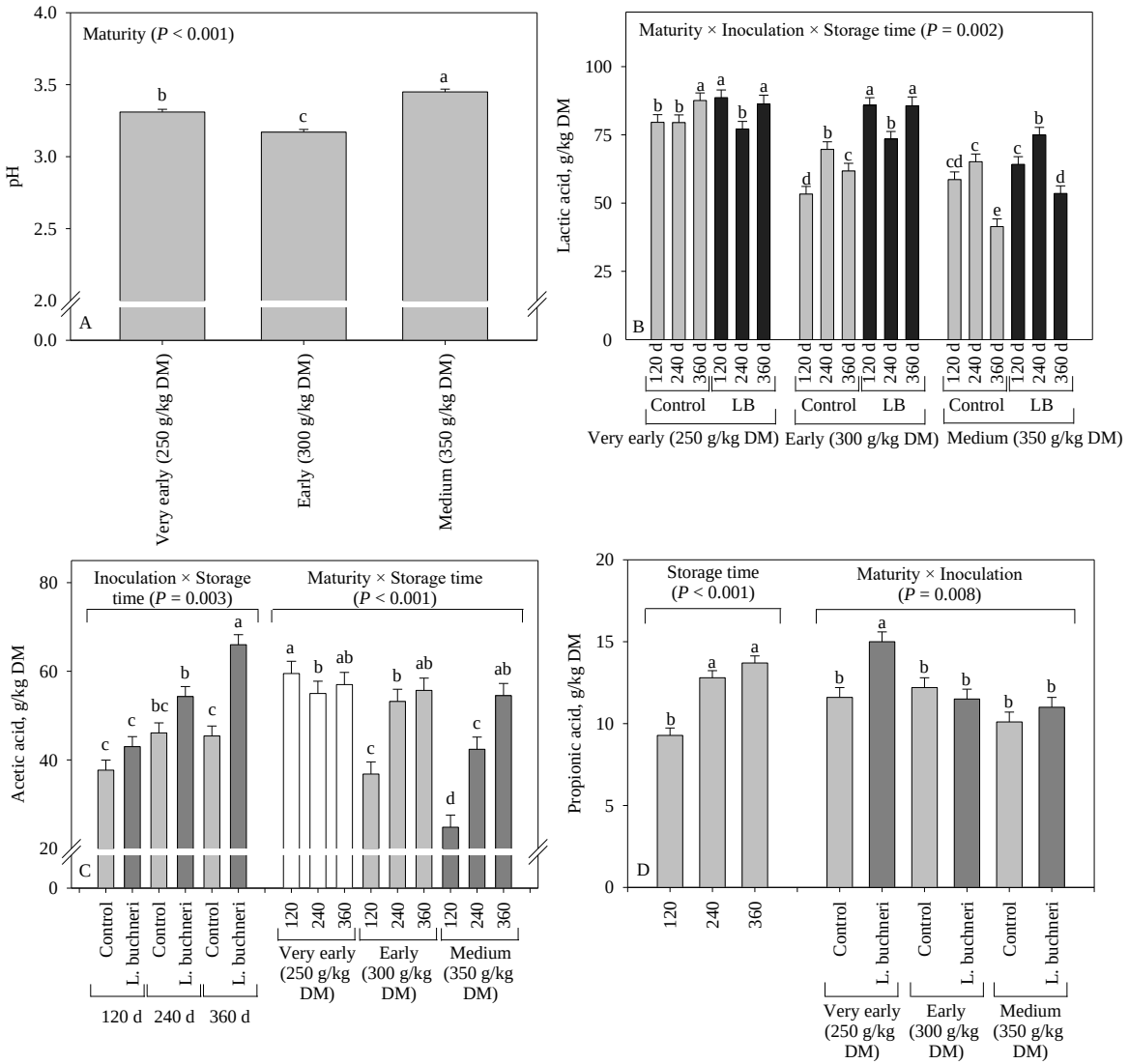

Fig. 7. Silage pH (A) and concentrations of lactic (B), acetic (C), and propionic (D) acids of corn silage as influenced by inoculation, maturity, storage time (days), and its interaction.

The aerobic stability of corn silage was affected by the interaction between inoculation and storage time, in which inoculation of corn silage with *L. buchneri* persistently increased ($P < 0.001$) the aerobic stability (+123 h on average compared to the control; Fig. 8A). Furthermore, the 2-way interaction (maturity × storage time) showed, in general, that more mature crop silage had higher aerobic stability (140 h; $P = 0.036$) than the others (118 and 48.5 h for those silages from very early and early harvest) and that the storage time had low impact on the aerobic stability of silages. There was a 3-way interaction for the aerobic deterioration, in which it was consistently lowered in the inoculated corn silages, with the exception of the silage produced at early harvest and stored for 120 d ($P < 0.001$; Fig. 8B).

There was an interaction between maturity and storage time for the estimated milk yield, and in general, the values increased ($P < 0.001$) as the maturity stage advanced (on average 875; 1,288 and 1,319 L/t of DM for silages produced at very early, early and medium harvest, respectively) and decreased as the storage time was prolonged (except for the silages produced at medium harvest; Fig. 9).

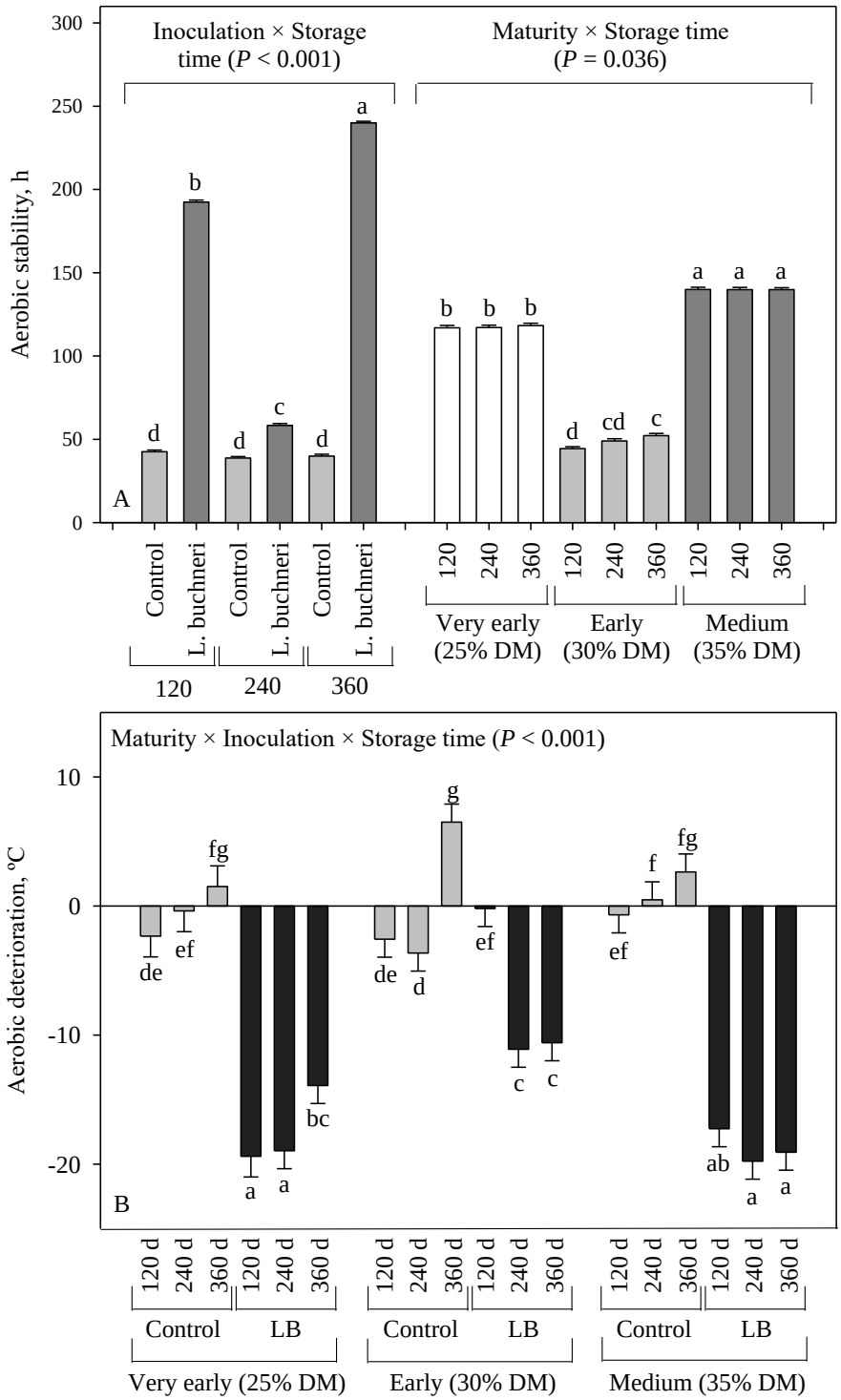

Fig. 8. Aerobic stability (A) and aerobic deterioration (B) of corn silage during the aerobic exposure period as influenced by the different interaction ways among maturity, inoculation, and storage time.

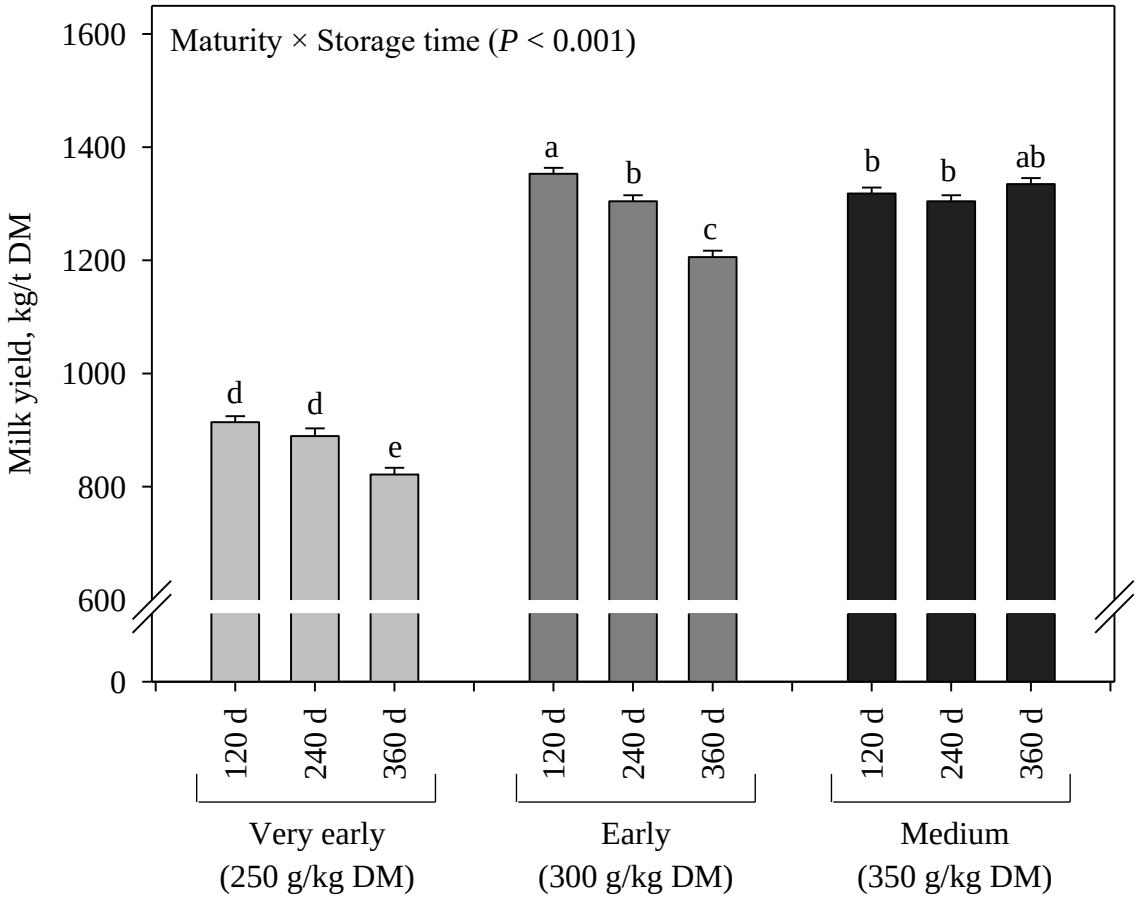

Fig. 9. Milk yield per tonne of DM from corn silage as influenced by inoculation and interaction between maturity and fermentation length (data estimated through the Milk2006 spreadsheet).

4. Discussion

In Brazil, corn silage represents the main forage source fed to dairy cows (Silva et al., 2019). As the majority of corn genotypes used for silage production in this country is flint type, strategies regarding corn silage management should focus on the improvements of starch utilization. In this regard, the current study was designed to investigate how maturity, bacterial inoculation, and storage length interact with each other in flint corn silage to obtain high-quality silage with enhanced starch-D. Agronomic features (DM yield and percentage of grains) and nutritional characteristics of the corn crop used in this study were typical of those reported under Brazilian conditions (Zopollatto et al., 2009; Oliveira et al., 2013; Rabelo et al., 2015; Silva et al., 2020), with the exception of the NDF content, which had a slight increase as the

maturity advanced rather than being diluted as a response of increasing percentage of grains in the whole-crop corn.

Several studies have shown that increasing storage length is a feasible strategy to enhance starch-D (Der Bedrosian et al., 2012; Windle et al., 2014; Da Silva et al., 2019). This occurs because prolamin, a protein matrix surrounding starch granules, is degraded mainly due to the proteolytic activity of bacteria and enzymes from its own plant (Junges et al., 2017). However, our results fail to support our initial hypothesis, which increasing storage time could enhance starch-D in more mature crops to a similar level as that observed in immature crop. This statement is based on the lack of an interaction between maturity and storage time for starch-D. Even though the concentration of soluble protein was similar between 240 and 360 d of storage (indicating similar proteolysis as well), corn silages stored for 360 d had a timid increase in starch-D (1.1%) in comparison to that stored for 240 d. Although significant, this response is of minor biological relevance.

The values relatively constant for starch-D likely can be attributed to the later storage interval assessed in this study (i.e., 120–360 d), which took into account only the plateau for gains in starch-D of corn silage. Our findings are in agreement with previous studies, which reported that the starch-D of both dent and flint corn silage remained constant after 45 d of storage (Der Bedrosian et al., 2012; Windle et al., 2014; Bueno et al., 2020). Indeed, broken line and segmented regression models showed that starch-D of corn silage was critically increased in the first 30 d of storage, and thereafter, the gains on starch availability were more moderate (Daniel et al., 2015; Bueno et al., 2020). Nevertheless, the ensiling process results in significant increases in starch-D, as well-documented before (Hoffman et al., 2011). For example, in this study, the starch-D of fresh forages harvested very early, early and medium were 657, 572, and 475 g/kg starch, respectively, and after 120 d of ensiling, the values increased to 696, 622, and 524 g/kg starch, respectively.

Inoculation of corn silage with *L. buchneri* increased the concentration of soluble protein, suggesting that prolamin could be degraded to a higher extent, as observed in HMC and rehydrated corn grain silage (Da Silva et al., 2018, 2019). Nevertheless, there was no effect of inoculation on the starch-D of corn silage. Moreover, the inoculated silages had increased concentrations of ammonia-N, but this variable is

indicative of deamination rather proteolysis. Our results differed from those of Da Silva et al. (2018, 2019) because the material examined was different (i.e., whole-crop corn silage × corn grain silage). Prolamin represents the most abundant protein class found in corn kernels (Holding, 2014), and shifts in the bacterial community towards higher proteolytic activity likely result in greater soluble protein accompanied of improved starch-D in HMC and rehydrated corn grain silage. However, whole-crop corn has protein classes in considerable amounts other than prolamin that is present in the leaf and stalk, such as enzymatic (Rubisco and phosphoenolpyruvate carboxylase), albumin, glutelin, and extensin (Sniffen et al., 1992; Boulter and Derbyshire, 2013). Thus, an increased concentration of soluble protein in whole-crop corn silage is not necessarily associated with greater proteolysis of prolamin.

Contrary to the small changes reported by prolonging the storage time, the starch-D of corn silage was dramatically reduced by maturity (694 g/kg starch at very early to 526 g/kg starch at medium harvest). This result is not surprising because there is an increase in corn grain vitreousness as the plant becomes more mature, especially in flint hybrids (Pereira et al., 2004). The vitreous endosperm is known to be hard and crystalline, with a continuous and abundant protein matrix surrounding the starch granules (Pereira et al., 2004), which is inversely related to ruminal starch disponibilization (Correa et al., 2002). A similar reduction in starch-D was observed by Der Bedrosian et al. (2012) working with normal and brown midrib hybrids harvested at 320 and 410 g/kg DM. However, as the whole-crop corn became more mature, the starch content and amount of digestible starch increased considerably. This result is particularly important because the starch accumulation may lead to the reduction of concentrate utilization inside the farm and the increased amount of digestible starch might result in increased milk yield.

It was also noted that advances in maturity increased aNDF content and decreased aNDF-D, which was expected. As the plant matures, there is an increase in fiber content accompanied by higher lignification of the cell wall, which is indigestible and therefore harmful fiber digestion (Jung and Allen, 1995).

Although the results from the literature are controversial, increasing the storage length was proposed to enhance NDF-D (Hallada et al., 2008). However, in the current study the aNDF-D was consistently reduced across the maturities examined as the storage

increased. This finding is associated with the acid hydrolysis of hemicellulose, caused mainly by the acidic environment within the silo as a consequence of fermentation (McDonald et al., 1991). This means that the hemicellulose disappearance led proportionally to an increase in the concentration of the indigestible fiber fraction in corn silages stored for a longer time, explaining the lower aNDF-D.

Furthermore, the aNDF-D increased following the bacterial inoculation of silages produced at early and medium harvest. The strain of *L. buchneri* used in this study was not assessed regarding its capacity to produce ferulic acid esterase (FAE). Nevertheless, it is well recognized that some strains of *L. buchneri* are able to produce FAE (Nsereko et al., 2008; Addah et al., 2012), an enzyme that usually leads to improved fiber digestion by releasing ferulic acid from cell-wall arabinoxylans and then increasing its susceptibility to microbial attachment (Kang et al., 2009). However, further studies are needed to confirm the hypothesis of FAE production by *L. buchneri* CNCM I-4323, since actually it is only a speculation because it was not measured. Additionally, inoculation of corn silage resulted in higher IVDMD, probably due to the increased aNDF-D found in this silage.

The inoculation of corn silage with *L. buchneri* increased the concentration of acetic acid in the silage produced from medium harvest. *Lactobacillus buchneri* is a heterofermentative lactic acid bacteria (LAB) that, under anaerobic conditions, metabolizes some quantity of lactic acid into acetic acid and other products (Oude Elferink et al., 2001), and then, inoculated silages usually have lower concentrations of lactic acid and higher acetic acid (Bernardi et al., 2019). However, in general, the inoculated silages had similar concentrations of lactic acid as observed in the control, but the causes for that are unclear.

The utilization of heterofermentative LAB such as *L. buchneri* often leads to increased DM loss in corn silage (Bernardi et al., 2019). This occurs because there is CO2 production in the heterofermentative pathway. In the current study, there was a 3-way interaction for DM recovery, but in general, inoculation did not decrease DM recovery of corn silage as expected, likely because the lactic acid was not lowered. Moreover, all the silages had good DM recovery (i.e., > 900 g/kg DM). However, the recovery of digestible DM was slightly reduced by inoculation.

Harvesting more mature whole-crop corn resulted in a less intense fermentation, which can be seen by the lower concentrations, in general, of lactic and acetic acid for the corn silage produced with 350 g/kg DM. This occurs because increasing maturity leads to the reduction of water activity that is available for the metabolism of microorganisms, and then, the growth of microorganisms is depressed (McDonald et al., 1991). Moreover, silages produced at early and medium harvest had increased acetic acid concentrations as the storage time increased. This indicates that in longer storage periods, the population of heterofermentative bacteria probably plays a more important role in the fermentation process because it remains fairly active (McDonald et al., 1991; Kleinschmit and Kung, 2006). Despite increases in acetic acid as storage increased, there was no improvement in the aerobic stability of corn silage stored for a longer time. It is known that well-fermented corn silage usually has low aerobic stability under tropical conditions because elevated temperatures favor the growth of yeasts (Ashbell et al., 2002), which initiate the aerobic deterioration of silage by using lactic acid as a substrate. In this regard, *L. buchneri* has been successfully used to improve the aerobic stability of silages (Bernardi et al., 2019), which was confirmed in this study. This bacterium inhibits the growth of yeasts and molds by increasing the acetic acid concentration in the silage, which has antifungal properties (Driehuis et al., 1999; Danner et al., 2003; Kleinschmit and Kung, 2006) and then, decrease the aerobic deterioration of silage, as observed in the current study.

In this study, the estimated milk yield obtained through the Milk2006 spreadsheet (Shaver et al., 2006) was most directed towards determining the ideal range of maturity stages in which whole-crop corn should be harvested. This statement is based on two points: 1) the Milk2006 spreadsheet did not consider the improvements in aerobic stability from bacterial inoculation and the consequent reduction in silage spoilage after the silos are opened, recognizably as the main benefit of using *L. buchneri*; and 2) the Milk2006 spreadsheet did not take into account starch-D. The lower milk yield observed as the storage time increased can be attributed to the reductions in CP content and aNDF-D. For the principal purpose of using the Milk2006 spreadsheet, milk yield was significantly increased (on average, +428 L/t of DM) when silages were produced at early and medium harvest compared to those produced at very early harvest. This result can be attributed mainly to the starch accumulation in

corn silages produced with plants harvested later. It is worth noting that our findings meet the recommendation of DM content ranging from 300 to 370 g/kg as being the ideal interval for whole-crop corn harvest in tropical conditions. This recommendation is based on 1) agronomic advantages such as increased DM yield and percentage of grains, 2) benefits for the ensiling process, since a lower amount of water is transported from the field to the silo and DM loss through effluent production is reduced, 3) benefits to the fermentation process, considering that the growth of undesirable microorganisms (e.g., Clostridia and Bacilli) is avoided or at least impaired by increasing DM content of forage, and 4) nutritional advantages such as higher starch accumulation in the grain.

5. Conclusion

The storage for a longer time (i.e., >120 d) with the goal of increasing silage digestibility did not occur. In spite of reducing silage digestibility, the harvest of whole-crop flint corn with 300 to 350 g/kg DM is desirable to have higher DM yield and starch accumulation, which lead to increased milk yield. Inoculation with *L. buchneri* is necessary to preserve the silage against aerobic deterioration.

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CHAPTER 3

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

CAPÍTULO 3 - THE GROWTH PERFORMANCE OF LAMBS IS IMPROVED BY INCREASING THE STORAGE LENGTH OF CORN SILAGE

Abstract

Our objective was to investigate the impact of inoculation and storage length of flint corn silage on the metabolic and production responses of lambs. A flint corn hybrid was harvested with 315 g dry matter (DM)/kg and ensiled in concrete pipe silos (n = 2) without (control) or with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage for 120, 240 and 360 d. Such silages were used to formulate six different diets. Sixty non-castrated male Dorper × Santa Inês lambs were used in the feeding program (n = 10). Additionally, eight ruminally cannulated Dorper × Santa Inês crossbred lambs were used to measure the ruminal fermentation (n = 4). Extending the storage of corn silage from 120 to 360 d increased the contents of soluble protein (P = 0.030; 415 to 490 g/kg CP) and ammonia-N (P = 0.047; 82.5 to 114 g/kg total N). Compared to the control, inoculation resulted in higher (P < 0.05) concentration of lactic (+19.9%) and acetic acid (+38.6%), and lower butyric acid (–25.3%), ammonia-N (–23.5%), and DM loss (–36.2%). The aerobic stability of corn silage was also improved by inoculation (P = 0.002; +160 h compared to the control). An interaction between storage length and inoculation was reported for feed intake, and lambs had increased DMI (P < 0.001) by feeding the inoculated silage stored for 120 d. Overall, the total-tract starch digestibility increased (P = 0.041) with longer storage (120 d = 94.4%; 240 d = 95.7%; 360 d = 96.5%). However, the total VFA was unchanged (P > 0.05) while the ruminal molar proportion of propionate decreased from 22.8 to 20.7 mM/100 mM in lambs fed corn silage stored for 120 and 360 d (P = 0.004). Lengthening silage storage from 120 to 240 and 360 d tended (P = 0.06) to increase the ADG of lambs by 4.6% and 10.2%, respectively. Moreover, the feed efficiency was substantially improved (P = 0.008) by 5% and 14% when the silages were stored for 240 and 360 d compared to 120 d. In opposite, inoculation had no effect (P > 0.05) on ADG and feed efficiency of lambs. Moreover, only few parameters of carcass and meat traits of lambs were altered by storage length and inoculation, but it were of minor biological importance. In conclusion, the productivity responses of lambs were enhanced by increasing the

storage length of flint corn silage, but inoculation with *L. buchneri* did not contribute to improve animal performance.

Key words: animal performance, flint corn silage, *Lactobacillus buchneri*, ruminal fermentation, starch digestion, storage length

1. Introduction

Extending the storage of corn silage was found to increase the in vitro starch digestibility (ivSD; Der Bedrosian et al., 2012; Windle et al., 2014). Such benefit has been associated with the breakdown of protein matrix surrounding the starch granules, which is a physiochemical impediment to starch digestion by difficulting microbial attachment in the rumen (Owens et al., 1986; McAllister et al., 1994; Hoffman et al., 2011). The proteolysis that occurs during fermentation explains the disruption of protein matrix and the proteolytic activity from bacteria and plant enzymes is responsible to 90% of this process (Junges et al., 2017). But in spite of such benefits on ivSD, few studies have been conducted to examine the impact of this strategy on the animal response. In one of those studies, dairy cows tended to produce 1.2 kg/d more milk without changing in dry matter intake (DMI) by increasing the storage of reconstituted sorghum grain silages (RSGS) from 30 to 90 d (Santos et al., 2019). It is worth highlighting that the potential benefits of extending silage storage on the animal performance are particularly important for countries where flint genotypes are predominantly used for livestock, such as in Brazil. Flint corn grains contain higher proportion of vitreous endosperm, which is negatively correlated with ivSD (Correa et al., 2002; Pereira et al., 2004). Thus, the limited starch digestion could be overcome by lengthening silage storage. In this regard, corn silage should be stored for at least 120 d to maximize starch digestion (Daniel et al., 2015).

Furthermore, recent studies showed that high-moisture corn silage (HMC) and rehydrated corn grain silage had lower concentration of prolamin and higher in situ DM degradation when *Lactobacillus buchneri*, a heterofermentative lactic acid bacterium (LAB), was added on silage (Da Silva et al., 2018, 2019). The authors suggested that such changes in prolamin concentration were related to shifts in the microbial

community within the silo caused by inoculation, likely directing towards those bacteria with higher proteolytic activity. However, this hypothesis needs further confirmation.

Based on this context, we aimed to investigate the impact of the prolonged storage and inoculation of flint corn silage with *L. buchneri* on the metabolic and performance responses of lambs. It was primarily hypothesized that extending silage storage might increase the apparent digestibility of starch, resulting in increased growth performance of lambs; secondarily, inoculation of corn silage may further contribute to increase starch digestibility.

2. Material and Methods

2.1. Ethics statement

All procedures adopted in this study were performed according to Ethical Principles in Animal Experimentation from the National Council for Animal Experiment Control (CONCEA) and were approved by the Ethics Committee on the Use of Animals (CEUA) from São Paulo State University (UNESP) at a regular meeting (Protocol No. 006764/17).

2.2. Crop harvest and ensiling procedure

A flint corn hybrid (2B 710 PW, Dow AgroSciences, São Paulo, SP, Brazil) was planted at a sowing density equivalent to 54,000 seeds/ha in 0.90-m rows in fields at São Paulo State University (at Jaboticabal, SP, Brazil: 21°150S, 48°180W; altitude 615 m). One week prior to planting, herbicides (4 L Zapp®/ha and 0.5 L Select®/ha; Syngenta, Matão, SP, Brazil) and mineral oil (0.5 L/ha) were applied to the field. The sowing date was 10 November 2016, and the soil was classified as Haplustox. The fields were fertilized with 350 kg/ha of 8-28-16 (N–P–K) at planting. Thereafter, on 17 November 2016, an additional fertilizer, 300 kg/ha of 30-0-10 (N–P–K), was applied after a week of corn growth, and a further 350 kg/ha of urea was applied after 4 weeks of corn growth on 7 December 2016. Herbicides (3 L Zapp®/ha and 2 L Atrazine®/ha; Syngenta, Matão, SP, Brazil) and insecticide (0.25 L Engeo Pleno S®/ha; Syngenta, Matão, SP, Brazil) were applied after 4 weeks of corn growth on 5 December 2016. Fungicide (0.5 L Priori Xtra®/ha; Syngenta, Matão, SP, Brazil), insecticide (0.15 L Ampligo®/ha; Syngenta, Matão, SP, Brazil) and mineral oil (0.5 L/ha) were also applied

after 5 weeks of corn growth on 12 December 2016. The climate where the corn was cultivated is classified as ‘Aw’ (Rolim et al., 2007) and characterized as tropical with a wet summer season and dry winter season.

On 14 February 2017, whole-crop corn (97-days growth) was randomly harvested in different locations in the field at 315 g of whole-plant DM/kg as fed at a stubble height of 20 cm using a pull-type New Pecus forage harvester (Nogueira, São João da Boa Vista, SP, Brazil). Forage was cut to 10 mm, and kernels were processed. Thereafter, six piles of corn forage were individually treated either with water (5 L/t; control) or with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g of fresh forage (inoculated; Lallemand Animal Nutrition, Goiânia, GO, Brazil). The inoculant was dissolved in distilled water (5 L/t) and sprayed onto fresh forage during the filling of the silos.

Twelve concrete pipe silos (1.58 m3; circumference = 120 cm; height = 140 cm) were each filled with approximately 1,000 kg of corn forage on the same day. Six silos were randomly filled with inoculated forage, and the other six were filled with untreated forage. Forage packing was achieved with the application of human pressure, and the bulk density among the silos was assumed to be the same (~630 kg fresh forage m3) by the end of filling. Silos were sealed with black-on-white polyethylene film (200-µm thick) (Electro Plastic, São Paulo, SP, Brazil) and stored at an ambient temperature for 120 (22.5 ± 2.19°C), 240 (22.3 ± 2.54°C), and 360 days (22.8 ± 2.37°C). Four net bags containing 4.89 ± 0.44 kg each of the respective fresh chopped corn (i.e., untreated or inoculated) were buried in the central part of the concrete pipe silo in 4 depths ~30 cm apart during the filling of the silo (Ashbell and Kashanchi, 1987). Forage allocated in each net bag was sampled to determine the chemical composition of whole-crop corn. During the unloading of the silo, according to the bags were encountered, they were removed from the silage mass and weighed to determine the DM loss. Furthermore, all silage of each bag was carefully homogenized to assess the chemical composition and fermentation end-products. Both forage and silage samples were stored at −20°C for further analysis.

2.3. Aerobic stability

Aerobic stability was determined by placing a silage sample (4.50 ± 0.41 kg) from each net bag in a plastic basin of 10 L capacity and kept in a room at ambient temperature (20–25°C). The silage temperature was measured every half an hour by using a datalogger (ESCORT Intelligent MINI; Escort Console, Buchanan, VA, USA) placed in the center of the mass for 10 d. The ambient temperature was also measured every half hour by two dataloggers placed near the buckets. Aerobic stability was defined as the number of hours that the silage temperature remained stable before increasing more than 2°C above the ambient temperature. Aerobic deterioration (°C) was defined as the sum of the daily temperature increases above the ambient temperature during the first 5 d of aerobic exposure (Conaghan et al., 2010).

2.4. Animal management and feeding

After silos were opened, the silages described earlier were used to formulate six total mixed rations (TMR) daily, as follows: 1) control silage stored for 120 d (C120); 2) inoculated silage stored for 120 d (LB120); 3) control silage stored for 240 d (C240); 4) inoculated silage stored for 240 d (LB240); 5) control silage stored for 360 d (C360); and 6) inoculated silage stored for 360 d (LB360). Diets were composed of 350 g/kg respective corn silage and 650 g/kg concentrate on a DM basis (Table 1) and were balanced to meet the nutrient requirements of lambs gaining 200 g/d (NRC, 2007). Feed ingredients (silage and concentrate) used to formulate TMR were sampled twice a week for DM determination and chemical analyses. Silage was obtained by sampling four locations from the feeding face of each silo and bulked into a single sample on each sampling day. Samples for chemical analyses were processed to form composite samples (n = 8), and were stored at −20°C until further analyses. The DM of ingredients measured weekly was used to adjust the proportion of ingredients in the TMR.

Table 1. Ingredients proportion and chemical composition of the diets offered to lambs in the feeding program.

Item*	120 d		240 d		360 d	
	Control[1]	*L. buchneri*	Control	*L. buchneri*	Control	*L. buchneri*
Ingredient proportion, g/kg DM						
Corn silage	350	350	350	350	350	350
Concentrate[2]	650	650	650	650	650	650
Chemical composition, g/kg DM						
DM, g/kg as fed	694	693	692	695	689	681
OM	954	952	953	947	952	963
CP	167	167	167	165	167	168
EE	28.1	27.9	28.3	28.1	28.2	27.9
aNDF	209	210	216	210	219	209
ADF	92.2	92.0	95.7	96.3	98.8	91.2
Starch	378	372	379	364	379	380
Particle size distribution, %						
> 19 mm	7.00	8.00	4.40	5.20	5.80	5.20
8–19 mm	33.6	32.3	37.6	38.6	37.6	39.7
4–8 mm	16.8	17.8	18.6	17.8	17.8	18.1
< 4 mm	42.6	41.9	39.4	38.4	38.8	37.0

[1]Corn forage was treated at ensiling either without (control) or with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g fresh forage.

[2]The commercial concentrate (Bell Peso Ovinos; Trouw Nutrition, Mirassol, SP, Brasil) was composed of (on DM basis) 208.5 g CP/kg; 42.7 g non-protein nitrogen/kg; 30.5 g EE/kg; 51.7 g ash/kg; 29 g crude fiber/kg; 8 g Ca/kg; 5,800 mg P/kg; 1,400 mg Na/kg; 7,700 mg K/kg; 1,500 mg Mg/kg; 3,200 mg Cl/kg; 30.7 mg Zn/kg; 11 mg Cu/kg; 31 mg Mn/kg; 0.15 mg Co/kg; 0.15 mg I/kg; 0.15 mg Se/kg.

*DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; aNDF = neutral detergent fiber; ADF = acid detergent fiber.

Sixty non-castrated male Dorper × Santa Inês lambs (initial body weight (BW) = 23.0 ± 2.5 kg; age = 4 months) were used in the feeding trial and each lamb was housed individually in 0.5 m^2 wooden pens equipped with a feed bunk and a water bowl. As the storage length of silage composed part of the diets and the opening of the silos was necessarily performed in different times, three groups of 20 lambs (described above) were randomly assigned (*n* = 10) to the control or inoculated diet for each storage assessed (i.e., 120, 240, and 360 d). In this regard, the feeding trial examining the C120 and LB120 diets occurred from 29 May 2017 to 19 August 2017 (82 d of duration). The C240 and LB240 diets were fed from 16 October 2017 to 06 January 2018 (82 d of duration), and the C360 and LB360 diets were fed from 19 February 2018 to 09 May 2018 (79 d of duration). During the feeding period the air temperature and humidity was recorded. Lambs were fed once daily (07:00) *ad libitum* (producing approximately 5% orts), and given free access to drinking water. Orts were weighed every morning and samples of offered feed and orts were collected twice a week and stored at –20°C for later analyses. Orts were used to calculate DMI daily, and their chemical composition was taken in consideration when apparent digestibility was calculated. Five fresh samples of each TMR were sampled to measure the particle size distribution using the Penn State Particle Separator (three-sieve model with 4-mm sieve; Jones and Heinrichs, 2013).

A 15 d period was allocated for the lambs to adapt to the diets based on a previous study assessing the growth performance of lambs in our laboratory (Basso et al., 2014). In this period, lambs were fed corn silage and concentrate in a ratio 80:20 (on DM basis) for the first 5 d of adaptation period, and thereafter concentrate was incorporated into the diet at 15% intervals over the five subsequent days until a forage:concentrate ratio of 35:65 was attained. The initial and final BW was measured after a 16-h fast and average daily gain (ADG) was calculated by subtracting the initial BW from the final BW and dividing the difference by the time in which lamb remained in the trial. Feed efficiency (gain:feed) was determined by dividing ADG by DMI.

2.5. Measurement of apparent digestibility

Apparent digestibility was calculated indirectly using the indigestible neutral detergent fiber (iNDF) as a marker to estimate fecal output (Valente et al., 2011). Fecal

grab samples (~30 g) were collected from each lamb for six consecutive days on days 20–25 of the feeding program (Pina et al., 2006). Samples of silage, concentrate, and orts were also collected daily during this period. Samples were composited across the 6-d period for chemical analyses and were stored at −20°C before analysis.

Fecal output and apparent digestibility were calculated using the Eqs. 1 and 2, respectively:

$$\text{Fecal output (g/d)} = \text{iNDF intake (g)/fecal iNDF (\%)} \times 100 \quad (1)$$

$$\text{Apparent digestibility (g/kg)} = [\text{DMI (g)} - \text{fecal yield (g)}]/\text{DMI (g)} \times 100 \quad (2)$$

2.6. Determination of carcass and meat traits

Eight lambs were slaughtered by cerebral concussion (TEC 10 PC) followed by jugular and carotid venesection when the adaptation phase (15 d) was finished to obtain the initial carcass weight. This value was used to calculate the carcass gain during the experiment. Similarly, sixty lambs were slaughtered when they reached a final BW of 34.2 ± 1.18 kg. Pre-harvest handling was in accordance with accepted animal welfare practices, and slaughtering procedures followed the Food of Animal Origin Sanitary and Industry Inspection (Brasil, 1997). Carcasses were split into two parts, and hot carcass weight (HCW) was recorded following removal of kidney, heart, and pelvic fat tissues. All carcasses were chilled for approximately 24 h at 6°C and then re-weighed to determine the cold carcass weight (CCW). Hot carcass yield (HCY) and cold carcass yield (CCY) were calculated by dividing the HCW and CCW by fasted BW, respectively, and multiplying by 100. The 12th rib fat thickness (RFT) and 12th rib longissimus muscle area (LMA) were measured on the left side of each carcass. The LMA was traced on transparencies and later measured with a planimeter, whereas RFT measurements were taken 3/4 the length ventrally over the longissimus muscle using a digital paquimeter (Greiner et al., 2003).

After slaughter, the initial pH was measured (triplicate) in the longissimus lumborum between the 12th and 13th ribs in the left half of the carcass of each lamb using a pH-meter with automatic endpoint and buffer recognition, as well as temperature compensation equipped with a penetrating electrode (Model Testo® 205, Testo-Direct Inc.). Final pH was recorded following 24 h post-mortem chilling of the carcass.

The longissimus muscle was removed completely from the right (for use in physical-chemical analyses) side of each carcass to estimate thawing and cooking losses (CL). These same samples were also used to assess the Warner-Bratzler shear force (WBSF). All longissimus muscle samples were vacuum-packaged and stored at –18°C prior to analyses.

For the determination of meat color, a transversal cut was made through the longissimus muscle 30 min prior to readings to expose the myoglobin to oxygen (Cañeque and Sañudo, 2000). A Minolta Chroma Meter CR-400 colorimeter (Minolta Camera Co., Osaka, Japan) was used to measure the L* a* b* space; in this space, L* indicates brightness, and a* and b* represent the chromaticity coordinates, as follows: the axis that runs from –a* to +a* varies from green to red, and the axis that runs from –b* to +b* var¬ies between blue and yellow. The colorimeter was calibrated against white and black standards prior to sample analysis. Readings were made in triplicate for each sample, and the average values of which were recorded.

Steaks were thawed at 10°C in a BOD incubator for 12 h and oven-broiled in an electric oven at 170°C, with the CL defined as the difference between the weight of the steaks before and after cooking.

To determine the WBSF, internal temperatures of the steaks were monitored with a digital thermometer attached to a 20-gauge copper-constantan thermocouple (Omega Engineering, Stamford, CT) implanted in the center of each steak. When the internal temperature reached 35°C, the steak was turned and allowed to reach an internal temperature of 71°C before removal from the oven. The cooked WBSF steaks were cooled at room temperature. Six round cores (1.27 cm diameter) were removed from each steak, and checked for visible fat and connective tissue, which if present were removed (Wheeler et al., 1995). Each core was sheared once perpendicularly to the fiber direction using a Warner-Bratzler shear machine of 1.016 mm at a speed of 300 mm/min (Texture Analyser TAXT2i; Stable Micro Systems Ltd., Godalming, UK).

To determine water-holding capacity (WHC), a 0.5 g steak sample was placed on filter paper between two acrylic plates, and a 10 kg weight was placed over the plates for 5 min (Honikel and Hamm, 1994); WHC was defined as the difference between the initial and final weights of the steaks (Hamm, 1986).

Samples used in the chemical analyses (in natura) were grinded and homogenized for analyses of moisture, protein, collagen, ether extract (EE), and ash in a Near-infrared spectroscopy (NIRS; FOSS FoodScanTM), in order to determine the chemical composition of each longissimus sample.

2.7. Measurement of ruminal fermentation

Eight ruminally cannulated Dorper × Santa Inês crossbred lambs (initial body weight = 28.7 ± 2.2 kg; age = 6 months), each fitted with a 50.8-mm silicone ruminal cannula, were used in a completely randomized design. Each lamb was housed individually in a 0.9 × 2.0-m pen fitted with a feed bunk and a water bowl. As described earlier to the lambs used in the feeding program, firstly, each lamb was randomly assigned to the C120 or LB120 diet; thereafter, they were randomly assigned to the C240 or LB240 diet, and for last, to the C360 or LB360 diet. Lambs were fed once a day (07:00) ad libitum (producing approximately 5% orts), with free access to drinking water. For each storage length assessed, the trial was carried for 15 d. A 14 d period was allocated for the lambs to adapt to the diets.

On day 15 of each period, a 50 mL sample of ruminal fluid was collected from each lamb before feeding (0 h), and at 3, 6, 9, 12 and 18 h post-feeding. Ruminal fluid was squeezed through four layers of cheesecloth, and pH was immediately measured using a pH meter (model MA522, Marconi Laboratory Equipment, Piracicaba, SP, Brazil). To inhibit microbial activity, 1 mL H_2SO_4 diluted with distilled water (1:1 v/v) was added to the ruminal fluid (50 mL) in an acid: rumen fluid ratio of 0.01:1 v/v. The resulting solution was stored at −20°C until volatile fatty acid (VFA) and ammonia-N analyses could be carried out.

2.8. Sample preparation and chemical analyses

Twenty-five grams of each sample of forage or silage were mixed with 225 mL of distilled water and blended in a Phillips Walita blender (Walita, Varginha, MG, Brazil) for 1 min at the highest setting, and filtered through two layers of cheesecloth. The pH of the filtrate was measured immediately by using a pH meter (model MA522, Marconi Laboratory Equipment, Piracicaba, SP, Brazil). After the pH was measured, the filtrate was stored at −20°C for subsequent analysis of lactic acid and volatile fatty acids

(acetic, propionic and butyric acids) using a high-performance liquid chromatograph (HPLC; Shimadzu model Prominence, Shimadzu Corp., Kyoto, Japan) equipped with a UV/VIS detection system and a refractive index detector (SPD-20). An apolar column (C-18 model Shimpack VP-ODS; 4.6 mm × 250 mm) was used at 35°C for chromatographic separation. The polar mobile phase consisted of a 20-mM buffer phosphate solution in pH 2.5 and acetonitrile was used as apolar solvent. The presence of the acid was detected by UV absorbance (210 nm). Ammonia N was measured by distillation according to the AOAC (1996; method no. 941.04).

Samples of forage, silage, concentrate, orts, and feces were oven-dried (at 55°C for 72 h) and processed in a knife mill (Willey mill model 4; Arthur H. Thomas Company, Philadelphia, PA) before being ground through a 1 mm screen and analyzed for DM (105°C for 12 h) and ash (500°C for 5 h) according to the AOAC (1996; methods no. 930.15 and 923.03, respectively). Silage DM content was corrected for volatile compounds according to Weissbach (2009). The EE was determined according to the procedures described by the AOAC (1996; method no. 920.39). The total nitrogen (TN) was measured by rapid combustion by using a LECO Analyzer (model F528 N; LECO Corp., St. Joseph, MI, USA); crude protein (CP) was calculated as TN × 6.25. Soluble protein was determined following the procedures described by Licitra et al. (1996). The neutral detergent fiber (aNDF) and acid detergent fiber (ADF) were sequentially determined in an ANKOM200 Fiber Analyzer (ANKOM Technology Corporation, Fairport, NY, USA) following the procedures described by Mertens (2002) and AOAC (1996; method no. 973.18), respectively. For aNDF analysis, a heat-stable α-amylase was used without sodium sulfite. The aNDF and ADF were expressed inclusive of residual ash. Starch was determined by an enzymatic-colorimetric assay using the acetate buffer in the gelatinization solution (Hall, 2009). The iNDF was determined by incubating F57 bags (ANKOM) containing silage (n = 8 per treatment), concentrate (i.e. ground corn and soybean meal; n = 3 for each), orts (n = 10 per treatment), and feces (n = 10 per treatment) samples in triplicate in the rumen of two Nellore steers for 264 h (a total of 102 bags for each animal) (Casali et al., 2008). Afterward, samples were removed from the rumen of the Nellore steers and analyzed for aNDF.

Ruminal ammonia-N was determined by distillation with 2N KOH according to Fenner (1965). Aliquots of strained ruminal fluid collected were thawed in a refrigerator

overnight and centrifuged (20,000g) for 30 min at 4○C, and the supernatant was analyzed for VFA using a gas chromatograph (Shimadzu model GC2014, Shimadzu Corp., Kyoto, Japan) equipped with an HP-INNOWax capillary column (30 m × 0.32 mm; Agilent Technologies, Colorado Springs, CO, USA) at an initial temperature of 80○C for 3 min followed by heating at a rate of 20○C/min until a final temperature of 240○C was achieved.

2.9. Statistical analyses

Silage variables were analyzed as a completely randomized design (n = 2). Inoculation (1 DF), storage length (2 DF), and their interactions were considered fixed effects, while the error was considered as random effect. As the storage length composed part of the treatments investigated in this study and it could be not blocked, the data of feed intake, apparent digestibility, growth performance, and carcass traits (n = 10) were analyzed as a completely randomized design. A previous study used similar approach for this kind of experiment (González and Rodríguez, 2003). Inoculation (1 DF), storage length (2 DF), and their interactions were considered fixed effects, while the lambs were considered as random effect. Data of ruminal fermentation was analyzed as a completely randomized design (n = 4) with repeated measurements over time (ruminal samples were taken 0, 3, 6, 9, 12, and 18 h post-feeding). All variables were analyzed using the MIXED procedure of SAS software (v. 9.0; SAS Institute Inc., Cary, NC, USA). Homogeneity of the data was verified using the UNIVARIATE procedure of SAS. Studentized residuals were plotted against the predicted values using the plot procedure to analyze data for outliers. Outliers were identified and deleted if absolute values of Studentized residuals exceeded ± 3. Several covariance structures were tested and those that generated the lowest Akaike information criterion values for ruminal fermentation were selected (Heterogeneous compound symmetry (CSH), Huynh-Feldt (HF), and Variance components (VC) were those that best fit the data). Initial BW and temperature and humidity of the air were used as covariates for all animal measurements; however, the covariate was removed from the model if not significant ($P > 0.05$; St-Pierre, 2001). Differences between means were compared using the PDIFF option of the LSMEANS command, which is based on the Fisher's F-protected least significant difference test (multiple t-test

comparisons). Significant differences were declared at $P \leq 0.05$ and trends were discussed when $0.05 < P \leq 0.10$.

3. Results

3.1. Effect of inoculation and storage length on fermentation patterns and aerobic stability of corn silage

The whole-crop corn used in this study was harvested with 315 g DM/kg as fed and had, on average, the following chemical composition (on DM basis) prior to ensiling: ash = 36.9 g/kg; CP = 96.5 g/kg; aNDF = 487 g/kg; ADF = 252 g/kg; and starch = 282 g/kg (Table 2). After the silos were opened, we reported a substantial reduction in DM loss (–36.2%; P = 0.011) of corn silage inoculated with *L. buchneri* (59.2 vs. 92.8 g/kg DM in the control). There was an interaction between inoculation and storage length for silage pH, ammonia-N, lactic, acetic, and propionic acids. Inoculation decreased the pH (P = 0.016) in the silage stored for 240 d, but it was similar with the control at 120 and 360 d of storage. Overall, the concentration of ammonia-N increased (P = 0.047) by increasing the silage storage (82.5, 96.3 and 114 g/kg total N for 120, 240 and 360 d, respectively), while it was systematically decreased (–23.5%) due to inoculation. Inoculation increased the lactic acid of corn silage by 19.9% and the highest values were reported after 240 d of storage (P = 0.022). Acetic acid was also increased following silage inoculation (+38.6%; P = 0.002) and in general, the concentration of this acid increased over storage (32.6, 56.8 and 72.8 g/kg DM for 120, 240 and 360 d, respectively). Overall, propionic acid increased due to extending silage storage and inoculation altered its concentration only in silages stored for 240 d (P = 0.027). Inoculation decreased the concentration of butyric acid by 25.3% (0.222 vs. 0.297 g/kg DM in the control; P = 0.013). Inoculation consistently improved (P = 0.002) the aerobic stability (+160 h compared to the control) and decreased (P < 0.001) the aerobic deterioration of corn silage (2.2 vs. 19.2○C in the control silage).

The DM and CP contents of corn silage decreased as the storage length increased (P < 0.001; Table 2). Compared to 120 d of storage, ash contents decreased after 240 d and increased after 360 d of storage (P = 0.012). There was an interaction between inoculation and storage for the contents of soluble protein, aNDF, ADF, and

starch. Overall, soluble protein increased (P = 0.030) by increasing the storage of corn silage (415, 468, and 490 g/kg CP after 120, 240, and 360 d, respectively). The aNDF content decreased as the storage increased, but such reductions were less pronounced due to inoculation in the silages stored for 240 and 360 d (P = 0.003). Overall, inoculation decreased the ADF content, while this variable increased due to prolonging the storage (P = 0.050). Despite the interaction reported for starch, this variable remained relatively constant throughout the storage lengths evaluated (31.6–32.3 g/kg DM), and the inoculation only caused a little difference on it when the corn silages were stored for 360 d (P = 0.006). The EE was unaffected by treatments (P ≥ 0.09).

Table 2. Characteristics of whole-corn crop prior and after ensiling (data are given in g/kg DM, unless otherwise stated) as influenced by bacterial inoculation and fermentation length (n=2).

Item	120 d		240 d		360 d		SEM	*P*-value[2]		
	Control[1]	*L. buchneri*	Control	*L. buchneri*	Control	*L. buchneri*		SL	I	SL × I
Characteristics of whole-crop corn prior to ensiling										
DM, g/kg as fed	311	310	319	321	315	311	1.01			
Ash	37.2	37.7	36.9	38.1	35.0	36.7	0.550			
CP	96.2	96.3	96.2	97.0	96.5	96.8	1.50			
Soluble protein, g/kg CP	393	391	391	391	391	391	1.94			
Ammonia-N, g/kg total N	32.1	32.1	32.3	32.1	32.6	31.5	0.190			
EE	33.3	32.4	34.3	34.6	33.8	33.2	0.210			
aNDF	490	487	485	487	486	487	1.54			
ADF	251	252	252	251	252	251	0.810			
Starch	283	283	280	282	281	284	1.48			
pH	6.15	6.16	6.10	6.13	6.11	6.04	0.028			
Characteristics of whole-crop corn after ensiling										
Fermentation and aerobic stability pattern										
DM loss	82.4	56.0	84.9	58.5	111	63.0	11.5	0.31	0.011	0.58
pH	3.51^{ab}	3.48^{b}	3.56^{a}	3.49^{b}	3.50^{ab}	3.53^{ab}	0.011	0.16	0.034	0.016
Ammonia-N, g/kg total N	91.2^{cd}	73.8^{e}	110^{b}	82.5^{de}	131^{a}	97.9^{c}	2.42	<0.001	<0.001	0.047
Lactic acid	69.1^{c}	78.4^{b}	80.2^{b}	95.2^{a}	55.3^{d}	71.7^{c}	0.950	<0.001	<0.001	0.022
Acetic acid	27.2^{e}	38.0^{d}	50.7^{c}	62.8^{b}	58.0^{bc}	87.6^{a}	1.58	<0.001	<0.001	0.002
Propionic acid	10.6^{c}	11.6^{c}	14.7^{b}	17.2^{a}	17.1^{a}	16.8^{a}	0.370	<0.001	0.011	0.027
Butyric acid	3.10	2.35	3.10	2.30	2.70	2.00	0.270	0.35	0.013	0.98
Aerobic stability, h	31.0	240	49.0	178	36.0	178	36.0	0.72	0.002	0.53
Aerobic deterioration, °C	20.9	0.435	14.7	3.44	22.0	2.71	1.80	0.26	<0.001	0.08
Chemical composition										
DM, g/kg as fed	324	322	317	318	309	306	1.96	<0.001	0.32	0.53
Ash	41.9	40.0	39.0	40.8	42.3	44.3	0.770	0.012	0.34	0.08

CP	77.2	79.9	70.6	72.3	61.9	68.7	1.03	<0.001	0.005	0.10
Soluble protein, g/kg CP	403^{d}	426^{cd}	484^{ab}	452^{bc}	474^{ab}	505^{a}	9.40	<0.001	0.39	0.030
EE	28.6	28.1	24.6	28.3	25.1	26.7	0.980	0.10	0.09	0.19
aNDF	495^{a}	492^{b}	477^{d}	488^{c}	461^{f}	468^{e}	0.590	<0.001	<0.001	0.003
ADF	280^{c}	276^{c}	308^{ab}	299^{b}	316^{a}	303^{b}	1.44	<0.001	<0.001	0.050
Starch	320^{ab}	312^{b}	319^{ab}	327^{a}	322^{a}	316^{b}	1.66	0.014	0.13	0.006

$^{a-f}$Means in the same row with different letters differed significantly ($P \leq 0.05$).
[1]Corn forage was treated at ensiling either without (control) or with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g fresh forage.
[2]SL = storage length; I = inoculation; SL × I = interaction between storage length and inoculation.
*DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; aNDF = neutral detergent fiber; ADF = acid detergent fiber.

3.2. Feed intake, apparent digestibility and growth performance of lambs

There was an interaction between inoculation and storage length for feed intake (Table 3). Lambs consumed more ($P < 0.001$) DM, OM, and CP by feeding the LB120 diet in comparison with C120 diet. Inoculation increased ($P = 0.028$) the NDF intake when the corn silage was stored for 120 and 240 d, but not for 360 d. Inoculation increased ($P \leq 0.003$) the ADF and starch intake only when the corn silage was stored for 120 d. The DM digestibility was not changed by treatments ($P = 0.11$), although numerically the inoculation increased it by 10 percent units in comparison with the control silage stored for 120 d. The starch digestibility was affected by the interaction between inoculation and storage length, but in general, it increased ($P = 0.041$) with longer storage (120 d = 94.4%; 240 d = 95.7%; 360 d = 96.5%). Compared to 120 d of storage, the ADG of lambs tended to increase ($P = 0.06$) by 4.6% and 10.2% when the corn silages were stored for 240 and 360 d (0.216, 0.226 and 0.238 kg/d for 120, 240 and 360 d of storage, respectively). Moreover, the feed efficiency was improved ($P = 0.008$) by 5% and 14% when the silages were stored for 240 and 360 d compared to 120 d.

Table 3. Feed intake, apparent digestibility, and growth performance of lambs fed diets containing either corn silage untreated or inoculated being stored for different times (n=10).

Item*	120 d		240 d		360 d		SEM	*P*-value[2]		
	Control[1]	*L. buchneri*	Control	*L. buchneri*	Control	*L. buchneri*		SL	I	SL × I
Intake, kg/d										
DM	1.01^{c}	1.29^{a}	1.10^{bc}	1.16^{b}	1.08^{bc}	1.12^{bc}	0.031	0.26	<0.001	<0.001
OM	0.945^{c}	1.21^{a}	1.03^{bc}	1.08^{b}	1.01^{bc}	1.05^{bc}	0.028	0.23	<0.001	<0.001
CP	0.157^{c}	0.205^{a}	0.170^{bc}	0.182^{b}	0.165^{bc}	0.176^{bc}	0.005	0.10	<0.001	<0.001
NDF	0.224^{b}	0.302^{a}	0.245^{b}	0.293^{a}	0.215^{b}	0.244^{b}	0.008	<0.001	<0.001	0.028
ADF	0.104^{b}	0.152^{a}	0.137^{a}	0.150^{a}	0.127^{ab}	0.136^{a}	0.006	0.032	<0.001	0.003
Starch	0.423^{b}	0.511^{a}	0.443^{b}	0.461^{b}	0.444^{b}	0.453^{b}	0.010	0.17	<0.001	<0.001
Apparent digestibility, %										
DM	76.1	86.4	80.7	78.1	82.7	84.6	2.93	0.36	0.20	0.11
Starch	94.2^{c}	94.5^{c}	95.3^{b}	96.1^{ab}	96.6^{a}	96.3^{a}	0.287	<0.001	0.18	0.041
Growth performance										
Initial BW, kg	23.3	22.6	23.5	22.4	22.8	23.2	0.960	1.00	0.57	0.71
Final BW, kg	34.1	34.2	34.2	34.2	34.2	34.2	0.485	1.00	0.97	0.98
ADG, kg/d	0.208	0.224	0.216	0.236	0.246	0.229	0.009	0.06	0.83	0.42
Feed efficiency	0.211	0.173	0.203	0.200	0.229	0.209	0.008	0.008	0.63	0.32
Feedlot days	51.9	51.8	49.5	50.0	46.3	48.0	2.41	0.24	0.59	0.39

[a-c]Means in the same row with different letters differed significantly ($P \leq 0.05$).
[1]Corn forage was treated at ensiling either without (control) or with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g fresh forage.
[2]SL = storage length; I = inoculation; SL × I = interaction between storage length and inoculation.
*DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber.

3.3. Ruminal fermentation of lambs

Inoculation of corn silage and storage length did not affect ($P > 0.05$) the concentration of total VFA and pH in the ruminal fluid of lambs; however, these parameters were altered ($P < 0.001$) by the time in which ruminal fluid was sampled with higher values of total VFA and lower pH occurring between 6 and 12 h post-feeding (Figure 1). Inoculation decreased ($P < 0.001$) the molar proportion of acetate and propionate by 3.0% and 10.9%, while butyrate and isobutyrate were increased ($P < 0.001$) by 32.8% and 12.5%, respectively (Figure 2A). The molar proportion of propionate decreased from 22.8 mM/100 mM in lambs fed corn silage stored for 120 d to 20.7 mM/100 mM on average for lambs fed corn silage stored for 240 and 360 d ($P = 0.004$; Figure 2B). Compared to 120 d of storage, butyrate increased from 11.8 to 13.2 mM/100 mM and isobutyrate increased from 1.2 to 1.6 mM/100 mM after 360 d of silage storage ($P < 0.05$; Figure 2B). The time post-feeding altered ($P < 0.001$) the molar proportions of acetate, propionate, butyrate, and isobutyrate (Figure 3). The molar proportions of valerate and isovalerate also increased ($P < 0.001$) by increasing the storage of corn silage (Figure 4A). There was an interaction between inoculation and time post-feeding for valerate and isovalerate ($P < 0.05$); there was no obvious impact of inoculation on the molar proportion of valerate (Figure 4B), but isovalerate consistently increased by inoculation (Figure 4C). Compared to 120 d of storage, the ruminal concentration of ammonia-N increased ($P = 0.005$) by 7.8% at 240 d, while the storage for 360 d produced intermediate value (Figure 5A). Ammonia-N was also affected by the time post-feeding in which the highest value was observed after 3 h of feeding ($P < 0.001$; Figure 5B).

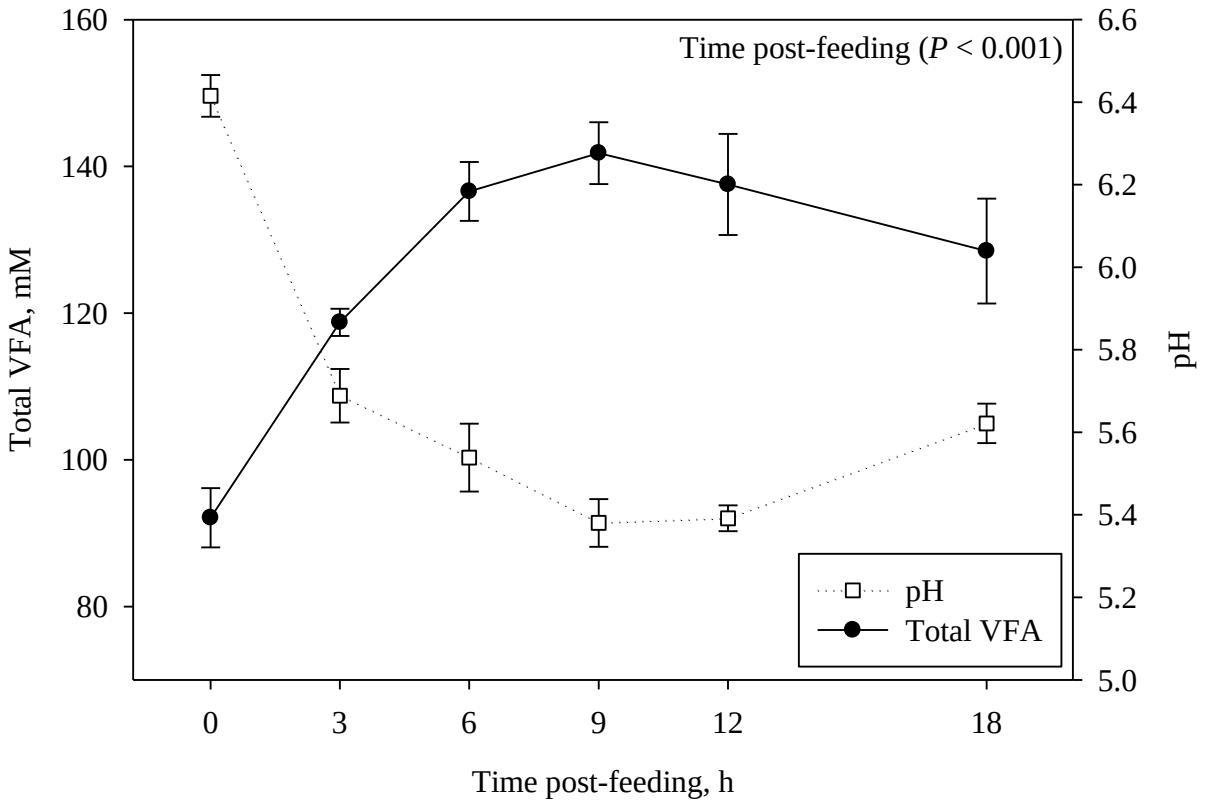

Figure 1. Effect of time post-feeding on total volatile fatty acid concentration and pH in the ruminal fluid of lambs fed diets containing either untreated or inoculated corn silage stored for different times (n = 4).

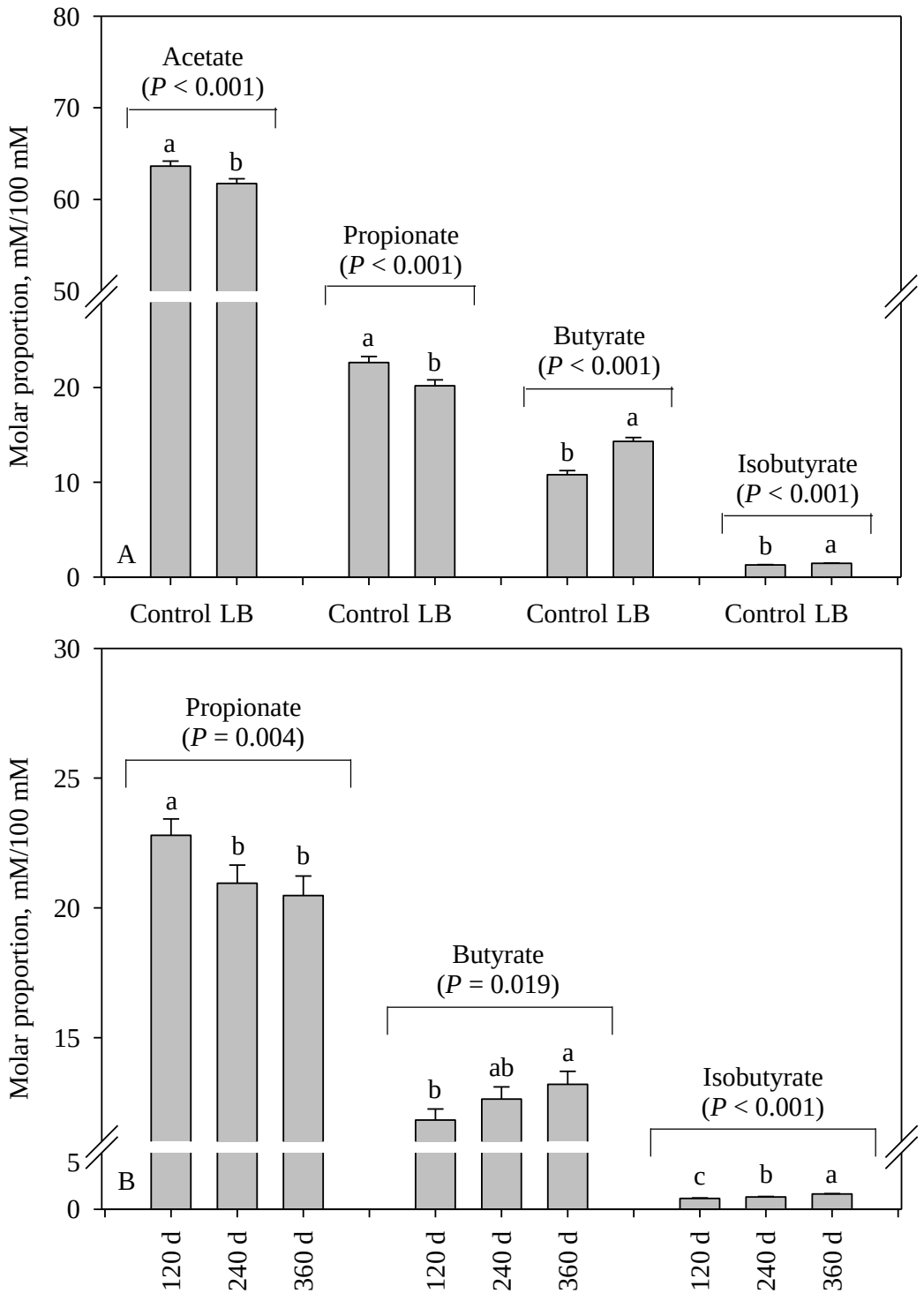

Figure 2. Molar proportion of acetate, propionate, butyrate, and isobutyrate in the ruminal fluid of lambs as affected by the inoculation (A) and storage length (B) of corn silage (LB = *Lactobacillus buchneri*; *n* = 4).

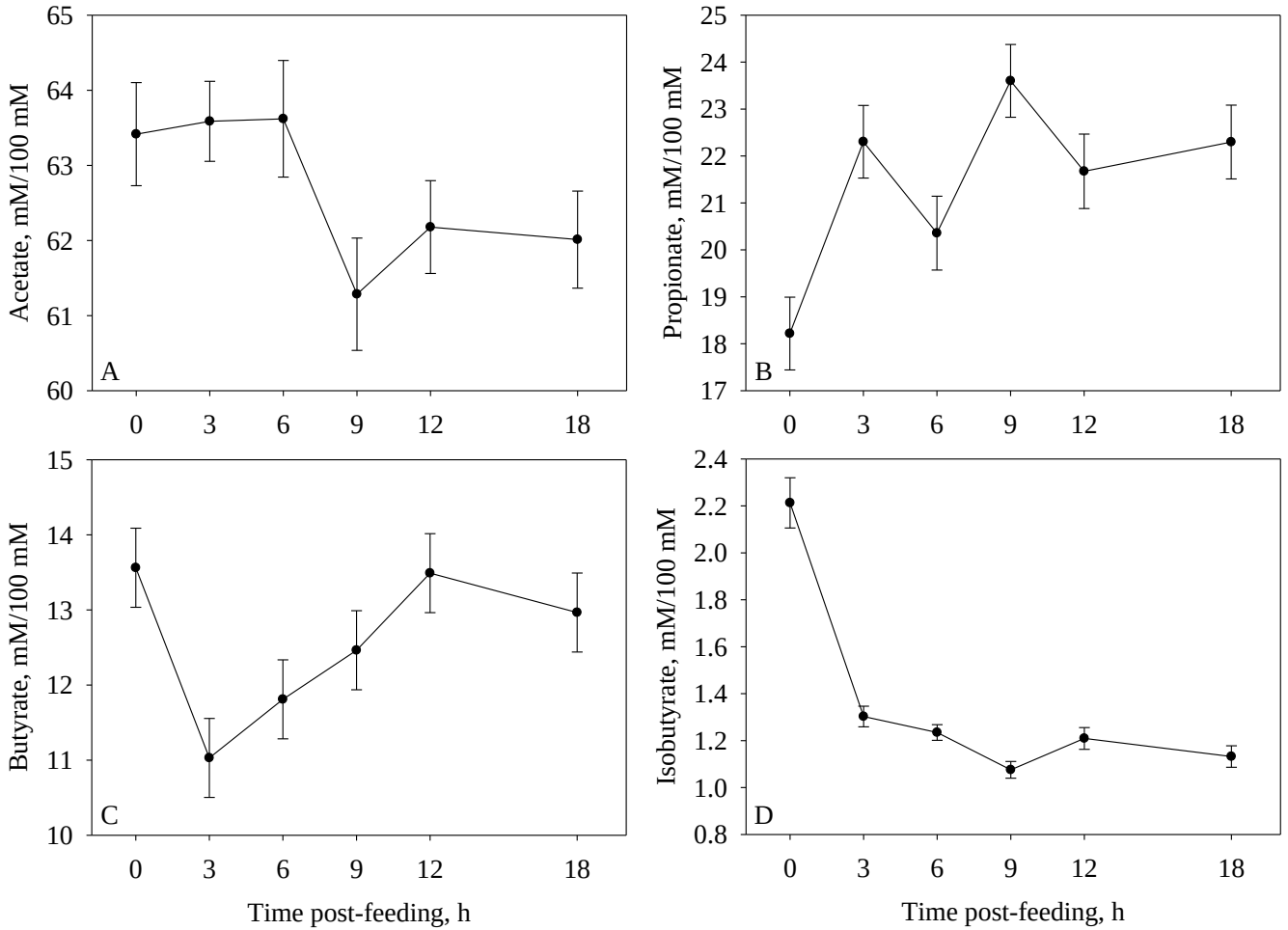

Figure 3. Molar proportion of acetate (A), propionate (B), butyrate (C), and isobutyrate (D) in the ruminal fluid of lambs fed diets containing either untreated or inoculated corn silage stored for different times (effect of time post-feeding: $P < 0.001$; $n = 4$).

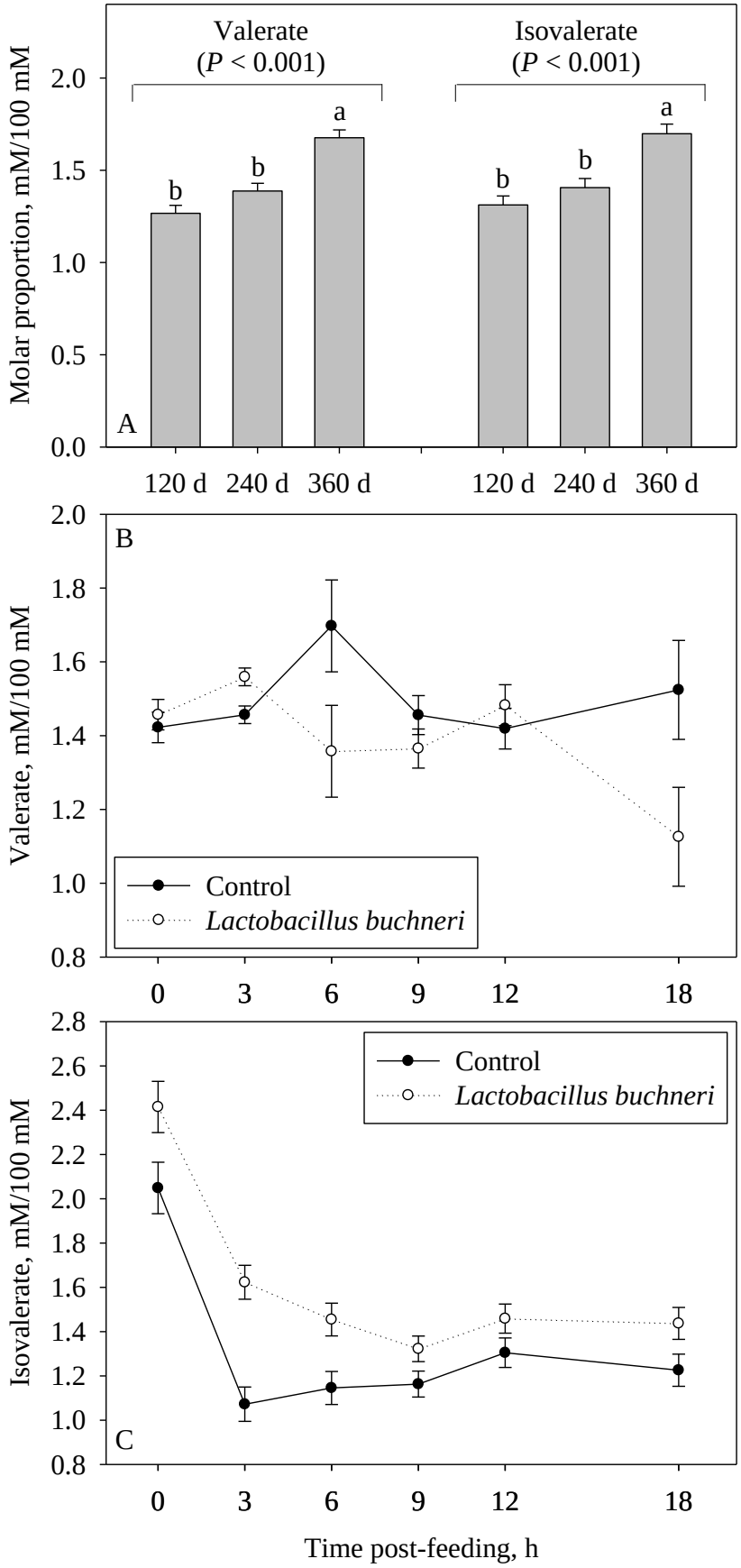

Figure 4. Effect of storage length on the molar proportion of valerate and isovalerate (A) and the interaction between inoculation and time post-feeding for valerate (B; P = 0.008) and isovalerate (C; P = 0.027) in the ruminal fluid of lambs (n = 4).

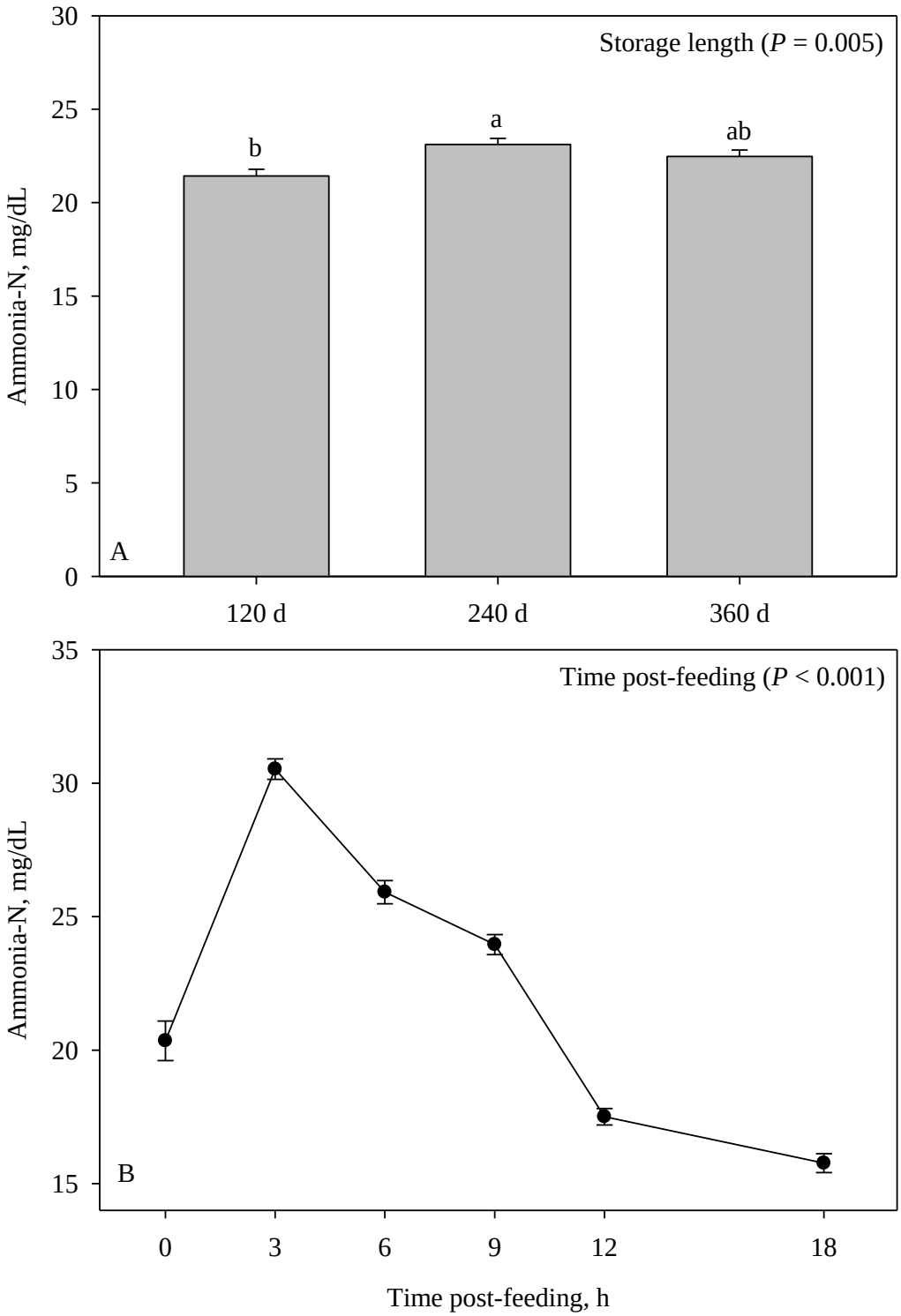

Figure 5. Effect of storage length (A) and time post-feeding (B) on the concentration of ammonia-N in the ruminal fluid of lambs (*n* = 4).

3.4. Carcass and meat traits of lambs

The initial HCW was higher ($P < 0.001$) in lambs fed the C240 and LB240 diets, while those lambs fed the C360 and LB360 had lower initial HCW (Table 4). Other carcass traits (final HCW, carcass gain, CCW, HCY, CCY, RFT, and LMA) were not altered by treatments ($P > 0.05$). Lambs fed the diets containing corn silage stored for 120 d had higher moisture in meat ($P = 0.015$), while the storage of corn silage for 240 and 360 d resulted in lower moisture. Contents of ash, protein, fat, and collagen were not modified by treatments ($P > 0.05$). The TL increased as the silage storage increased ($P = 0.002$). Inoculation of corn silage resulted in higher ($P = 0.002$) initial (6.38 vs. 6.34) and final pH (5.50 vs. 5.46) of meat in comparison to the control. The TL increased ($P = 0.012$) from 4.84% to 4.95% due to inoculation. The CL, WHC, and WBSF were not changed by treatments ($P > 0.05$). Inoculation of corn silage increased the meat luminosity ($P < 0.001$; 41.4 vs. 39.5 in the control) and decreased the intensity of red ($P = 0.042$; 14.8 vs. 15.5 in the control). The intensity of yellow was not altered in meat of lambs ($P > 0.05$).

Table 4. Carcass and meat traits of lambs fed diets containing either corn silage untreated or inoculated being stored for different times (*n* = 10)

Item*	120 d		240 d		360 d		SEM	*P*-value[2]		
	Control[1]	*L. buchneri*	Control	*L. buchneri*	Control	*L. buchneri*		SL	I	SL × I
Características da carcaça										
Initial HCW, kg	9.32	9.32	9.39	9.39	9.02	9.02	0.005	<0.001	1.00	1.00
Final HCW, kg	17.0	16.9	16.8	17.1	17.2	16.8	0.255	0.99	0.87	0.31
Carcass gain, kg	7.60	7.59	7.42	7.77	8.19	7.75	0.255	0.24	0.87	0.31
CCW, kg	16.5	16.4	16.3	16.7	16.8	16.3	0.243	0.99	0.71	0.26
HCY, %	49.8	49.5	49.1	50.2	50.3	49.0	0.793	1.00	0.86	0.31
CCY, %	48.5	48.1	47.7	48.9	49.0	47.6	0.803	1.00	0.74	0.30
RFT, mm	4.10	4.30	4.26	3.82	3.51	4.53	0.320	0.82	0.32	0.09
LMA, cm^2	14.3	14.1	13.8	14.7	14.1	14.4	0.722	1.00	0.59	0.73
Chemical composition of *longissimus muscle*, %										
Moisture	77.2	77.1	73.5	73.9	74.4	74.4	0.500	0.015	0.68	0.47
Ash	2.57	2.44	2.51	2.54	2.37	2.73	0.216	0.98	0.62	0.52
Protein	20.9	21.0	20.9	21.0	21.3	20.7	0.156	0.97	0.50	0.07
Fat	1.43	1.50	1.61	1.17	1.41	1.60	0.199	0.83	0.73	0.26
Collagen	1.62	1.69	1.70	1.65	1.78	1.60	0.076	0.87	0.38	0.26
Meat traits										
Initial pH	6.33	6.39	6.34	6.36	6.34	6.39	0.016	0.71	0.002	0.50
Final pH	5.46	5.51	5.47	5.49	5.46	5.51	0.014	0.71	0.002	0.56
TL, %	4.12	4.23	4.59	4.71	5.82	5.91	0.176	0.002	0.012	0.96
CL, %	28.4	28.5	28.5	28.4	28.6	28.3	0.251	1.00	0.68	0.72
WHC, %	54.5	54.4	53.8	53.8	54.3	54.4	0.821	0.75	1.00	0.99
WBSF, kgf	3.35	3.27	3.36	3.26	3.26	3.29	0.202	0.99	0.78	0.95
Meat color										
L*	39.5	41.2	39.5	41.4	39.5	41.7	0.559	0.91	<0.001	0.91

a*	15.7	14.9	15.7	14.6	15.1	14.9	0.401	0.82	0.042	0.60
b*	1.45	1.46	1.47	1.46	1.41	1.47	0.043	0.86	0.61	0.68

[a-c]Means in the same row with different letters differed significantly ($P \leq 0.05$).
[1]Corn forage was treated at ensiling either without (control) or with *Lactobacillus buchneri* CNCM I-4323 at 1×10^5 cfu/g fresh forage.
[2]SL = storage length; I = inoculation; SL × I = interaction between storage length and inoculation.
*HCW = hot carcass weight; CCW = cold carcass weight; HCY = hot carcass yield; CCY = cold carcass yield; RFT = rib fat thickness; LMA = *Longissimus* muscle area; TL = thawing loss; CL = cooking loss, WHC = water-holding capacity, WBSF = Warner-Bratzler shear force; L* = luminosity (0 = black, 100 = white); a* = index from green (−) to red (+); b* = index from blue (−) to yellow (+).

4. Discussion

4.1. Fermentation and aerobic stability of corn silage

Prolonged storage (> 120 d) of corn silage and its interaction with inoculation using *L. buchneri* was investigated in the current study as a strategy to improve starch disponibilization. The results revealed that both contents of soluble protein and ammonia-N were increased as the storage length was longer. This is not surprise because during fermentation these variables may increase as a response to proteolysis and deamination, respectively (McDonald et al., 1991). Increased soluble protein and ammonia-N are particularly important responses in this case because they have been well-correlated with degradation of protein matrix, being considered good indicators of ivSD (Der Bedrosian et al., 2012; Ferraretto et al., 2015). The degradation of the protein matrix that surrounds the starch granules must improve the microbial attachment, leading to the higher starch degradation in the rumen (McAllister et al., 1994).

A study showed that the proteolytic activity from bacteria and plant enzymes plays a major contribution (90%) to the proteolysis in silo (Junges et al., 2017), and may explain the rapid gains on ivSD occurring in the first 30 d of fermentation of corn silage (Daniel et al., 2015). Nevertheless, the ivSD continues to be improved after this period, but in a moderate way (Daniel et al., 2015) as a consequence of the proteolysis that takes place due to longer storage, as observed in this study. Thus, increasing the storage length of corn silage might be a crucial strategy to improve the starch digestibility, which is particularly important to Brazil. In this country, the majority of corn hybrids available for ensiling are flint genotypes, which recognizably exhibit low ivSD due to the higher vitreousness of the corn grain (Correa et al., 2002; Pereira et al., 2004).

Despite the increased concentration of ammonia-N by prolonging the storage of corn silage, its concentration was consistently reduced by inoculation. However, inoculation did not produce a clear response on the contents of soluble protein. This suggests that changes in fermentation pattern of corn silage caused by inoculation likely impaired the action of microorganisms capable of deaminate amino acids. For example, microorganisms such as clostridia are responsible to increase deamination into the silo (McDonald et al., 1991; Pahlow et al., 2003), and inoculation probably

decreased its population as the butyric acid (a typical fermentation end-product of this microorganism) was also reduced. This resulted in better protein preservation compared to the control silage. Hence, the lower concentration of ammonia-N and inconclusive responses on soluble protein in the inoculated silage may reject our initial hypothesis that *L. buchneri* could improve the starch digestibility in whole-crop corn silage stored for longer periods, a response noted previously in HMC and rehydrated corn grain silages (Da Silva et al., 2018, 2019). Probably the controversial results found in our study compared with that of Da Silva et al. (2018, 2019) can be attributed to the different materials investigated (whole-crop corn silage vs. corn kernel silages), which may display different microbial communities as well.

There was an interaction between inoculation and storage length for aNDF and ADF, in which inoculation did not produce obvious results. In contrast, the aNDF decreased while ADF increased according to the silage storage was longer. This result is likely attributed to the acid hydrolysis of hemicellulose during fermentation (McDonald et al., 1991). Despite the interaction reported for starch content, the little changes caused in this variable are probably of minor biological importance considering that the values remained relatively constant (31.6–32.3 g/kg DM).

The fermentation pattern of corn silage was altered by the interaction between inoculation with *L. buchneri* and storage length. Overall, inoculation of corn silage increased the concentration of acetic acid by 38.6%. This response is explained by the fact that *L. buchneri* is a heterofermentative LAB able in metabolizing lactic acid into acetic acid and 1,2-propanediol under anaerobic conditions (Oude Elferink et al., 2001). Moreover, acetic and propionic acids persistently increased as the storage of corn silage increased, while lactic acid decreased at 360 d. As known, there is a microbial succession according the fermentation process takes place, and heterofermentative LABs might be predominant after prolonged storage times (Lin et al., 1992; Drouin et al., 2019). For example, *L. buchneri*-like strains can remain fairly active even after months of storage (Kleinschmit and Kung, 2006). In this case, the epiphytic population of heterofermentative LAB may be responsible to convert lactic acid into acetic acid explaining the lowered concentration of lactic acid after 360 d of storage, since inoculation resulted in increased concentration of lactic acid. This statement is based on the fact that some epiphytic strains of LAB are able in utilize

lactic acid anaerobically for growth whether glucose is limiting during fermentation (Lindgren et al., 1990). Furthermore, strains of bacteria such as Lactobacillus diolivorans, an indigenous bacteria found in corn silage, are able to metabolize 1,2-propanediol (product of *L. buchneri* metabolism) into 1-propanol and propionic acid (Krooneman et al., 2002), and this may explain the increased concentration of propionic acid by extending the silage storage.

Despite the increased acetic acid in the inoculated silage, the concentration of lactic acid was also surprisingly increased (+19.9%) due to inoculation. Although we do not have a clear response for such result, increased concentration of lactic acid following inoculation with *L. buchneri* was previously reported (Arriola et al., 2011). As described earlier, inoculation of corn silage decreased the concentration of butyric acid (0.222 vs. 0.297 g/kg DM in the control). This response is desirable because the pathway used by clostridia to produce butyric acid leads to 51% of DM loss in the process (Pahlow et al., 2003). Thus, the increased concentration of lactic acid and lowered butyric acid may explain the lower DM loss found in the inoculated silage. Although the DM loss in corn silage is often increased following inoculation with *L. buchneri* because the additional CO2 produced in the heterofermentative pathway during fermentation (Bernardi et al., 2019), controversial results have been reported. For example, the inoculation of corn silage with *L. buchneri* substantially decreased fermentative loss (50 vs. 149 g/kg DM in the control) in mini-silo (Arriola et al., 2011).

Inoculation with *L. buchneri* consistently improved the aerobic stability of corn silage, which may be associated with the higher amount of acetic acid in the inoculated silage. Acetic acid has antifungal properties and it has been well-correlated with increased aerobic stability (Danner et al., 2003). Under tropical conditions, the yeast populations can overgrowth during the feedout phase because the elevated temperature (Ashbell et al., 2002), and its control through the *L. buchneri* is a crucial strategy to avoid higher spoilage, mainly if silage removal from the silo's face is low. Moreover, despite the increased concentrations of acetic and propionic acids in corn silage stored for longer periods, the aerobic stability was not affected.

4.2. Growth performance of lambs

To the best of our knowledge, the current study is the first one investigating the effect of increasing the storage length of corn silage on the metabolic and production responses of lambs. Feeding diets containing corn silage stored for longer time confirmed our initial hypothesis that the apparent digestibility of starch should be improved in lambs. Compared to 120 d, the storage of corn silage for 240 and 360 d increased the starch digestibility by 1.3 and 2.1 percentage units, respectively. As discussed earlier, the protein matrix is a physiochemical impediment to starch digestion and its breakdown during fermentation likely provided ruminal bacteria easy access to starch granules, resulting in higher digestion (Owens et al., 1986; McAllister et al., 1994; Junges et al., 2017). The gains observed on starch digestibility in this study are slightly lower than that reported in dairy cows (Santos et al., 2019), possibly by differences in the material and interval of storage investigated. The authors reported that starch digestibility increased from 86.9% to 89.3% when the cows consumed diets containing RSGS stored for 30 d and 90 d, respectively. In contrast, the improvements on starch digestibility found in our study are much lower than that usually observed in in vitro conditions (5 to 15%; Der Bedrosian et al., 2012; Windle et al., 2014; Kung et al., 2018). However, it is worth noting that substantial improvements on ivSD in corn silage has been reported in the first 30 d of fermentation and thereafter the gains are more moderate (Daniel et al., 2015). As the current study investigated only times of silage storage over the moderate curve for improvements on starch digestibility, it is possible that comparing shorter storages (i.e., < 30 d) against longer storages (i.e., > 120 d) may result in higher gains on starch digestibility, as well as improved animal performance. Nevertheless, this hypothesis needs further confirmation.

The ruminal pH and total VFA were not affected by treatments. These findings are in agreement with the unchanged ruminal fermentation in dairy cows fed RSGS stored for different periods (Santos et al., 2019). Nevertheless, in spite of increased starch digestibility as a response to increasing the storage of corn silage, an unexpected reduction in the molar proportion of propionate was reported in lambs fed the 240-d and 360-d diets compared to the 120-d diet. Taking into account the intake and digestibility of starch (Table 3), the expected consumption of digestible starch by lambs should be 0.44, 0.43, and 0.43 kg/d for the silages stored for 120, 240, and 360

d, which may partially explain why propionate was not increased. Similar inconsistency was reported earlier by Santos et al. (2019). In contrast, our study showed increased molar proportions of butyrate, valerate, and branched-chain fatty acids by extending the silage storage, which can allow higher microbial protein synthesis (MPS). An in vitro fermentation system demonstrated that branched-chain fatty acids and valeric acid supplementation led to increased MPS (Cummins and Papas, 1985).

Extending the storage resulted in higher soluble protein content of corn silage, but ruminal ammonia-N was increased only in lambs fed silages stored for 240 d in comparison to 120 d. The storage for 360 d produced intermediate value. Lengthening silage storage is expected to increase the rate of starch degradation in the rumen, leading to the higher ruminal use of ammonia-N (Santos et al., 2019) for MPS. Additionally, the rumen is a complex ambient and the ammonia accumulation is dependent of interactions between dietary protein solubility, diet energy content, ruminal fermentation, microbial community and growth, and host (Clark et al., 1992; Lana et al., 1998; Charmley, 2001). This means that the higher dietary soluble protein will not always be accompanied of increased concentration of ruminal ammonia-N because it is dependent of other factors as well.

Despite the improved starch digestibility, the DM digestibility was not significantly changed by treatments. However, inoculation produced an important difference numeric in DM digestibility when lambs were fed diets containing the corn silage stored for 120 d (86.4 vs. 76.1% in the control). The increased DM digestibility (+10 percentage units) may explain the increased feed intake reported for lambs fed the LB120 diet in comparison to the C120 diet. Alterations on feed intake and in vivo digestibility following silage inoculation have been attributed to changes in chemical composition of silages, including the fermentation end-products (Winters et al., 2001; Rabelo et al., 2018). Nevertheless, there were not substantial changes in chemical composition of corn silage inoculated with *L. buchneri* and stored for 120 d that justify such effect on digestibility. *Lactobacillus buchneri* has been used with the goal to decrease silage spoilage, and this benefit was clearly observed in the current study. Nevertheless, the causes for the enhanced digestibility and the interaction noted between inoculation and storage length for feed intake in the 120-d diet is difficult to explain, once the same results were not reported in lambs fed the 240-d and 360-d

diets. Furthermore, inoculation of corn silage did not enhance the ADG and feed efficiency. These results agree very well with those of Bernardi et al. (2019), who showed through a meta-analytical approach that inoculation of corn silage with *L. buchneri* did not change the growth performance of sheep.

Lengthening silage storage from 120 d to 240 and 360 d tended to increase the weight gain (+4.6% and +10.2%, respectively) of lambs. Such result may be explained by the higher digestibility of starch (and other nutrients, which were not measured) and MPS, although the latter was not measured in this study. It is well-recognized that MPS in the rumen is largely driven by the amount of energy derived from ruminal fermentation of carbohydrates, but is also influenced by other factors such as ruminal pH and bacterial requirements for N (Russell, 1998). As ruminal pH was not affected by treatments, a higher energy available for microbial growth could be obtained through the increased starch digestibility. However, such hypothesis about MPS needs to be investigated further. As a consequence, the feed efficiency was improved by 5% and 14% when the silages were stored for 240 and 360 d compared to 120 d. Despite of absence of statistical differences, the enhanced feed efficiency and ADG resulted numerically in less time needed for animal slaughter due to lengthening silage storage (51.9, 49.8 and 47.2 d for 120, 240 and 360 d of storage, respectively). Such result has important practical implications because this means reduction of costs associated with feeding and labor in the feedlot. Although, to date, there is no other study examining the impact of storage length on animal weight gain, our findings are supported by Santos et al. (2019). In that study, increasing the storage of RSGS from 30 to 90 d allowed the cows to produce 1.2 kg/d more milk without changing DMI. The authors noted that the higher starch and protein digestibility were responsible to explain 88.6% of the difference in milk yield.

The meat of lambs fed inoculated silages had characteristics such as increased pH, TL, and luminosity and decreased intensity of red. Nevertheless, the changes caused by inoculation were little with minor biological importance or did not exist for the majority of variables. These findings are supported by previous studies (Addah et al., 2014; Rabelo et al., 2016; Lara et al., 2018), which showed that silage inoculation is not expected to cause significant changes in meat of small and large ruminants.

With exception of moisture and TL, the carcass and meat traits of lambs were not altered by increasing the storage of corn silage. Probably this result may be associated with the interval of storage examined in this study, which covered only longer times and then, there is not expected to find big differences in meat quality in this case.

In summary, the current study detected a substantial improvement on ADG and feed efficiency in lambs by increasing the storage length of flint corn silage. It is worth noting that such results were obtained through a moderate inclusion of silage in the diet of lambs (35% on DM basis) and also the interval of storage length assessed (120 to 360 d) was over the curve wherein the expected gains on starch digestibility are more timid (Daniel et al., 2015). Once the majority of corn hybrids available in Brazilian market for silage production are flint type and its vitreousness is a constraint for increased starch digestibility, extending the storage length of flint corn silage was proven to be a huge strategy to attain improved starch digestibility with significant impact on lambs' performance. Such results mean that farmers should open the silos only after longer storage of corn silage with the goal to improve animal performance, with consequent impact on the farm profitability. This information is particularly important because in some cases Brazilian farmers have opened the silos earlier than desirable (< 30 d of storage) and therefore, the benefits of extending storage on starch availability will be not achieved.

5. Conclusion

The productivity responses of lambs are enhanced by increasing the storage length of flint corn silage, a response likely arising from the improved silage digestibility and MPS. Despite the increased aerobic stability of corn silage, inoculation with *L. buchneri* did not contribute to improve animal performance.

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