TATIANE FERNANDES

CHARACTERIZATION OF REHYDRATED SORGHUM AND CORN GRAIN SILAGES WITH ENZYMES AND A MODEL OF STARCH RUMINAL DEGRADABILITY IN FEEDSTUFFS

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

Prof.ª Carla Luiza da Silva Ávila

Orientadora

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Fernandes, Tatiane .
Characterizationof rehydrated sorghum and corn grain silages with enzymes and a model of starchruminal degradability in feedstuffs / Tatiane Fernandes. - 2018.
140 p.

Orientador(a): Carla Luiza da Silva Ávila.
Coorientador(a): Marcos Neves Pereira, Rosane Freitas Schwan, Luiz Felipe Ferraretto.
Tese (doutorado) - Universidade Federal de Lavras, 2018.
Bibliografia.

1. Bacillus. 2. ruminal degradability. 3. reconstituted corn. I. Ávila, Carla Luiza da Silva. II. Pereira, Marcos Neves. III. Schwan, Rosane Freitas. IV. Ferraretto, Luiz Felipe. V. Título.

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CHARACTERIZATION OF REHYDRATED SORGHUM AND CORN GRAIN SILAGES WITH ENZYMES AND A MODEL OF STARCH RUMINAL DEGRADABILITY IN FEEDSTUFFS

CARACTERIZAÇÃO DAS SILAGENS DE GRÃOS DE SORGO E MIILHO REIDRATADOS COM ADIÇÃO DE ENZIMAS E MODELO DE DEGRADAÇÃO RUMINAL DO AMIDO EM ALIMENTOS

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

Aprovada em 05 de março de 2018.
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LAVRAS - MG

2018

Aos meus pais, Vanderlei e Neusa, pelo maior amor do mundo

Dedico

AGRADECIMENTOS

Ao “SENHOR” que me conduz pelos caminhos da vida.

Aos meus pais Vanderlei e Neusa Fernandes, que amo incondicionalmente, pelo amor, ensinamentos, dedicação, educação, paciência e todas as oportunidades que eu tive em minha vida.

Às minhas irmãs Anne e Deise Fernandes, pela paciência, amizade e amor durante toda a minha vida.

À minha pequena e linda sobrinha Júlia, por alegrar as nossas vidas.

A minha família pela paciência e compreensão que tiveram durante os momentos ausentes e difíceis em que deixei de compartilhar alegria e carinho.

À Universidade Federal de Lavras, em especial aos Programas de Pós-Graduação em Zootecnia e Microbiologia Agrícola, por fornecer o aporte necessário para o desenvolvimento deste trabalho.

À University of Florida por me receber como aluna no programa sanduiche, e dar suporte à realização deste trabalho.

Ao Conselho Nacional de Pesquisa – CNPq, pela concessão da bolsa de estudos, apoiando financeiramente a realização desta pós-graduação.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pela concessão de bolsa no programa de doutorado sanduiche.

À Professora Carla Luiza da Silva Ávila, pela orientação, ensinamentos, paciência, compreensão e principalmente exemplo de pessoa e profissionalismo.

Aos meus coorientadores Marcos Neves Pereira, Luiz Felipe Ferraretto e Rosane Shwan, pelo apoio e dedicação.

À Renata Apocalypse Nogueira Pereira, pela disponibilidade, apoio e auxilio.

À Beatriz Carvalho por todas as broncas e puxões de orelha, mas principalmente pelo apoio e dedicação durante a condução dos trabalhos e sem dúvida pelo exemplo de pessoa forte e batalhadora.

Aos colegas dos grupos de estudo Grupo do Leite, NEFOR e NEFER, obrigado por compartilharem conhecimentos, dividirem os trabalhos e somarem os bons momentos.

Aos meus mais que colegas, amigos com quem partilhei muitos momentos Paola Spasiani, Fernanda Cavalari, Felipe Amaro, Fernanda Gomes, Josiane Santos, Karina Toledo, Fabiana Cardoso, Júlia Dias, Eveline Alves, Leilane Oliveira, Jessica Gusmão, Cátia Feranandes, Daviane Costa, Maria Helena, Douglas Duarte, Eugenio Barbosa.

Aquelas com quem dividi muito mais do que o aluguel, dividi os melhores momentos e também as piores frustrações, por todo apoio, companheirismo e amizade Stéfanny Martins, Patrícia Rodriguez, Viviane Reis, Mariana Vianna, Agatha Neves, Victoria Rocha, Jessica, Ana.

A todos aqueles que de alguma forma colaboraram para que este trabalho se tornasse uma realidade.

Só levo a certeza que muito pouco eu sei.

Ou nada sei.

Almir Sater

RESUMO GERAL

Artigo 1. Os objetivos deste estudo foram avaliar o efeito de dois amyloglicosidases e duração de armazenamento de grãos de sorgo ou milho reidratado e ensilados. Os grãos foram reidratados (65% de MS) antes de ensilar. Dosagens de amiloglicosidase AMG (AMG, Novozymes) e GAM (Sanferm rendimento, Novozymes) foram de 0,35 mL/kg de grão. Tratamentos foram um fatorial de 2 x 3 x 2, sendo grão (sorgo vs. milho), enzima (CTL vs AMG vs GAM), e duração (30 d vs 180 d) com 6 repetições. A degradação in situ de MS foi avaliada com tempos de incubação de 0 (lavagem), 3, 6, 12, 18 e 48 h em 3 vacas canuladas no rúmen. Reidratação e ensilagem de sorgo ou milho com adição de amiloglicosidase resultaram em fermentação adequada, como evidenciado pelo pH e concentração ácido láctico. Aumento da perda de MS, da hidrolise de amido e a proporção de MS como fração A, sem alteração no kd ou degradabilidade efetiva a 6.5% de taxa de passagem. Sendo necessário maior tempo de fermentação para melhorar a digestibilidade da MS.

Artigo 2. O objetivo deste estudo foi identificar a diversidade de microrganismos e a relação entre esses microrganismos com características de sorgo e milho reidratado e ensilados com adição de amiloglicosidase. Para silagem foi utilizado o mesmo procedimento do artigo 1. Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry foi utilizado para identificação. Sete espécies de bactérias do ácido láctico (BAL) foram isoladas com uma população média de 3,84 e 5.74 ufc/g aos 30 e 180 dias de fermentação, respectivamente. A diversidade de bactérias formadoras de esporos aeróbias (SAB) foi de 20 espécies, com uma população média de 3,18 e 2.72 ufc/g aos 30 e 180 dias de fermentação, respectivamente. A população de BAL e maior parte do SAB foram correlacionadas com ácidos e perdas de nutrientes e negativamente correlacionadas com teor de pH, DM e amido. Três SAB foram correlacionadas com conteúdo prolamina. A

fermentação do grão reidratado é dominada por BAL, com alta população, mas SAB possuem maior diversidade. A maior parte dos microrganismos envolveram-se em perdas de MS e produção de ácidos.

Artigo 3. Os objetivos deste estudo foram determinar: 1) o método mais adequado para estimar a fração rapidamente degradável (A); 2) um ponto de tempo para medir a fração de indigestível (C); e 3) a viabilidade de utilizar menos tempo-pontos para estimar a taxa de desaparecimento fracionária de amido (kd) de grãos de milho maduro moídos através de 4 tamanhos (1, 2, 4 e 6 mm) de peneira. Fração A foi determinada por seis procedimentos diferentes. Incubação ruminal *in-situ* foi realizada nos tempos 48, 72, 96 e 120 h para determinar a fração C e nos tempos 0 (máquina de lavar), 3, 6, 12, 18, 24 e 48 h para determinar a cinética do desaparecimento do amido. Modelos foram usados com 2 ou 3-pools e kd foi determinada pela inclinação do logaritmo linear 'ln' do resíduo de cada tempo de incubação como proporção da amostra incubada. Fração A foi afetada pela moagem tamanho; mas não pelo método de lavagem. Amostras moídas em 6mm tinha maior fração C do que outros tamanhos de moagem com 48, 72 ou 96 h; mas não em 120 h. O modelo afetou valores de fração B unicamente. Como maior tamanho de moagem, observou-se maior fracções B e C, mas menor kd e ERD. Com base na análise de correlação, o modelo 2-pools, e os tempos de incubação de 48 h, 3 e 0 foram adequados para avaliar a cinética de degradação ruminal do amido nos alimentos para animais. Incubação ruminal *in situ* a 120h evidenciou a falta de uma fração C de amido (0,13% de amido). Determinação da fração A enxaguando em uma máquina de lavar e incubação ruminal *in situ* por 0, 3 e 48 h para a cinética de degradação de amido usando um modelo de 2-pools são sugeridos para alimentos ricos em amido. Tamanho de moagem afetou a cinética de degradação de amido.

Palavras chave: *Bacillus*, degradabilidade ruminal, Lactobacillus, milho reconstituído.

GENERAL ABSTRACT

Paper 1. The objectives of this study were to evaluate the effect of two amyloglucosidases and duration of storage of sorghum or corn kernel rehydrated and ensiled. Kernels were rehydrated (65% of DM) before ensiled. Dosages of amyloglucosidase AMG (AMG, Novozymes) and GAM (Sanferm Yield, Novozymes) were 0.35 mL/kg of kernel. Treatments were a 2 x 3 x 2 factorial combination of G (Sorghum vs. Corn), A (CTL vs. AMG vs. GAM), and duration (30 d vs. 180 d) with 6 replicates. The *in situ* DM degradation was evaluated with incubation times of 0 (bag wash), 3, 6, 12, 18, and 48 h in 3 rumen cannulated cows. Rehydration and ensiling of sorghum or corn with addition of amyloglucosidase resulted in adequate fermentation, as evidenced by pH and lactic acid concentration. Increased DM loss, hydrolyze of starch, and the proportion of DM as fraction A, without alteration on kd or ERD 6.5. Is necessary long time of fermentation to improve DM digestibility.

Paper 2. The objective of this study was to identify microorganism diversity and relationship between those microorganisms with characteristics of rehydrated sorghum or corn silages with addition of amyloglucosidase. For silage were used the same procedure of paper 1. Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry and PCR-based to identification. Seven species of lactic acid bacteria (LAB) were isolated with an average population of 5.74 and 3.84 ufc/g at 30 and 180 days of fermentation, respectively. The spore forming aerobic bacteria (SAB) diversity was 20 species, with an average population of of 3.18 and 2.72 ufc/g at 30 and 180 days of fermentation, respectively. The population of LAB and most part of SAB were corelated with acids and nutrient losses and negatively correlation with pH, DM and starch content. Three SAB were corelated with prolamin content. The fermentation of rehydrated kernel was

dominating by LAB, with highly population, but had greater diversity of SAB. The most part of microorganisms were involved in acids production, DM and starch losses.

Paper 3. The objectives of this study were to determine: 1) the most adequate method to estimate the rapidly degradable fraction (**A**); 2) a time-point to measure the undigestible fraction (**C**); and 3) the viability of using fewer time-points to estimate starch fractional disappearance rate (**kd**) of mature corn grain ground through 4 grinding sizes (1, 2, 4, and 6-mm). Fraction A was determined by six different procedures. Ruminal in situ incubations were performed at 48, 72, 96, and 120 h to determine fraction C, and at 0 (washing machine), 3, 6, 12, 18, 24, and 48 h to determine the kinetics of starch disappearance. Models were used with either 2 or 3-pools and kd was determined by the linear slope of the 'ln' of bag residues as a proportion of incubated samples over time. Fraction A was affected by grinding size; but not by washing method. Samples ground at 6-mm had greater fraction C than other grinding sizes at 48, 72 or 96 h; but not at 120 h. Model affected fraction B values solely. Greater fractions B and C, but reduced kd and ERD were observed as grinding size increased. Based on correlation analysis the 2-pool model, and the incubation times of 0, 3, and 48 h were suitable to evaluate ruminal starch degradation kinetics in feedstuffs. Ruminal in situ incubation at 120 h highlighted the lack of a fraction C of starch (0.13% of starch). Determination of fraction A by rinsing in a washing machine, and ruminal in situ incubations of 0, 3, and 48 h for starch degradation kinetics using a 2-pool model are suggested for starchy feedstuffs. Grinding size affected starch degradation kinetics.

Key words: *Bacillus*, ruminal degradability, Lactobacillus, reconstituted corn.

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PRIMEIRA PARTE

1 INTRODUÇÃO

O milho (*Zea mays*) e o sorgo (*Sorghum bicolor* (L.) Moench) estão entre os principais cereais utilizados na alimentação animal como fonte de amido (CRUZ; NUCIO, 2002). Os grãos de milho e sorgo são compostos por aproximadamente 72% de amido (PAES, 2006), mas híbridos de sorgo apresentam maior variação na digestibilidade e fermentação ruminal do amido que os híbridos de milho (STOCK, 1999). A digestibilidade do amido é influenciada por vários fatores tais como o tamanho das partículas, processamento de cereais, método de armazenamento (Firkins et al., 2001; Ferraretto et al., 2013), maturidade na colheita, teor de umidade, duração do armazenamento no silo (Hoffman et al., 2011; Ferraretto e Shaver, 2012) e tipo de endosperma de milho (Correa et al., 2002).

As principais diferenças entre sorgo e milho se referem ao tipo e distribuição de proteínas que circundam o amido no endosperma (CRUZ; NUCIO, 2002). Os grãos de milho e sorgo apresentam em média 7-15% de proteína, sendo que 70-90% são consideradas como proteínas de reserva, as prolaminas, chamadas de zeínas no milho e kafirinas no sorgo (BOYER; HANNAH, 2001; LANRY et al., 2005; NUNES et al., 2005; WANISKA; ROONEY, 2000). As prolaminas estão localizadas principalmente no endosperma e possuem grande quantidade de prolina e aminas (HERMAN; LARKINS, 1999). A prolina é a responsável pela característica hidrofóbica das prolaminas, tornando-a não solúvel em água e formando uma barreira hidrofóbica que envolve os grânulos de amido (MCALLISTER et al., 1990) e que impede a ação das enzimas que degradam o amido, reduzindo a degradação.

A silagem de grão reidratado consiste, basicamente, na hidratação do grão maduro moído, o que propicia sua fermentação e armazenamento como silagem (PEREIRA et al., 2013). A moagem, reidratação e ensilagem são formas de processamento, que associadas podem aumentar a digestibilidade do amido (CASTRO, 2017). A silagem de milho reidratado é uma forma de contrapor o efeito negativo da textura dura do endosperma sobre a digestibilidade do amido em grãos no estágio maduro de maturação. Durante a ensilagem ocorre proteólise por enzimas microbianas das prolaminas que envolvem os grânulos de amido (HOFFMAN et al., 2011), o que é capaz de aumentar a proporção da degradação que ocorre no rúmen (BITENCOURT, 2012).

O processo de fermentação da silagem é considerado como uma metabiose, envolvendo o desenvolvimento de microrganismos de diversos gêneros e espécies que são influenciados pelo pH, pelo potencial de oxirredução e pelos substratos presentes no material ensilado (PEREIRA; SANTOS, 2006). Caracterizar a microbiota da silagem pode auxiliar na compreensão da fermentação da silagem, possibilitando a intervenção do processo de fermentação e da melhora da qualidade da silagem (CARVALHO et al., 2016). Há indícios que o tipo de microrganismo presente na silagem pode modificar a intensidade da proteólise e influenciar a degradação ruminal (SILVA et al., 2018).

A ensilagem de milho ou sorgo reidratado resulta em melhoras na digestibilidade do amido, porém, os maiores ganhos ocorrem com o longo período de ensilagem (CARVALHO et al., 2016), esses mesmos autores observaram que maiores perdas ocorrem nos maiores tempos de fermentação. O uso de enzimas como aditivo no momento da ensilagem pode resultar em rápida disponibilização do amido para a degradação (FERRARETTO et al., 2015). A amiloglicosidase é uma enzima com potencial de auxiliar a disponibilização do amido, pois não se liga ao amido apenas pelas extremidades, mas também desestrutura as ligações helicoidais facilitando a hidrólise por outras enzimas como a α-amilase (ZHANG; DHITAL; GIDLEY, 2013).

Entender como as características dos alimentos podem afetar o local de digestão ou a degradação do amido é essencial para melhorar o desempenho dos animais (Allen, 2000). Desta forma o uso de técnicas para mensurar a degradação do amido é necessário. A técnica de degradabilidade *in situ* foi proposta por Mehrez e Orskov (1977) e consiste no desaparecimento da amostra de alimento acondicionada em sacos de nylon e incubados no rúmen por diferentes períodos de tempo. É relatado o uso de diferentes tamanhos de partícula de amostra submetida à incubação, com variações de 1,5 a 3,0 mm para concentrado (VANZANT et al., 1998). Os tempos de incubação são dependentes do substrato e características do alimento que se pretende avaliar, existindo tempos críticos de incubação próximos a parte “mais sensível” da curva de degradação (MICHALET-DOREAU; OULD-BAH, 1992). Os modelos utilizados para estimativa da taxa de degradação devem ser adequados ao encaixe nos dados de degradação, assim como devem descrever de forma adequada a biologia de degradação (VANZANT et al., 1998).

Neste contexto, objetivou-se caracterizar o sorgo e o milho reidratados e ensilados com adição de amiloglicosidase; identificar e correlacionar os microrganismos presentes na silagem com o perfil de fermentação; determinar o melhor método de lavagem para determinar a fração

solúvel; determinar o tempo de incubação para estimar o amido indigestível; determinar os melhores tempos de incubação para estimar a taxa de degradação do amido; gerar um banco de dados com as taxas de degradação de alimentos concentrados.

2 REFERENCIAL TEÓRICO

2.1 Disponibilidade de amido nos cereais: sorgo e milho

Os cereais constituem a principal fonte de alimentos no mundo. O milho é o principal cereal cultivado nas regiões tropicais e é consumido, basicamente, como fonte energética (PEREIRA, 2006). O amido é a principal forma de armazenamento de energia para a germinação da semente, onde o endosperma (tecido de armazenamento) é constituído, basicamente, de proteínas e grânulos de amidos. O aproveitamento do amido é afetado por sua composição, interações entre amido e proteína, integridade celular do grânulo de amido, presença de fatores antinutricionais como o tanino e a forma física do alimento fornecido (ROONEY; PFLUGFELDER, 1986).

2.1.1 Milho e sorgo

O milho (*Zea mays*) e o sorgo (*Sorghum bicolor* (L.) Moench) estão entre os principais cereais utilizados na alimentação animal, como fonte de amido (CRUZ; NUCIO, 2002). Os grãos de milho e sorgo são compostos por aproximadamente 72 % de amido (PAES, 2006), porém apresentam diferenças quanto à digestibilidade e à taxa de degradação do amido, sendo inferior para o sorgo em relação o milho (CRUZ; NUCIO, 2002).

Aproximadamente 70 % do milho produzido no mundo é destinado à alimentação animal, podendo chegar à 85% nos países mais desenvolvidos (PAES, 2006). A utilização do sorgo como substituto ao milho é vantajosa principalmente em regiões regiões áridas e semi-áridas. O sorgo apresenta maior resistência ao estresse hídrico, em função do seu sistema radicular bem desenvolvido o que permite absorção de água nas camadas mais profundas do solo, além da menor superfície foliar comparada ao milho, resultando em menor perda de água por transpiração (SERRANO, 1971).

O milho, que tem como provavel local de origem o México, possui grãos, geralmente, amarelos ou brancos, com variações de cor entre vermelho e preto (FERNANDES, 2014). De acordo com Paes (2006) o grão de milho possui quatro principais estruturas físicas. Endosperma é a estrutura de armazenamento de energia, que corresponde por 83% do grão, sendo constituído principalmente de amido (88%) e proteína de reserva (8%). Gérmen é a estrutura reprodutiva, que representa 11% do grão, sendo rico em lipídeos, minerais, proteínas e açúcares. Pericarpo que

possui a função de proteção fisica do grão, representando 5% do peso do grão, constituído basicamente de hemicelulose e celulose. Ponta é o local de inserção do grão no sabugo, representa 2% da estrutura física do grão, sendo composta por material lignocelulósico (PAES, 2006).

O sorgo é originário de regiões de clima tropical, provavelmente da África, mas há evidências indicam que teve dispersão independente em duas regiões, na Índia, além da África (RIBAS, 2008). Não suporta temperaturas baixas, necessitando de temperaturas acima de 20°C para um bom desenvolvimento (RODRIGUES FILHO et al., 2006). Entretanto, adapta-se às situações de déficit hídrico e baixa fertilidade dos solos (RIBAS, 2010). O grão de sorgo é composto por pericarpo, endosperma e gérmen. O pericarpo é o revestimento fibroso, com função de proteção física, correspondendo a 5% do grão. O endosperma onde está estocado o amido, correspondendo a 84%. O gérmen, que é a estrutura germinativa, rica em lipídeos, proteínas de reserva, enzimas e minerais, representando 10% do grão (ROONEY; SERNA-SALDIVAR, 1991).

Os híbridos de sorgo apresentam maior variação na digestibilidade e fermentação do amido, que os híbridos de milho. Sendo que essa variação é resultante das condições de cultivo deste grão, pois os híbridos de sorgo, muitas vezes são cultivados em regiões mais quentes e com baixa disponibilidade de água (STOCK, 1999). Além de fatores climáticos e do tipo de hibrido, a composição e a forma física, as interações entre proteína e amido, a integridade celular do grânulo de amido e os fatores antinutricionais, como a presença de taninos, que afetam a digestibilidade do amido. O sorgo apresenta maior proporção de endosperma periférico, região mais densa do endosperma e resistente à penetração de água, com maior proporção de proteína, tornando-o mais resistente à degradação enzimática (ROONEY; PFLUGFELDER, 1986).

2.1.2 Textura do endosperma

Há uma variedade de genótipos de milho com o intuito de atender as exigências de diferentes regiões quanto aos fatores ambientais, à produtividade, resistência à pragas e doenças, ciclo vegetativo e o tipo do endosperma do grão (ZOPOLLATTO, 2007). Da mesma forma, buscando aumentar a produtividade e a qualidade do grão de sorgo, tem sido realizado melhoramento de variedades, principalmente à partir da introdução de características do endosperma amarelo, que garantem resistência à seca e aumento da qualidade do grão (MILLER, 1995).

Como relatado anteriormente, o endosperma corresponde à aproximadamente 83% do peso seco do grão de milho ou sorgo (PAES, 2006; ROONEY; SERNA-SALDIVAR, 1991), sendo constituído principalmente de amido organizado na forma de grânulos (ECKHOFF; PAULSEN, 1996). O grânulo de amido possui função de reserva e é constituído por dois tipos de cadeias helicoidais de glicose, amilose e amilopectina. A amilose constitui 20 a 30 % do amido do granulo, é um polímero linear, formado por aproximadamente 600 unidades de α-D glicose, com ligações α-1,4 entre as unidades (BULÉON et al., 1998). A amilopectina constitui 70 a 80% do amido do grânulo, formado por cadeias grandes e ramificadas em conformação alfa-hélice, com ligações α-1,4 entre as unidades acrescidas de ligações α-1,6 a cada 20 a 25 moléculas de glicose, formando as ramificações (BULÉON et al., 1998). Grânulos de amido são formados por moléculas de amilose e amilopectina, unidas por pontes de hidrogênio, resultando em uma estrutura organizada (VAN SOEST, 1994), formados pela deposição de anéis de crescimento, que contém regiões amorfas, compostas principalmente por amilose, e regiões cristalinas compostas principalmente por amilopectina (NOCEK; TAMINGA, 1991). A organização dos grânulos de amido é muito complicada e muito dependente da origem botânica da planta (BULÉON et al., 1998). A digestibilidade do amido é, em geral, inversamente proporcional ao seu conteúdo de amilose (ROONEY; PFLUGFELDER, 1986).

No Brasil, há predominância por produção de semente de milho com endosperma do tipo *flint*, ou seja, endosperma duro, que permite maior resistência à pragas e à doenças. São resistentes à quebra mecânica durante a colheita, secagem, transporte e armazenamento e também são menos susceptíveis à colonização por fungos e ao ataque de insetos durante o armazenamento (KAMRA, 2005). O milho de textura macia ou farinácea, predominantes na América do Norte, tem maior propensão à quebra mecânica, o que é desejável no processo de produção de silagem ou durante a moagem na indústria (CORREA et al., 2002; GIUBERTI et al., 2013).

A textura do grão é determinada pelo cultivar, sendo uma avaliação visual da proporção das regiões (vítria ou farinácea) do endosperma (RIBAS et al., 2007). A vitreosidade é uma forma objetiva de mensurar a textura do endosperma, sendo uma medida que determina a proporção entre o endosperma duro (vítreo) em relação ao endosperma total. A vitreosidade no estágio maduro de maturação foi em média 72,3% para híbridos duro e em média 48,1% para híbridos de textura macia, em trabalho realizado com grãos de diferentes híbridos de milho (PHILIPPEAU; MICHALET-DOREAU, 1997). Já no que diz respeito ao sorgo, segundo Rooney; Pflugfelder,

(1986) este grão apresenta, de maneira geral, maior proporção do chamado endosperma periférico (vítreo), região densa e resistente à penetração de água. Porém, em estudo mais recente, realizado com 33 cultivares produzidos por empresas melhoradoras no Brasil, 81.8 % dos cultivares apresentaram endosperma de textura média à média-macia, ou seja com 50% ou menos de vitreosidade, obtido por avaliação visual (ANTUNES, 2007).

A vitreosidade do endosperma do grão pode ser obtida indiretamente pela mensuração da densidade (CORREA et al., 2002), e por mensuração do teor de prolamina na semente (HOLDING, 2014). Essas avaliações são vantajosas, pois permitem mensurar a vitreosidade em grãos processados ou ensilados, pela prolamina (LOPES, 2016). Ou a densidade para o grão inteiro, sendo uma forma vantajosa de obtenção indireta da vitreosidade pois demanda menos tempo de trabalho manual (CORREA et al., 2002; DOMBRINK-KURTZMAN; BIETZ, 1993).

No endosperma vítreo, os grânulos de amido estão fortemente incrustados no arcabouço protéico formado pela matriz protéica espessa e contínua, e pelos corpos protéicos (SULLINS; ROONEY, 1975). No endosperma farináceo, a matriz protéica encontra-se presente na forma de lâminas delgadas, é descontínua e está fracamente incrustada aos grânulos de amido, o que torna o amido mais disponível ao ataque enzimático (SULLINS; ROONEY, 1975; SHULL et al., 1990).

Ao se considerar aspectos relacionados à nutrição animal, quanto menor a porção do endosperma vítreo, maior é a digestibilidade do amido presente no grão, em contrapartida, quanto maior a vitreosidade do grão, maior é a resposta ao processamento do grão (CORREA et al., 2002; PHILIPPEAU; MICHALET-DOREAU, 1997).

2.1.3 Matriz proteica e prolaminas

Os grãos de milho apresentam em média 9-14% de proteína, sendo que 70-90% são consideradas como proteínas de reserva. As proteínas do milho podem ser classificadas em albuminas, que representam 3-5% da proteína total; globulinas, com 10-20% da proteína total; glutelinas, representando aproximadamente 25% da proteína total; e as prolaminas, chamadas de zeínas no milho, que representam aproximadamente 52% do conteúdo de proteína total no milho (BOYER; HANNAH, 2001; LANRY et al., 2005).

As proteínas do grão de sorgo variam entre 7% a 15% no grão inteiro, sendo considerado aproximadamente 82 % de proteína de reserva (WANISKA; ROONEY, 2000). Podem ser

divididas em globulinas, representando cerca de 10% das proteínas; as albuminas com cerca de 8% do total de proteínas; as glutelinas compõem cerca de 25% do da proteína; e as prolaminas, chamadas de kafirinas no sorgo, compõem 57% do total de proteínas (NUNES et al., 2005; WANISKA; ROONEY, 2000).

A matriz protéica é constituída por glutelinas, que são proteínas solúveis somente em soluções alcalinas, já os corpos protéicos, os quais são constituídos por prolaminas, são solúveis apenas em substâncias alcoólicas (butanol mais mercaptoetanol) (CHANDRASHEKAR; MAZHART, 1999). Com relação às proteínas localizadas no gérmen, as albuminas são solúveis em água e as globulinas em soluções salinas (BOYER; HANNAH, 2001).

Além das diferenças de tamanho e forma do grão, as principais diferenças entre sorgo e milho se referem ao tipo e distribuição de proteínas que circundam o amido no endosperma (CRUZ; NUCIO, 2002). As zeínas do milho compreendem quatro grupos estruturalmente distintos: alfa, beta, gama e delta-zeínas, que podem ser separadas com base no padrão eletroforético e no peso molecular (SHEWRY; HALFORD, 2002). A nomenclatura proposta para as kafirinas do sorgo é baseada nos padrões protéicos de polipeptídeos com peso molecular semelhante ao grupo das zeínas em milho, em alfa, beta, gama-kafirinas (SILVA, 2015).

No milho, as alfas-zeínas começam a se acumular, como lóculos discretos, dentro de uma matriz de gama e beta-zeínas, nos corpos protéicos. Ocasionalmente, os lóculos de alfa-zeínas fundem-se e preenchem o centro do corpo protéico (LENDING; LARKINS, 1989). Beta e gama-zeínas formam uma camada mais ou menos contínua na periferia, mas, manchas pequenas de beta e gama-zeínas permanecem no interior. As delta-zeínas são encontradas, principalmente, no centro dos corpos protéicos, junto com as alfa-zeínas (ESEN; STELLER, 1992). Os polipeptídeos das diferentes classes de zeínas variam, em tamanho, de 10 kDa a 27 kDa. As alfa-zeínas possuem pesos moleculares de 19 e 22 kDa, correspondendo à aproximadamente 70% da fração total de zeínas (LOPES, 1993; SHOTWELL; LARKINS, 1989). As beta-zeínas são proteínas de 14kDa e correspondem à aproximadamente 15% da fração de zeína (PEDERSEN et al., 1986). As gama-zeínas apresentam peso molecular de 16 e 27 kDa, correspondendo à 20% das zeínas totais, podendo chegar até a 50% (KIRIHARA ET AL., 1988; ORTEGA; BATES, 1983). A delta-zeína, possui 10 kDa e é uma proteína pequena que apresenta 130 aminoácidos de comprimento (KIRIHARA et al, 1988).

No sorgo as alfas-kafirina tem uma localização central no corpo da proteína e é envolvida por beta e gama-kafirina, encontrado ao redor da proteína. Esse arranjo estrutural da kafirina afeta a sua digestibilidade (WONG et al., 2010). As kafirinas podem formar polímeros que se ligam à pontes de dissulfeto, componentes estruturais de proteínas, fortalecendo e diminuindo a digestibilidade das proteínas (DUODU et al., 2002). Apesar de solúvel em água, a gama-kafirina não se solubiliza em estado nativo (no grão), exatamente pela polimerização entre as cadeias polipeptídicas (BELTON et al., 2006). As alfas-kafirinas, compõe cerca de 65-85% do total kafirinas e possuem peso molecular de 23 KDa (WATTERSON et al.,1993; WONG et al., 2010). A beta-kafirina possui peso molecular de 20 KDa e corresponde de 7 a 8 % do total de kafirina (RODRIGUES et al., 2009; WATTERSON et al.,1993). A gama-kafirina possui peso molecular de 28 KDa e corresponde de 9 a 12% das kafirinas (RODRIGUES et al., 2009; WATTERSON et al.,1993).

As prolaminas estão localizadas principalmente no endosperma, possuem grande quantidade de prolina e aminas, tendo como principal função fornecer nitrogênio durante o desenvolvimento inicial da planta (HERMAN; LARKINS, 1999). A prolina é a responsável pela característica hidrofóbica das prolaminas, tornando-a não solúvel em água e formando uma barreira hidrofóbica que envolve os grânulos de amido. A adesão microbiana aos grânulos de amido ocorre com maior facilidade no endosperma farináceo, que possui menos prolamina (MCALLISTER et al., 1990). O teor de prolamina na semente aumenta com a maturidade da planta e é maior em híbridos com endosperma de alta vitreosidade (HOLDING, 2014). Simpson (2001) sugere que a acidificação do endosperma torna as prolaminas mais susceptíveis à clivagem, pois as prolaminas são solúveis em ácido lático e acético (LAWTON, 2002). Essa clivagem pode ser facilitada e estimulada pela ensilagem.

2.1.4 Processamento e tamanho de partícula

O termo processamento de grãos se refere à métodos de preparação destes para serem utilizados na alimentação animal. A moagem grosseira, a quebra ou a laminação do grão são formas menos intensas de processamento; enquanto que a floculação, a extrusão e a reconstituição são métodos mais intensos (CRUZ; NUCIO, 2002). A moagem é mundialmente usada visando

aumentar a digestibilidade do amido, por ser relativamente barata, comparado com outros processos, podendo ser realizada na própria fazenda (LOPES, 2016).

Considera-se o milho moído mais fino possível, como tamanho ótimo de partícula para maior digestibilidade (HOFFMAN; SHAVER, 2011). A redução do tamanho de partícula resulta em aumento da área de superfície, aumentando assim a área disponível para a adesão dos microrganismos ruminais nos grânulos de amido e para a digestão enzimática (McALLISTER et al., 1993). O pericarpo é a camada externa com função de proteção do grão de milho, e a adesão de microrganismos ruminais ao pericarpo é mínima comparada ao endosperma (MCALLISTER et al., 1990). Com o processo de moagem o pericarpo é quebrado, tornando o endosperma acessível aos microrganismos.

Um dos efeitos do processamento de grãos é a mudança no sítio de digestão de amido do intestino delgado para o rúmen, com concomitante aumento nas porcentagens degradadas em ambos compartimentos. A digestão do amido ocorrendo preferencialmente no rúmen resulta em em maior produção de ácidos graxos voláteis e proteína microbiana para o animal, além do amido que chega ao intestino ter maior digestibilidade quando o grão é processado. O resultado final é maior suprimento de energia e proteína metabolizável para o animal e portanto, melhor desempenho (Huntington, 1997). Devido à sua menor digestibilidade, o sorgo é o grão mais responsivo aos métodos de processamento, fazendo com que ocorram aumentos significativos em seu valor alimentar e resposta em desempenho animal (CRUZ; NUCIO, 2002). A moagem, reidratação e ensilagem são formas de processamento, que associadas podem aumentar a digestibilidade do amido (CASTRO, 2017).

2.2 Ensilagem de grãos reidratados

A ensilagem é uma prática de conservação de alimentos que consiste na fermentação dos açúcares solúveis por bactérias do ácido lático (BAL) (MUCK et al., 2010), levando à rápida redução do pH (McDONALD, 1991) e consequentemente, a inibição do desenvolvimento de microrganismos indesejados (HENDERSON, 1993). A ensilagem de grãos como milho e sorgo, tornou-se uma prática comum em diversas propriedades brasileiras, em busca da melhoria da digestibilidade do amido e facilidade da estocagem (GOBETTI et al., 2013), possibilitando melhor

utilização do milho brasileiro, que possui endosperma duro de alta vitreosidade e baixa digestibilidade para bovinos (CORREA et al., 2002).

A silagem de grão reidratado consiste, basicamente, na hidratação do grão maduro moído, o que propicia sua fermentação e armazenamento como silagem (PEREIRA et al., 2013). O grão é colhido em estágio de maturação maduro (10-14% de umidade), é reidratado simultaneamente à moagem, com água para que atinja umidade entre 30 e 40% da matéria natural, ideal para a fermentação de grãos (GOBETTI et al., 2013), e a ensilagem é realizada. Esse processamento permite colher o grão com uma janela de colheita maior que a silagem de grão úmido, mais comumente utilizado. O grão não precisa ser cultivado na propriedade, podendo ser adquirido em época de safra, quando o valor do grão no mercado é baixo (LOPES, 2016). Dispensa a necessidade de grandes investimentos em silos para secagem e armazenamento de grãos secos e o milho pode ser armazenado na propriedade (FERNANDES, 2014). Possui como principal vantagem o ganho em digestibilidade do amido, pois durante a ensilagem ocorre a quebra das prolaminas por ação das enzimas microbianas (HOFFMAN et al., 2011), o que é capaz de aumentar a proporção da degradação que ocorre no rúmen (BITENCOURT, 2012).

A silagem de milho reidratado é uma forma de contrapor o efeito negativo da textura dura do endosperma sobre a digestibilidade do amido em grãos no estágio maduro de maturação. Os processamentos usados nos grãos ricos em amido normalmente visam aumentar a degradação ruminal desse nutriente (HALE, 1973). A magnitude da alteração promovida pelo processamento é inversamente proporcional à digestibilidade do grão não processado. Sendo assim, o efeito da ensilagem do milho maduro reidratado é mais pronunciado no milho com endosperma duro quando comparado ao milho com endosperma farináceo (ANDRADE FILHO et al., 2010), assim como o grão que mais responde ao processamento é o de sorgo, seguido pelo de milho e demais cereais, que tem alta degradação na forma não processada (THEURER, 1986).

2.2.1 Perfil fermentativo

O processo de fermentação da silagem é complexo, sendo considerado como uma metabiose, ou seja, envolve o desenvolvimento simultâneo e sucessivo de microrganismos de diversos gêneros e espécies que dependem principalmente do pH, do potencial de oxirredução e do tipo e quantidade de substratos presentes no material ensilado (PEREIRA; SANTOS, 2006). O

processo de ensilagem está baseado em princípios básicos de conservação, são eles: baixo pH e anaerobiose (JOBIM; NUSSIO, 2013). A velocidade com que esses princípios são alcançados é determinante para garantir o bom armazenamento dos grãos.

A condição de anaerobiose é dependente de diferentes processos. Sendo otimizada a partir de estratégias tecnológicas no momento do abastecimento, compactação e vedação do silo. Os processos metabólicos, tanto da forragem que está sendo armazenada, por meio da respiração e atuação de enzimas da própria planta, assim como de microrganismos presentes no material ensilado, que consomem o oxigênio retido entre as partículas na fase inicial de ensilagem também irão contribuir na anaerobiose da silagem (BERNARDES; WEINBERG, 2013; JOBIM; NUSSIO, 2013).

A rápida redução do pH é dependente de características da planta, como capacidade tampão e carboidratos solúveis disponíveis. Além disso, a população de microrganismos presentes no material no momento da ensilagem, sejam eles epifíticos ou introduzidos com o uso de inoculantes, irá consumir os carboidratos solúveis, produzindo ácidos orgânicos, responsáveis pela redução do pH (JOBIM; NUSSIO, 2013). Na silagem de milho maduro reidratado sem o uso de inoculante o tempo de consumo dos carboidratos solúveis pode variar, sendo de 5 dias para milho moído em crivo de 3 mm (CARVALHO et al., 2016) e 21 dias para milho moído em crivo 12 mm (FERNANDES, 2014). Já a redução no pH ocorreu de forma mais lenta atingindo pH 4,68 entre 15 e 30 dias e pH próximo a 4,2 após 210 dias de ensilagem, para milho moído a 3 mm, (CARVALHO et al., 2016), e pH próximo a 5, com 21 dias de ensilagem, em milho moído em crivo 12 mm, permanecendo com pH acima de 4,5 até 120 dias (FERNANDES, 2014).

Em silagens inoculadas com bactérias do ácido lático a redução do pH foi mais significativa, reduzindo para pH próximo a 3.7 em 247 dias, para milho moído em crivo de 3 e 9 mm (CASTRO, 2017). Em silagens inoculadas com *Lactobacillus plantarum* e *Pediococcus acidilactici* o pH ficou próximo a 4, para silagens inoculadas com *L. buchneri* o pH ficou próximo a 4,5, com 124 dias de ensilagem (Silva et al., 2018).

Os principais ácidos produzidos durante o processo de ensilagem são lático, acético, propiônico e butírico (KUNG; SHAVER, 2001). Além destes, existem vários ácidos que são produzidos durante o processo de fermentação, como isobutírico, valérico, isovalérico, succínico e fórmico málico (McDONALD et al., 1991). Na silagem de milho reidratado o aumento do ácido lático ocorreu à partir do dia 15 até dia 90 de ensilagem e o aumento no ácido propiônico ocorreu

após 60 dias de ensilagem, enquanto que alterações nos ácidos málico e succínico foram observadas após 90 dias de ensilagem, e os ácidos isobutírico, butírico e isovalérico ficaram abaixo dos níveis detectáveis (CARVALHO et al., 2016). Dentre os ácidos orgânicos formados durante a fermentação no processo de ensilagem, o ácido lático pode gerar menor perda de matéria seca (MS), pois sua produção é energeticamente mais eficiente e tem maior potencial em reduzir o pH (ROOKE; HATFIELD, 2003). Desta forma o uso de inoculantes à base de bactérias homofermentativas para a silagem de milho reidratado pode ser mais interessante.

A quebra das prolaminas durante o processo de ensilagem, resulta em aumento na concentração de amônia ao longo da ensilagem. Esse processo foi observado por Carvalho et al., (2016) com aumento de 2,2 unidades percentuais em silagens com 280 dias e por Fernandes (2014) com aumento de 2,5 unidades percentuais com 120 dias de ensilagem. Quando a silagem de milho reidratado foi avaliada em períodos curtos esse aumento no teor de amônia não foi tão evidenciado Ferraretto et al. (2018) observaram aumento de 0,09 % no teor de amônia, de silagens ensiladas por 28 dias. Silva et al. (2018) observaram aumento na concentração de NH_3 e degradação ruminal de silagem de milho reidratado e inoculado com *L. buchneri*, mas não observaram esta variação em silagens inoculadas com uma combinação de *L. plantarum e P. acidilactici*, indicando que o tipo de microrganismo adicionado na silagem pode modificar a intensidade da proteólise.

2.2.2 Microbiologia da silagem

Diferentes microrganismos estão presentes no processo de ensilagem, sendo que alguns são considerados benéficos ao processamento, como as bactérias ácido láticas (BAL) que são os principais responsáveis pela rápida queda no pH. Mas outros microrganismos considerados indesejados estão presentes no material ensilado, como bactérias da família *Enterobactericea* e gênero *Clostridium*, e também fungos filamentosos e leveduras, os quais se não forem controlados durante a fermentação, podem trazer consequências negativas para a qualidade da silagem (PAHLOW et al., 2003). Caracterizar a microbiota da silagem pode auxiliar na compreensão da fermentação da silagem, possibilitando a intervenção do processo de fermentação e melhora da qualidade da silagem (CARVALHO et al., 2016).

Há poucos relatos da população de microrganismos presentes na silagem de grãos reidratados. Carvalho et al. (2016) estudaram a microbiologia da silagem de grão de milho

reidratado do 0 aos 280 dias de ensilagem. As espécies de bactérias do ácido lático, *Enterococcus casseliflavus* e *Pediococcus pentosaceus* foram as únicas bactérias láticas identificadas antes da ensilagem e apenas o *P. pentosaceus* manteve-se na silagem até os 30 dias de fermentação. *Enterococcus casseliflavus*, *Enterococcus faecium*, *Enterococcus gilvus*, *Lactococcus lactis*, *Leuconostoc mesenteroides* e *Leuconostoc pseudomesenteroides* estavam presentes na silagem entre 5 e 15 dias de ensilagem. *Lactobacillus plantarum* foi detectado esporadicamente nos dias 5, 15, 60 e 280 de ensilagem, com população máxima de 6.48 log de UFC por g, aos 60 dias. *Weissella cibaria* e *Weissella paramesenteroides* foram identificadas com 5 e 15 dias de fermentação. *Lactobacillus paracasei* estava presente entre 30 e 150 dias de fermentação, apresentando redução na população (7,71 para 5,70 log de UFC), neste período. *Lactobacillus rhamnosus* e *Lactobacillus buchneri* foram identificados aos 60 dias de fermentação. Ainda esses autores identificaram as seguintes *leveduras Issatchenkia orientalis* (sinônimo de *Pichia kudriavzevii* e *Candida krusei*), *Meyerozyma guilliermondii* (previamente identificada como *Pichia guilliermondii*), *Wickerhamomyces anomalus* (sinônimo de *Pichia anomala* e *Hansenula anomala*) e *Hyphopichia burtonii* (sinônimo de *C. chodatii*).

A identificação de microrganismos provenientes da silagem tem evoluído ao longo do tempo, passado por técnicas de identificação por características morfológicas e bioquímicas, nas quais era necessária a utilização de chaves de identificação (ROGOSA, 1970) Posteriormente, com o desenvolvimento de técnicas de extração, amplificação e sequenciamento do ácido desoxirribonucleico (DNA), foi possível a identificação pela sequência gênica dos microrganismos. A maioria destas técnicas utiliza a reação da cadeia polimerase (PCR) para fazer muitas cópias de uma porção do DNA dos microrganismos (MUCK, 2013). O primer de amplificação é desenvolvido para uma fração altamente conservada da região 16S do RNA ribossomal, que é a base para a identificação das bactérias, já outras porções da região 16S são variáveis entre as espécies, permitindo a classificação (MUCK, 2013). O uso da espectrometria de massa por tempo de dispersão / ionização assistida por matriz (MALDI-TOF MS) é um novo método de identificação de microrganismos baseado no perfil de proteínas da célula, utilizado por Carvalho et al. (2016), para identificação de microrganismos presentes na silagem. Quando compara o método MALDI-TOF ao método fenotípico e ao sequenciamento do DNA, constata-se o MALDI-TOF como uma técnica mais rápida, requerendo menor volume de amostra e menor custo de reagentes (DEC et al., 2014).

2.2.2.1 Bactérias do ácido lático

As bactérias do ácido lático (BAL) são assim classificadas em função do seu metabolismo, em razão da capacidade de produzir ácido lático como principal produto do seu metabolismo. Seis gêneros deste grupo de bactérias estão normalmente presentes no processo de ensilagem, sendo eles: *Latobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Lactoccocus* e *Streptococcus* (KUNG JUNIOR, 2009). Ainda em função do metabolismo, as BAL podem ser classificadas em três grupos: as homofermentativas, que produzem somente ácido lático à partir da fermentação de hexoses, pela via glicolítica e não são capazes de utilizar pentoses via pentose fosfato; heterofermentativas facultativas que são normalmente classificadas como homofermentativas pois usam a mesma via glicolítica para a fermentação de glicose, mas fermentam pentoses em determinadas condições, produzindo etanol para manter o balanço redox via pentose fosfato e há ainda as heterofermentativas obrigatórias, que não utilizam a via glicolítica para metabolizar hexoses e sim a via fosfoquetolase, fermentam diferentes açucares e produzem principalmente lactato, etanol, CO_2, adenosina trifosfato (ATP) e água a partir da glicose, e lactato, acetato, 2-manitol, CO_2, ATP e água a partir da frutose pela via das pentoses fosfato (MADIGAN et al., 2014; PAHLOW et al., 2003). O uso de BAL no processo de silagem tem efetividade para acelerar a fermentação, com produção elevada de ácido láctico e redução do pH, reduzindo o metabolismo de microrganismos indesejados e melhorando o desempenho dos animais (BARNES et al., 2007).

Como visto anteriormente, o sequenciamento da região 16S é comumente utilizado para identificação de BAL (MUCK, 2013). Porém algumas espécies de BAL apresentam a sequência de genes da região 16S semelhante, resultando em identificação com mais de 99% de similaridade para mais de uma espécie. Desta forma, a associação de mais de uma técnica de identificação pode ser necessária para correta identificação de algumas espécies. Por exemplo, *P. pentosaceus* e *P. acidilactici* são proximamente relacionados quanto à base genética, mas as bactérias *P. pentosaceus* não crescem à 50°C, enquanto que *P. acidilactici* tem habilidade de crescer à 50°C (TANASUPAWAT; KOZAKI; KOMAGATA, 1993). Outras BAL que não são diferenciadas em função do sequenciamento de genes são *Lactobacillus pentosus*, *L. paraplantarum* e *L. plantarum*. Porém, *L. pentosus* pode ser diferenciado pela inabilidade em produzir ácido à partir da melezitose (CURKE; HUBERT; BRINGEL, 1996), enquanto que o *L. plantarum* pode ser distinto pela

habilidade de produzir ácido à partir do dulcitol (BRINGEL; QUENEE; TAILLIEZ, 2001). *L. buchneri* e *L. brevis* são geneticamente relacionados, porém *L. buchneri* tem a habilidade de fermentar melezitose (ROGOSA et al., 1970).

2.2.2.2 Bactérias formadoras de esporos

As bactérias formadoras de esporos envolvem diferentes gêneros de microrganismos e em geral são considerados indesejáveis para o processo de ensilagem (ADESOGAN; QUEIROZ, 2009).

São bactérias Gram positivas, anaeróbicas facultativas, formadoras de esporos, que são resistentes à altas temperaturas, podendo sobreviver ao processo de pasteurização do leite (PAHLOW et al., 2003). Logo, a presença destes microrganismos na silagem de vacas leiteiras, por exemplo, torna-se um entrave para a sanidade da produção de leite, já que em condições de contaminação deste produto, estes microrganismos, em especial o *Bacillus cereus*, podem produzir toxinas que são prejudiciais para a saúde humana, além de reduzir o tempo de prateleira de derivados lácteos (PAHLOW et al., 2003).

No processo de ensilagem Pahlow et al. (2003) sugerem que os *Bacillus* sejam os primeiros a se desenvolver após as leveduras consumirem o lactato, iniciando o processo de degradação. As principais espécies já registradas em silagens são *B. cereus*, *B. lentus*, *B. firmus*, *B. sphaericus*, *B. licheniformis* e *B. polymyxa* (GIFFEL et al., 2002). Paenibacillus macerans e Bacillus megaterium são espécies de formadores de esporos recentemente identificadas em silagens (MUCK, 2013).

Assim como ocorre com as BAL, os *Bacillus* também apresentam grupos que não podem ser distintos pelo sequenciamento da região 16S. Desta forma a utilização de testes bioquímicos pode auxiliar na correta identificação. *Bacillus atrophaeus*, *B. mojavensis*, *B. subtilis* e *B. vallismortis* não podem ser diferenciados pela região do 16S (ROBERTS et al., 1996), mas podem ser diferenciados pelo teste de produção de ácido à partir do sorbitol, melibiose e amido (RUIZ-GARCIA et al., 2005). *B. sonorensis* e *B. licheniformis* são proximamente relacionados com base na região 16S, porém podem ser diferenciados pela incapacidade que o *B. sonorensis* apresenta em crescer no meio contendo 5, 7 ou 10% de NaCl (PALMISANO et al., 2001). *B. safensis*, *B. pumilus*, *B. aerophilus*, *B. altitudinis* e *B. stratosphericus* são geneticamente próximos, mas podem ser diferenciados pelos testes de utilização de citrato, utilização de D-sorbitol e melibiose como

fonte de carbono (SATOMI at al., 2006; SHIVAJI et al., 2006). Para a diferenciação do grupo *Bacillus cereus* é necessário avaliação da composição de ácidos graxos da bactéria (NAKAMURA, 1998).

Os formadores de esporos do gênero *Lysinobacillus* também necessitam da combinação de testes fenotípicos para a identificação. *Lysinobacillus boronitolerans*, *L. fusiformis* e *L. sphaericus* necessitam dos testes de crescimento em NaCl a 7% e hidrolise da ureia para serem diferenciados (AHMED et al., 2007).

2.2.3 Aumento da degradação ruminal do amido e matéria seca

A digestão de carboidratos fibrosos e não fibrosos no rúmen e a digestão de carboidratos não fibrosos nos intestinos é a principal fonte de energia em dietas para ruminantes. Um dos principais nutrientes em dietas de vacas leiteiras de alta produção é o amido proveniente do milho, e o seu processamento tem grande impacto sobre a degradação ruminal, por reduzir a interação entre a matriz proteica e os grânulos de amido (FERRARETTO; CRUMP; SHAVER, 2013; FIRKINS et al, 2001). Durante a ensilagem ocorre a quebra das prolaminas por ação das enzimas microbianas possibilitando maior degradação ruminal do amido (HOFFMAN et al., 2011). No processo de ensilagem do grão úmido, ocorre hidratação da matriz protéica, perda de organização e rompimento de células do endosperma, promovendo rompimento parcial da matriz protéica (SULLINS; ROONEY, 1971).

Carvalho et al. (2016) observaram aumento na digestibilidade *in vitro* da MS do milho reidratado, com 7 h de incubação, a partir de 30 até 180 dias de ensilagem, e com 3 h de incubação após 90 dias de ensilagem. Lopes (2016) observou aumento na digestibilidade *in vitro* da MS do milho reidratado, dos 30 para 90 dias de fermentação, sem diferença entre 90 e 120 dias, quando as amostras foram incubadas por 7 e 18 h. Castro (2017) observou maior degradação da MS em amostras de milho reidratado e ensilado por 247 dias em comparação ao milho seco moído, aumentando 39,6 unidades percentuais na fração A, e aumento de 60,5; 57,7; 44,2; 19,5 unidades percentuais nos tempos 3, 6, 18 e 48 h de incubação, para o milho ensilado em comparação ao milho seco. Esses resultados demonstraram que o efeito da ensilagem do grão reidratado é mais pronunciado nas primeiras horas de incubação ruminal e é necessária a fermentação da silagem por longos períodos para alterar a degradabilidade ruminal.

Fernandes (2014) avaliando a degradabilidade ruminal do amido com 12 e 24 h de incubação observou que com o aumento dos dias de ensilagem houve aumento na degradação do amido, sendo que as maiores alterações na degradação do amido ocorreram entre 0 e 21 dias de ensilagem. Arcari et al. (2016) demostraram aumento constante na fração A e na degradação efetiva do amido ao longo do tempo de fermentação (3 a 330 dias).

Em trabalho avaliando a ensilagem de sorgo reidratado, Huck; Kreikemeier; Bolsen, (1999) observaram aumento da NH_3 ao longo da fermentação, indicando que ocorreu hidrólise da proteína, e um possível aumento na digestibilidade deste material. Estes autores recomendam a reidratação do sorgo para que atinja pelo menos 35% de umidade. Silva et al., (2014) observaram aumento de 4 unidades percentuais na degradabilidade efetiva para o sorgo reidratado (40% de umidade) e ensilado por 30 dias, em relação ao sorgo seco. Igarasi et al. (2008) obtiveram resultados de desempenho de bovinos jovens, em confinamento, semelhantes entre o sorgo e o milho reidratado, indicando que a ensilagem do sorgo reidratado pode equiparar à degradação do amido do sorgo ao milho, quando ambos são reidratados ensilados.

Tem sido demonstrado que as ensilagens de milho ou sorgo reidratado resultam em melhoras na digestibilidade do amido, porém os maiores ganhos ocorrem com longos períodos de ensilagem (CARVALHO et al., 2016), o que nem sempre é possível nas propriedades. Entretanto, o uso de enzimas como aditivo no momento da ensilagem pode resultar em rápida disponibilização do amido para a degradação (FERRARETTO et al., 2015) auxiliando na melhora da digestibilidade, sem aumentar o período de ensilagem.

2.2.4 Utilização de enzimas

As enzimas são catalizadores biológicos altamente específicos. São proteínas produzidas pelos organismos vivos que aceleram reações químicas de forma seletiva, reduzindo o gasto de energia de ativação e o tempo da reação. Possuem especificidade e seletividade com relação às substâncias com que interagem, sendo que a capacidade de reação é regulada pela quantidade de substrato disponível, concentração de produto formado, além da interação com outros metabólitos do meio (Ray; Rosell, 2017).

O sítio ativo é a fração da enzima onde o composto será conectado, antes de ser quebrado, neste local há grupos químicos ionizáveis. Desta forma, o pH e a temperatura do meio podem

interferir na conformação do sítio de ligação, aumentando ou reduzindo a atividade catalítica. Geralmente a máxima atividade catalítica é alcançada nas mesmas condições de ótimo crescimento do microrganismo que produz a enzima. Em função disto uma mesma α-amilase pode degradar com mais eficiência o amido proveniente de um tipo de grão, quando comparado a outra fonte de amido (PLANCHOT et al., 1995).

A hidrólise do amido é uma reação heterogênea, envolvendo o substrato sólido (amido) e a enzima em meio líquido. As enzimas iniciam a degradação do amido penetrando em poros do grânulo e o processo de hidrólise ocorre no sentido radial, da periferia para o centro do grânulo, formando novos canais, que resultam em aumento da área de superfície (ZHANG; DHITAL; GIDLEY, 2013). Durante o processo de degradação do amido, as amilases (α 1-4 glicano hidrolases) são as enzimas responsáveis pela digestão do amido. No processo digestivo, as ligações α 1-4 glicosídicas são hidrolisadas pela α-amilase, dentro da molécula do amido, gerando maltoses e dextrinas ramificadas e lineares. A β-amilase e glicoamilase atacam os resíduos de glicose terminais, produzindo maltose e glicose, respectivamente. As amiloglicosidases hidrolisam as dextrinas, produzindo glicose como processo final da digestão (MOREIRA, 1993).

A conformação do grânulo de amido nos cereais é uma barreira para a atividade enzimática (ZHANG; DHITAL; GIDLEY, 2013), o processo de ensilagem pode facilitar a quebra das pontes de hidrogênio entre amido e prolaminas, acelerando o processo de degradação por ação enzimática e ou pela disponibilização do amido para ataque dos microrganismos ruminais. As amilases podem disponibilizar substrato para o desenvolvimento de BAL por meio da hidrólise parcial do amido (KUNG, 2014).

2.2.4.1 Amiloglicosidase

Amiloglicosidase tem nome sistemático de 4-α-D-glicano-glicohidrolase, e o nome comum, de glicano-1,4-alfaglicohidrolase, mas também é conhecida como glicoamilase, γ-amilase, lisossomal-α-glicosidase, maltase ácida, exo-1,4-α-glicosidase, glicose amilase. Na comissão internacional IUBMB (International Union of Biochemistry and Molecular Biology) recebe o código EC 3.2.1.3. É uma exo-enzima que libera unidades de glicose a partir da extremidade não redutora da amilose, amilopectina e glicogênio (MANERA; MEINHARDT; KALIL, 2011) e sua reação é de hidrólise das ligações (1-4) e (1-6) α-D-glicose, com liberação de

β-D-glicose. É a única enzima amilolítica com capacidade de hidrolisar ao mesmo tempo as ligações α-1,4 e α-1,6 (PANDEY et al., 2000).

Os gêneros *Aspergillus*, *Rhizopus* e *Endomyces* são destacados como os principais gêneros produtores de amiloglicosidase (SOCCOL et al., 2005). *Aspergillus niger*, *A. fumigatus*, *A. saitri*, *A. terreus*, *A. foetidus*; *Rhizopus foetidus* e *R. delemar*, são espécies de fungos utilizados para a produção de amiloglicosidase (PANDEY et al., 2005), destacando os fungos filamentosos *Aspergillus niger* e *Rhizopus sp.* como principais produtores (SILVEIRA et al., 2006). A amiloglicosidase obtida do cultivo de *Aspergillus niger*, tem temperatura ótima de ação de 40 à 50ºC e em solução com pH entre 4,8 a 5 (BRENDA, 2016). Mas de acordo com Gates (2010) a amiloglicosidase produzida à partir de *Aspergillus niger*, tem temperatura ótima de ação de 60ºC em solução com pH 4,8; enquanto que a a amiloglicosidase produzida à partir de *Rhizopus sp.* tem temperatura ótima de ação de 55ºC em solução com pH 4,5.

Recentes estudos indicam que a amiloglicosidase não só se liga ao amido pelas extremidades, mas também desestrutura as ligações helicoidais facilitando a hidrólise por outras enzimas como a amilase (ZHANG; DHITAL; GIDLEY, 2013). Além disso, estes mesmos autores relataram que em sistemas *in vitro*, α-amilase é inibida por oligossacarídeos e a amiloglicosidase degrada esses compostos em glicose, reduzindo a inibição. Gutiérrez et al. (2005) avaliaram o efeito da amiloglicosidase sobre a digestibilidade da MS do milho e do sorgo. A digestibilidade da MS foi maior com adição da enzima na concentração 90 µl, com valores de 53,21% sem enzima e 76,33% da MS no milho com enzima, e 58,52% sem enzima e 81,11% da MS no sorgo com enzima.

2.3 Degradação ruminal do amido

O interesse em melhorar a digestibilidade do amido consumido por vacas leiteiras foi estimulado pelo elevado custo dos grãos de cereais. O aumento na digestibilidade do amido resulta em maior rendimento de leite e de proteína, e melhora a eficiência alimentar (Firkins et al., 2001). A digestibilidade do amido em todo trato digestivo de vacas leiteiras varia de 70 a 100% (Firkins et al., 2001; Ferraretto et al., 2013) e é influenciado por vários fatores tais como o tamanho das partículas, tratamento de cereais, método de armazenamento (Firkins et al., 2001; Ferraretto et al., 2013), maturidade do milho à colheita, teor de umidade, duração da fermentação no silo (Hoffman

et al., 2011; Ferraretto e Shaver, 2012) e o tipo de endosperma do milho (Taylor e Allen., 2005; Lopes et al., 2009). Entender como características dos alimentos podem afetar o local de digestão ou a degradação do amido é essencial para a produção leiteira comercial, pois a manipulação da degradação do amido pode alterar o consumo dos animais (Allen, 2000), e consequentemente a produção de leite.

Já a degradação ruminal do amido pode variar de 50 a 90 % entre híbridos de milho (MOHARRERY; LARSEN; WEISBJERG, 2014). Metabolizar o amido no rúmen é mais eficiente para o ruminante, pois o amido que escapa do rúmen é digerido no intestino delgado e absorvido como glicose, ou metabolizado anaerobicamente no intestino grosso à ácidos graxos voláteis (AGV), ou perdido nas fezes. Os ruminantes apresentam inibição da secreção de amilase pancreática quando um fluxo grande de amido passa para o intestino delgado, reduzindo a capacidade de digestão do amido neste local (HARMON; YAMKA; ELAM, 2004). Quando aumenta a quantidade de amido que escapa da digestão intestinal, ocorre fermentação de amido no intestino grosso e a proteína microbiana produzida é perdida nas fezes. Entretanto o aumento da degradação de amido no rúmen pode resultar em acúmulo de AGV no fluído ruminal, induzindo baixo pH (acidose ruminal). Desta forma, torna-se importante otimizar a degradação ruminal de amido, e através do conhecimento da cinética de degradação pode-se evitar distúrbios metabólicos como a acidose. Diferentes técnicas têm sido propostas com o objetivo de avaliar a digestibilidade ou degradabilidade dos alimentos: digestibilidade *in vivo*, *in situ* e *in vitro*.

2.3.1 Método de avaliação *in situ*

A técnica de degradabilidade *in situ* foi proposta por Mehrez e Orskov (1977) e consiste no desaparecimento da amostra de alimento acondicionada em sacos (nylon ou material sintético), incubados no rúmen por diferentes períodos de tempo, estimando de forma rápida e simples, a degradabilidade ruminal. A precisão dos parâmetros obtidos por meio dessa técnica, está associada às condições ótimas de temperatura, pH, tamponamento e substratos que a mesma oferece (ASSIS et al., 1999).

O nylon tem sido utilizado como material padrão para confecção dos sacos utilizados na técnica *in situ* (NOCEK, 1988). Esses devem ser resistentes a temperaturas elevadas, com porosidade que permita o contato dos microrganismos no interior dos sacos propiciando a

degradação do alimento, remoção dos produtos finais da degradação, mas também devem proporcionar redução das perdas de amostras não degradadas (VAN HELLEN; ELLIS, 1977), simulando o que acontece no rúmen. De acordo com Vanzant et al. (1998) a variação na porosidade de 40 a 60 µm é adequada para a avaliação *in situ*.

Estabelecer o tamanho de partícula do alimento a ser incubado é importante, mas complicado, pois, a partícula deve ser similar àquela obtida pela mastigação do animal, sem favorecer perdas de material não degradado. Diferentes tamanhos são recomendados na literatura, sendo possível observar variações de 1,5 a 3,0 mm para concentrado e 1,5 a 5,0 mm para forragem (CASALI et al., 2008; FORTALEZA et al., 2009; SILVA et al., 2007; VANZANT et al., 1998). A moagem tem diferentes efeitos sobre a distribuição do tamanho de partícula de diferentes alimentos, o que pode resultar em alterações na estimativa da degradabilidade (MICHALET-DOREAU; CERNEAU, 1991). Já a quantidade de amostra incubada, deve fornecer resíduo suficiente para que, após a degradação, seja possível a execução das análises químicas desejadas. Sem desrespeitar a relação entre a quantidade de amostra e área útil do saco. A recomendação é que esta relação seja inferior à 10mg/cm^2, podendo ser aumentado o número ou tamanho de sacos, caso o resíduo seja pequeno (VANZANT et al., 1998).

Existem dois métodos para estimar a incubação de sacos de nylon no rúmen. No primeiro, os sacos de nylon são colocados no rúmen simultaneamente e recuperados em diferentes tempos. Esse método é amplamente empregado nas pesquisas (EDMUNDS et al., 2012; PEYRAT et al., 2014). No segundo, os sacos de nylon são inseridos no rúmen em diferentes tempos e retira-se simultaneamente (JONKER et al., 2011; CAO et al., 2009). A aplicação de um ou outro método é dependente de alguns fatores como: identificação adequada dos sacos que serão retirados a cada tempo, para que esse processo ocorra da forma mais rápida possível; padronização dos métodos de lavagem, para que não tenha variação entre um e outro horário de retirada dos sacos. Caso não seja possível o controle destes fatores, recomenda-se a inserção em diferentes tempos e retirada simultânea dos sacos (VANZANT et al., 1998).

2.3.2 Estimativa da taxa de degradação e tempos de incubação

A variável de maior influência nos procedimentos da técnica *in situ* é o tempo de incubação ruminal. Com base nisso, tem-se discutido sobre o tempo de incubação que permita representar

melhor as frações degradáveis das amostras. Casali et al. (2008) sugeriram 240 horas de incubação para obtenção de estimativas das frações indigestíveis e 264 horas para avaliação de FDAi. Azevedo et al. (2012), em estudos com subprodutos, recomendam 72 horas de incubação para predição de frações digestíveis de fibra em detergente neutro *in vitro* e *in situ*. Para a estimativa das frações degradáveis da MS há muita variação entre os autores, Haese et al. (2017) utilizaram cinco tempos de incubação variando de 2 a 24 horas de incubação, enquanto Lee et al. (2016) utilizaram nove tempos de incubação variando de 0 a 48 horas. Entretanto, tempos mais longos também foram utilizados por outros autores, tais como sete tempos de incubação ao longo de 3 a 336 horas (VISSER et al., 1996).

Os tempos de incubação são dependentes do substrato e características do alimento que se pretende avaliar, existindo tempos críticos de incubação próximos a parte "mais sensível" da curva de degradação (MICHALET-DOREAU; OULD-BAH, 1992). Para nutrientes de degradação mais rápida, como por exemplo amido, tempos menores de incubação são requeridos. Dieho et al. (2017) utilizaram seis tempos de incubação, variando de zero até 72 horas. Já Hristov et al. (2007) utilizaram seis tempos de incubação variando de zero a 24 horas de incubação. Porém, a maioria dos estudos que avaliou a degradação do amido utilizou como tempo máximo de degradação 48 horas de incubação ruminal, com seis tempos de degradação variando entre zero e 48 h (CORREA et al., 2002; MALAN; RAFFRENATO, 2016; PHILIPEAU; MICHALET, 1998).

Os modelos utilizados para estimativa da taxa de degradação devem ser adequados ao encaixe nos dados de degradação para descrever de forma adequada a biologia de degradação (VANZANT et al., 1998). Cuidados com a adequação do modelo são necessários, pois pode-se obter diferentes tamanhos de pool e/ou taxas de degradação diferentes para mesma curva de degradação ao se aplicar diferentes modelos (VANZANT et al., 1998). Por exemplo, o uso do ''Lag time'' (tempo considerado entre a entrada do alimento no rúmen e início da degradação ou passagem para o omaso) é necessário para a estimativa da degradação de forragens que possuem um tempo de retenção no rúmen. Para alimentos concentrados, que estão aptos a passar para o omaso, não há a necessidade de utilizar o ''Lag time'' no modelo (KLOPFENSTEIN et al., 2001). Estratégias de modelagem simples podem oferecer alguns benefícios em relação à facilidade de aplicação sem grandes prejuízos na exatidão ou precisão das estimativas (VANZANT et al., 1998).

O uso de um ou mais pools no modelo de degradação deve-se às características dos nutrientes e alimentos, devendo-se adotar o modelo que descreva biologicamente o que acontece

na degradação. Para a degradação do amido alguns autores consideram apenas a presença de dois pools, fração solúvel e potencialmente degradável, desconsiderando a existência da fração indigestível (HERRERA-SALDANA; HUBER; POORE, 1990; HRISTOV et al., 2007).

2.4 REFERÊNCIAS

ADESOGAN, A.T.; QUEIROZ, O.C.M. **Silage pathogenicity and implications for the ruminant production chain**. In: International Simposium on forage quality and conservation, 2009, São Pedro, Procedings…p.225-241, 2009.

AHMED, I.; YOKOTA, A.; YAMAZOE, A.; FUJIWARA, T. Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and Bacillus sphaericus to *Lysinibacillus sphaericus* comb. nov. **International Journal of Systematic and Evolutionary Microbiology**. v.57, p.1117–1125, 2007.

ALLEN, M. S. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. **Journal of Dairy Science**, v. 83, p. 1598–1624, 2000.

ANDRADE FILHO, R. et al. Degradabilidade ruminal *in situ* de grãos de milho maduros do tipo flint ou dentado, secos ou reconstituídos e ensilados. In: REUNIÃO ANUAL DA SOCIEDADE BRASILEIRA DE ZOOTECNIA, 47, 2010, Salvador, BA. **Anais**..., 2010.

ANTUNES, R. C.; RODRIGUEZ, N. M.; GONÇALVES, L. C.; RODRIGUES, J. A. S.; BORGES, I.; BORGES, A. L. C. C.; SALIBA, E. O. S. Composição bromatológica e parâmetros físicos de grãos de sorgo com diferentes texturas do endosperma. **Arq. Bras. Med. Vet. Zootec**., v.59, n.5, p.1351-1354, 2007.

ARCARI, M. A.; MARTINS, C. M. M. R.; TOMAZI, T.; SANTOS, M. V. Effect of the ensiling time of hydrated ground corn on silage composition and *in situ* starch degradability. **Braz. J. Vet. Res. Anim. Sci**., São Paulo, v. 53, n. 1, p. 60-71, 2016.

ASSIS, M.A.; SANTOS, G.T.; CECATO, U. et al. Degradabilidade *in situ* de gramíneas do gênero Cynodon submetidas ou não a adubação nitrogenada. **Acta Scientiarum**, v.21, n.3, p.657- 663, 1999.

BARNES, R.F.; NELSON, J.C.; MOORE K.J.; COLLIN., M. Forages: **The Science of Grassland Agriculture.** 6th Edn., Wiley, p.791, 2007.

BELTON, P. S.; DELGADILLO, I.; HALFORD, N. G.; SHEWRY, P. R.; Kafirin structure and functionality. **J. Cereal Sci**. v. 44, pp. 272-286, 2006.

BERNANRDES, T. F.; WEINBERG, Z. **Aspectos associados ao manejo da ensilagem**. In. REIS, R. A.; BERNARDES, T. F.; SIQUEIRA, G. R. **Forragicultura: ciência, tecnologia e gestão de recursos forrageiros**. Jaboticabal: Maria de Lourdes Brandel – ME, 671-680, 2013.

BITENCOURT, L.L. **Respostas de vacas leiteiras à substituição de milho moído por milho rehidratado e ensilado ou melaço de soja**. Lavras: Federal University of Lavras. 2012.

BOYER, C. D.; HANNAH, L. C. **Kernel mutants of corn**. In: HALLAUER, A. (Ed.). Specialty corns. 2. ed. Boca Raton, FL: CRC, 2001. p. 1-32.

BRENDA. **The comprehensive Enzyme Informatio System**, 2016. Disponível em: <http://www.brenda-enzymes.org>

BRINGEL, F.; QUÉNÉE, P.; TAILLIEZ, P. Polyphasic Investigation of the Diversity within *Lactobacillus plantarum* Related Strains Revealed Two *L. plantarum* subgroups. **System. Appl. Microbiol.** v.24, p.561–571, 2001.

BULÉON, A.; COLONNA, P.; PLANCHOT, V.; BALL, S. Starch granules: structure and biosynthesis. **International Journal of Biological Macromolecules**, v.23, p.85-112, 1998.

CAO, Z.J.; ANDERSON, J.L.; KALSCHEUR, K.F. Ruminal degradation and intestinal digestibility of dried or wet distillers grains with increasing concentrations of condensed distillers solubles. J**. Ani. Sci**., v. 87, p. 3013–3019, 2009.

CARVALHO, B. F., C. L. S. AVILA, T. F. BERNARDES, M. N. PEREIRA, C. SANTOS, AND R. F. SCHWAN. Fermentation profile and identification of lactic acid bacteria and yeasts of rehydrated corn kernel silage. **J. Appl. Microbiol**. 122: 589-600. 2016.

CASTRO, L. P. **Lactation performance of dairy cows fed rehydrated and ensiled corn grain differing in particle size and inclusion in the diet**. Dissertação (Mestrado), Universidade Federal de Lavras, 2017, 71p.

CASALI, A.O.; DETMANN, E.; VALADARES FILHO, S.C. et al. Influência do tempo de incubação e do tamanho de partículas sobre os teores de compostos indigestíveis em alimentos e fezes bovinas obtidos por procedimentos *in situ*. **R. Bras. Zootec**., v.37, n.2, p.335-342, 2008.

CHANDRASHEKAR, A.; MAZHAR, H. The biochemical basis and implications of grain strength in sorghum and maize. **J. Cereal Sci**., v.30, p.193-207, 1999.

CORREA, C. E. S.; SHAVER, R. D.; Pereira, M.N.; Lauer, J.G.; Kohn, K. Relationship between corn vitreousness and ruminal *in situ* starch degradability. **Journal of Dairy Science**, v. 85, p. 3008– 3012, 2002.

CRUZ, G. M.; NUCCIO, C. M. B. **Sorgo na alimentação de bovinos.** II Simpósio sobre Ingredientes na Alimentação Animal CBNA - Uberlândia, MG. 2002.

CURK, M. C.; HUBERT, J. C.; BRINGEL, F. *Lactobacillus paraplantarum* sp. nov., a New Species Related to *Lactobacillus plantarum*. **International Journal of Systematic Bacteriology**. v.46, n.2, p.595-598, 1996.

DEC, M.; URBAN-CHMIEL, R.; GNAT, S.; PUCHALSKI, A.; WERNICKI, A. Identification of Lactobacillus strains of goose origin using MALDI-TOF mass spectrometry and 16S-23S rDNA intergenic spacer PCR analysis. **Res Microbiol**. v.165, p.190–201, 2014.

DIEHO, K.; VAN DEN BOGERT, B.; HENDERSON, G.; BANNINK, A.; RAMIRO-GARCIA, J.; SMIDT, H.; DIJKSTRA, J. Changes in rumen microbiota composition and *in situ* degradation kinetics during the dry period and early lactation as affected by rate of increase of concentrate allowance. **Journal of Dairy Science**. v.100 n.4, p. 2695–2710, 2017.

DOMBRINK-KURTZMAN, M. A.; BIETZ, J. A. Zein composition in hard and soft endosperm of maize. **Cereal Chemistry**, v. 70, p. 105–108, 1993.

DUODU, K. G.; NUNES, A.; DELGADILLO, I.; PARKER, M. L.; MILLS, E. N. C.; BELTON, P. S.; TAYLOR, J. R. N. Effect of grain structure and cooking on sorghum and maize in vitro protein digestibility. **Journal of Cereal Science**, v. 35, p. 161–174, 2002.

ECKHOFF, S. R.; PAULSEN, M. R. Maize. In HENRY, R. J. KETTLEWELL, P. S. (Ed.). **Cereal grain quality**. London:Chapman & Hall, v.3, p. 77-89, 1996.

EDMUNDS, B.; SÜDEKUM, K.H.; SPIEKERS, H. et al. Estimating ruminal crude protein degradation of forages using *in situ* and *in vitro* techniques. **Ani. Feed Sci. Tech**., n. 175, p. 95–105, 2012.

ESEN, A.; STETLER, D. A. Immunocytochemical localization if delta-zein in the protein bodies of maize endosperm cells. **American Journal of Botany**, Columbus, v. 79, n. 3, p. 243-248, Mar. 1992.

FERNANDES, J. **Influência de genótipo, maturidade e tempo de armazenamento na qualidade de silagens de grão de milho com alta umidade**. Dissertação(Mestrado), Escola Superior de Agricutura "Luiz de Queiroz". Piracicaba, 97p, 2014.

FERRARETTO, L.F., CRUMP, P.M.; SHAVER, R.D. Effect of cereal grain type and corn grain harvesting and processing methods on intake, digestion, and milk production by dairy cows through a meta-analysis. **Journal of Dairy Science**, v. 96, p. 533-540. 2013.

FERRARETTO, L. F.; FREDIN, S. M.; SHAVER, R. D. Influence of ensiling, exogenous protease addition and bacterial inoculation on fermentation profile, nitrogen fractions and ruminal in vitro starch digestibility in rehydrated and high-moisture corn. **Journal of Dairy Science**. v.98, p.7318-7327, 2015.

FERRARETTO, L. F.; SILVA FILHO, W. I.; FERNANDES, T.; KIM, D. H.; SULTANA, H. Effect of ensiling time on fermentation profile and ruminal in vitro starch digestibility in

rehydrated corn without or with varied concentrations of wet brewer's grains. **Journal of Dairy Science**, 2018.

FERRARETTO, L.F.; TAYSOM, k.; TAYSOM, D.M.; SHAVER, R.D.; HOFFMAN, P.C. Relationships between dry matter content, ensiling, ammonia-nitrogen, and ruminal in vitro starch digestibility in high-moisture corn samples. **Journal of Dairy Science**, v. 97, p. 3221-3227. 2014.

FERRARETTO, L. F.; SHAVER, R. D. Meta-analysis: Impact of corn silage harvest practices on intake, digestion and milk production by dairy cows. **Prof. Animal Science**, v. 28, p. 141–149. 2012.

FIRKINS, J.L., EASTRIDGE, M.L.; ST-PIERRE, N.R.; NOFTSGER, S.M. Effects of grain variability and processing on starch utilization by lactating dairy cattle. **Journal of Animal Science**, v. 79, p. E218-E238. 2001.

FORTALEZA, A.P.S.; SILVA, L.D.F.; RIBEIRO, E.L.A. et al. Degradabilidade ruminal *in situ* dos componentes nutritivos de alguns suplementos concentrados usados na alimentação de bovinos. **Semina: Ciências Agrárias**, v. 30, n. 2, p. 481-496, 2009.

FREDIN, S.M., FERRARETTO, L.F.; AKINS, M.S.; HOFFMAN, P.C.; SHAVER, R.D. Fecal starch as an indicator of total-tract starch digestibility by lactating dairy cows. **Journal of Dairy Science**, v. 97, p. 1862-1871, 2014.

GATES, R. Enzymes and Reagents for Ethanol Research. In Technical content: **Enzymes and Reagents for Alternative Energy**. v.5, n.5, 2010.

GIUBERTI, G.; GALLO, G.; MOSCHINI, M.; CERIOLI, C.; MASOERO F. Evaluation of the impact of maize endosperm vitreousness on in vitro starch digestion, dry matter digestibility and fermentation characteristics for pigs. **Animal Feed Science and Technology**, v. 186, n. 1–2, p. 71–80, 2013.

GIFFEL, M. C. T. et al. Bacterial spores in silage and raw milk. **Antonie van Leeuwenhoek**, Amsterdam, v. 81, n. 1-4, p. 625-630, Dec. 2002.

GOBETTI, S.T.C.; NEUMANN, M.; OLIBONI, R.; OLIVEIRA, M.R. Utilização de silagem de grão úmido na dieta de animais ruminantes. **Ambiência,** 9 225-239. 2013.

GONZALES, C.; DELGADO, O.; BAIGORI, M.; ABATE, C.; DE FAGUEROA, L. I. C.; CALLIERI, D. A. Ethanol production from native cassava starch by a mixed culture of Endomycopsis fibuligera and Zymomonas mobilis. **Acta Biotechnology**, v.18, n.2, p.149-155, 1998.

GUTIÉRREZ, C. et al. Effect of exogenous amylase or glucoamylase dose on *in situ* ruminal digestion of corn and sorghum. **Journal of Applied Animal Research**, v. 27, n. 1, p. 7–10, 2005.

HAESE, E.; MÖHRING, J.; STEINGASS, H.; SCHOLLENBERGER, M.; RODEHUTSCORD, M. Effect of dietary mineral phosphorus and phytate on *in situ* ruminal phytate disappearance from different concentrates in dairy cows. **Journal of Dairy Science**. v.100, n.5, p. 3672–3684, 2017.

HALE, W. H. Influence of processing on the utilization of grains (starch) by ruminants. **Journal of Animal Science**, 37, 1075-1984. 1973.

HARMON, D. L.; YAMKA, R. M.; ELAM, N. A. Factors affecting intestinal starch digestion in ruminants: A review. Can. J. Anim. Sci., v. 84, p. 309–318, 2004.

HENDERSON, N. Silage additives. **Animal Feed Science and Technology**, 45, 35-56. 1993.

HERMAN, E. M.; LARKINS, B. A. Protein storage bodies and vacuoles. **The plant cell**. Rockville, v.11, n.4, p.601-614, 1999.

HERRERA-SALDANA, R. E.; HUBER, J. T.; POORE, M. H. Dry Matter, Crude Protein, and Starch Degradability of Five Cereal Grains. **Journal of Dairy Science**. v.73, n.9, p.2386-2393, 1990.

HOFFMAN, P. C. et al. Influence of ensiling time and inoculation on alteration of the starch-protein matrix in high-moisture corn. **Journal of Dairy Science**, v. 94, n. 5, p. 2465–2474, 2011.

HOLDING, D. R. Recent advances in the study of prolamin storage protein organization and function. **Frontiers in plant science**, v. 5, n. June, p. 276, 2014.

HRISTOV, A. N.; ZAMAN, S.; VANDERPOL, M.; SZASZ, P.; HUBER, K.; GREER, D. Effect of a saponin-based surfactant and aging time on ruminal degradability of flaked corn grain dry matter and starch. **J. Anim. Sci**. v.85, p.1459–1466, 2007.

HUCK, G. L.; KREIKEMEIER, K. K.; BOLSEN, K. K. Effect of Reconstituting Field-Dried and Early-Harvested Sorghum Grain on the Ensiling Characteristics of the Grain and on Growth Performance and Carcass Merit of Feedlot Heifers. **J. Anim. Sci.** v.77, p.1074–1081, 1999.

HUNTINGTON, G. B.; HARMON, D. L.; RICHARDS, C. J. Sites, rates, and limits of starch digestion and glucose metabolism in growing cattle. **Journal of Animal Science**, v. 84 Suppl, p. 14–24, 2006.

IGARASI, M. S.; ARRIGONI, M. B.; SOUZA, A. A.; SILVEIRA, A. C.; MARTINS, C. L.; OLIVEIRA, H. N. Desempenho de bovinos jovens alimentados com dietas contendo grão úmido de milho ou sorgo. **R. Bras. Zootec**., v.37, n.3, p.513-519, 2008.

JOBIM, C. C.; NUSSIO, L. G. Princípios básicos da fermentação na silagem. In. REIS, R. A.; BERNARDES, T. F.; SIQUEIRA, G. R. **Forragicultura: ciência, tecnologia e gestão de recursos forrageiros**. Jaboticabal: Maria de Lourdes Brandel – ME, 649-660, 2013.

JONKER, A.; GRUBER, M.Y.; WANG, Y. Modeling degradation ratios and nutrient availability of anthocyanidin accumulating Lc-alfalfa populations in dairy cows. **J. Dairy Sci**., v. 94, p. 1430–1444, 2011.

KIRIHARA, J. A.; HUNSPERGER, J. P.; MAHONY, W. C.; MESSING, J. W. Differential expression of a gene for a methionine-rich storage protein in maize. **Molecular General Genetics**, New York, v. 211, n. 3, p. 477-487, Mar. 1988.

KLOPFENSTEIN, T. J.; MASS, R. A.; CREIGHTON, K. W.; PATTERSON, H. H. Estimating forage protein degradation in the rumen. **J. Anim. Sci**. v.79(E. Suppl.), p.E208–E217, 2001.

KUNG JUNIOR., L.; SHAVER, R. Interpretation and use of silage fermentation analysis reports. **Focus on Forage**, v.3, n.13, p.1-5, 2001.

KUNG JNIOR, L. **Effects of microbial additives in silages: facts and perspectives**, In: NUSSIO, L.G.; ZAPOLLATTO, M. International symposium on forage quality and quality, Proceedings… Piracicaba, 280p, 2009.

KUNG, L.; WINDLE, M. C.; WALKER, N. The effect of an exogenous protease on the fermentation and nutritive value of high-moisture corn. **Journal of Dairy Science**, v. 97, n. 3, p. 1707–1712, mar. 2014.

LANDRY, J.; DAMERVAL, C.; AZEVEDO, R.A.; DELHAYE, S. Effect of the opaque and floury mutation on the accumulation of dry matter and protein fractions in maize endosperm. **Plant Physiology and Biochemistry**, Paris, v.43, n.6, p549-556, 2005.

LAWTON, J. W. Zein: A history of processing and use. **Cereal Chem**. v.79, p.1–18, 2002.

LEE, Y. H.; AHMADI, F.; CHOI, D. Y.; KWAK, W. S. *In situ* ruminal degradation characteristics of dry matter and crude protein from dried corn, high-protein corn, and wheat distillers grains. **Journal of Animal Science and Technology.** v.58, n.33, p.2-7, 2016.

LENDING, C. R.; LARKINS, B. A. Changes in the zein composition of protein bodies during of maize development. **The plant cell**, Rockiville, v. 1, n. 10, p. 1011-1023, Oct. 1989.

LOPES, J. C., SHAVER, R. D.; HOFFMAN, P. C.; AKINS, M. S.; BERTICS, S. J.; GENCOGLU, H.; COORS, J. G. Type of corn endosperm influences nutrient digestibility in lactating dairy cows. **Journal of Dairy Science**, v. 92, p. 4541–4548. 2009.

LOPES, M. A. LARKINS, B. Endosperm Origin, development, and function. **The plant cell**, Rockville, v. 5, n. 10, p. 1383-1399, Oct. 1993.

LOPES, N. M. **Efeito de enzimas sobre a digestibilidade ruminal da silagem do grão de milho reidratado**. Tese (Doutorado), Universidade Federal de Lavras, 2016.

MADINGAN, M. T.; MARTINKO, J.; BENDER, K.; BUCKLEY, D; STAHL, D. **Brock Biology of Microoganisms**. 14. ed. Porto AlegreÇ Artmed, 2010.

MALAN, E.; RAFFRENATO E. Effects of corn particle size and neutral detergent fiber:starch ratio on in vitro neutral detergent fiber degradability. **J. Anim. Sci.** v.94, E-Suppl. 5/**J. Dairy Sci**. v. 99, E-Suppl. 1. 2016.

MANERA, A. P.; MEINHARDT, S.; KALIL, S. J. Purificação de amiloglicosidase de *Aspergillus niger*. **Semina: Ciências Agrárias.** v.32, n.2, p.651-658, 2011.

McALLISTER, T. A.; RODE, L. M.; MAJOR, D. J.; CHENG, K.-J.; BUCHANAN-SMITH, J. G. Effect of ruminal microbial colonization on cereal grain digestion. **Canadian Journal of Animal Science**, v. 70, p. 571-579, 1990.

McALLISTER, T. A. PHILLIPPE, C. R.; RODE, L. M.; CHENG, K. J. Effect of the Protein Matrix on the Digestion of Cereal Grains by Ruminal Microorganisms'. **Journal of Animal Science**, v. 71, p. 205–212, 1993.

McDONALD, P.; HENDERSON, A.R. and HERON, S.J.E. **The biochemistry of silage**. Marlow, Chalcomb Publication. 1991.

MEHREZ, A.Z.; ORSKOV, E.R. A study of the artificial fiber bag technique for determination the digestibility of feeds in the rumen. **J. Agric. Sci.,** v.88, n.1, p.645, 1977.

MICHALET-DOREAU, B.; CERNEAU, P. Influence of foodstuff particle size on *in situ* degradation of nitrogen in the rumen. **Anim. Feed Sci. Technol**. v.35, p.69−81, 1991.

MICHALET-DOREAU, B.; OULD-BAH. M. Y. In vitro and in sacco methods for the estimation of dietary nitrogen degradability in the rumen: a review. **Anim. Feed Sci. Technol**. v.40, p.57−86, 1992.

MILLER, F. Alternative marketing opportunities for sorghum. In: 19TH **Biennial grain sorghum research & utilization conference**, Lubbock, Texas, 1995. Proceedings. Lubbock: NATIONAL GRAIN SORGHUM PRODUCERS, 1995. p. 120.

MOREIRA, I. **Valor nutritivo e utilização do milho e soja integral processados a calor na alimentação de leitões.** Viçosa, MG: Universidade Federal de Viçosa. Tese (Doutorado em Zootecnia) - Universidade Federal de Viçosa, 1993. 145p.

MOHARRERY, A.; LARSEN, M.; WEISBJERG, M. R. Starch digestion in the rumen, small intestine , and hind gut of dairy cows – A meta-analysis. **Animal Feed Science and Technology**, v. 192, p. 1–14, 2014.

MUCK, R.E. Recent advances in silage microbiology. **Agricultural and Food Science**. v.22, p.3-15, 2013.

MUCK, R.E. Silage microbiology and its control through additives. **Revista Brasileira de Zootecnia**, 39, 183-191. 2010.

NAKAMURA, L. K. Bacillus pseudomycoides sp. nov. **International Journal of Systematic Bacteriology**. v.48, p.103 1-1 035, 1998.

NOCEK, J. E.; KOHN, R. A. *In situ* particle size reduction of alfalfa and timothy hay as influenced by form and particle size. **J. Dairy Sci**. v.71, p.932−945, 1988.

NOCEK, J. E.; TAMMIMGA, S. Site of digestion of starch in the grastrointestinal tract of dairy cows and its effect on milk yield and composition. **Journal of Dairy Science**, Lancaster, v.74, n.8, p. 3598-3629, 1991.

NUNES, A.; CORREIA, I.; BARROS, A.; DELGADILLO, I. Characterization of kafirin and zein oligomers by preparative sodium dodecyl sulfate − Polyacrylamide gel electrophoresis. **Agricultural and Food Chemistry**, v. 53, p. 639–643, 2005.

ORTEGA, E. I.; BATES, L. S. Biochemical and agronomic studies of two modified hard endosperm opaque-2 maize (Zea mays) populations. **Cereal Chemistry**, St. Paul, v. 60, n. 2, p. 107-111, Mar./Apr. 1983.

PAES, M. C. D. Aspectos Físicos, Químicos e Tecnológicos do Grão de Milho. **Circular Tecnica Embrapa**. 75, p. 16, 2006.

PAHLOW, G.; MUCK, R. E.; DRIEHUIS, F.; OUDE ELFERINK, S. J. W. H.; SPOELSTRA, S.F. Microbiology of ensiling. In BUXTON, D. R.; MUCK, R. E.; HARRISON, J. H. **Silage science and technology**. Madison: American Society of Agronomy; Crop Science Society of America; Soil Science Society of America, chap 2, p. 31-93, 2003.

PALMISANO, M. M.; NAKAMURA, L. K.; DUNCAN, K. E.; ISTOCK, C. A.; COHAN, F. M. *Bacillus sonorensis* sp. nov., a close relative of *Bacillus licheniformis*, isolated from soil in the Sonoran Desert, Arizona. **International Journal of Systematic and Evolutionary Microbiology** v.51, p.1671–1679, 2001.

PANDEY, A.; POONAM, N.; SOCCOL, C. R.; SOCCOL, V. T.; SINGH, D.; MOHAN, R. Advances in microbial amylases. **Biotechnology and Applied Biochemistry**, v.31, n.2, p.135-152, 2000.

PEDERSEN, K.; ARGOS, P.; NARAYANA, S. L. V.; LARKINS, B. A. Sequence analysis and characterization of a maize gene encoding a high sulphur zein protein of Mr 15,000. **The Journal of Biological Chemistry**, Baltimore, v. 261, n. 14, p. 62779-6284, 1986.

PEYRAT, J.; NOZIÈRE, P.; LE MORVAN, A. et al. Effects of ensiling maize and sample conditioning on *in situ* rumen degradation of dry matter, starch and fibre. **Ani. Feed Sci. Tech.,** n. 196, p. 12–21, 2014.

PEREIRA, M.N; PEREIRA, R.A.N.; BITENCOURT, L.L.; DIAS JÚNIOR, G.S.; LOPES, N.M.; ZACARONI, O.F. Silagem de milho reidratado na alimentação do gado leiteiro. **Informe Agropecuário**, 34, 27-33. 2013.

PEREIRA, R.C. **Relação entre características estruturais e bioquímicas e a textura do grão de milho**. Tese (Doutorado em Genética e Melhoramento de plantas) Universidade Federal de Lavras, Lavras, MG, 2006. 54 p.

PEREIRA, O.G.; SANTOS, E.M. **Microbiologia e o processo de fermentação em silagens**. In: SIMPÓSIO SOBRE MANEJO ESTRATÉGICO DA PASTAGEM. 3., Viçosa: UFV, 2006. Anais... Viçosa: UFV, p. 393-430, 2006.

PHILIPPEAU, C.; MICHALET-DOREAU, B. Influence of genotype and stage of maturity of maize on rate of ruminal starch degradation. **Animal Feed Science and Technology**, v. 68, n. 1–2, p. 25–35, 1997.

PLANCHOT, V. et al. Extensive degradation of native starch granules by alpha-amylase from aspergillus fumigatus. **Journal of Cereal Science**, v.21, p.163–171, 1995.

REZENDE, A. V., C. H. S. RABELO, R. M. VEIGA, L. P. ANDRADE, C. J. HARTER, F. H. S. RABELO, F. C. BASSO, D. A. NOGUEIRA, AND R. A. REIS. 2014. Rehydratation of corn grain with acid whey improves the silage quality. **Anim. Feed Sci. Technol**. 197: 213-221.

REY, R.C.; ROSELL, C.M. Microbial Enzyme Technology in Food Applications. P.520, 2017.

RIBAS, M.N. **Avaliação agronômica e nutricional de híbridos de sorgo com capimsudão, normais e mutantes bmr - portadores de nervura marrom**. Tese (Doutorado em zootecnia). Universidade Federal de Minas Gerais, Escola de Veterinária, Belo Horizonte. 140 f. 2010.

RIBAS, M. N.; GONÇALVES, L. C.; HIBRAHIM, G. H. F.; RODRIGUEZ, N. M.; BORGES, A. L. C. C.; BORGES, I. Consumo voluntário e digestibilidade aparente de silagem de milho com diferentes vitreosidades. **Revista Brasileira de Milho e Sorgo**, Sete Lagoas, v.6 p. 104-115, 2007.

RIBAS, P.M. **Cultivo do sorgo**. 2008. Disponível em: http://www.cnpms.embrapa.br/publicacoes/sorgo_4_ed/plantio-plantio.html Acesso em: 03 de fevereiro de 2018.

ROBERTS, M. S.; NAKAMURA, L. K.; COHAN, F. M. *Bacillus vallismortis* sp. nov., a Close Relative of *Bacillus subtilis*, Isolated from Soil in Death Valley, California. **International Journal of Systematbica Cteriology**, v.46, n.2, p.470-475, 1996.

RODRIGUES FILHO, O., FRANÇA, A.F.S., OLIVEIRA, R.P., OLIVEIRA, E.R., ROSA, B.; SOARES, T.V.; MELLO, S.Q.S. Produção e composição de quatro híbridos de sorgo forrageiro (Sorghum bicolor L. Moench) submetidos a três doses de nitrogênio. **Ciência Animal Brasileira, Goiânia**, v.7, n.1, p.37-48, 2006.

RODRIGUES, W. A.; MAGALHÃES, P. C.; PAIVA, E.; DOS SANTOS, F. G. Padrões eletroforéticos das proteínas de reserva em grãos de sorgo com presença e ausência de tanino. **Ciência e Agrotecnologia**, v. 33, p. 1972–1977, 2009.

ROGOSA, M. Characters used in the classification of *Lactobacilli.* **International Journal of Systematic Bacteriology**. v.20, n.4, p.519-533, 1970.

ROOKE, J. A.; HATFIELD, R. D. Biochemistry of ensiling. In BUXTON, D. R.; MUCK, R. E.; HARRISON, J. H. **Silage science and technology**. Madison: American Society of Agronomy; Crop Science Society of America; Soil Science Society of America. p. 251-304, 2003.

ROONEY, L. W.; PFLUGFELDER, R. L. Factors affecting starch digestibility with special emphasis on sorghum and corn. **Journal of Animal Science**, Champaing, v.63 p. 1607-1623, 1986.

ROONEY, L. W.; SERNA-SALDIVAR, S. O. **Sorghum, in Handbook of Cereal Sciece Thechnology** (eds K.J. LORENZ; K. KULP), Marcel Dekker, New York, NY, p 233-270, 1991.

RUIZ-GARCIA, C.; QUESADA, E.; MARTINEZ-CHECA, F.; LLAMAS, I.; URDACI, M. C.; BEJAR V. *Bacillus axarquiensis* sp. nov. and *Bacillus malacitensis* sp. nov., isolated from river-mouth sediments in southern Spain. **International Journal of Systematic and Evolutionary Microbiology**. v.55, p.1279–1285, 2005.

SATOMI, M.; LA-DUC, M. T.; VENKATESWARAN, K. *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. **International Journal of Systematic and Evolutionary Microbiology**. v.56, p.1735–1740, 2006.

SERRANO, J.M.R. **El sorgo híbrida despierta interés en las Américas**. La Hacienda, Kissimmée, v.66, n.5, p. 36-37, 1971.

SHEWRY, P. R.; HALFORD, N. G. Cereal seed storage proteins: structures, properties and role in grain utilization. **Journal of Experimental Botany**, v. 53, p. 947–958, 2002.

SHIVAJI, S.; CHATURVEDI, P.; SURESH, K.; REDDY, G. S. N.; DUTT, C. B. S.; WAINWRIGHT, M.; NARLIKAR, J. V.; BHARGAVA, P. B. *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. **International Journal of Systematic and Evolutionary Microbiology**. v.56, p.1465–1473, 2006.

SHOTWELL, M. A.; LARKINS, B. A. The biochemistry and molecular biology of seed storage proteins. In: MARCUS, A. (Ed.). **The biochemistry of plant**: a comprehensive treatise. San Diego: Academic, v. 15, p. 296-345. 1989.

SHULL, J.M.; CHANDRASHEKAR, A.; KIRLEIS, A.W.; EJETA, G. Development of sorghum [Sorghum bicolor (L.) Moench] endosperm in varieties of varying hardness. **Food Struct**., v.9, p.253-267, 1990.

SILVA, J.F.; LEÃO, M.I. **Fundamentos de nutrição dos ruminantes**. Piracicaba: Livroceres, 1979. 380p.

SILVA, P.A.; VALADARES FILHO, S.C.; VALADARES, R.F.D. et al. Valor energético do capim-elefante em diferentes idades de rebrota e estimativa da digestibilidade in vivo da fibra em detergente neutro. **Arq. Bras. Med. Vet. Zootec**., v.59, n.3, p.711-718, 2007.

SILVEIRA, S. T.; OLIVEIRA, M. S.; COSTA, J. A. V.; KALIL, S. J. Optimization of glucoamylase production by Aspergillus niger in solid-state fermentation. **Applied Biochemistry and Biotechnology**, v.128, n.2, p.131-139, 2006.

SILVA, N. **Quantificação de amido e proteínas totais em grãos de sorgo [Sorghum bicolor (L.) Moench - Família: Poaceae] visando à alimentação humana**. Dissertação (Mestrado), Universidade de Brasília, 2015, 72p.

SILVA, J. S.; BORGES, A. L. C. C.; LOPES, F. C. F.; SILVA, R. R.; VIEIRA, A. R.; DUQUE, A. C. A.; BORGES, I.; RODRIGUES, J. A. S.; GONÇALVES, L. C. Degradabilidade ruminal *in situ* do sorgo grão em diferentes formas de reconstituição. **Arq. Bras. Med. Vet. Zootec**., v.66, n.6, p.1822-1830, 2014.

SILVA, N. C.; NASCIMENTO, C. F.; NASCIMENTO, F. A.; DE RESENDE, F. D.; DANIEL, J. L. P.; SIQUEIRA, G. R. Fermentation and aerobic stability of rehydrated corn grain silage treated with different doses of Lactobacillus buchneri or a combination of Lactobacillus plantarum and Pediococcus acidilactici. **Journal of Dairy Science.** Vol. 101 No. 5, 2018.

SIMPSON, D. J. Proteolytic degradation of cereal prolamins—the problem with proline. **Plant Science**, v. 161, n. 5, p. 825–838, 2001.

SOCCOL, C. R. et al. Glucoamylase. In: **Enzyme Technology**. New Delhi: Asiatec Publishers Inc., p. 221-230.
2005.

STOCK, R.A. Nutritional benefits of specialty grain hybrids in beef feedlot diets. **Journal of Animal Science**, v. 77, Suppl. 2, p. 208-212, 1999.

SULLINS, R.D.; ROONEY, L.W. Light and scanning electron microscopy studies of waxy and nonwaxy endosperm sorghum varieties. **Cereal Chem**., v.52, p.361366, 1975.

SULLINS, R.D.; ROONEY, L.W.; RIGGS, J.K. Physical changes in the kernel during reconstitution of sorghum grain. **Cereal Chem**., v.48, p.567-575, 1971.

TANASUPAWAT, S.; OKADA, S.; KOZAQ, M.; KOMAGATA, K. Characterization of Pediococcus pentosaceus and Pediococcus acidilactici Strains and Replacement of the Type Strain of P. acidilactici with the Proposed Neotype DSM 20284. **International Journal of Systematic Bacteriology**, v.43, n.2, p.860-863, 1993.

TAYLOR, C. C.; M. S. ALLEN. Corn Grain Endosperm Type and Brown Midrib 3 Corn Silage: Site of Digestion and Ruminal Digestion Kinetics in Lactating Cows. **Journal of Dairy Science**, v. 88, p. 1425–1433, 2005.

THEURER, C.B. Grain processing effects on starch utilization by ruminants. **Journal of Animal Science**, 63, 1649-1662. 1986.

VAN HELLEN, R.W.; ELLIS, W.C. Samples container porosities for rumen *in situ* studies. **J. Ani. Sci**., v.44, p.141-146, 1977.

VAN SOEST, P. J. **Nutritional ecology of the ruminant**. 2nd ed. Ithaca: Cornell University Press, 1994. 476p.

VANZANT, E. S.; COCHRAN, R.C.; TITGEMEYER, E. C. Standardization of *In situ* Techniques for Ruminant Feedstuff Evaluation. **J. Anim. Sci**. v.76, p.2717–2729, 1998.

VISSER, H.; KLOP, A.; VAN DER KOELEN, C. J.; VAN VUUREN, A. M. Starch Supplementation of Grass Harvested at Two Stages of Maturity Prior to Ensiling: Intake, Digestion, and Degradability by Dairy Cows. **Journal of Dairy Science**. v.81, n.8, p.2221-2227, 1998.

WANISKA, R. D.; ROONEY, L. W. Chapter 4.1 Structure and chemistry of the sorghum caryopsis. **Sorghum: Origin, History, Technology, and Production**, p.649–688 2000.

WATTERSON, J. J.; SHULL, J. M.; KIRLEIS, A. W. Quantification of α-, β- e ɣ-Kafirina in vitreous and opaque endosperm of sorghum bicolor. **Cereal Chemistry**, v. 70, p. 452–457, 1993.

WONG, J. H.; MARX, D. B.; WILSON, J. D.; BUCHANAN, B. B.; LEMAUX, P. G.; PEDERSEN, J. F. Principal component analysis and biochemical characterization of protein and starch reveal primary targets for improving sorghum grain. **Plant Science**, v. 179, p. 598–611, 2010.

ZOPOLLATTO, M. **Avaliação do efeito da maturidade de cultivares de milho (Zea mays L.) para silagem sobre a produtividade, composição morfológica e valor nutritivo da planta e seus componentes.** Tese (Doutorado em Agronomia) - Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, 2007. 210f.

ZHANG, B.; DHITAL, S.; GIDLEY, M. J. Synergistic and Antagonistic Effects of α - Amylase and Amyloglucosidase on Starch Digestion. **Biomacromolecules**, v.14, p.1945–1954, 2013.

SEGUNDA PARTE

3 ARTIGO 1: Addition of exogenous amyloglucosidases to rehydrated corn and sorghum kernel silage at two durations of storage

Artigo formatado de acordo com normas da revista Grass and Forage Science

Disponível em: http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1365-2494/homepage/ForAuthors.html

Addition of exogenous amyloglucosidases to rehydrated corn and sorghum kernel silage at two durations of storage

Amyloglucosidase in rehydrated kernel silage

Acknowledgments

Appreciation is extended to Drs. Schwan (Department of Biology, Federal University of Lavras, Lavras, MG) for the opportunity to use their laboratory equipment during this experiment; and MCs Gomide (EPAMIG, Agricultural Research Corporation of Minas Gerais State, TrêsPontas, MG) for provide the animals to this experiment. The authors are also grateful to the National Research Council (CNPq; Brasilia, DF, Brazil) and Foundation for Research Support of the State of Minas Gerais (FAPEMIG; MG, Brazil) for the grants to the authors.

Abstract

The objectives of this study were to evaluate the effect of two amyloglucosidases and duration of storage of sorghum or corn kernel rehydrated and ensiled. Kernels were rehydrated (65% of DM) before ensiled. Dosages of amyloglucosidase AMG (AMG, Novozymes) and GAM (Sanferm Yield, Novozymes) were 0.35 mL/kg of kernel. Treatments were a 2 x 3 x 2 factorial combination of G (Sorghum vs. Corn), A (CTL vs. AMG vs. GAM), and duration (30 d vs. 180 d) with 6 replicates. The *in situ* DM degradation was evaluated with incubation times of 0 (bag wash), 3, 6, 12, 18, and 48 h in 3 rumen cannulated cows. The fractional rate of degradable

Fraction B (kd) was determined by the slope of linear regression of the natural logarithm of bag residue as a proportion of the initial incubated mass over time. The effective ruminal degradation (ERD6) was calculated based on soluble fraction (A), degradable fraction (B), kd, and passage rate (kp) defined as 6.5% h^{-1}: A + B [kd / (kd + kp)]. Rehydration and ensiling of sorghum or corn with addition of amyloglucosidase resulted in adequate fermentation, as evidenced by pH and lactic acid concentration. Increased DM loss, hydrolyze of starch, and the proportion of DM as fraction A, without alteration on kd or ERD 6.5. Is necessary long time of fermentation to improve DM digestibility. The ensilage of sorghum improved the quality of kernel making it like corn.

Key words: amyloglucosidase, degradation rate, enzymes, fermentation profile, kernel, silage

Introduction

Rehydrated kernel silage consists of milled mature kernel rehydration, which provides adequate conditions for storage and fermentation as silage (Andrade et al., 2010; Carvalho et al., 2016). Digestibility of starch is impaired by hydrophobic starch-protein matrix surrounding starch granules which reduced microbial attachment and fermentation in the rumen (Giuberti et al., 2014). During the ensiling process, proteolysis of the prolamins involving the starch granules occurs by the action of microbial proteases (Hoffman et al., 2011), and thus, an increase of the extent of degradation that occurs in the rumen for starch of corn and sorghum (Ferraretto et al., 2015; Aguerre et al., 2015).

Improvements in starch digestibility occurs with a prolonged storage period, but at the expense of increased DM loss (Carvalho et al., 2016). The use of exogenous enzymes at ensiling

were previously reported to increase the availability of starch after a short period of storage (Ferraretto et al., 2015). Enzymes such as amylases and amyloglucosidase breakdown the starch chain to monosaccharaides and disaccharides, increasing the rate of starch digestion in the rumen (Zhang et al., 2013). However, the optimal temperature and pH for enzymatic activity may change depending on the growing conditions of the microorganism that produces it (Gates, 2010). For sorghum, those enzymes had more expressive results as the starch-protein matrix is more resistant than the corn matrix (Theurer, 1986). However, the action of these enzymes may be affected by pH and temperature conditions of the silage (Motta, 2011).

Therefore, experimental objectives were to evaluate the effect of two amyloglucosidases produced under different condition, and storage length on fermentation profile, and *in situ* degradability of sorghum or corn kernels rehydrated and ensiled. We hypothesized that the addition of exogenous amyloglucosidases would improve the degradability of starch after a short term length of storage.

Materials and methods

Kernel and ensiling conditions

Mature sorghum (Dow 1G220, 84.9% DM on as fed) and corn (Dow 2B707, 87.3% DM on as fed) kernels were ground through a 1 mm mesh diameter screen using a stationary mill Nogueira TN-8 (Nogueira Máquinas Agrícolas, São João da Boa Vista, SP). Ground kernels were rehydrated to a targeted DM concentration of 65%. Approximately 4 kg of rehydrated kernel was ensiled in experimental polyvinyl chloride mini-silos (10 cm in diameter and 60 cm in length). Experimental silos were sealed with tight lids containing Bunsen type valves for gas release.

One amyloglucosidase unit (AGU) is defined as the amount of enzyme that cleaves 1 µmol of maltose per minute under standard assay conditions (JECFA, 2002). Enzymes used were: 1) amyloglucosidase from *Aspergillus niger* with high activity at 40 to 50 ^{o}C and pH 4.8 to 5, with 300 AGU mL^{-1} (AMG; Novozymes Latin America Ltda, Araucária, PR, Brasil), or 2) amyloglucosidase from *Aspergillus niger* with adequate activity at 55^{o}C and pH 4.5, with 138 AGU mL^{-1} (GAM; Sanferm Yield, Novozymes Latin America Ltda, Araucária, PR, Brasil). Treatments were cereal kernel type (Sorghum vs. Corn), enzyme (control [CON] vs. AMG vs. GAM), and duration of silage storage (30 vs. 180 d). Experiment was conducted as randomized blocks, each block corresponded to a day of ensiling, with three replicates in each block. The factorial arrangement consisted of 2 cereal kernel types x 3 enzymes x 2 storage length. The silage was treated with a dosage of 0.35 mL of enzyme kg^{-1} of kernel, according to the recommendation of the manufacturer.

Each silo was packed to achieve a packing density of 950 kg m^{-3} of fresh matter, and the weight of empty and full silos was recorded. The silos were stored at room temperature (25 ± 1.5 °C) and protected from sunlight and rain. After 30 and 180 days of ensiling, the full silos were weighed and opened. The total loss of DM was calculated using weights of the DM contents of fresh forage and silage.

Analytical procedures

After the opening, samples were frozen at -20°C for 5 days for inactivation of enzymes. The DM contents of each sample were determined according AOAC (1990). NDF by filtration in porous crucibles with heat stable alpha-amylase and sodium sulfite (Van Soest et al., 1991), and the ether extract (EE) as in AOAC International (2012). The starch content was measured by ground samples in a boll mill (SPlabor, Presidente Prudente, SP, Brazil) and analyzed for starch by an enzymatic method with thermostable α-amylase (Termamyl, Novozymes Latin America

Ltda, Araucária, PR, Brasil) and amyloglucosidase (AMG; Novozymes Latin America Ltda, Araucária, PR, Brasil) enzymes, with free and enzymatically released glucose measured. Values from a separate determination of free glucose were subtracted to give values for enzymatically released glucose. Starch concentration was calculated as enzymatically released glucose multiplied by 0.9, as described by Hall (2015). The prolamine content was quantified according to method of Nellis et al. (2013), with use of zein as standard.

Samples (100g) were dry-sieved for 10 min with a Bertel shaker (Bertel Indústria Metalúrgica Ltda., Caieiras, Brazil) with sieves of square diagonals of 4,750, 4,000, 2,000, 1,200, 600, 300, and 150 µm and pan. The proportion of particles retained on each sieve was determined. The geometrical mean particle size (GMPS, µm) and surface area (cm^2/g) were calculated as in Baker and Herrman (2002).

To obtain the aqueous extract, a 25-g sample of silage was blended in 225 mL of sterile water and homogenized in a shaker for 1 min. The pH of each sample was then determined using a pH meter (DIGIMED® DM 20 Potentiometer, Digicrom Instrumentos, SP, Brazil). Aqueous extracts (50 mL) were acidified with 250 µL of 50% (vol/vol) H_2SO_4 and frozen prior to analysis of fermentation end products. The acidified aqueous extracts were analyzed for ethanol, acetic, butyric, propionic, and lactic acids, and 1,2-propanediol by high-performance liquid chromatography according to the method described by Carvalho et al. (2012) using an ion-exclusion column from Shimadzu (Shim-pack SCR-101H; 7.9 mm x 30 cm) operating at 50°C and the mobile phase 100 mM perchloric acid solution with a flow rate of 0.6 mL min^{-1}. Aliquots of 50 ml of aqueous extracts were utilized for reading determined the ammonia concentration, using an ion selective electrode coupled to a multiparameter (High Performance Ammonia Ion Selective; Thermo Fisher Scientific Inc., Waltham, MA) as described by Carvalho et al. (2016).

Measurement of *in situ* ruminal DM disappearance were performed with six incubation time-points 0, 3, 6, 12, 18, and 48 h. Two empty bags were put together to correct for DM adhesion at each incubation time. Dry samples without ground, were weighted (0.5045 ± 0.0026 g) in 5 x 5 cm polyester cloth bags (bags were made by a single piece of 5 x 10 cm, which were folded, and heat sealed) tied by a plastic clamp on its last opening. Three rumen cannulated crossbred Holstein x Gyr cows in mid lactation (26.3 ± 3.6 kg of milk) were used (Ethics Committee of Federal University of Lavras, code 072/2015). Cows were fed with corn silage ad libitum and 10 kg of DM from a concentrate composite of (DM basis): Crude protein 24%, Crude fiber 8%, Ash 14%, Ether extract 2%, Fiber insoluble in detergent acid 13%. After removal, bags were immersed in cold water for 15 min to stop fermentation. The 0 h and the incubated bags were washed with cold tap water until rinse water was clear.

The model to estimate the fractional degradation rate (kd) was a two-pool model comprised of a fast degrading fraction (A) and a slow degrading fraction (B). An indigestible residue was not included in the model. Fraction A was the bags at 0 h, and a fractional degradation rate was determined using the other incubation times. The kd was obtained by the slope of the natural logarithm of each time point residue as a percentage of the incubated, which resulted in a linear regression. Effective rumen degradability (ERD) was calculated as: A + B [kd/(kd + kp)], where: A = fraction A, B = fraction B (100 – fraction A), kd = fractional degradation rate, kp = fractional passage rate. The fractional passage rate (kp) used was calculated using the average experimental cow being fed experimental diet and using the CNCPS formula (Tylutki et al., 2008). The kp obtained was equal to 6.5 %/h.

Microbiological analyses

Enumeration of microorganisms was performed on the aqueous extract. To obtain the aqueous extract, a 25-g sample of silage was blended in 225 mL of peptone water (0.1%) and homogenized in a shaker for 10 min. A sequential 10-fold dilution was prepared. For enumeration of lactic acid bacteria (LAB), pour plating on the Man Rogosa Sharpe – Difco (MRS) agar plus nystatin (4 mL L^{-1}) was used. The plates were incubated at 30°C for 72 h. Colonies were counted on plates containing a minimum of 30 and a maximum of 300 colony-forming units (CFU).

Statistical analysis

Data were analyzed in a completely randomized block design using the PROC MIXED procedure of SAS 9.1; each block corresponded to day of ensilage, using the model $Y_{ijklm} = \mu + B_i + K_j + E_k + (KE)_{jk} + D_l + (KD)_{jl} + (ED)_{kl} + (KED)_{jkl} + e_{ijklm}$. Where, μ is the overall mean; B_i is the block effect (i = 2); K_j is the effect of kernel (j = corn, sorghum); E_k is the effect of enzyme(k = control, AMG, GAM); (KE) is the interaction effect of kernel × enzyme;D_l is the effect of day of ensiled (l = 30, 180 d); (KD) is the interaction effect of kernel × day; (ED) is the interaction effect of enzyme × day; (KED) is the triple interaction effect of kernel × enzyme × day, and e_{ijklm} is the experimental error. Statistical significance was considered at $P \leq 0.05$.

Results and Discussion

Unensiled sorghum and corn had similar chemical composition, with expressive differences observed for starch and prolamin content, with 11.07 and 2.05 percentage-units greater values for corn than sorghum, respectively (Table 1). Even though the same mesh diameter was used throughout the grinding process of kernels, the sorghum had smaller GMPS (129 vs. 211 µm) and greater surface area (77 vs. 60 cm^2 g^{-1}) than corn. There was interaction of enzyme and duration of storage on DM concentration ($P < 0.01$; Table 2). At 30 d of storage length the addition

of GAM decreases from 67.24 to 66.24% of DM, at 180 d both enzymes, AMG and GAM, decrease the DM concentration from 65.05 to 62.80 and 62.83% of DM, respectively. A reduction in DM content during the fermentation period is a result of nutrient hydrolysis and the corresponding release of CO_2 and free water (Pahlow et al., 2003); the use of enzymes may have improved this process. Also, the DM content reduction can be related to the methodology used, oven-drying, which excludes the volatile components (Woolford, 1984), resulting in loss of dry matter during oven-drying silages (Wilson et al., 1964). The DM loss was greater in corn than sorghum. There was an interaction between enzyme addition and days of storage length for DM loss ($P < 0.01$); lessened DM loss was observed for CON at 30 d and increase during the ensiling period, reaching a maximum of 5.61%, 11.43 and 9.64% of DM CON and ensiled with enzyme AMG and GAM, respectively, by day 180. According to Rezende et al. (2014), substantial losses of DM during fermentation are arising from the low soluble carbohydrates (WSC) and low amount of acids produced. The DM loss of CON is similar to results of Carvalho et al. (2016) but increased with the addition of amyloglucosidase. The enzyme α-amylase is inhibited by oligosaccharides whereas amyloglucosidase degrades these compounds (Zhang et al., 2013), and hence increases starch degradation, followed by increase starch and DM losses. Increased DM and starch losses were observed for corn compared with sorghum. Sorghum had greater proportion of the vitreous endosperm, a dense region and resistant to water penetration (Rooney and Pflugfelder, 1986), which likely compromises starch availability.

Starch concentration decreased from 67.38% of DM at 30 d to 59.63% of DM at 180 d ($P < 0.01$; Table 2). Corn had increased starch loss than sorghum. Starch loss increased along with storage length. Addition of AMG and GAM increased starch loss (10.64%, 20.34% and 25.26%, respectively for CON, AMG and GAM). For free glucose, there was an interaction between kernel,

enzyme, and duration of ensiling ($P < 0.01$; Figure 1). Addition of AMG, increased free glucose at 180 d for sorghum; corn had more free glucose than sorghum at 180 d. Both enzymes (AMG and GAM) increase free glucose at 180 d in rehydrated corn kernel silage. The starch loss is a result of hydrolyses of starch to glucose, and was not a real loss of DM content, because when the starch content reduced the content of glucose increase.

The longer duration of ensiling reduced 36.21% the amount of prolamin and increased 37.81% the ammonia concentration ($P < 0.01$). Neither kernel type nor the addition of amyloglucosidase affected prolamin degradation. Rehydrated sorghum kernel silage had greater concentration of ammonia than rehydrated corn kernel silage. The increase of ammonia concentrations coincided with reduced prolamin concentrations (Hoffman at al., 2011). According to Junges et al. (2017), besides proteolytic activity of microorganisms, kernel enzymes may contribute on proteolysis in rehydrated corn kernel silage.

Amyloglucosidase increased Fraction A on Deg 0 ($P = 0.02$; 33.7, 38.2, and 36.6 for CON, AMG and GAM, respectively), but had no effect on DM degradation at other incubation times and ERD 6.5%. Corn had greater kd than sorghum ($P < 0.01$), but lower DM degradation up to 6 h of ruminal incubation and ERD 6.5%. The smaller particle size of kernel sorghum (Table 1) may explain this response. Longer storage increased kd in 0.13%-unit from day 30 to day 180, ruminal degradation at all incubation times, with increase of up to 11% on degradation (6h), being the smallest increase occurred with 48 hours (2,9%). Ferraretto et al. (2015) observed a 13.2%-units increase in *in vitro* starch digestibility for rehydrated and ensiled corn compared with unensiled corn. Also, Carvalho et al. (2016) observed increase on *in vitro* DM digestibility at 7 h for rehydrate and ensiled corn, with long storage length. The reduction of prolamin subunits that cross-link

starch granules during the ensiling process may explain the increase in degradation (Hoffman et al., 2011).

The addition of amyloglucosidase (GAM) altered the pH reduction ($P = 0.03$), the pH at 30 d remained high in rehydrated sorghum kernel silage with GAM. Total acid concentration was increased with longer duration of storage length ($P < 0.01$), with consequent reduction in pH to less than 3.80. Rehydrated corn kernel silage had lower pH than sorghum. There was an interaction between kernel type and enzyme addition ($P = 0.02$), since the use of the enzyme GAM resulted in higher pH (4.38) in sorghum, without difference for corn. Therefore, pH value suggested an adequate fermentation, as enterobacteria and bacilli will be inhibited once pH drops below 4.5 to 5.0 (Muck, 2010).

There was an interaction between kernel and duration of silage for total acids ($P = 0.02$); sorghum at 180 d of storage length had greater concentration of total acids, but not at other storage length (Table 3). For propionic and butyric acid there was an interaction among kernel type, enzyme addition and duration of fermentation ($P < 0.01$); a tendency for acetic acid was also observed. The same behavior was observed for both propionic and butyric acids. At 180 d sorghum CON had increased in this acids concentration compared with other treatments. Propionic and butyric acid concentration was increased with the addition of GAM compared with AMG in sorghum, at 180d (Figure 2B). For corn at 180 d there were no differences between enzymes, and for CON there were no increased in this acids between 30 and 180 days of storage. Carvalho et al. (2016) also observed increased the concentration of propionic acid with long fermentation, but the concentration of acids was greater than observed in this study. Concentration of butyric acid may indicate the activity of *Clostridium*, because this acid is part of primary products of *Clostridium*

metabolism (Muck, 2010). Besides *Clostridium*, other microorganisms as yeasts or *Bacillus* can produced butyric acid in low quantities (McDonald et al., 1991).

There was interaction between kernel and enzyme to 1,2 propanediol ($P < 0.01$; Table 3). In sorghum the addition of GAM increased the concentration, but no difference between AMG and CON. Corn was unaffected by the addition of enzymes. There was also an interaction between kernel and duration of storage length ($P = 0.03$). The concentration of 1,2 propanediol was increased only in sorghum at 180 d. The degradation of lactic acid by L. buchneri occurs in anaerobic conditions and resulting in the production of acetic acid and 1,2-propanediol (Oude Elferink et al., 2001; Heinl et al., 2012). The concentration of acetic acid tends ($P = 0.08$) to same behavior of propionic acid. A kernel by enzyme by duration interaction was observed for ethanol concentration (Table 3), with increased concentration at 180 d when GAM was added in sorghum and corn, or when AMG was added in corn.

The population of lactic acid bacteria (LAB) was greater ($P < 0.01$) in corn (6.49 log per g) than sorghum (5.07 log per g) at 30 d of storage length but reduced at 180 d for both kernel types. The duration of fermentation reduced the count of LAB, and the use of the enzyme GAM in sorghum and of both enzymes in corn resulted in reduction of the population to undetectable levels at 180 d of storage length. The population of LAB is higher than undesirable micro-organisms but is slow than compared with population related by Carvalho et al. (2016).

Rehydration and ensiling of sorghum or corn kernel with addition of exogenous amyloglucosidase resulted in adequate fermentation, as evidenced by pH and lactic acid concentration. Increase DM loss, hydrolyze of starch, and fraction A, without alteration on kd or DM degradability. These results suggest that longer storage length is warranted to improve DM

degradability of rehydrated kernel silage. The ensilage of sorghum improved the quality of kernel making it similar to corn.

References

Aguerre, M., Cajarville, C., & Repetto, J.L. (2015). Impact of water addition, germination ensiling and their association on sorghum grain nutritive value. Animal Feed Science and Technology. 205, 75-81.

Andrade Filho, R., R. B. Reis, M. N. Pereira, and M. Antenor. (2010). Degradabilidade ruminal *in situ* de grãos de milho maduros do tipo flint ou dentado, secos ou reconstituídos e ensilados. Anais da 47a reunião anual da sociedade brasileira de zootecnia. Anais...Salvador, BA.

AOAC International. (2012). Official Methods of Analysis. 19th ed. AOAC International, Arlington, VA.

Baker, S., and T. Herrman. (2002). Evaluating particle size. MF-2051. Kansas State Univ., Manhattan.

Carvalho, B.F., Ávila, C.L.S., Pinto, J.C., Pereira, M.N., & Schwan, R.F. (2012) Effects of propionic acid and Lactobacillus buchneri (UFLA SIL 72) addition on fermentative and microbiological characteristics of sugar cane silage treated with or without calcium oxide. Grass Forage Science, 67, 462–471.

Carvalho, B.F., Ávila, C.L.S., Bernardes, T.F., Pereira, M.N., Santos, C., & Schwan, R.F. (2016). Fermentation profile and identification of lactic acid bacteria and yeasts of rehydrated corn kernel silage. Journal of Applied Microbiology. 122, 589-600.

JECFA. (2002). Joint FAO/WHO Expert Committee on Food Additives. Compendium of food additive specifications, addendum 9. FAO Food and Nutrition Paper, No. 52, Add. 9, 2002, p 193.

Gates, R. (2010). Enzymes and Reagents for Ethanol Research. In Technical content: Enzymes and Reagents for Alternative Energy. 5,1-10.

Giuberti, G., Gallo, A., Masoero, F., Ferraretto, L. F., Hoffman, P. C., & Shaver, R. D. (2014). Factors affecting starch utilization in large animal food production system: A review. Starke. 66, 72–90.

Ferraretto, L. F., Fredin, S. M., & Shaver, R. D. (2015). Influence of ensiling, exogenous protease addition and bacterial inoculation on fermentation profile, nitrogen fractions and ruminal in vitro starch digestibility in rehydrated and high-moisture corn. Journal of Dairy Science.98, 7318-7327.

Hall, M. B. (2015). Determination of dietary starch in animal feeds and pet food by an enzymatic-colorimetric method: Collaborative study. Journal ofAssociation of Official Analytical Chemists. 98, 397-409.

Heinl S., Wibberg D., Eikmeyer F., Szczepanowski R., Blom J., Linke B., Goesmann A., Grabherr R., Schwab H., Puhler A. and Schluter A. (2012) Insights into the completely annotated genome of *Lactobacillus buchneri* CD034, a strain isolated from stable grass silage. Journal of Biotechnology, 161, 153– 166.

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

Junges, D., G. Morais, M. H. F. Spoto, P. S. Santos, A. T. Adesogan, L. G. Nussio, and J. L. P. Daniel. (2017). Short communication: Influence of various proteolytic sources during fermentation of reconstituted corn grain silages. J. Dairy Sci. 100:9048-9051.

Mcdonald, P., Henderson, A.R., & Heron, S.J.E. (1991) The biochemistry of silage. Marlow, Chalcomb Publication.

Motta, V. T. (2011). Bioquímica Básica. 2. ed. Rio de Janeiro, RJ: Medbook.

Muck, R.E. (2010) Silage microbiology and its control through additives. Revista Brasileira de Zootecnia, 39, 183-191.

Nellis, S.E., Hoffman, P.C., & Shaver, R. D. (2013) A modified method to quantify prolamin proteins in dry and high-moisture corn. Journal of Dairy Science, 96, 4647-4652.

Novozymes (2015). Healthier fermentation and healthier profits with NovozymesSanferm® Yield, 2015, from http://www.novozymes.com/en/solutions/food-and-beverages/casestudies-on-Food-Beverages/Pages/Healthier-fermentation-and-healthier-profits-with-Novozymes-Sanferm®-Yield.aspx.

Oude Elferink S.J.W.H., Krooneman J., Gottschal J.A., Spoelstra S.F. and Faber F. (2001) Anaerobic conversion of lactic acid to acetic acid and 1,2propanediol by *Lactobacillus buchneri.* Applied and Environmental Microbiology, 67, 125–132.

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

Rezende, A. V., Rabelo, C. H. S., Veiga, R. M., Andrade, L. P., Harter, C. J., Rabelo, F. H. S., Basso, F. C., Nogueira, D. A., & Reis, R. A. (2014). Reydratation of corn grain with acid whey improves the silage quality. Animal Feed Science and Technology. 197, 213-221.

Rooney, L. W.; Pflgfelder, R. L. (1986). Factors affecting starch digestibility with special emphasis on sorghum and corn. Journal of Animal Science, Champaing, v.63 p. 1607-1623,

Theurer, C.B. (1986) Grain processing effects on starch utilization by ruminants. Journal of Animal Science, 63, 1649-1662.

Tylutki, T. P., Fox, D. G., Durbal, V. M., Tedeschi, L. O., Russell, J. B., Van Amburgh, M. E., Overton, T. R., Chase, L. E., & Pell, A. N. (2007). Cornell Net Carbohydrate and Protein System: A model for precision feeding of dairy cattle. Animal Feed Science and Technology. 143, 174-202.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

Wilson, R. F., J. M. A. Tilley, and A. Mar1a. (1964) Comparison of oven drying and toluene distillation in the determination of the dry-matter content of silage. J. Sci. Fd Agric. 15:197-200.

Woolford, M. K. (1984) The silage fermentation. New York: Marcel Dekker, 2 ed. 350 p.

Zhang, B., Dhital, S., & Gidley, M. J. (2013). Synergistic and Antagonistic Effects of α - Amylase and Amyloglucosidase on Starch Digestion. Biomacromolecules. 14, 1945–1954.

Table 1. Nutrient composition and particle size of ground sorghum and corn kernel before ensiling

	Sorghum	Corn
DM, % of as fed	90.2 ± 0.1	89.6 ± 0.2
NDF, % of DM	17.5 ± 1.1	18.3 ± 0.9
ADF, % of DM	5.5 ± 1.8	4.5 ± 0.6
Starch, % of DM	57.5 ± 2.5	68.6 ± 1.1
Free Glucose, % of DM	3.2 ± 1.8	4.0 ± 2.3
EE, % of DM	1.8 ± 0.2	4.0 ± 0.6
Ash, % of DM	1.3 ± 0.2	1.0 ± 0.2
Prolamin, % of starch	7.4 ± 1.2	9.4 ± 2.5
Ruminal fractional disappearance rate of Fraction B, % h^{-1}	3.1 ± 0.4	3.3 ± 0.6
Effective ruminal disappearance ERD 6.5[2], % of DM	51.4 ± 3.7	48.6 ± 3.6
Geometrical mean particle size,[1] µm	129.0 ± 1.4	210.6 ± 45.7
% retained on sieve		
4000 µm	0.0 ± 0.0	0.1 ± 0.1
2000 µm	1.6 ± 2.3	0.1 ± 0.2
1200 µm	2.8 ± 4.7	9.0 ± 13.3
600 µm	33.2 ± 7.4	30.2 ± 8.5
300 µm	31.5 ± 6.1	41.8 ± 13.5
150 µm	19.0 ± 7.1	14.0 ± 11.5
Bottom pan	12.0 ± 0.6	4.9 ± 2.9
Surface area, cm^2/g	76.7 ± 0.4	59.5 ± 3.8

[1]Kansas State University: MF-2051. Baker & Herman (2002)

[2]ERD = Fraction A + Fraction B × [kd / (kd + kp), kp estimated by the Cornel Net Carbohydrate and Protein System, 7.47 % h^{-1} (Tylutki et al., 2008)

Table 2. Dry matter (DM), starch, glucose, prolamin, and ammonia-N content, DM loss and starch during ensiling, and kinetics of ruminal DM degradation of rehydrated corn or sorghum kernel silage with 30 or 180 d, and addition of amyloglucosidase (AMG), amyloglucosidase (GAM), and Control (CON)

Kernel (K)	Sorghum						Corn							*P*		
Enzyme (E)	CON		AMG		GAM		CON		AMG		GAM		SEM	K	E	D
Duration (D)	30	180	30	180	30	180	30	180	30	180	30	180				
DM, % of fresh	66.96	64.91	66.50	62.68	66.34	62.79	67.51	65.18	66.74	62.91	66.14	62.86	0.838	0.34	<0.01	<0.01
DM loss, % of DM	0.64	4.28	3.85	10.51	3.04	9.21	3.24	6.94	4.26	12.36	3.76	10.07	1.756	<0.01	<0.01	<0.01
Starch, % of DM	62.92	51.28	58.90	48.79	56.96	48.02	64.07	61.16	57.14	49.33	55.81	50.73	3.741	0.23	<0.01	<0.01
Starch loss, % of starch	2.04	12.15	4.27	22.08	12.36	28.48	10.77	17.58	19.35	35.65	25.35	34.45	4.43	<0.01	<0.01	<0.01
Glucose free, % of DM	0.653	1.821	2.254	4.295	2.804	2.569	2.190	0.547	3.495	6.115	2.428	2.985	0.6441	0.02	<0.01	<0.01
Prolamin, % Starch	8.21	7.21	8.83	6.19	8.04	7.33	9.01	7.01	7.76	5.96	8.68	5.39	1.091	0.42	0.37	<0.01
Ammonia-N, % of DM	0.058	0.122	0.050	0.088	0.061	0.118	0.046	0.093	0.046	0.079	0.041	0.070	0.0130	<0.01	0.10	<0.01
Deg 0, % of DM	35.03	39.97	37.63	45.33	38.74	41.89	25.05	34.67	30.83	38.90	30.24	35.42	2.207	<0.01	0.02	<0.01
Deg 3, % of DM	43.26	51.28	44.30	50.69	45.30	51.32	32.82	47.44	36.20	47.63	37.29	45.67	2.717	<0.01	0.80	<0.01
Deg 6, % of DM	47.01	57.43	47.02	55.18	48.10	54.46	39.08	57.51	40.04	51.73	41.57	52.69	3.393	0.03	0.76	<0.01
Deg 12, % of DM	52.62	59.04	53.29	58.92	56.46	60.61	47.48	61.83	51.14	60.29	52.77	58.87	3.040	0.42	0.66	<0.01
Deg 18, % of DM	57.86	65.28	59.40	62.78	61.41	65.83	53.71	68.33	56.72	65.77	56.67	62.25	2.434	0.28	0.98	<0.01
Deg 48, % of DM	85.14	88.19	85.78	87.52	86.22	87.91	84.40	89.37	84.78	88.05	84.04	87.23	1.274	0.51	0.89	<0.01
kd, % h^{-1}	3.05	3.23	3.10	3.11	3.12	3.23	3.32	3.61	3.23	3.35	3.22	3.27	0.161	<0.01	0.42	0.06
ERD 6.5%[1], % of DM	55.69	59.98	57.65	62.82	58.56	61.03	50.18	57.95	53.67	59.68	53.03	56.99	1.782	<0.01	0.17	<0.01

[1] Effective degradation of DM assuming kp of 6.5%/h. ERD 6.5% = A + B * [kd / (kd + kp)].
Significantly interaction was shown in figure.

Table 3. Silage pH, lactic acid bacteria (LAB), organic acids and alcohol content of rehydrated corn or sorghum kernel silage with 30 or 180 d, and addition of amyloglucosidase (AMG), amyloglucosidase (GAM), and Control (CON)

Kernel (K)	Sorghum						Corn							*P*		
Enzyme (E)	CON		AMG		GAM		CON		AMG		GAM		SEM	K	E	D
Duration (D)	30	180	30	180	30	180	30	180	30	180	30	180				
pH	4.18	3.74	4.12	3.72	4.38	3.80	4.05	3.71	4.15	3.76	4.14	3.71	0.119	0.02	0.03	<0.01
Lactic, % of DM	4.18	12.99	6.08	9.52	5.03	15.04	8.46	7.06	3.00	7.80	3.73	6.37	4.075	0.05	0.64	< 0.01
Propionic, % of DM	0.11	3.56	0.07	0.89	0.09	1.49	0.08	0.98	0.04	0.70	0.03	0.56	0.475	< 0.01	< 0.01	< 0.01
Acetic, % of DM	0.10	0.11	0.02	0.07	0.03	0.15	0.03	0.06	0.01	0.05	0.01	0.04	0.028	< 0.01	0.02	< 0.01
Butyric, % of DM	0.00	0.72	0.00	0.24	0.00	0.46	0.00	0.06	0.02	0.19	0.05	0.17	0.084	< 0.01	0.07	< 0.01
Total acids, % of DM	4.40	17.38	6.18	10.73	5.16	17.14	8.57	8.16	3.07	8.74	3.83	7.13	2.511	0.02	0.40	< 0.01
1,2 propanodiol	2.00	3.66	1.54	2.95	1.88	5.19	1.64	2.05	1.06	2.14	0.62	1.28	0.911	< 0.01	0.51	< 0.01
Ethanol	0.53	0.67	0.70	1.20	0.61	0.87	0.71	0.73	0.65	1.43	0.81	1.59	0.155	< 0.01	<0.01	< 0.01
LAB, log CFU/g	5.07	3.52	5.22	2.32	4.92	<2.0	6.83	3.53	6.62	<2.0	6.02	<2.0	0.458	<0.01	<0.01	<0.01

Significantly interaction was shown in figure.

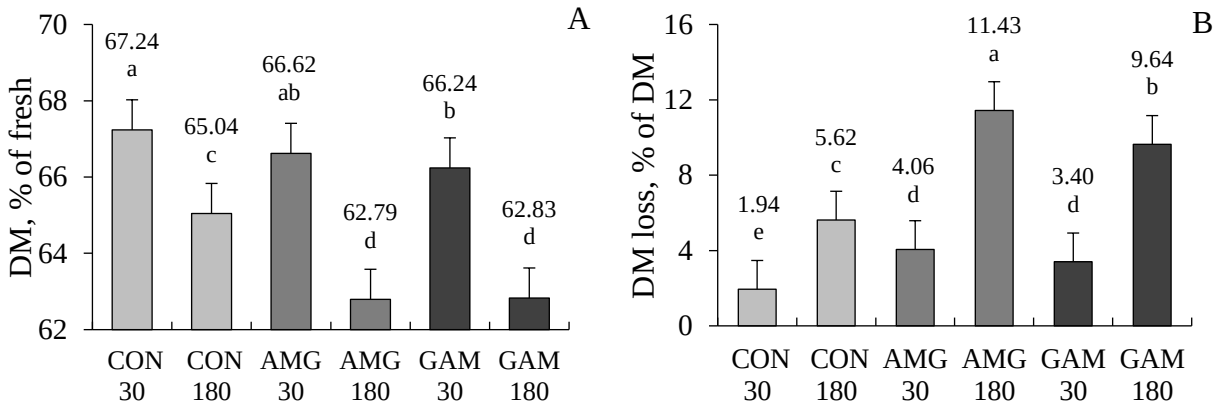


Figure 1. Interaction between amyloglucosidase addition, and duration of storage length on DM (panel A; $P < 0.01$; SEM = 0.942) in rehydrated sorghum or corn kernel silage. Interaction between amyloglucosidase addition, and duration of storage length on DM loss (panel B; $P < 0.01$; SEM = 1.983) in rehydrated sorghum or corn kernel silage. Treatments were rehydrated sorghum or corn kernel ensiled with amyloglucosidase (AMG or GAM) or without (C), and duration of storage length (30 or 180 days).

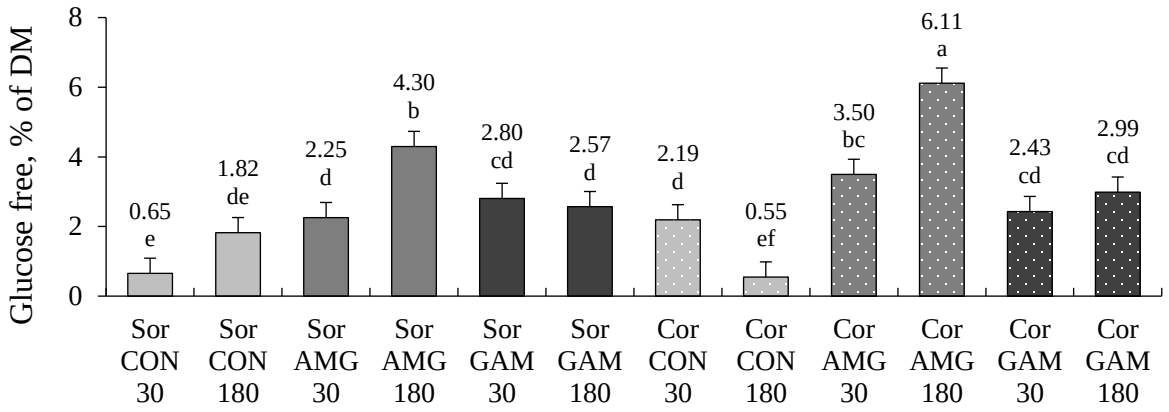


Figure 2. Interaction between kernel, amyloglucosidase addition, and duration of storage length on free glucose ($P < 0.01$; SEM = 0.594) in rehydrated sorghum or corn kernel silage. Treatments were rehydrated sorghum (Sor) or corn (Cor) kernel ensiled with amyloglucosidase (AMG or GAM) or without (C), and duration of storage length (30 or 180 days).

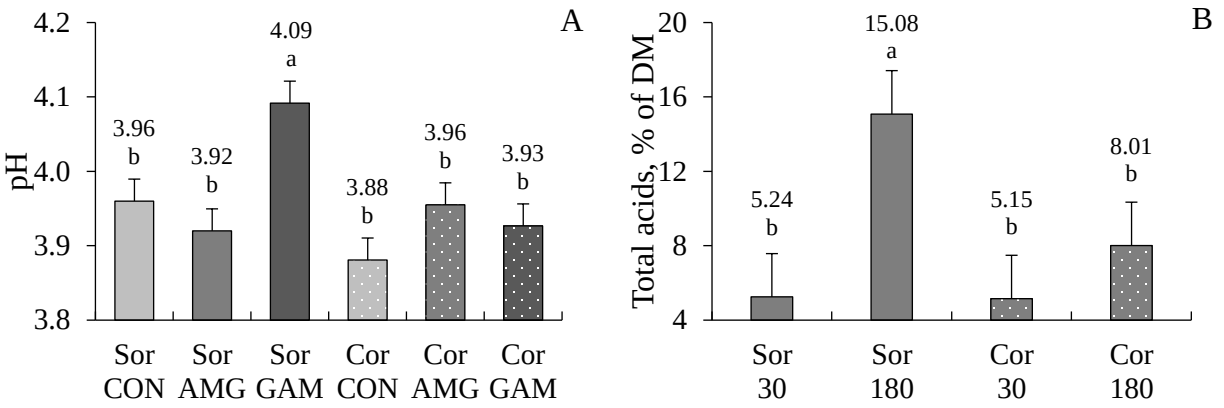


Figure 3. Interaction between kernel and amyloglucosidase addition length on pH (panel A; *P* = 0.02; SEM = 0.249) in rehydrated sorghum or corn kernel silage. Interaction between kernel and and duration of storage length on pH (panel B; *P* = 0.02; SEM = 2.463) in rehydrated sorghum or corn kernel silage. Treatments were rehydrated sorghum (Sor) or corn (Cor) kernel ensiled with amyloglucosidase (AMG or GAM) or without (C), and duration of storage length (30 or 180 days).

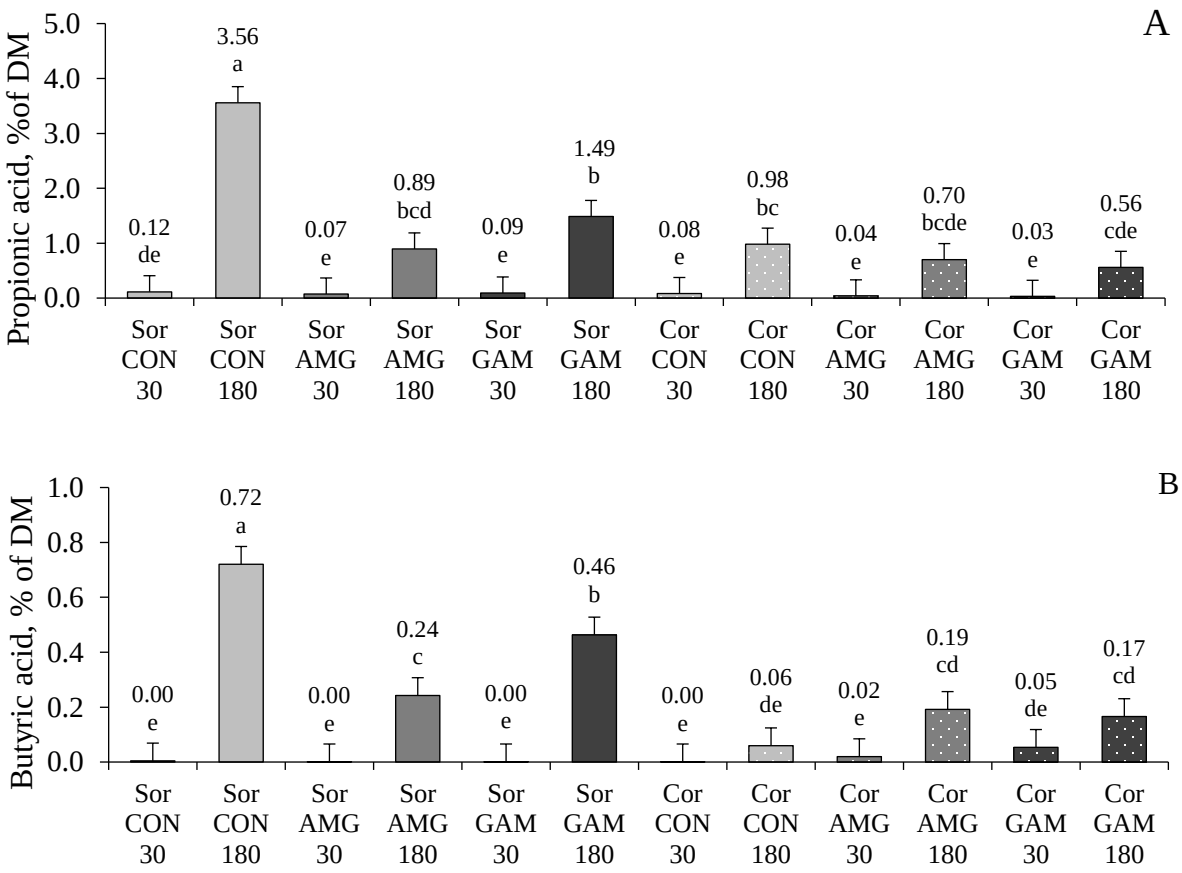


Figure 4. Interaction between kernel, amyloglucosidase addition, and duration of storage length on propionic acid (panel A; $P = 0.02$; SEM = 0.3878), and butyric acid (panel B; $P < 0.01$; SEM = 0.0684) in rehydrated sorghum or corn kernel silage. Treatments were rehydrated sorghum (Sor) or corn (Cor) kernel ensiled with amyloglucosidase (AMG or GAM) or without (C), and duration of storage length (30 or 180 days).

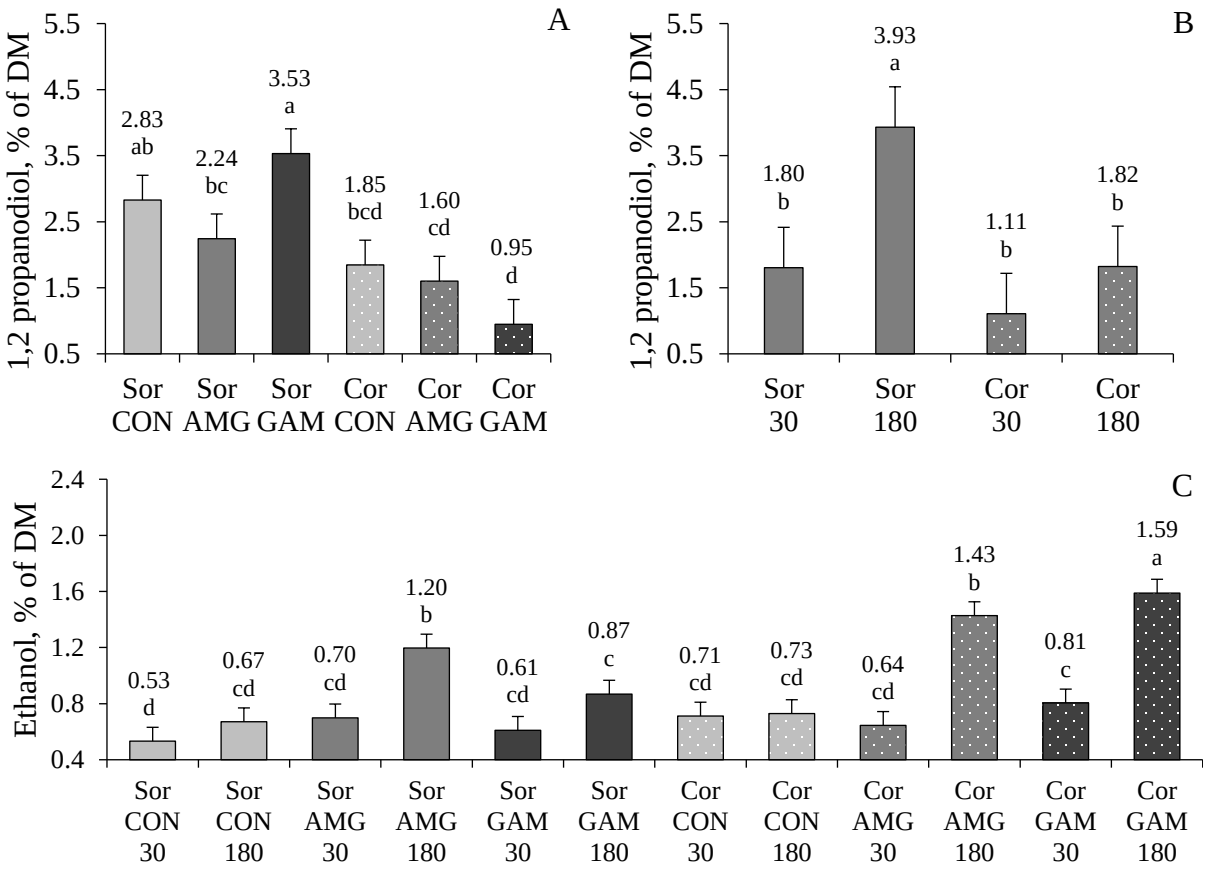


Figure 5. Interaction between kernel, amloglucosidase addition, and duration of storage length on ethanol (P = 0.04; SEM = 0.1263), and butyric acid (panel B; P < 0.01; SEM = 0.0684) in rehydrated sorghum or corn kernel silage. Treatments were rehydrated sorghum (Sor) or corn (Cor) kernel ensiled with amyloglucosidase (AMG or GAM) or without (C), and duration of storage length (30 or 180 days).

4 ARTIGO 2: Microbiota in corn and sorghum kernels rehydrated and ensiled with amyloglucosidase addition

Artigo formatado de acordo com normas da revista Journal of Dairy Science

Disponível em:

http://www.journalofdairyscience.org/pb/assets/raw/Health%20Advance/journals/jods/2016_SF.pdf

Interpretative Summary: Fernandes et al. During the kernel storage, several biochemical processes occur. Characterizing the microbial communities and understand those activity in silage fermentation can help to improve ensilage process. The objective of this study was to identify the microbiota diversity and relationship between those microorganisms with characteristics of rehydrated sorghum or corn silage with addition of amyloglucosidase. The fermentation of rehydrated kernel was dominating by LAB, with highly population, but had greater diversity of SAB. The most part of microorganisms were involved in acids production, DM and starch losses.

Running Head: *Microorganisms in sorghum and corn kernels silages*

Lactic acid bacteria and spore forming aerobic bacteria in sorghum and corn kernels rehydrated and ensiled with amyloglucosidase addition

ABSTRACT

The objective of this study was to identify lactic acid bacteria (**LAB**) and spore forming aerobic bacteria (**SAB**) diversity and relationship between those microorganisms with characteristics of rehydrated sorghum or corn silages with addition of amyloglucosidase. Kernel was rehydrated (65% of DM) and ensiled. Dosage of amyloglucosidase **AMG** (AMG, Novozymes) and **GAM** (Sanferm Yield, Novozymes) was 0.35 mL/kg of kernel. Treatments were a 2 x 3 x 2 factorial combination of kernel (Sorghum vs. Corn), enzyme (CTL vs. AMG vs. GAM), and duration (30 d vs. 180 d) with 6 repetitions. Matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry and PCR-based identification were utilized to identify LAB and SAB. Seven species of LAB were isolated with an average population of 5.74 and 3.84 ufc/g at 30 and 180 days of fermentation, respectively. The SAB diversity was 20 different species, with an average

population of of 3.18 and 2.72 ufc/g at 30 and 180 days of fermentation, respectively. The greatest diversity of micro-organisms was present with 30 days of fermentation. The population of LAB and most part of SAB were corelated with acids and nutrient losses and negatively correlation with pH, DM and starch content. Three SAB were corelated with prolamin content. The fermentation of rehydrated kernel was dominating by LAB, with highly population, but had greater diversity of SAB. The most part of microorganisms were involved in acids production, DM and starch losses.

Palavras chave: fermentation, kernel silage, microorganisms

INTRODUCTION

The rehydration and ensilage of corn and sorghum kernels, are practices that pursuit improving the digestibility of starch (Gobetti et al., 2013), making better use of the Brazilian corn, which has hard endosperm of high vitreousness, with lower digestibility for cattle (Correa et al., 2002) and improve the quality of sorghum starch. A way to counteract the negative effect of hard texture of the endosperm on the digestibility of starch kernels in the mature stage. The processing of kernels, which are a source of starch, usually aim to improve ruminal degradation of starch (Hale, 1973).

During the kernel storage several biochemical processes occur, causing modifications in the material, and thus in the nutritional, chemical and microbiological quality of these silages. The prolamins involving the starch granules are hydrolyzed, by microbial enzymes (Hoffman et al., 2011). The proteolysis from microbial enzymes corresponds to 60.4% of proteolysis which occurs during the fermentation process of silage (Junges et al., 2017). However, we do not know which species of micro-organisms are involved in this process, since LAB has low proteolytic activity (McDonald et al., 1991). Addition of inoculant (*L. buchneri*) can altered the hydrolysis of prolamin and improve digestibility of starch, but when inoculant based on *L.plantarum* and *P. pentosaceus* were added, did not change digestibility of starch (Silva et al., 2018). The use of exogenous enzymes as additive at ensilage can reduce the fermentation duration for improve digestibility (Ferraretto et al., 2015). The amyloglucosidase hydrolyses starch molecule to monosaccharaides and disaccharides, and disrupts the helical links facilitating the hydrolysis by other enzymes (Zhang et al., 2013). The availability of glucose can change the microorganisms diversity during silage fermentation.

In this way, characterizing the microbial communities present in silage may help to understand silage fermentation, to enables improve the quality of silages by intervene in the fermentation process (Carvalho et al., 2016). The fermentation microbiota of reiterated corn silage was already characterized by Carvalho et al., (2016), but there are no studies detailing the microbiota involved during ensiling of rehydrated sorghum kernel or evaluate the relationship between microbiota and starch degradation. The aim of this study was to identify microbiota diversity and to evaluate the relationship between microorganisms and improvement dry matter degradation during storage length of rehydrated sorghum or corn with addition of amyloglucosidase.

MATERIALS AND METHODS

Kernel and ensilage conditions

The samples for microorganism evaluation, and data (Table 1), used in this study were derived from previous experiment conducted by Fernandes et al., (Artigo 1). The enzyme used in the ensilage was AMG - Amyloglucosidase from *Aspergillus niger* had high activity at 40 to 50ºC and pH 4.8 to 5, with 300 AGU mL^{-1} (Novozymes Latin America Ltda, Araucária, PR, Brasil) or GAM -amyloglucosidase from *Aspergillus niger* had good activity at 55ºC and pH 4.5, with 138 AGU mL^{-1} (Sanferm Yield, Novozymes Latin America Ltda,Araucária, PR, Brasil). Treatments were kernel type (Sorghum vs. Corn), enzyme (control-CON vs. amyloglucosidase-AMG vs. amyloglucosidase-GAM), and duration of silage storage (30 vs. 180 d), was carried out in randomized blocks, each block corresponded to a day of ensilage, with three replicates in each block.

Microbiological analyses

Enumeration of micro-organisms was obtained from the aliquot of the aqueous extract. To obtain the aqueous extract, a 25-g sample of kernel silage was blended in 225 mL of peptone water (0.1%) and homogenized in a shaker for 10 min. Sequential 10-fold dilution was prepared.

Yeasts and filamentous fungi were enumerated on Dichloran Rose Bengal Chloramphenicol Medium (DRBC, Difco, Becton Dickinson, Sparks, MD, USA), were incubated at 28 °C for 72 h. For enumeration of lactic acid bacteria (LAB), pour plating on the Man Rogosa Sharpe – Difco (MRS) agar plus nystatin (4 mL L^{-1}) was used. For enumeration of spore forming aerobic bacteria (SAB), a sample of aqueous extracts was maintained at 80°C for 10 min, and further decimal dilutions were plated onto Nutrient Agar medium (HiMedia Laboratories, Mumbai, India). The plates were incubated at 30°C for 72 h. Colonies were counted on plates containing a minimum of 30 and a maximum of 300 colony-forming units (CFU).

After counting the bacteria grown in MRS medium and Nutrient Agar medium, the square root of each morphotype were isolated and subjected to the tests of Gram stain, cellular shape, catalase production, oxidase production and motility. Subsequently subjected to gas production from MRS (Drosinos et al., 2006).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis was used to group and identify the bacteria according to their protein profile. Measurements were performed with an ultrafleXtreme (Bruker Daltonics; Bremen, Germany) following the procedure prescribed by Carvalho et al. (2016).

The 258 strains isolated from MRS medium and 108 SAB strains were analyzed using MALDI-TOF MS. Following the cluster analysis of the MALDI-TOF MS spectrum.

For identification of *Pediococcus pentosaceus* and *Ped. acidilactici* the growing test at 50°C was accomplished (Tanasupawat et al., 1993). The *Lactobacillus pentosus*, *Lac. paraplantarum* and *Lac. plantarum*, were separated by acid production from melezitose and dulcitol test (Bringel et al., 2001; Curke et al., 1996). For *Lac. buchneri* and *Lac. brevis* the test of melezitose fermentation was accomplished (Rogosa et al., 1970).

For SAB other test were used to identification. Acids from sorbitol, melibiose and starch were used for separate *Bacillus atrophaeus*, *Bac. mojavensis*, *Bac. subtilis* and *Bac. vallismortis* (Ruiz-Garcia et al., 2005). Growth at medium with concentration of 5, 7, and 10% of NaCl for identification of *Bac. sonorensis* and *Bac. licheniformis* (Palmisano et al., 2001). Citrate utilization, and D-sorbitol and melibiose as carbon source were used for characterization of *Bac. pumilus*, *Bac. safensis*, *Bac. aerophilus*, *Bac altitudinis*, and *Bac stratosphericus* (Satomi at al., 2006; Shivaji et al., 2006). Growth at medium with concentration of 7% of NaCl also used for *Lysinobacillus boronitolerans*, *Lys. fusiformis* and *Lys. sphaericus* (Ahmed et al., 2007).

Statistical analysis

The principal component analyses (PCA) were performed using STATISTICA 8.2 (Cary, CN, USA, 2002) for grouping the data of silage characteristics, fermentation end products, and BAL and SAB community. For analyses the PCA silage characteristics and fermentation end products were used as active data, and population of bacteria was used as supplementary data, to identify the comportment of bacteria related with silage characteristic (Vicini, 2005). Average of data obtained in different treatments from Fernandes et al. (Artigo 1) in silages of sorghum or corn, with addition of two kinds of amiloglucosidase and ensiled for 30 and 180 days.

RESULTS

A total of 258 isolates were selected from MRS media and were characterized in terms of cell morphology and biochemical features (Table 2) and them, a total of 108 isolates were selected from AN media using the same characteristics (Table 3). The population of yeast and filamentous fungi remained below the detection level. After purification, the isolates were analyzed and grouped according to their protein expression profile by MALDI-TOF MS, for each rehydrated kernel (sorghum or corn) silage and each isolation media (Figure 1, 2, 3, and 4), each group corresponding a one specie, or more than one specie closely related. Seven different LAB species were identified from the MRS medium (Table 2). Twenty different SAB species were identified from AN medium (Table 3).

Lactobacillus plantarum was present at 30 days of silage, in all treatments (CON, AMG, and GAM) and in both kernel silages, but greater population in corn kernel silage 5.07 log UFC/g than sorghum kernel silage 4.13 log UFC/g. At 180 days of fermentation the population of *Lac. plantarum* remained below 2 log UFC/g (Table 4). The identification of *Lac. plantarum* was made by acid production from melezitose and none production from dulcitol (Table 2). Some strains of *Pediococcus acidilactici* and *Ped. pentosaceus* were grouped near *Lac. plantarum* and *Lac. brevis*, most of the strains identified as *Ped. acidilactici* was grouped separately in the sorghum silage (Figure 1), but in the corn silage *Ped. acidilactici* and *Ped. pentosaceus* were grouped together (Figure 2). For correct identification between *Ped. acidilactici* and *Ped. pentosaceus* the growth at 50°C was used, because the *Ped. acidilactici* can growth in this temperature and *Ped. pentosaceus* not. The population of *Ped. acidilactici* and *Ped. pentosaceus* were greater in corn than in sorghum, and *Ped. acidilactici* was present at 180 days of CON corn silage but reduce below detectable in other treatments.

The *Lac. brevis* and *Lac. buchneri* isolates from silages with 30 days of fermentation were grouped together by protein expression profile evaluated in MALDI-TOF MS, the acid production from melezytose was used for differentiation between this strain, *Lac. brevis* from silages with 180 days of fermentation were grouped separated, tow group for sorghum and one group for corn. One distinct group of MALDI TOF was not identify by processed with the MALDI Biotyper 3.0. This group was present only in sorghum silage at 180 days of fermentation, with low population 1.3 log UFC/g (Table 4). The *Lac. parabuchneri* was only identified from CON corn silage at 180 days of fermentation, with low population 0.82 log UFC per g.

Three groups of SAB (*Bac. kochii*, *Bac. velezensis*, *Pae. lautus*), present only in sorghum silage were not identified by MALDI Biotyper 3.0 (Figure 3), for identification of this strains the sequence of 16S RNA gene was necessary. The other group in sorghum dendogram was formed by *Lysinobacillus boronitolerans* and *Lys. sphaericus* (Figure 3), for the corn silage the MALDI Biotyper 3.0 was grouped both species of *Lysinobacillus* in the same group (Figure 4). *Lysinobacillus boronitolerans* cannot hydrolyzes the urea and growth in NaCl 7%, and *Lys. sphaericus* cannot hydrolyzes the urea and cannot growth in NaCl 7%, these tests were used for identification of this species (Table 3). From species of *Bacillus megaterium*, *Bac. circulan, Paenibacillus macerans, Pae. rhizosphaerae, Bac. claussi*, *Bac. oleronius*, only one strain was identified in sorghum silage. Only one strain of *Pae. lautus*, *Bac. circulans*, *Brevibacillus invocatus* (Previous named as *Bacillus invocatus*), and *Bac. vallismortis* were identified in corn silage.

The *Bac. atrophaeus* and *Bac. vallismortis* were grouped together in more than one group of sorghum and corn silage dendogram (Figures 3 and 4), for correct identification tests of acid

production from sorbitol, melibiose and starch (Table 3). Most part of isolated of *Bac. atrophaeus* specie, 68% (strains 46, 47, 54, 58, 60, 75-78), were from corn silage at 30 days of fermentation (Table 5).

One group was identified as *Bac. horneckiae* present only in sorghum silage CON and treated with AMG enzyme, at 30 days of fermentation (Table 5). The *Bac. cereus* group were present in sorghum silage CON and in sorghum and corn treated with AMG enzyme, at 30 days of fermentation (Table 5). *Bac. licheniformis*, were present in sorghum silage at 30 days of fermentation on treatment with AMG enzyme, and at 30 and 180 days of fermentation on treatment with GAM enzyme. Only one strain of *Bac. licheniformis*, was present in CON corn silage at 30 days (Table 5). The growth at medium with concentration of 5, 7, and 10% of NaCl was used to confirm the identification of *Bac. licheniformis* (Table 3).

Bac. pumilus were divided in tow groups in sorghum silage (Figure 3) and one group in corn silage (Figure 4), this is the only specie present in all treatments and fermentation days for both silages. For correct identification of *Bac. pumilus* the test of citrate utilization, and D-sorbitol and melibiose as carbon source were used. The *Brevibacillus brevis* (Previous named as *Bacillus brevis*) were only identified in sorghum silage treated with AMG, at 180 days of fermentation, and in the corn silage this specie was present in all treatments and days, except at 180 days of CON.

The factor 1 and factor 2 of principal components explained 51.60 and 22.09%, respectively, of the total variance (Figure 5). The PCA of treatments based on variables show that corn and sorghum with 30 days of fermentation are grouped more closely than with 180 days of fermentation (Figure 5). The principal characteristics that grouped sorghum silages with 30 days of fermentation (Figure 5) were the pH, DM and starch content of silage (Figure 6). The

sorghum silage CON and with addition of GAM at 180 days of fermentation, were more correlated with acids, and 1,2-propanodiol (Figure 5 and 6). The silages of corn with addition of amyloglucosidase (AMG and GAM) were more correlated with DM loss, starch loss, free glucose and ethanol content.

The species of SAB *Brevibacillus invocatus*, *Paenibacillus glucanolyticus* (Previous named as *Bac. glucanolyticus*), and *Bre. brevis* had positive correlation with prolamin content and content of prolamin had negatively correlation with starch loss, free glucose, kd and ethanol (Figure 6).

The group 1 of not identified SAB and *Bacillus horneckiae* were closely related with DM loss. The *Bac. pumilus* was the specie of SAB with biggest population but did not shown correlation with characteristic of rehydrated kernel silage. The *Bac. licheniphormis* were closely related with starch loss, glucose and ethanol content. The soluble fraction (fraction A) of DM was correlates with microorganisms and acids accumulation (Figure 6).

DISCUSSION

There are few studies evaluating the microorganism species in rehydrated kernel, Carvalho et al. (2016) identified the LAB and yeasts diversity of rehydrated corn kernel silage, and Carvalho (2014) evaluate the bacterial community by Denaturing Gradient Gel Eletrophoresis technique, and PCR products sequence, by Ilumina MiseqTMSystem. To our knowledge, this study is the first identification of microorganisms of rehydrated sorghum silage, and the first study to identify the SAB in the rehydrated corn and sorghum kernel silage.

In sorghum and corn silages the species Lac. plantarum and *Ped. acidilactici* are present in all treatments at first days of fermentation, but not on the long fermentation. These species had

a negative correlation with pH, DM and starch contents that is beneficial for silage quality, since we want low pH value in silage and the lowest concentration of starch indicates that most of the starch was made available to the ruminal degradation, by hydrolyses to glucose. Those microorganisms are used as inoculant in the different silages for reducing the pH and increasing the preservation of DM (EFSA, 2012; 2014). The *Ped. pentosaceus* shown low population in comparation of *Lac. plantarum* and *Ped. acidilactici*, however were reported as responsible for the start of lactic fermentation in rehydrated corn kernel silage (Carvalho et al., 2016). Strains of *Lac. buchneri* shown low correlation with acetic acid or 1,2 propanediol. Although, some studies report the inoculation of this microorganism can improve the stability of corn silages, by production of acetic acid (Kleinschmit; Kung, 2006) and 1,2 propanediol (Oude Elferink et al., 2001). At 180 days of fermentation the *Lac. brevis* dominated the fermentation; this specie was studied to improve the stability of aerobic exposure EFSA (2016).

The microorganisms *Bre. brevis*, *Bre. invocatus* and *Pae. glucanoliticus* are positive related to prolamin content, and had negative correlation to glucose, starch loss, ethanol, and kd. *Pae. glucanoliticus* hydrolyzed starch and produced acids from glucose (Alexander; Priest, 1989), but *Bre. brevis* and *Bre. Invocatus* do not ferment glucose or hydrolyze starch (Logan et al., 2002; Nakamura, 1991). According Panda et al. (2014) the genus *Brevibacillus* shown negligible amount of extracellular protease. This result indicated that *Bre. brevis* and *Bre. invocatus* were not involved in broke of prolamin content and making the starch lass susceptible to degradation by other microrganisms, as well *Pae. glucanoliticus* can consuming part of free glucose.

The *Bac. licheniformis* had correlation with starch loss and free glucose and had negatively correlation with prolamin. For *Bac. licheniformis* the protease enzyme is highly active

between pH 7.0 to 12.0, optimum around pH 10.0, but approximated 70% of these proteases remanding activity at pH 4.0, the amylase enzyme is highly active between pH 5.0 and 10.0 with optimum pH around 6.5, and 95% of these proteases remanding activity at pH 4.0 to 9.0 (HMIDET et al., 2009). Probably *Bac. licheniformis* favored the prolamine and starch breakage during the fermentation.

In silages with 180 days of fermentation the differences of treatments were more evident, and the silages cannot be grouped together. Silages with more acid production, sorghum CON and GAM (17.38 and 17. 14 % of DM of total acids), were grouped together and silages with more starch and DM loss, corn AMG and GAM (DM loss: 12.36 and 10.07 % DM; starch loss: 23.97 and 23.94 % of DM). The most of microorganisms of silage were involved in conversion of carbohydrates to acid, consequently generates losses during the metabolism of this and other compounds (McDonald et al., 1991), because this, most part of microorganism had relationship with acids and nutrient losses resulting in a formation of a microorganism group between acids group and starch and DM loss.

The correlation with fraction A and microorganisms and acids accumulation can be explained by Junges et al. (2017) by evaluating of proteolysis during fermentation of silage, the bacterial activity represents 60% and end-products, had lower contributions, approximately 5%.

CONCLUSIONS

The fermentation of rehydrated kernel was dominating by LAB, with highly population, but had greater diversity of SAB. The most part of microorganisms were involved in acids production, DM and starch losses.

ACKNOWLEDGMENTS

The authors are also grateful to the National Consul to Scientific and Technologic Development (CNPq; Brasilia, DF, Brazil) and Foundation for Research Support of the State of Minas Gerais (FAPEMIG; MG, Brazil) for the scholarship financial support.

REFERENCES

Ahmed, I.; A. Yokota, A. Yamazoe, and T. Fujiwara. 2007. Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and Bacillus sphaericus to *Lysinibacillus sphaericus* comb. nov. Int. J. Syst. Evol. Microbiol. 57:1117–1125.

Alexander B. and F. G. Priest. 1989. *Bacillus glucanolyticus*, a New Species That Degrades a Variety of p-Glucans. Int. J. Syst. Evol. Microbiol. 8:112-115.

Bringel, F.; P. Quénée, and P. Tailliez. 2001. Polyphasic Investigation of the Diversity within *Lactobacillus plantarum* Related Strains Revealed Two *L. plantarum* subgroups. System. Appl. Microbiol. 24:561–571.

Carvalho, B. F., C. L. S. Avila, T. F. Bernardes, M. N. Pereira, C. Santos, and R. F. Schwan. 2016. Fermentation profile and identification of lactic acid bacteria and yeasts of rehydrated corn kernel silage. J. Appl. Microbiol. 122:589-600.

Carvalho, B.F.; C.L.S. Ávila, J.C. Pinto, M.N. Pereira, and R.F. Schwan. 2012. Effects of propionic acid and *Lactobacillus buchneri* (UFLA SIL 72) addition on fermentative and microbiological characteristics of sugar cane silage treated with or without calcium oxide. Grass For. Sci. 67:462–471.

283 Carvalho, P. A. 2014. Influência do genótipo e maturidade na diversidade microbiológica em
284 milho grão para silagem. Dissertation (Mestre Animal Science and Pasture) Escola Superior
285 de Agricultura 'Luiz de Queiroz', Universidade de São Paulo, Piracicaba 95p.
286 Correa, C. E. S.; R. D. Shaver, M. N. Pereira, J. G. Lauer, and K. Kohn. 2002. Relationship
287 between corn vitreousness and ruminal *in situ* starch degradability. J. Dairy Sci., 85:3008–
288 3012.
289 Curk, M. C.; J. C. Hubert and F. Bringel. 1996. *Lactobacillus paraplantarum* sp. nov., a New
290 Species Related to *Lactobacillus plantarum.* Int. J. Syst. Evol. Microbiol. 46:595-598.
291 Gobetti, S.T.C.; M. Neumann, R. Oliboni, and M. R. Oliveira. 2013. Utilização de silagem de
292 grão úmido na dieta de animais ruminantes. Ambiência, 9:225-239.
293 Drosinos, E. H.; S. Paramithiotis, G. Kolovos, I. Tsikouras, And I. Metaxopoulos. 2006.
294 Phenotypic and technological diversity of lactic acid bacteria and staphylococci isolated
295 from traditionally fermented sausages in Southern Greece. Food Microbiol. 24:260-270.
296 EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), G.
297 Rychen, G. Aquilina, G. Azimonti, V. Bampidis, M. L. Bastos, G. Bories, A. Chesson, P.
298 Sandro Cocconcelli, G. Flachowsky, J. Gropp, B. Kolar, M. Kouba, S. L. Puente, M. Lopez-
299 Alonso, A. Mantovani, B. Mayo, F. Ramos, R. E. Villa, R. J. Wallace, P. Wester, R. Brozzi
300 and M. Saarela. 2016. Safety and efficacy of *Lactobacillus brevis* NCIMB 42149 as a silage
301 additive for all animal species. EFSA Journal. 14:4616.
302 EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). 2012.
303 Scientific Opinion on the safety and efficacy of *Lactobacillus plantarum* (NCIMB 41028)
304 and *Lactobacillus plantarum* (NCIMB 30148) as silage additives for all animal species.
305 EFSA Journal. 10:2529.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). 2014. Scientific Opinion on the safety and efficacy of *Pediococcus acidilactici* (NCIMB 30005) as a silage additive for all animal species. EFSA Journal. 12:3613.

Ferraretto, L. F.; S. M. Fredin, and R. D. Shaver. 2015. Influence of ensiling, exogenous protease addition and bacterial inoculation on fermentation profile, nitrogen fractions and ruminal in vitro starch digestibility in rehydrated and high-moisture corn. J. Dairy Sci. 98:7318-7327.

HALE, W. H. 1973. Influence of processing on the utilization of grains (starch) by ruminants. J. Anim. Sci. 37:1075-1984.

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

Junges, D.; G. Morais, M. H. F. Spoto, P. S. Santos, A. T. Adesogan, L. G. Nussio, and J. L. P. Daniel. 2017. Short communication: Influence of various proteolytic sources during fermentation of reconstituted corn grain silages. J. Dairy Sci. 100.

Kleinshmitt, C.; G. Morais, L. Custódio, J. Fernandes, M. C. Santos, J. L. P. Daniel, and L. G. Nussio. 2013. Performance of lactating dairy cows fed maize silage with increased dosages of *L. buchneri*. In: Proceedings of the 15th International Conference on Forage Conservation, High Tatras, Slovakia. p.141-142.

Logan, N. A.; G. Forsyth, L. Lebbe, J. Goris, M. Heyndrickx, A. Balcaen, A. Verhelst, E. Falsen, A. I. Ljungh, H. B. Hansson, and P. De Vos. 2002. Polyphasic identification of *Bacillus* and *Brevibacillus* strains from clinical, dairy and industrial specimens and proposal of *Brevibacillus invocatus* sp. nov. Int. J. Syst. Evol. Microbiol. 52:953–966.

McDonald, P.; A.R. Henderson, and S.J.E. Heron. 1991. The biochemistry of silage. Marlow, Chalcomb Publication.

Nakamura, L. K. 1998. *Bacillus pseudomycoides* sp. nov. Int. J. Syst. Evol. Microbiol. 48:1031-1035.

Novozymes 2015. Healthier fermentation and healthier profits with Novozymes Sanferm® Yield, 2015, from http://www.novozymes.com/en/solutions/food-and-beverages/casestudies-on-Food-Beverages/Pages/Healthier-fermentation-and-healthier-profits-with-Novozymes-Sanferm®-Yield.aspx.

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

Palmisano, M. M.; L. K. Nakamura, K. E. Duncan, C. A. Istock, F. M. Cohan. 2001. *Bacillus sonorensis* sp. nov., a close relative of *Bacillus licheniformis*, isolated from soil in the Sonoran Desert, Arizona. Int. J. Syst. Evol. Microbiol. 51:1671–1679.

Rogosa, M. 1970. Characters used in the classification of *Lactobacilli.* Int. J. Syst. Bact. 20:519-533.

Ruiz-Garcia, C.; E. Quesada, F. Martinez-Checa, I. Llamas, M. C. Urdaci, V. Bejar 2005. *Bacillus axarquiensis* sp. nov. and *Bacillus malacitensis* sp. nov., isolated from river-mouth sediments in southern Spain. Int. J. Syst. Evol. Microbiol. 55:1279–1285.

Satomi, M.; M. T. La-Duc, K. Venkateswaran. 2006. *Bacillus safensis* sp. nov., isolated from spacecraft and assembly-facility surfaces. Int. J. Syst. Evol. Microbiol. 56:1735–1740.

Shivaji, S.; P. Chaturvedi, K. Suresh, G. S. N. Reddy, C. B. S. Dutt, M. Wainwright, J. V. Narlikar, P. B. Bhargava. 2006. *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov.,

Bacillus stratosphericus sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. Int. J. Syst. Evol. Microbiol. 56:1465–1473.

Silva, N. C.; C. F. Nascimento, F. A. Nascimento, F. D. De Resende, J. L. P. Daniel, G. R. Siqueira. 2018. Fermentation and aerobic stability of rehydrated corn grain silage treated with different doses of *Lactobacillus buchneri* or a combination of *Lactobacillus plantarum* and *Pediococcus acidilactici*. J. Dairy Sci. 101:1-10.

Statistical Analysis System Institute. SAS user's guide. Version 8.2. Cary, 2002. 1 CD-ROM.

Tanasupawat, S.; S. Okada, M. Kozaq, K. Komagata. 1993. Characterization of *Pediococcus pentosaceus* and *Pediococcus acidilactici* Strains and Replacement of the Type Strain of *P. acidilactici* with the Proposed Neotype DSM 20284. Int. J. Syst. Bact. 43:860-863.

Zhang, B.; S. Dhital, and M. J. Gidley. 2013. Synergistic and Antagonistic Effects of α - Amylase and Amyloglucosidase on Starch Digestion. Biomacromolecules.14:1945–1954.

365 **Table 1.** Silage characterization, average of nutrient composition, degradation and fermentative
366 parameters of rehydrated sorghum and corn kernel silages with 30 and 180 days of storage and
367 with or without addition of amyloglucosidades

	Sorghum	Corn
DM, % of as fed	65.03 ± 1.74	65.22 ± 1.79
DM loss, % of DM	5.26 ± 3.47	6.77 ± 3.41
Starch, % of DM	54.48 ± 5.50	56.37 ± 5.24
Starch loss, % of DM	7.92 ± 5.56	16.35 ± 6.13
Free Glucose, % of DM	2.40 ± 1.09	2.96 ± 1.68
Prolamin, % of DM	14.22 ± 1.03	12.85 ± 1.64
NH_3, % of DM	0.08 ± 0.03	0.06 ± 0.02
Deg 0, %of DM	39.77 ± 3.25	32.52 ± 4.43
Kd, % of DM h^{-1}	3.14 ± 0.07	3.33 ± 0.13
pH	3.99 ± 0.25	3.92 ± 0.20
Lactic, % of DM	8.81 ± 4.08	6.07 ± 2.03
Acetic, % of DM	0.08 ± 0.04	0.03 ±0.02
Propionic, % of DM	1.04 ± 1.24	0.40 ± 0.37
Butyric, % of DM	0.24 ± 0.27	0.08 ± 0.07
1,2 propanodiol, % of DM	2.87 ± 1.26	1.46 ± 0.54
Ethanol, % of DM	0.76 ± 0.22	0.98 ± 0.38

368

Table 2. Characteristics of lactic acid bacteria isolated of rehydrated sorghum or corn kernel silages with 30 or 180 d, and addition of amyloglucosidase (AMG), amyloglucosidase (GAM), and Control (CON)

Identification	N	Gram	Shape	CA	OX	MO	GA	50	Melezitose	Dulcitol	Xilose
Lactobacillus brevis	102	-	Short and medium rod	-	-	-	29 (+)	ND	+	ND	ND
Lactobacillus buchneri	43	-	Short and medium rod	-	-	4 (+)	25 (+)	ND	-	ND	ND
Lactobacillus parabuchneri	2	-	Short and medium rod	-	-	-	-	ND	+	ND	+
Lactobacillus plantarum	25	-	Short and medium rod	-	-	-	-	ND	+	-	ND
Pediococcus acidilactici	76	3 (+)	Short and medium rod	-	-	1 (+)	10 (+)	+	ND	ND	ND
Pediococcus pentosaceus	7	-	Short and medium rod	-	-	1 (+)	1 (+)	-	ND	ND	ND
Not identificated	3	-	Medium rod	-	-	-	-	ND	ND	ND	ND

Gram: Gram strain; Shape: cellular shape; CA: catalase production; OX: oxidase production; MO: motility; GA: gas production from MRS; NI: total number of isolates. Positive (+); Negative (-).

374 **Table 3**. Characteristics of aerobic spore forming bacteria isolated of rehydrated sorghum or corn kernel silage with 30 or 180 d, and
375 addition of amyloglucosidase (AMG), amyloglucosidase (GAM), and Control (CON)

						Growth in the presence of			Utilization of:			Acids from:		
Identification	N	Gram	CA	OX	Urea hydrolyze	NaCl 5%	NaCl 7%	NaCl 10%	Citrate	Sorbitol	Melibiose	Starch	Sorbitol	Melibiose
Bac. atrophaeus	13	1 (-)	+	-	ND	+	+	+	+	+	+	-	+	-
Bac. cereus grupo	8	2 (-)	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bac. circulans	2	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bac. clausii	1	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bac. horneckiae	4	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bac. licheniformis	5	+	+	ND	ND	+	+	+	+	+	+	1 (+)	1 (-)	-
Bac. megaterium	1	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bac. oleronius	1	+	+	ND	ND	+	+	+	ND	+	+	-	-	-
Bac. pumilus	39	4 (-)	4 (-)	-	ND	+	+	+	+	-	-	2 (+)	4 (+)	2 (+)
Bac.vallismortis	3	+	+	ND	ND	+	+	+	+	+	+	-	-	-
Bre. brevis	8	3 (-)	4 (-)	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Bre. invocatus	1	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lys. boronitolerans	5	2 (-)	+	-	+	+	-	-	ND	ND	ND	ND	ND	ND
Lys. sphaericus	4	1 (-)	+	-	-	1 (-)	-	-	ND	ND	ND	ND	ND	ND
Pae. glucanolyticus	4	-	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pae. lautus	1	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pae. macerans	1	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pae. rhizosphaerae	1	-	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Not identificated 1	1	-	+	-	ND	+	+	+	+	+	-	+	-	+
Not identificated 2	4	+	1 (-)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

376

377

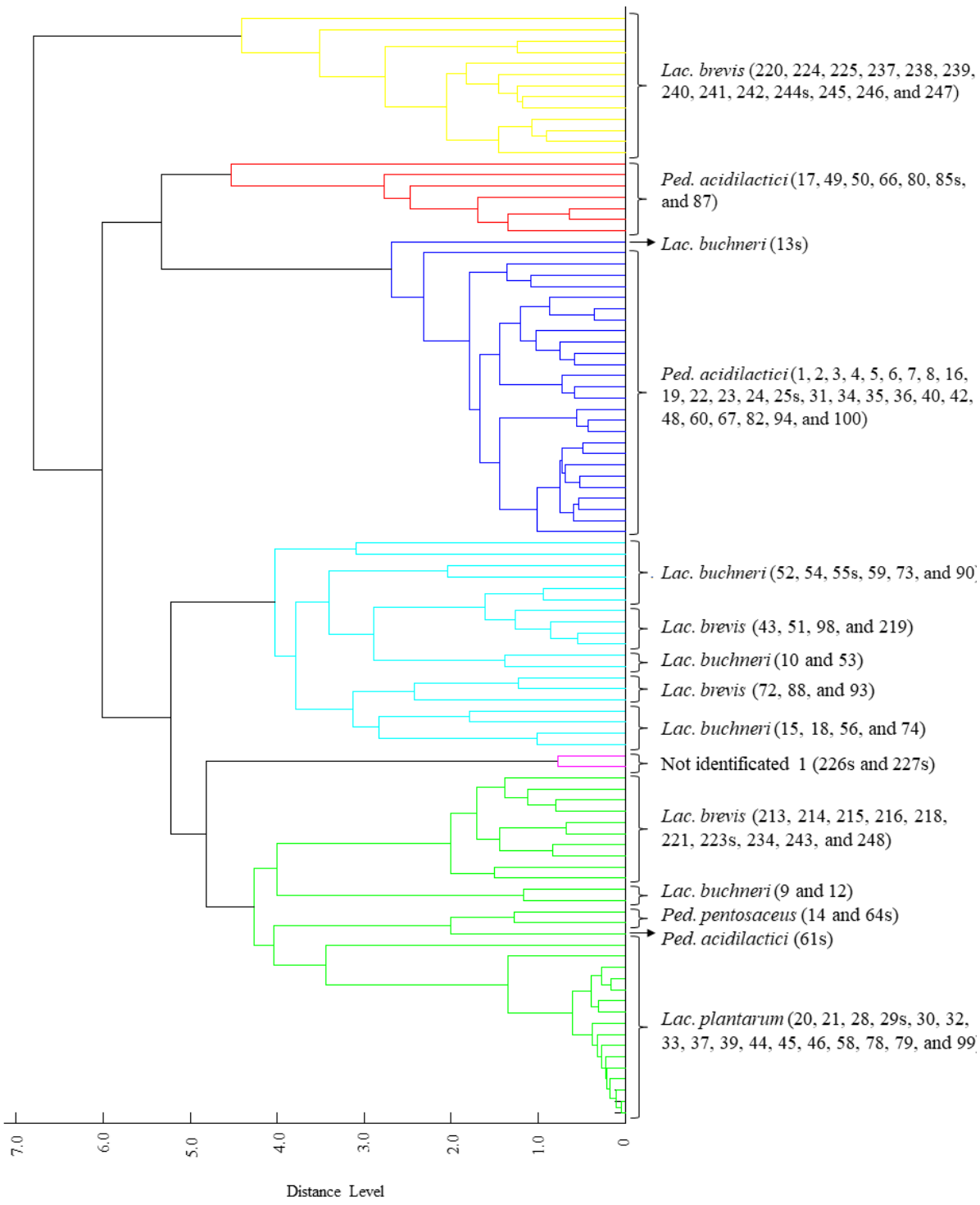


Figure 1. Dendrogram derived from a protein cluster analysis of lactic acid bacteria isolated of rehydrated sorghum kernel silage with 30 or 180 d, and addition of amyloglucosidase AMG or GAM, and control, in MRS agar. Numbers in brackets are the strain identification.

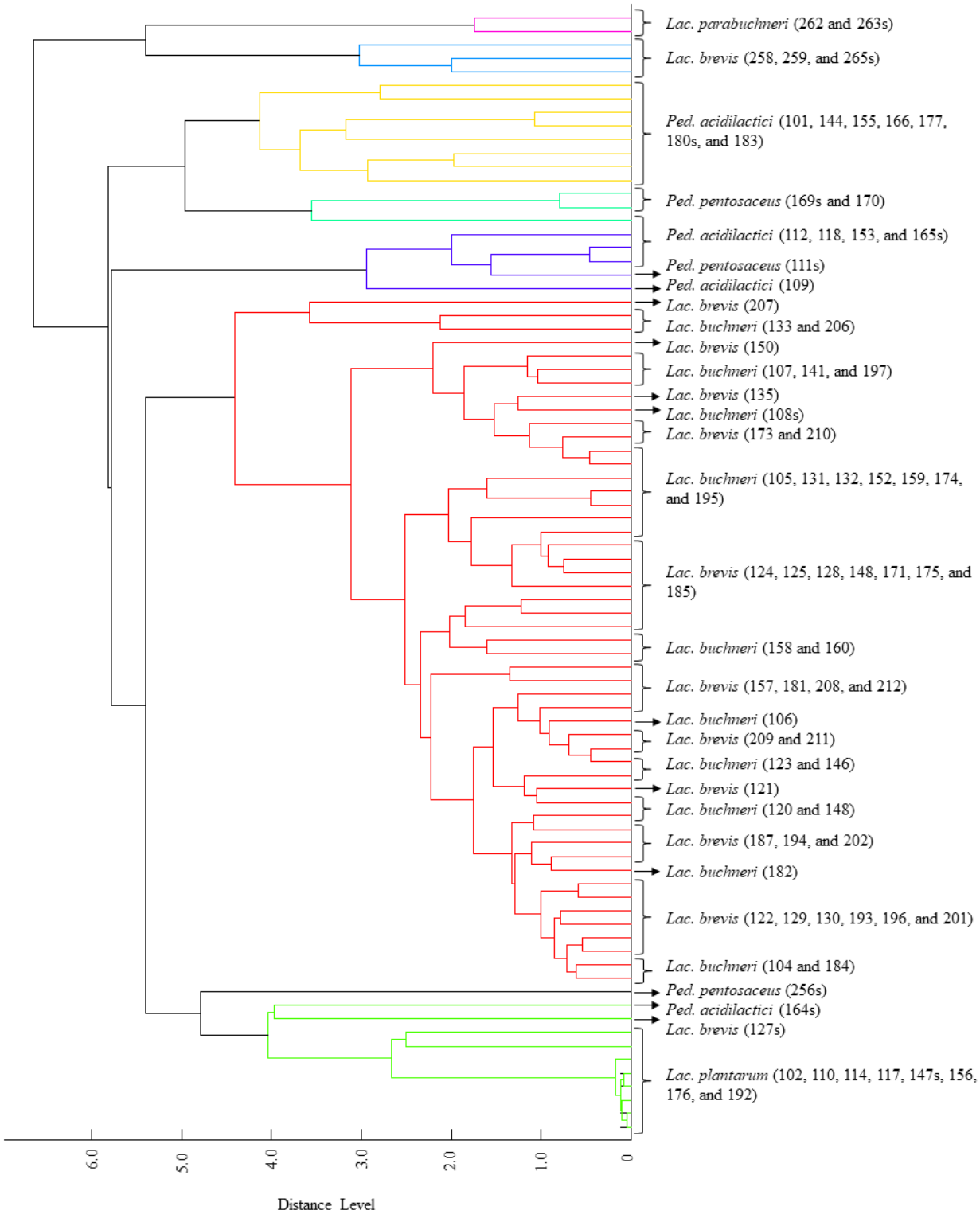
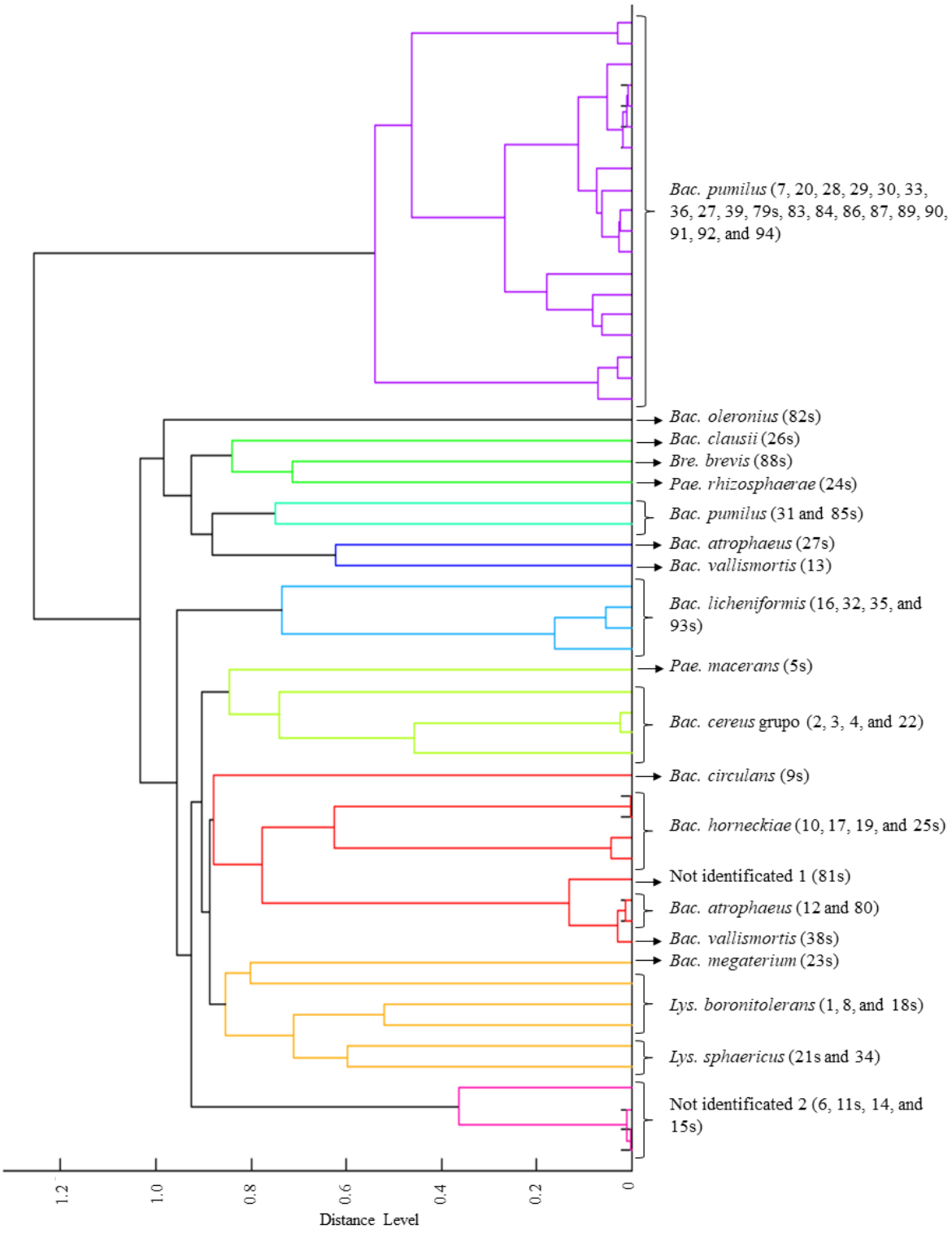
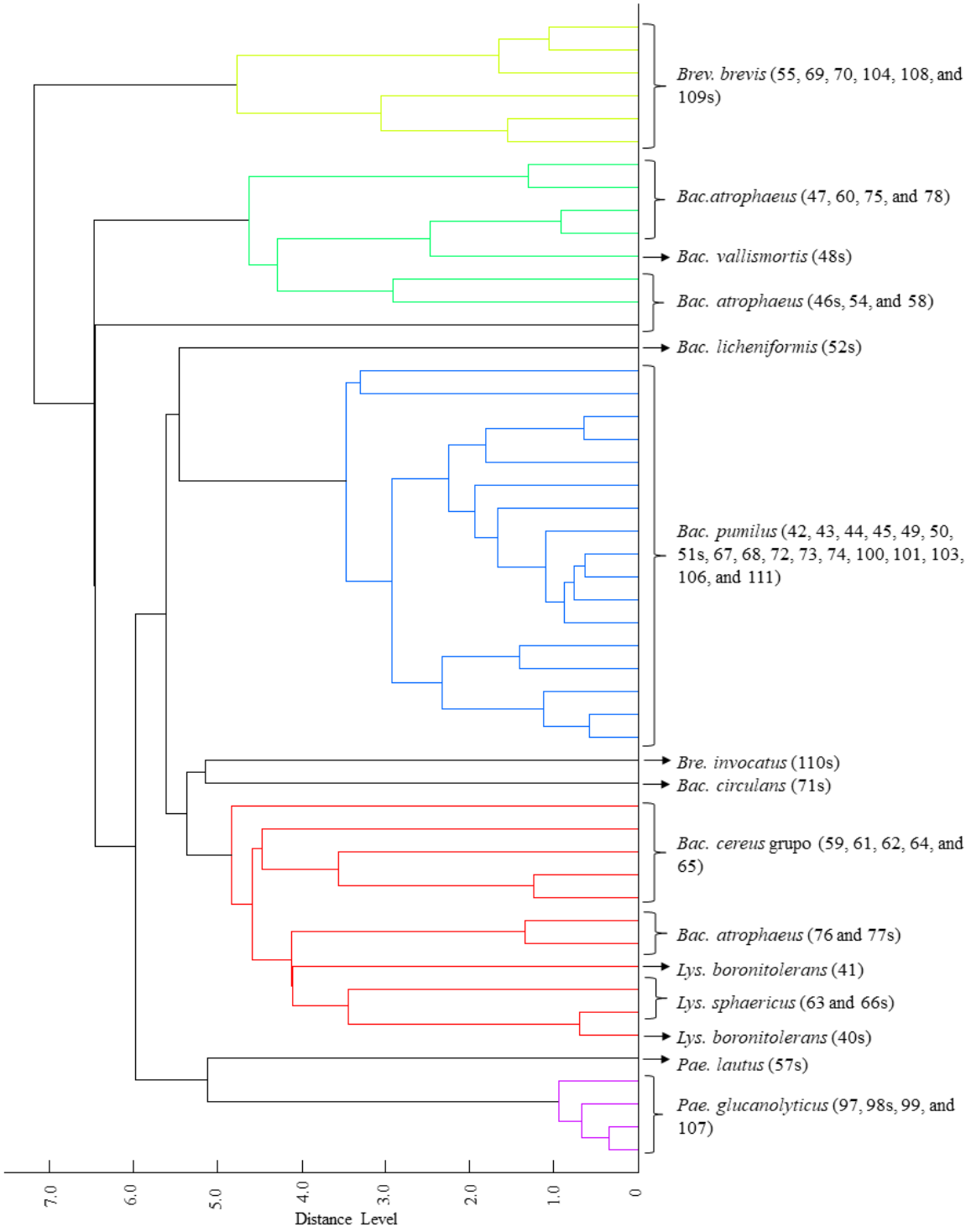


Figure 2. Dendrogram derived from a protein cluster analysis of lactic acid bacteria isolated of rehydrated corn kernel silage with 30 or 180 d, and addition of amyloglucosidase AMG or GAM, and control, in MRS agar. Numbers in brackets are the strain identification



386

387 **Figure 3.** Dendrogram derived from a protein cluster analysis of aerobic spore forming bacteria
388 isolated of rehydrated sorghum kernel silage with 30 or 180 d, and addition of amyloglucosidase
389 AMG or GAM, and control, in MRS agar. Numbers in brackets are the strain identification.



390

391 **Figure 4.** Dendrogram derived from a protein cluster analysis of aerobic spore forming bacteria
392 isolated of rehydrated corn kernel silage with 30 or 180 d, and addition of amyloglucosidase
393 AMG or GAM, and control, in MRS agar. Numbers in brackets are the strain identification.

394 **Table 4.** Population (Log) and identification of lactic acid bacteria isolated of rehydrated sorghum or corn kernel silage with 30 or 180
395 d, and addition of amyloglucosidase (AMG), amyloglucosidase (GAM), and Control (CON)

Grain	Sorghum						Corn					
Enzyme	CON		AMG		GAM		CON		AMG		GAM	
Duration	30	180	30	180	30	180	30	180	30	180	30	180
Lactobacillus brevis	< 2	3.57	4.00	< 2	4.14	< 2	6.31	4.11	6.03	< 2	5.25	< 2
Lactobacillus buchneri	4.60	< 2	< 2	< 2	3.98	< 2	6.41	< 2	6.12	< 2	4.92	< 2
Lactobacillus parabuchneri	< 2	< 2	< 2	< 2	< 2	< 2	< 2	0.82	< 2	< 2	< 2	< 2
Lactobacillus plantarum	3.82	< 2	4.74	< 2	3.82	< 2	5.96	< 2	5.12	< 2	4.12	< 2
Pediococcus acidilactici	4.91	< 2	4.98	< 2	4.81	< 2	5.77	< 2	6.44	< 2	5.02	< 2
Pediococcus pentosaceus	3.92	< 2	< 2	< 2	3.38	< 2	5.12	1.82	5.78	< 2	< 2	< 2
Not identificated 1	< 2	1.30	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
Total	5.14	3.57	5.20	< 2	4.99	< 2	6.79	4.11	6.77	< 2	5.58	< 2

396

397 **Table 5.** Population (Log) and identification of aerobic spore forming bacteria isolated of rehydrated sorghum or corn kernel silage with
398 30 or 180 d, and addition of amyloglucosidase (AMG), amyloglucosidase (GAM), and Control (CON)

Kernel	Sorghum						Corn					
Enzyme	CON		AMG		GAM		CON		AMG		GAM	
Duratin	30	180	30	180	30	180	30	180	30	180	30	180
Bacillus atrophaeus	< 2	2.00	2.30	< 2	< 2	< 2	2.82	< 2	2.30	< 2	2.85	< 2
Bacillus cereus group	2.54	< 2	2.00	< 2	< 2	< 2	< 2	< 2	2.74	< 2	< 2	< 2
Bacillus circulans	2.00	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	2.00	< 2
Bacillus clausii	< 2	< 2	2.00	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
Bacillus horneckiae	2.00	< 2	2.48	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
Bacillus licheniformis	< 2	< 2	2.00	< 2	2.30	2.00	2.00	< 2	< 2	< 2	< 2	< 2
Bacillus megaterium	< 2	< 2	2.00	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
Bacillus oleronius	< 2	2.00	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
Bacillus pumilus	2.18	2.60	2.00	2.60	2.90	2.60	3.08	2.30	2.30	2.48	2.70	2.00
Bacillus vallismortis	< 2	< 2	2.00	< 2	2.00	< 2	2.23	< 2	< 2	< 2	< 2	< 2
Brevibacillus brevis	< 2	< 2	< 2	2.00	< 2	< 2	2.30	< 2	2.00	2.00	2.00	2.40
Brevibacillus invocatus	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	2.18
Lysinobacillus boronitolerans	2.18	< 2	2.00	< 2	< 2	< 2	2.48	< 2	< 2	< 2	< 2	< 2
Lysinobacillus sphaericus	< 2	< 2	2.00	< 2	2.00	< 2	< 2	< 2	2.40	< 2	< 2	< 2
Paenibacillus glucanolyticus	< 2	< 2	< 2	< 2	< 2	< 2	< 2	2.48	< 2	2.00	< 2	< 2
Paenibacillus lautus	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	2.00	< 2	< 2	< 2
Paenibacillus macerans	2.18	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
Paenibacillus rhizosphaerae	< 2	< 2	2.00	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
Not reliable identification 1	2.40	< 2	2.30	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
Not reliable identification 2	< 2	2.00	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
Total	3.10	2.85	3.20	2.70	3.08	2.70	3.42	2.70	3.15	2.70	3.15	2.70

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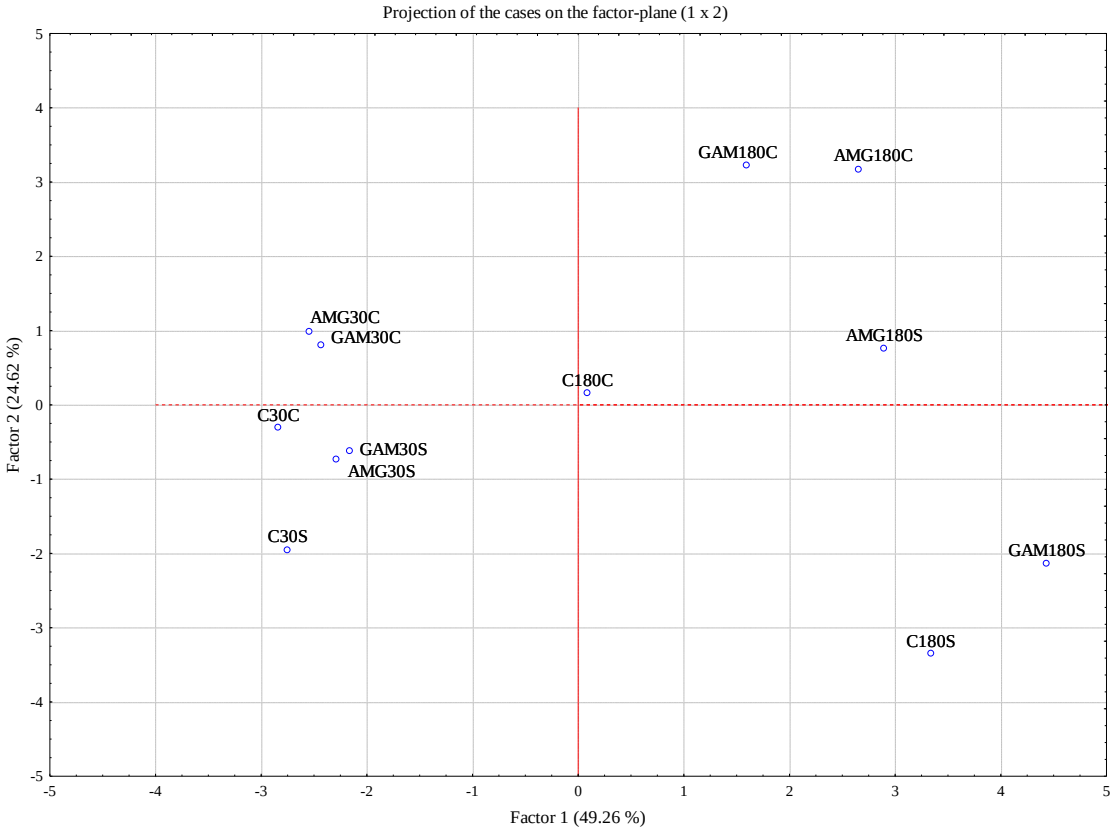

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402

403 **Figure 5**. Principal component analysis (PCA) treatments kernel (sorghum and corn), enzyme
404 (CON, AMG, and GAM) and duration of fermentation (30 and 180 days), grouped based on
405 nutrient values, fermentation end products, and population of bacteria. The factor 1 and factor 2 of
406 principal components explained 51.60 and 22.09%, respectively, of the total variance. The
407 numbers represent kernel, enzyme, and duration of fermentation: 1- Sorghum, CON, 30 d; 2-
408 Sorghum, AMG, 30 d; 3- Sorghum, GAM, 30 d; 4- Sorghum, CON, 180 d; 5- Sorghum, AMG,
409 180 d; 6- Sorghum, GAM, 180 d; 7- Corn, CON, 30 d; 8- Corn, AMG, 30 d; 9- Corn, GAM, 30 d;
410 10- Corn, CON, 180 d; 11- Corn, AMG, 180 d; 12- Corn, GAM, 180 d.

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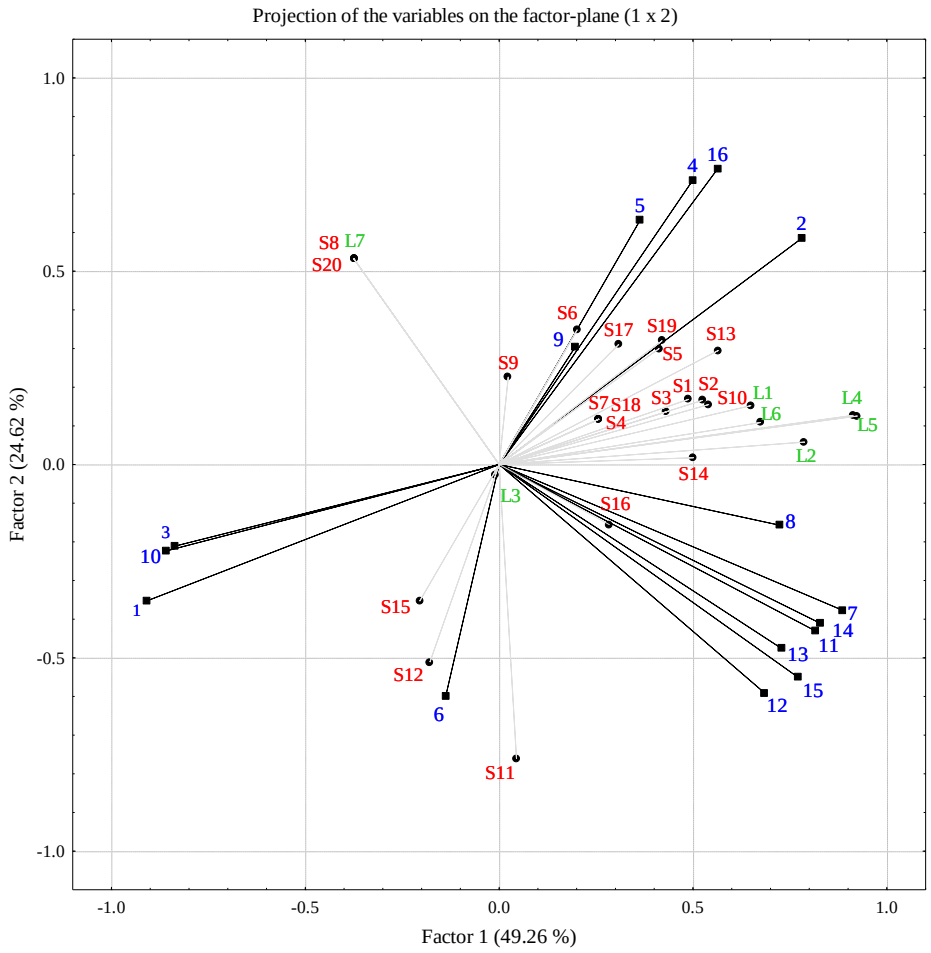

Figure 6. Principal component analysis (PCA) of nutrient values, fermentation end products, and population of acid lactic bacteria (L) and aerobic spore forming bacteria (S). The factor 1 and factor 2 of principal components explained 51.60 and 22.09%, respectively, of the total variance. The numbers represent the variables: 1- % of DM; 2- DM loss; 3- Starch; 4- Starch loss; 5- Free Glucose; 6- Prolamin; 7- NH_3; 8- Fraction A; 9- Kd; 10- pH; 11- Lactic; 12- Acetic; 13- Propionic; 14- Butyric; 15- 1,2 propanodiol; 16- Ethanol; and the microorganisms: L1- *Lactobacillus brevis*; L2- *Lactobacillus buchneri*; L3- *Lactobacillus parabuchneri*; L4- *Lactobacillus plantarum*; L5- *Pediococcus acidilactici*; L6- *Pediococcus pentosaceus*; L7- LAB not identificated; S1- *Bacillus atrophaeus*; S2- *Bacillus* cereus group; S3- *Bacillus circulans*; S4- *Bacillus clausii*; S5- *Bacillus horneckiae*; S6- *Bacillus licheniformis*; S7- *Bacillus megaterium*; S8- *Bacillus oleronius*; S9- *Bacillus pumilus*; S10- *Bacillus vallismortis*; S11- *Brevibacillus brevis*; S12- *Brevibacillus invocatus*; S13- *Lysinobacillus boronitolerans*; S14- *Lysinobacillus sphaericus*; S15- *Paenibacillus glucanolyticus*; S16- *Paenibacillus lautus*; S17- *Paenibacillus macerans*; S18- *Paenibacillus rhizosphaerae*; S19- SAB not identificated 1; S20- SAB not identificated 2.

5 ARTIGO 3: Effect of washing method, grinding size, and the determination of an undegraded fraction on *in situ* effective ruminal disappearance and disappearance rate of starch in mature corn grain

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

Disponível em:

http://www.journalofdairyscience.org/pb/assets/raw/Health%20Advance/journals/jods/2016_SF.pdf

Interpretative Summary: Fernandes et al. Starch is the most important source of fermentable energy for dairy cows; thus, an accurate prediction of ruminal starch degradation is necessary to improve lactation performance and health. The objective of this study was to evaluate the methodology used for ruminal in situ determination of fractions A, B, and C, and the models used to estimate ruminal fractional degradation rate of fraction B. Determination of fraction A by rinsing in a washing machine, and ruminal in situ incubations of 0, 3, and 48 h for starch disappearance kinetics using a 2-pool model (Fractions A and B) are suggested for starchy feedstuffs. Grinding size affects starch disappearance kinetics.

Running Head: *Model to estimate the starch ruminal disappearance*

Effect of washing method, grinding size, and the determination of an undigestible fraction on in situ degradation of starch in mature corn grain

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ABSTRACT

The objectives of this study were to determine: 1) the most adequate method to estimate the rapidly degradable fraction (**A**); 2) a time-point to measure the undigestible fraction (**C**); and 3) the viability of using fewer time-points to estimate starch fractional disappearance rate (**kd**) of mature corn grain ground through 4 grinding sizes (1, 2, 4, and 6-mm). Fraction A was determined by rinsing in a bucket or washing machine, rumen immersion followed by bucket or washing machine, and water immersion for 30-min followed by bucket or washing machine. Ruminal in situ incubations were performed at 48, 72, 96, and 120 h to determine fraction C, and at 0 (washing machine), 3, 6, 12, 18, 24, and 48 h to determine the kinetics of starch disappearance. Models were used with either 2 or 3-pools and kd was determined by the linear slope of the 'ln' of bag residues as a proportion of incubated samples over time. The effective ruminal disappearance (ERD) was calculated as: A + B [kd / (kd + kp)], kp = 7.47 % h^{-1}. Data were analyzed using PROC MIXED of SAS with the fixed effect of method (either washing or model), grinding size, and their interaction and the random effect of run or cow. Correlation between estimates calculated using all time-points or combinations of 2 and 3 time-points were determined using PROC CORR. Fraction A was affected by grinding size; but not by washing method. Samples ground at 6-mm had greater fraction C than other grinding sizes at 48, 72 or 96 h; but not at 120 h. Model affected fraction B values solely. Greater fractions B and C, but reduced kd and ERD were observed as grinding size increased. Based on correlation analysis the

2-pool model, and the incubation times of 0, 3, and 48 h were suitable to evaluate ruminal starch degradation kinetics in mature corn. Ruminal in situ incubation at 120 h highlighted the lack of a fraction C of starch (0.13% of starch). Determination of fraction A by rinsing in a washing machine, and ruminal in situ incubations of 0, 3, and 48 h for starch degradation kinetics using a 2-pool model are suggested for starchy feedstuffs. Grinding size affected starch degradation kinetics.

Key Words: soluble fraction, effective ruminal degradation, fractional degradation rate, starch sources

INTRODUCTION

Starch is a major energy source for both ruminant animals and ruminal microorganisms (Moharrery et al., 2014). However, ruminal in vivo starch digestibility varies from 25 to 95% of intake, which in turn may alter lactation performance and feed efficiency by dairy cows (Firkins et al., 2001; Ferraretto et al., 2013). This variance in ruminal starch digestibility could be explained by a myriad of factors, including intake level, starch source, endosperm texture, particle size, grain processing, storage method, harvesting maturity, enzyme supplementation, and duration of storage in the silo (Firkins et al., 2001; Ferraretto et al., 2013; Moharrery et al., 2014; Gallo et al., 2016; Andreazzi et al., 2018). However, it is difficult to account for this variation in nutritional models, since a standard method established to evaluate the ruminal disappearance of starch has not been defined (Peyrat et al., 2014). Considering that predicting total tract starch digestibility from fecal starch concentration is highly reliable (Fredin et al., 2014; Owens et al., 2016), an accurate estimation of ruminal starch digestibility would allow for the estimation of the proportion of starch intake digested in the intestines. Estimating the

partition in starch digestibility between rumen and intestines may be useful for ration formulation, since it is associated to the efficiency of energy usage, ruminal microbial synthesis, and the occurrence of ruminal acidosis.

The ruminal in situ digestibility method is one of the standard procedures used for determination of ruminal starch digestibility (Seifried et al., 2016). This procedure measures the disappearance of DM and nutrients from bags containing feed samples and incubated in the rumen across various time-points (Seifried et al., 2016). With the use of several time-points we could determine the soluble or rapidly degradable fraction (fraction A), the slowly degradable raction (fraction B), and the indigestible fraction (fraction C). However, this procedure is not standardized. Time-points of incubation have ranged from five (Dias Junior et al., 2016) to nine (Saifried et al., 2016), whereas the final time-point ranges from 24 h (Hristov et al., 2007; Dias Junior et al., 2016) to 336 h (Dieho et al., 2017). According to Hall and Mertens (2017), the NFC fraction of feedstuffs commonly fed to dairy cows have either minimal or no indigestible fraction. Moreover, the majority of starch models do not consider soluble and indigestible fractions (Lanzas et al., 2007; Volden and Larsen, 2011) nor do they separate starch from other carbohydrates (NRC, 2001).

The ruminal in situ technique may also be affected by other factors. For example, the sample particle size may interfere with the estimation of ruminal degradation (Benton et al., 2005) due to the potential loss of small particles through the pores of incubation bags (Dulphy 1999; Philippeau and Michalet-Doreau 1997). Furthermore, different methodologies applied during bag washing can affect the estimation of fraction A; some commonly used methods include rinsing in water (Herrera-Saldan et al., 1990; Lee et al., 2016), using a washing machine

(Dieho et al., 2017), and soaking in water for a specific amount of time (Batajoo and Shaver, 1997; Keim et al., 2013).

These differences during the estimation of the fractional degradation rate (**kd**) and thereby, the effective ruminal degradation (**ERD**) may yield different values for the same feedstuff. The lack of a standardized method makes the comparison among feeds analyzed by different research laboratories unfeasible and hinders the establishment of more precise prediction models. Therefore, experimental objectives were: (1) to evaluate washing methods to estimate fraction A of starch, (2) to determine a time-point to measure fraction C, (3) to determine the feasibility of using less time-points to estimate starch kd, and (4) to measure starch ERD and kd of various starch sources using the methodology defined by objectives 1, 2 and 3. We hypothesized that the use of a methodology with few incubation time-points to calculate starch ruminal degradation kinetics would yield the same ERD as the prediction performed with all time-points.

MATERIALS AND METHODS

A batch of 4 kg of dried mature dent corn was homogenized and allocated into 4 samples using a quartering technique: homogeneous samples were divided into 4 equal subsamples. Two subsamples were saved for later treatment, whereas the 2 other subsamples were re-homogenized and re-divided. The process was repeated until 4 subsamples of 500 g were prepared. Each subsample was assigned to a grinding size and ground to pass 1, 2, 4 or 6-mm sieves in a Wiley mill (A. H. Thomas Scientific, Philadelphia, PA). Each sample (82.4 ± 14.5 g; mean ± SD) was dry-sieved using a Tyler Ro-Tap Shaker (model RX-29, W.S. Tyler, Mentor, OH) using a set of 9 sieves (W.S. Tyler) with nominal square apertures of 6.70, 4.75, 3.35, 2.36, 1.70, 1.18, 0.59, 0.20, 0.15-mm and a pan (ASABE, 2007) to determine particle size distribution; GMPS (μm)

and surface area (cm^2 /g) were calculated using a log normal distribution (Baker and Herrman 2002).

Ruminal in situ procedures were conducted at the University of Florida Dairy Research Unit (Gainesville, FL) under a protocol approved by the University of Florida, Institute of Food and Agricultural Sciences, Animal Care Research Committee. Dacron polyester cloth bags (R1020, 10 × 20 cm and 50 ± 10 micro porosity; Ankom Technology, Macedon, NY) containing 5.04 ± 0.02 g of DM, yielding a ratio of sample mass per bag area of 16.6 mg/cm^2, were used to compare fraction A methods, to determine a time-point to measure fraction C, and to estimate kd of fraction B and ERD.

A comparison of 6 methodologies used to determine fraction A of DM and starch was performed. Three independent runs were conducted per method, and samples from each grinding size were used in duplicate within each run. Each run and method contained a blank bag to allow correction for infiltration of DM into sample bags. Methods were: 1) to rinse (6 ± 2 wash/bag) in a bucket with water (**B**); 2) to rinse in a washing machine using the rinse and spin cycle set with cold water for a 30-min cycle (**W**; Roper RTW4516F*, Whirlpool Corp., Benton Harbor, MI); 3) to insert bags in the rumen, remove immediately after moistened, soak in cold water for 15 min, and rinse in a bucket (**RB**); 4) to insert bags in the rumen, remove immediately after moistened, soak in cold water for 15 min, and rinse using a washing machine set as described for treatment 2 (**RW**); 5) to submerge bags in water (approximated 25°C; 1000 mL^{-1} bag) for 30 min, and rinse it in a bucket (**IB**); and 6) to submerge bags in water for 30 min, and rinse it using a washing machine set as described for treatment 2 (**IW**). All six methods were performed simultaneously for each run and all bags were dried together in a forced-air oven set at 60°C for 48 h. The mean DM degradation value of the 2 bags of each grinding size for each washing method was

calculated for each run. Residues from duplicates within each grinding size treatment for each fraction A method within run were composited and ground to pass the 1-mm sieve in a cyclone mill (UDY Corporation, Fort Collins CO) and analyzed for starch by an enzymatic method with thermostable α-amylase (Ankom Tchnology, Macedon, NY) and amyloglucosidase (Megazyme E-AMGDF, Bray, Co. Wicklow, Ireland) enzymes, with free and enzymatically released glucose measured. Values from a separate determination of free glucose were subtracted to give values for enzymatically released glucose. Starch concentration was calculated as enzymatically released glucose multiplied by 0.9, as described by Hall (2015).

To define the incubation time for fraction C determination, measurements of ruminal in situ DM degradation were performed for each grinding size using 3 ruminally-cannulated, midlactation, multiparous Holstein cows fed a TMR containing (DM basis) corn silage (38.2%), alfalfa hay (4.0%), dry ground shelled corn (27.3%), soybean meal (14.5%), citrus pulp (9.1%), minerals and supplements (6.8%). The in situ bags for each grinding size treatment for each time-point (48, 72, 96, and 120 h) were placed in a nylon laundry bag (30 × 40-cm) and then positioned in the ventral rumen of each cow. Each laundry bag was attached to the inside of the rumen cannula with a 50-cm long nylon rope and contained a rubber weight to ensure they remained submerged in the ruminal contents. Each laundry bag contained a blank bag to allow correction for infiltration of DM into sample bags. Bags were introduced into the rumen in reverse chronological order so that the removal of all bags occurred at the same time. After removal, samples were soaked in ice water for 15 min. The bags were then rinsed with cold tap water to remove adhering feed particles and washed in a commercial washing machine (Roper RTW4516F*, Whirlpool Corp., Benton Harbor, MI) using the rinse and spin cycle setting with cold water for 30-min cycle (7 min to fill water, 13 min for agitation, and 10 min to drain and

spin). Bags were dried together in a forced-air oven set at 60°C for 48 h. The mean DM disappearance value of the 2 bags within grinding size treatment and incubation time was calculated for each cow. Residues from each duplicate within grinding size, incubation time-point, and cow were composited, ground, and analyzed for starch as described previously.

Subsequently, the same cows and procedure described for fraction C determination were used to determine the kinetics of DM disappearance, but using different incubation time-points (3, 6, 12, 18, 24, and 48 h). Residues from each duplicate within grinding size, incubation time-point, and cow were composited, ground, and analyzed for starch as described previously. Two models were used to determine fraction B. The first model consisted of three fractions (A, B and C); a rapidly degrading fraction (A) estimated by washing bags in a washing machine, an undigested fraction (C) estimated from a ruminal in situ incubation at 120 h, and a slowly degrading fraction (B) estimated by the equation: B = 100 – A – C. The second model consisted of two fractions (A and B); fraction A was estimated as described for the first model, and fraction B was estimated by the equation: B = 100 – A. The second model assumed that there is no indigestible fraction (fraction C) in starch. Fractional disappearance rate (**kd**) was determined by the linear slope over time of the natural logarithm of each time-point residue as a percentage of the incubated material. This calculation was performed for each time-point of each sample in each cow, which resulted in a linear regression of fraction B for each model. The ERD was calculated as: A + B [kd / (kd + kp)], where: A = fraction A, B = fraction B, kd = fractional degradation rate of fraction B, kp = fractional passage rate. The kp rates used were estimated from the average experimental cow fed and TMR: 1) using the CNCPS equation, and was equal to 7.47% h^{-1} (Tylutki et al., 2008; **ERD 1**); 2) using the equation (7.3) from NorFor, which was

equal to 6.85% h^{-1} (Volden and Larsen, 2011; **ERD 2**); or 3) using the 16% h^{-1} as used by the Feed Grain V2 model (Hoffman et al., 2012; **ERD 3**).

Based on these data, the most suitable time-points (0, 3, 48, and 120 h) of ruminal in situ incubation were defined to measure fractions A, B, and C, as well as kd of fraction B and ERD from various starch sources. Ruminal in situ incubations were performed on 2-mm ground samples as described previously. Starch sources were barley grain, cassava root, cassava starch residue, corn flakes, corn kernels from silage (separated manually from whole-plant corn silage), mature corn grain, whole-plant corn silage, oat flakes, potato root, rehydrated and ensiled corn, rehydrated and ensiled sorghum, sorghum grain, sweet potato root, wheat bran, wheat flakes, whole wheat flour, and wheat grain. These ingredients were collected either in Brazil or in the United States.

Statistical analysis

Fraction A data were analyzed using PROC MIXED of SAS (version 9.4; SAS Institute, Inc., Cary, NC), the model included the fixed effects of wash procedure (B, W, RB, RW, IB, and IW), grinding size (1, 2, 4 and 6-mm), and their interaction, and the random effect of run. Fraction C and kinetics of DM and starch degradation data were also analyzed using PROC MIXED (SAS Institute, 2004). The model included the fixed effects of grinding size (1, 2, 4 and 6 mm), incubation time (48, 72, 96, and 120 for fraction C; or 0, 3, 6, 12, 18, 24, 48, and 120 for kinetic), and their interaction, and the random effect of cow.

For kd of fraction B and ERD estimates, data were analyzed using PROC MIXED of SAS (version 9.4; SAS Institute, Inc., Cary, NC), with a model inclusive of the fixed effects of grinding size (1, 2, 4 and 6-mm), model (2-pool or 3-pool), and the interaction of grinding size and model, with cow as a random effect. Means were determined using the least squares means

205 statement and treatment means were compared using the PDIFF option. Statistical significance
206 and trends were declared at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

207 Correlations and relationships between fractions A and C, degradation time-points, and
208 kd from DM and starch were determined using the CORR and REG procedures of SAS (version
209 9.4; SAS Institute, Inc., Cary, NC).

210 To determine if accurate kd estimates could be obtained with fewer ruminal in situ
211 incubation time-points, a subsequent data analysis was performed to analyze the correlation and
212 relationships between estimates calculated using all time-points 0 (fraction A), 3, 6, 12, 18, 24,
213 and 48 h and estimates calculated using combinations of three or two time-points were
214 determined using the CORR and REG procedures of SAS (version 9.4; SAS Institute, Inc., Cary,
215 NC).

216 **RESULTS AND DISCUSSION**

217 Nutrient composition and particle size distribution of ground corn grain are in Table 1.
218 Starch composition of corn was 65.9%, with a SD of 0.55 among different grinding size samples.
219 This variation is not biologically expressive, and it is likely related to the method used for starch
220 analyses; according to Hall (2015), the SD within sample of dry ground corn was 0.34. The
221 range in GMPS was 390 to 1,181-µm, for samples ground using sieves of 1 to 6-mm. Similarly,
222 Hoffman et al. (2012) reported a range of 378 to 1,410- µm when samples were ground using
223 sieves of 1 to 8-mm. The minimal amount of sample (0.1%) passing through the 150-µm sieve
224 (Table 1) suggests that the loss of particulate material when fraction A was measured was minor,
225 since bag porosity was 50-µm. According to López (2005), the loss of undegraded particulate
226 matter from bags leads to an overestimation of the soluble fraction.

Fraction A of DM and starch was reduced ($P < 0.01$) with increased grinding size (Figures 1A and 1C). Particle size interferes with nutrient degradation as increased grinding size reduces (Table 1) surface area and thereby may impair the attachment of ruminal microorganisms and the action of their enzymes (McAllister et al., 1993). Furthermore, with reduced surface area the amount of nutrient in contact with water is decreased, a plausible explanation for the reduction of 13.77 and 14.98%-units in the soluble fraction of DM and starch, respectively (Figures 1A and 1C).

The estimation of the fraction A of DM differed ($P < 0.01$) among washing procedures (Figure 1B) with samples determined by IB having the greatest fraction A (26.15% vs. 24.43% of DM, on average). However, the fraction A of starch was unaffected ($P = 0.82$) by washing procedure. Interestingly, the fraction A of DM was 10.2%-units numerically greater, on average, than fraction A of starch. Perhaps there is a difference in solubility of other components of DM, as soluble carbohydrates or nitrogenous components, which potentially alters the Fraction A estimates from varying washing procedures. The soluble and mechanical particle losses may account for a considerable proportion of nutrients, particularly of N (Nocek, 1988). Therefore, the use of the washing machine is recommended as the use of a mechanical procedure could eliminate inherent variation in washing intensity between and within technicians (De Boer et al., 1987). The linear relationship between fraction A of DM and starch is shown in Figure 2 ($P = 0.01$, $r^2 = 0.75$); the slope was similar to one, indicating that a constant adjustment factor [Fraction A, % of starch = - 12.647 + (1.098 × Fraction A, % of DM)] may be used for the estimation of the fraction A of starch based on values of the fraction A of DM. Furthermore, the correlation of fraction A of DM and starch was 0.87 ($P < 0.01$, Figure 2), which is similar to the correlation of 0.85 reported by Ramsey et al. (2001).

An interaction between grinding size and incubation time was observed ($P = 0.01$; Figure 3A) for fraction C of DM; greater fraction C was observed when using the 6-mm grinding size compared with other treatments at 48 and 72 h, and compared with 1-mm at 96 h of incubation. However, fraction C of DM was similar among grinding size treatments at 120 h. Therefore, the determination of fraction C using an incubation time-point of 120 h is recommended regardless of the grinding size used. An interaction between grinding size and incubation time was also observed ($P = 0.01$; Figure 3B) for fraction C of starch. At 48 h of incubation, samples ground at 6-mm had the greatest, at 4-mm intermediate, and 1- and 2-mm the lowest values of fraction C; after 72 h of incubation fraction C was greater for 6-mm compared with other treatments; and at 96 or 120 h grinding size did not affect fraction C. Overall, fraction C of DM and starch demonstrated greater variation when samples were ground at greater particle size; samples ground at 1 or 2-mm were more homogeneous. Fraction C of DM had numerically greater values than fraction C of starch throughout all time-points. This is likely related to fraction C of DM containing fibrous components such as pericarp and tip cap compared to solely starch. These components are resistant to bacterial attachment (Huntington, 1996) whereas NFC are virtually 100% degradable in the rumen (Hall and Mertens, 2017). Fraction C of DM and starch were related ($P < 0.01$), but relationship was not adequate ($r^2 = 0.66$; Figure 4) based on the slope and intercept values. These results suggest that despite the relationship, accurate determination of fraction C requires starch analysis.

The DM and starch disappearance had a similar pattern throughout ruminal in situ incubation time-points (Figure 5) and a time effect was observed ($P < 0.05$) with disappearance increasing logarithmically. Interestingly, despite the difference among most time-points, 3- and 6 h of incubation were similar. A grinding size by time interaction was observed for starch ($P =$

0.05) but not for DM ($P = 0.40$) ruminal degradation. Samples ground at 1-mm had greater starch disappearance than 6-mm from 0 to 48 h, than 4-mm at 0, 12, 18 and 24 h, and compared with 2-mm only at 24 h. Likewise, starch disappearance of samples ground at 2-mm were greater than 6-mm from 6 to 48 h, and compared with 4-mm at 12 and 18 h. Samples ground at 4-mm had greater starch disappearance than 6-mm at 18 and 24 h of incubation. A strong positive relationship between ruminal in situ degradation of DM and starch was observed ($P < 0.01$, $r^2 = 0.97$; Figure 6). However, the adjustment factor underestimates the starch disappearance in the initial hours of incubation and overestimate for the late incubation time-points. This is likely related to the loss of particulate matter and hence overestimation of DM disappearance at early time-points (López, 2005), and the contamination with microorganisms which may underestimate the DM disappearance at late time-points (Vanzant et al., 1998).

Ruminal disappearance kinetics of DM and starch are in Table 2. Although the effects of grinding size ($P < 0.01$) were observed for all DM and starch parameters, model ($P < 0.01$) and interaction between grinding size and model were observed solely on fraction B. The kd and ERD 3 of DM had a tendency ($P = 0.09$) to decrease when the model without fraction C was used. These data suggest that a model of 2-pools is adequate to determine starch digestion kinetics. Previously, Herrera-Saldana et al. (1990) used a 2-pools model to estimate the kd of DM, starch, and crude protein of cereal grains.

The relationship between kd of DM and starch was positive ($P < 0.01$, $r^2 = 0.88$; Figure 7). Although the high correlation between the two kd measurements was high, the intercept was not close to zero, and slope was not close to 1. Possibly, samples ground at 6-mm or greater, required that microorganisms degraded protein and other components prior to accessing the starch granule (McAllister et al., 1993; Hoffman et al., 2011). The correlation coefficients of kd

296 estimated with all time-points of ruminal in situ incubation (0, 3, 6, 12, 18, 24, and 48 h) and the
297 combination of two or three time-points are in Table 3. All kd calculated with the 48 h
298 incubation time-point had linear correlation coefficients greater than 0.99, except for the kd
299 estimated with incubations for 24 and 48 ($r = 0.938$), suggesting that the 48 h incubation is
300 required for accurate determination of the kd. The highest linear correlation coefficient (0.998)
301 was obtained with combination of 0, 3, and 48 h of incubations, with intercept close to zero, and
302 slope close to one. Another combination which could be used is 0, 6, and 48 h, as it presents
303 similar characteristics. According to Vanzant et al. (1998), the use of simple modeling strategies
304 may offer some benefits relative to ease of application without large compromises in the
305 accuracy or precision of estimates.

306 Based on previous results, a 2-pool model, and incubation times of 0, 3, and 48 h were
307 used to determine the ruminal kinetics parameters of starchy feedstuffs. These results are in
308 Table 4. Ruminal in situ incubation at 120 h highlighted that a fraction C does not exist or could
309 be considered null for most feedstuffs as the residue amount is not biologically meaningful.
310 Herrera-Saldana (1990) found different concentration of starch (% of DM), fraction A (%), and
311 kd (% h^{-1}), respectively, for barley 64.3, 66.2, and 14.7; corn 75.7, 21.0, and 6.43; oats 58.1,
312 96.6, and 7.05; sorghum 71.3, 3.5 and 5.34; and wheat 70.3, 78.2, and 19.28. These values are
313 slightly higher than our data. This is likely related to grinding size as Herrera-Saldana ground
314 samples at 1-mm, whereas in this study a 2-mm sieve was used. In addition, their procedure used
315 to measure starch did not consider free glucose. Our methodology considered both free and
316 released glucose (Hall, 2015). The variation (SD = 18.8) in the ERD estimate ranged from 25.47
317 to 97.67%, with an average of 71.85%. This variation was higher than the observed (SD = 12.2)
318 in the meta-analysis by Ferraretto and Shaver (2015) which summarized ruminal in vivo starch

digestibility data ranging from 24.0 to 66.4% of the starch intake for dairy cows fed on TMR. However, the variation in the present study is similar to the variation (SD = 18.5) of values obtained in another meta-analysis conducted with in vivo data with a range of 22.4 to 94.2% and an average of 68.1% of ruminal starch digestibility (Moharrery et al., 2014). These data suggest that the in situ procedure may represent the variation of ruminal starch degradation of feedstuffs, similarly to the in vivo evaluation. It should be emphasized that the calculated range in ruminal digestibility of starch in feedstuffs, although biologically coherent, assumes a constant kp for all starch sources. The concentration of starch in the diet, DMI, and source of starch are known factors affecting starch kp and the role of the intestines on total tract starch digestibility (Oba and Allen, 2003). The use of a constant starch kp for all feedstuffs is a major imprecision in nutritional models attempting to predict ruminal starch degradation and its quantification under different feeding scenarios is a plausible field for research aiming at improving the accuracy of starch ERD predictions for ration formulation.

CONCLUSIONS

Although no effects of methodology were observed for starch fraction A in the present study, the use of a washing machine for determination of fraction A may be the most effective at reducing the potential variation between various technicians. Our results underscore that ruminal in situ incubation at 120 h is necessary to determine fraction C of DM, but not for starch because the residues were minimal (0.13% of starch, on average among all grinding sizes) and a model with 2-pools represents the starch degradation kinetics. As expected, grinding size affected all fractions, disappearance rate, and effective ruminal disappearance. A strong correlation was observed between 0, 3, and 48 h of incubation and the whole set of time-points to determine starch fractional disappearance rate suggesting that the use of fewer time-points is feasible.

ACKNOWLEDGMENTS

Appreciation is extended to Drs. A. T. Adesogan and D. Vyas (Department of Animal Sciences, University of Florida, Gainesville, FL) and their group for the opportunity to use their laboratory equipment during this experiment; and to Mr. Todd Pritchard (University of Florida Dairy Research Unit, Gainesville, FL) for assisting with the use of cannulated cows. The authors are also grateful to the Brazilian Federal Agency for Post-Graduate Education (CAPES; Brasilia, DF, Brazil) and National Research Council (CNPq; Brasilia, DF, Brazil) for the grants to the authors.

REFERENCES

Andreazzi, A. S. R., M. N. Pereira, R. B. Reis, R. A. N. Pereira, N. N. Morais Júnior, T. S. Acedo, R. G. Hermes, and C. S. Cortinhas. (2018). Effect of exogenous amylase on lactation performance of dairy cows fed a high starch diet. J. Dairy Sci. (in presse).

ASABE. 2007. Method of determining and expressing fineness of feed materials by sieving. ANSI/ASAE S319.4:646–649. Am. Soc. Ag. Biol. Eng., St. Joseph, MI.

Baker, S., and T. Herrman. 2002. Evaluating particle size. MF-2051. Kansas State Univ., Manhattan.

Batajoo, K. K., and R. D. Shaver. 1997. *In situ* dry matter, crude protein, and starch degradabilities of selected grains and by-product feeds. Anim. Feed Sci. Technol. 71:165-176.

Benton, J. R., T. J. Klopfenstein, and G. E. Erickson. 2005. Effects of Corn Moisture and Length of Ensiling on Dry Matter Digestibility and Rumen Degradable Protein. Nebraska Beef Cattle Reports. Paper 151. http://digitalcommons.unl.edu/animalscinbcr/151.

De Boer, G., J. J. Murphy, and J. J. Kennelly. 1987. A modified method for determination of *in situ* rumen degradation of feedstuffs. Can. J. Anim. Sci. 67:93−102.

Dias Junior, G. S., L. F. Ferraretto, G. G. S. Salvati, L. C. de Resende, P. C. Hoffman, M. N. Pereira, and R. D. Shaver. 2016. Relationship between processing score and kernel-fraction particle size in whole-plant corn silage. J. Dairy Sci. 99:2719–2729.

Dieho, K., B. Van den Bogert, G. Henderson, A. Bannink, J. Ramiro-Garcia, H. Smidt, and J. Dijkstra. 2017. Changes in rumen microbiota composition and *in situ* degradation kinetics during the dry period and early lactation as affected by rate of increase of concentrate allowance. J. Dairy Sci. 100:2695–2710.

Dulphy, J. P., C. Demarquilly, R. Baumont, M. Jailler, L. L'Hotelier, and C. Dragomir. 1999. Study of modes of preparation of fresh and conserved forage samples for measurement of their dry matter and nitrogen degradations in the rumen. Ann. Zootechnol. 48:275–288.

Ferraretto, L. F., P. M. Crump, and R. D. Shaver. 2013. Effect of cereal grain type and corn grain harvesting and processing methods on intake, digestion and milk production by dairy cows through a meta-analysis. J. Dairy Sci. 96:533–550.

Ferraretto, L. F., and R. D. Shaver. 2012. Effect of corn shredlage on lactation performance and total tract starch digestibility by dairy cows. Prof. Anim. Sci. 28:639–647.

Ferraretto, L. F., and R. D. Shaver. 2015. Effects of whole-plant corn silage hybrid type on intake, digestion, ruminal fermentation, and lactation performance by dairy cows through a meta-analysis. J. Dairy Sci. 98:1–14.

Ferreira, G., and D. R. Mertens. 2005. Chemical and physical characteristics of corn silages and their effects of in vitro disappearance. J. Dairy Sci. 88:4414–4425.

386 Firkins, J. L., M. L. Eastridge, N. R. St-Pierre, and S. M. Noftsger. 2001. Effects of grain
387 variability and processing on starch utilization by lactating cattle. J. Anim. Sci. 79: E218-
388 E238.
389 Fredin, S.M., L.F. Ferraretto, M.S. Akins1, P.C. Hoffman, R.D. Shaver. 2014. Fecal starch as an
390 indicator of total-tract starch digestibility by lactating dairy cows. J. Dairy Sci. 97:1862–
391 1871.
392 Gallo, A., G. Giuberti, and F. Masoero. 2016. Gas production and starch degradability of corn
393 and barley meals differing in mean particle size. J. Dairy Sci. 99:4347–4359.
394 Hall, M. B. 2015. Determination of dietary starch in animal feeds and pet food by an enzymatic-
395 colorimetric method: Collaborative study. J. Assoc. Off. Anal. Chem. 98:397-409.
396 Hall, M. B., and D. R. Mertens. 2017. A 100-Year Review: Carbohydrates—Characterization,
397 digestion, and utilization. J. Dairy Sci. 100:10078–10093.
398 Herrera-Saldana, R. E., J. T. Huber, and M. H. Poore. 1990. Dry matter, crude protein, and starch
399 degradability of five cereal grains. J. Dairy Sci. 73:2386–2393.
400 Hristov, A. N., S. Zaman, M. VanderPol, P. Szasz, K. Huber, and D. Greer. 2007. Effect of a
401 saponin-based surfactant and aging time on ruminal degradability of flaked corn grain dry
402 matter and starch. J. Anim. Sci. 85:1459–1466
403 Hoffman, P. C., N. M. Esser, R. D. Shaver, W. K. Coblentz, M. P. Scott, A. L. Bodnar, R J.
404 Schmidt, and R. C. Charley. 2011. Influence of ensiling time and inoculation on alteration of
405 the starch-protein matrix in high-moisture corn. J. Dairy Sci. 94:2465-2474.
406 Hoffman, P. C., R. D. Shaver, and D. R. Mertens. 2012. Feed Grain V2.0 Background and
407 development guide. Accessed Dec. 5, 2017. https://shaverlab.dysci.wisc.edu/spreadsheets.

Huntington, G. B. 1997. Starch utilization by ruminants: From basics to the bunk. J. Anim. Sci. 75:852–867.

Keim, J. P., X. Valderrama, D. Alomar, and I. F. López. 2013. *In situ* rumen degradation kinetics as affected by type of pasture and date of harvest. Sci. Agric. 70:405-414.

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

Lopez, S. 2005. In vitro and *In situ* techniques for estimating digestibility. Pages 87-111 in Quantitative Aspects of Ruminant Digestion and Metabolism. Dijkstra, J., J. M. Forbes, J. France, 2nd ed. CABI puplishing, London, UK.

McAllister, T. A., R. C. Phillippe, L. M. Rode, and K.-J. Cheng. 1993. Effect of the protein matrix on the digestion of cereal grains by ruminal microorganisms. J. Anim. Sci. 71:205–212.

Moharrery. A., M. Larsen, and M. R. Weisbjerg. 2014. Starch digestion in the rumen, small intestine, and hind gut of dairy cows - A meta-analysis. Anim. Feed Sci. Technol. 192:1–14.

Nocek, J. E. 1988. *In situ* and Other Methods to Estimate Ruminal Protein and Energy Digestibility: A Review. J. Dairy Sci. 71:2051-2069.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. National Academies Press, Washington, DC.

Oba, M., and M. S. Allen. 2003. Effects of corn grain conservation method on ruminal digestion kinetics for lactating dairy cows at two dietary starch concentrations. J. Dairy Sci. 86:184-194.

Owens, C.E., R.A. Zinn, A. Hassen, N.F. Owens. 2016. Mathematical linkage of total-tract digestion of starch and neutral detergent fiber to their fecal concentrations and the effect of site of starch digestion on extent of digestion and energetic efficiency of cattle. Prof. Anim. Sci. 32:531–549.

Peyrata, J., P. Nozièrea, A. Le Morvana, A.Férard, P. V. Protin, and R. Baumon. 2014. Effects of ensiling maize and sample conditioning on *in situ* rumen degradation of dry matter, starch and fibre. Anim. Feed Sci. Technol. 196:12–2.

Philippeau, C., and B. Michalet-Doreau. 1997. Influence of genotype and stage of maturity of maize on rate of ruminal starch degradation. Anim. Feed Sci. Technol. 68:25–35.

Ramsey, P. B., G. W. Mathison, L. A. Goonewardene. 2001. Relationships between rumina dry matter and starch disappearance and apparent digestibility of barley grain. Anim. Feed Sic. Technol. 94:155-170.

Seifried, N., H. Steinga, W. Schipprack, and M. Rodehutscord. 2016. Variation in ruminal *in situ* degradation of crude protein and starch from maize grains compared to in vitro gas production kinetics and physical and chemical characteristics. Arch. Anim. Nutr. 70:333–349.

Tylutki, T. P., D. G. Fox, V. M. Durbal, L. O. Tedeschi, J. B. Russell, M. E. Van Amburgh, T. R. Overton, L. E. Chase, and A. N. Pell. 2007. Cornell Net Carbohydrate and Protein System: A model for precision feeding of dairy cattle. Anim. Feed Sci. Technol. 143:174-202.

Vanzant, E. S., R. C. Cochran, and E. C. Titgemeyer. 1998. Standardization of *In situ* Techniques for Ruminant Feedstuff Evaluation. J. Anim. Sci. 76:2717–2729.

451 Volden, H., and M. Larsen. 2011. Digestion and metabolism in the gastrointestinal tract. In:
452 Volden, H. (Ed.), NorFor – The Nordic Feed Evaluation System. EAAP Publication No.
453 130. Wageningen Academic Publishers, Wageningen, The Netherlands, pp.59–80.
454

455 **Table 1**. Dry matter and starch concentrations, particle size distribution, geometric mean particle
456 size (GMPS), and surface area of dry corn ground at varying grinding sizes

	Grinding size			
	1-mm	2-mm	4-mm	6-mm
Dry matter, % of as fed	89.5	89.5	89.5	89.3
Starch, % of DM	65.8	66.5	65.2	66.1
Free Glucose, % of DM	0.63	0.71	0.63	0.68
Sieves,[1] µm	--- % of particles retained, DM basis ---			
3350	0.0	0.0	0.1	3.9
2360	0.3	0.1	1.6	24.5
1700	0.3	0.1	13.9	35.7
1180	0.7	17.9	55.7	21.6
600	80.0	73.5	22.7	11.0
300	17.0	6.2	4.3	2.3
150	1.6	1.9	1.5	0.9
Bottom pan	0.1	0.1	0.1	0.1
GMPS, µm	390	468	746	1,181
Surface area, cm^2/g	41.0	39.2	34.2	29.0

457

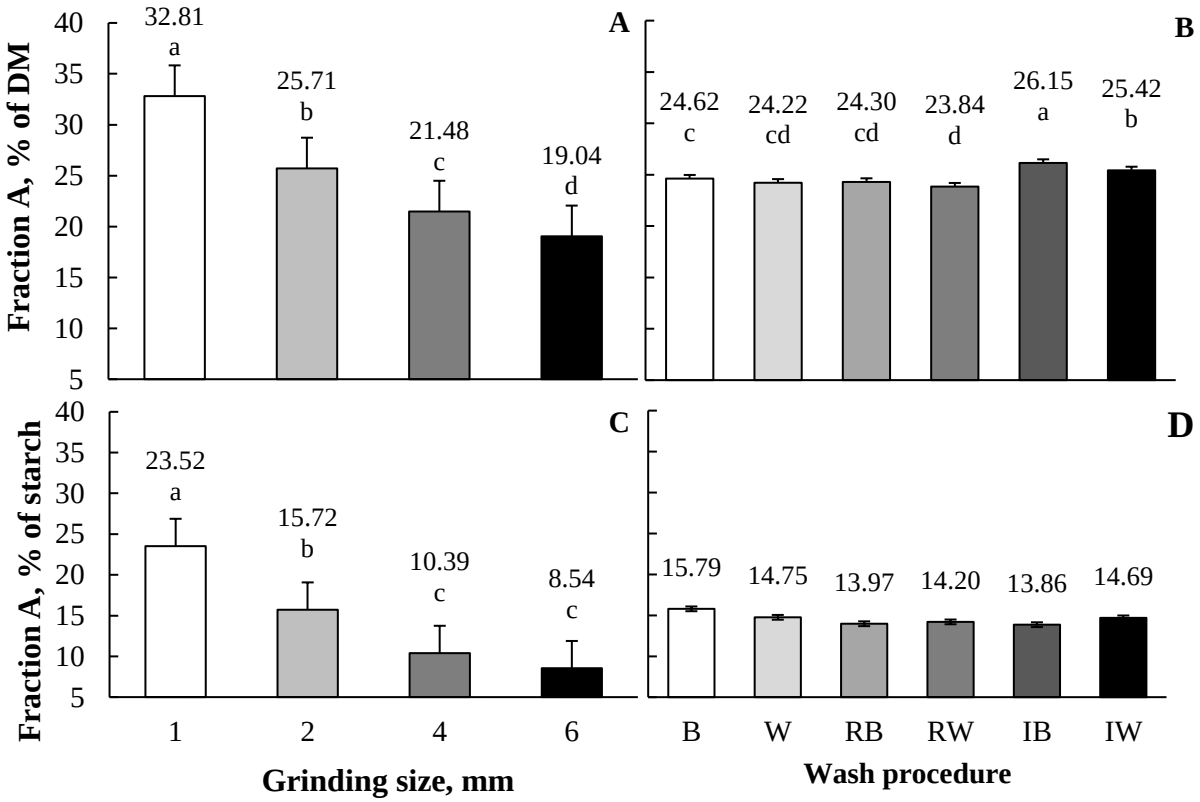

Figure 1. Fraction A of DM (Panels A and B) and starch (Panels C and D) of dry corn ground at different grinding sizes (1-mm, □; 2-mm, ▫; 4-mm, ▪; and 6-mm, ■) and determined by different washing procedures [rinsing in a bucket (B) □, rinsing in a washing machine (W) ▫, inserting in the rumen and rinsing in a bucket (RB) ▫, inserting in the rumen and rinsing in the washing machine (RW) ▪, immersing in warm water during 30 min and rinsing in a bucket (IB) ▪, and immersing in warm water during 30 min and rinsing in the washing machine (IW) ■]. For fraction A of dry matter: effects of grinding size ($P = 0.001$), washing method ($P = 0.001$), and interaction between grinding size and washing method ($P = 0.77$), SEM = 0.480. For fraction A of starch: P effects of grinding size ($P = 0.001$), washing method ($P = 0.82$), and interaction between grinding size and washing method ($P = 0.94$), SEM = 2.140. Means with different letters differ at $P \leq 0.05$ by pairwise t-test.

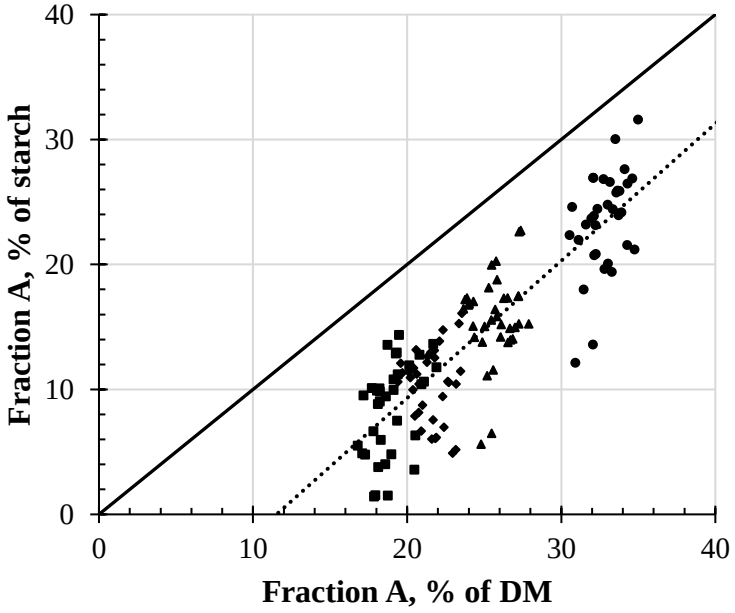

Figure 2. Relationship of the instantaneously degradable Fraction A of DM and starch of dry corn ground at different grinding sizes (1-mm ●, 2-mm ▲, 4-mm ◆, and 6-mm ■) and determined by different washing procedures. Prediction equation: Y = - 12.647 + 1.098 X; n = 144, root mean square error = 15.63, $R^2 = 0.75$, $P < 0.001$.

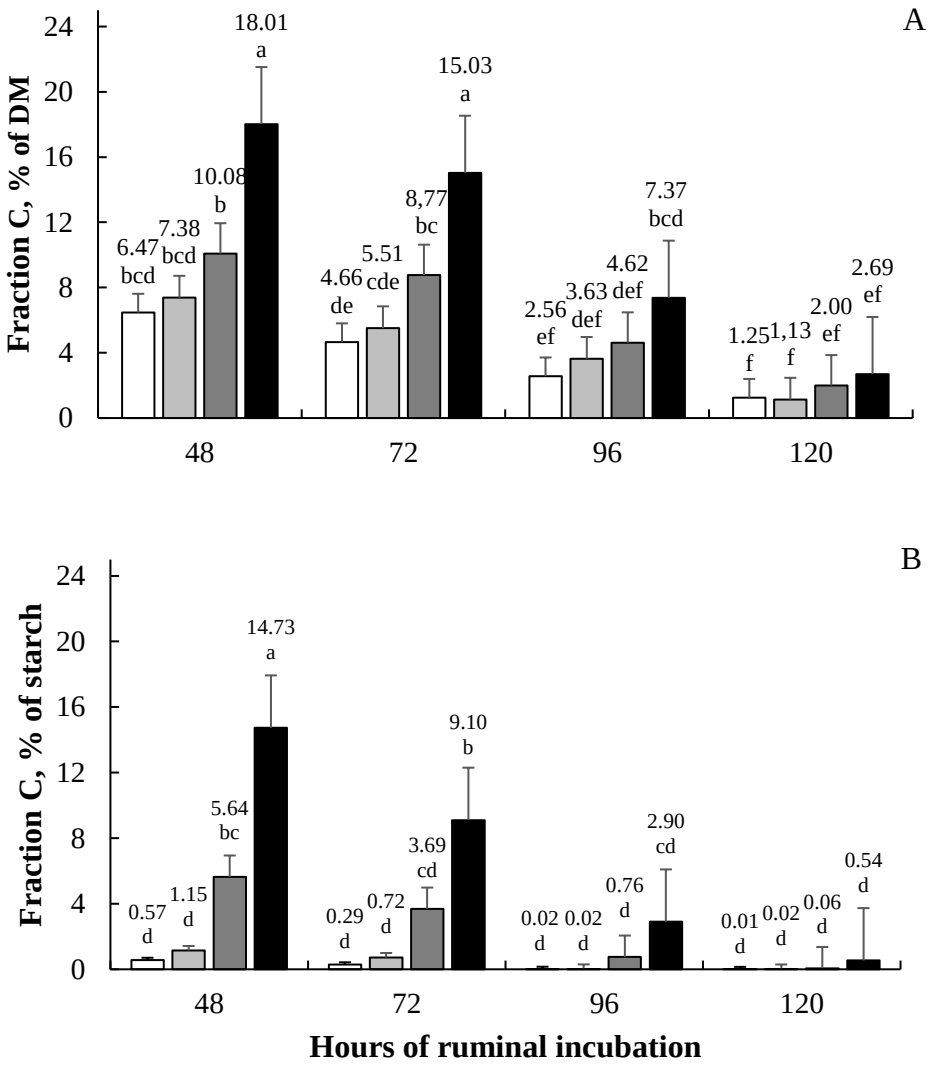

Figure 3. Fraction C of DM (% of DM; Panel A) and starch (% of starch; Panel B) of dry corn ground at different grinding sizes (1-mm □, 2-mm ▫, 4-mm ▪, and 6-mm ■) and incubated for different time-points of ruminal *in situ* disappearance (48, 72, 96, and 120-h). For Panel A: effects of grinding size ($P = 0.001$), incubation time ($P = 0.001$), and interaction between grinding size and incubation time ($P = 0.01$), SEM = 1.893. For Panel B: effects of grinding size ($P = 0.001$), incubation time ($P = 0.001$), and interaction between grinding size and incubation time ($P = 0.001$), SEM = 1.960. Means with different letters differ at $P \leq 0.05$ by pairwise t-test.

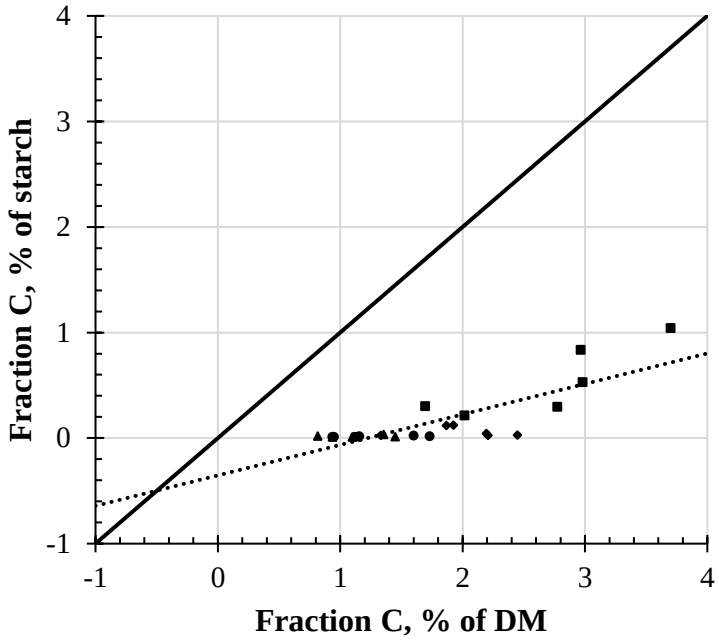

Figure 4. Relationship of the Fraction C of DM and starch (residue of the 120-h ruminal *in situ* incubation) of dry corn ground at different grinding sizes (1-mm ●, 2-mm ▲, 4-mm ◆, 6-mm ■). Prediction equation: Y = - 0.3532 + 0.2889 X; n = 24, root mean square error = 0.27, R^2 = 0.66, *P* < 0.001.

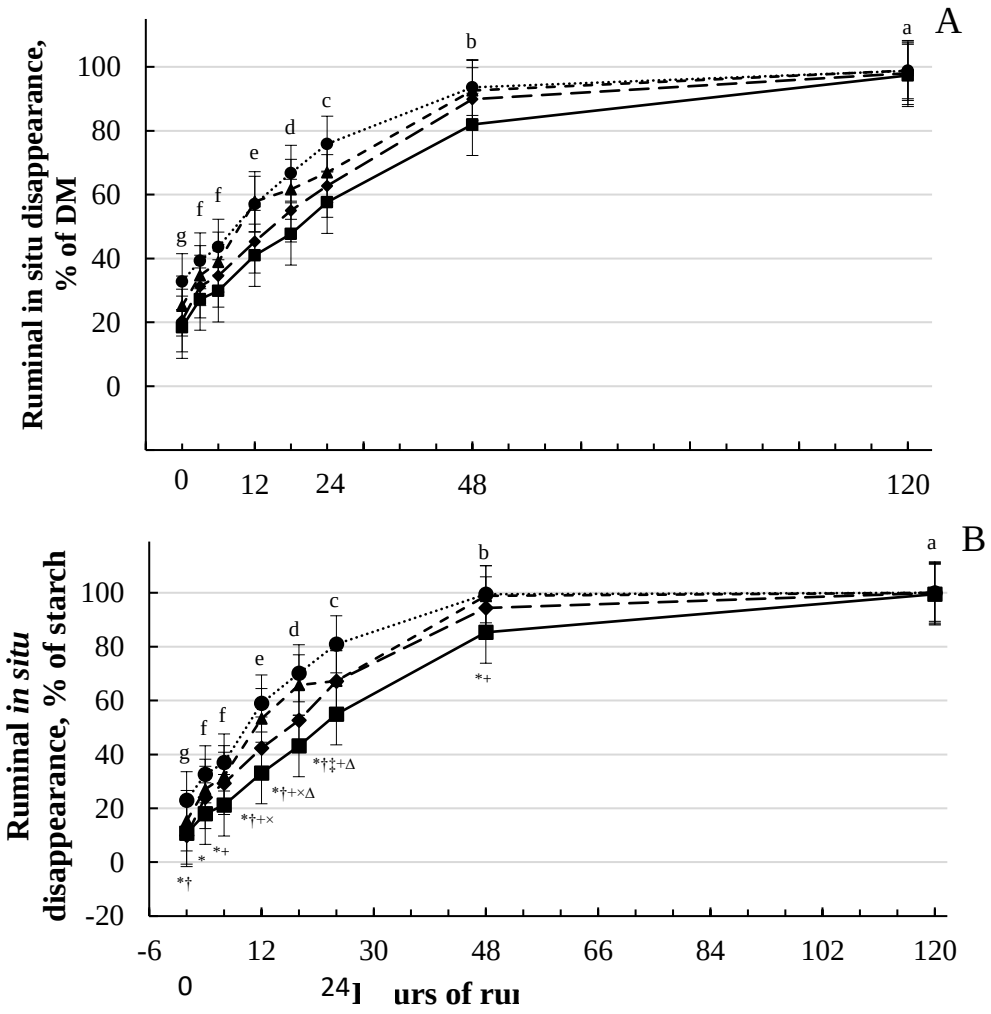

Figure 5. Effect of grinding size (1-mm ●, 2-mm ▲, 4-mm ◆, and 6-mm ■) on ruminal *in situ* DM (% of DM; Panel A) and starch (% of starch; Panel B) disappearance at 0, 3, 6, 12, 18, 24, 48, and 120-h of incubation of dry ground corn. Time 0 degradation was the disappearance measured using the washing machine procedure. For Panel A: Effects of grinding size ($P = 0.001$), incubation time ($P = 0.001$), and interaction between grinding size and incubation time ($P = 0.40$), SEM = 4.285. For Panel B: Effects of particle size ($P = 0.001$), incubation time ($P = 0.001$), and interaction between particle size and incubation time ($P = 0.05$), SEM = 4.264. Means for incubation times with different letters differ at $P \leq 0.05$ by pairwise t-test. Means for mesh diameter with different symbols differ at $P \leq 0.05$ by pairwise t-test (1-mm vs. 6-mm *, 1-mm vs. 4-mm †, 1-mm vs. 2-mm ‡, 2-mm vs. 6-mm +, 2-mm vs. 4-mm ×, 4-mm vs. 6-mm Δ).

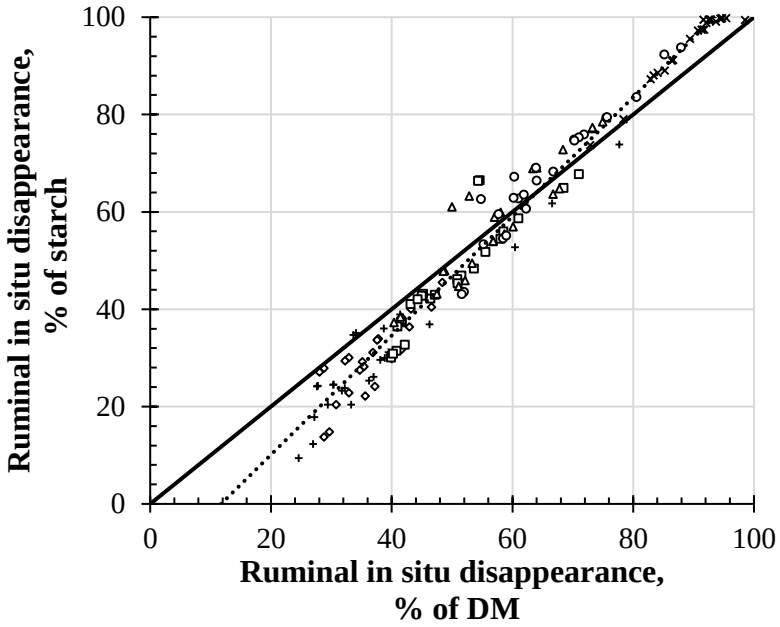

Figure 6. Relationship of ruminal disappearance of DM and starch of dry corn ground at different grinding sizes (1, 2, 4, and 6-mm) and incubated *in situ* for 3-h (+), 6-h (◇), 12-h (□), 18-h (▵), 24-h (○), and 48-h (×). Prediction equation: Y = - 14.394 + 1.2245 X; n = 144, root mean square error = 59.89, $R^2 = 0.97$, $P < 0.001$.

Table 2. Fractions A, B, and C[1], fractional disappearance rate of Fraction B (kd), effective ruminal disappearance (ERD[2]) estimated with differing ruminal fractional passage rates (kp) of dry corn ground at different grinding sizes (GS; 1, 2, 4, and 6-mm) and subjected to ruminal *in situ* incubations for 0, 3, 6, 12, 18, 24, and 48-h. Two (Fractions A and B) or 3-pool models (Fractions A, B, and C) were used to estimate the ruminal disappearance parameters.

	Mesh Diameter									*P-value*		
	1-mm		2-mm		4-mm		6-mm		SEM			
	3-Pool	2-Pool	3-Pool	2-Pool	3-Pool	2-Pool	3-Pool	2-Pool		GS	Model	GS*Model
Dry matter												
Fraction A, %	32.75	32.75	25.10	25.10	20.56	20.56	18.46	18.46				
Fraction B, %	66.00	67.25	73.77	74.90	77.44	79.44	78.85	81.54	0.138	< 0.01	< 0.01	< 0.01
Fraction C, %	1.25		1.13		2.00		2.69		0.188	< 0.01		
kd, % h^{-1}	5.39	4.99	5.06	4.75	4.89	4.37	3.43	3.16	0.297	< 0.01	0.09	0.98
ERD 1[3-], % of DM	59.09	59.59	53.66	54.10	48.82	49.64	40.46	42.58	1.163	< 0.01	0.26	0.88
ERD 2[4], % of DM	60.48	61.00	55.20	55.65	50.42	51.24	41.95	44.06	1.189	< 0.01	0.27	0.88
ERD 3[5], % of DM	48.10	48.69	41.64	42.19	36.53	37.50	29.66	31.87	0.841	< 0.01	0.09	0.74
Starch												
Fraction A, %	23.00	23.00	15.40	15.40	9.92	9.92	10.69	10.69				
Fraction B, %	76.98	77.00	84.58	84.60	90.02	90.08	88.77	89.30	0.072	< 0.01	0.01	0.01
Fraction C, %	0.02		0.02		0.06		0.54		0.098	0.01		
kd, % h^{-1}	10.72	10.65	9.61	9.56	6.24	6.22	3.98	3.92	0.406	< 0.01	0.86	0.99
ERD 1, % of starch	68.05	67.95	62.50	62.43	50.22	50.23	40.64	41.03	1.020	< 0.01	0.94	0.99
ERD 2, % of starch	69.65	69.55	64.28	64.21	52.13	52.14	42.38	42.77	1.052	< 0.01	0.94	0.99
ERD 3, % of starch	53.67	53.57	46.83	46.76	34.83	34.69	27.69	28.11	0.749	< 0.01	0.91	0.98

[1]A = soluble fraction, B = slowly degradable fraction, C = undigested fraction; Fraction C was the residue of a 120-h incubation.

[2]ERD = Fraction A + Fraction B × [kd / (kd + kp)]

[3]ERD 1 = kp estimated by the Cornel Net Carbohydrate and Protein System, 7.47 % h^{-1} (Tylutki et al., 2008)

[4]ERD 2 = kp estimated by the Nordic Feed Evaluation System, 6.85 % h^{-1} (Volden and Larsen, 2011)

[5]ERD 3 = kp of 16.0 % h^{-1} (Hoffman et al., 2012)

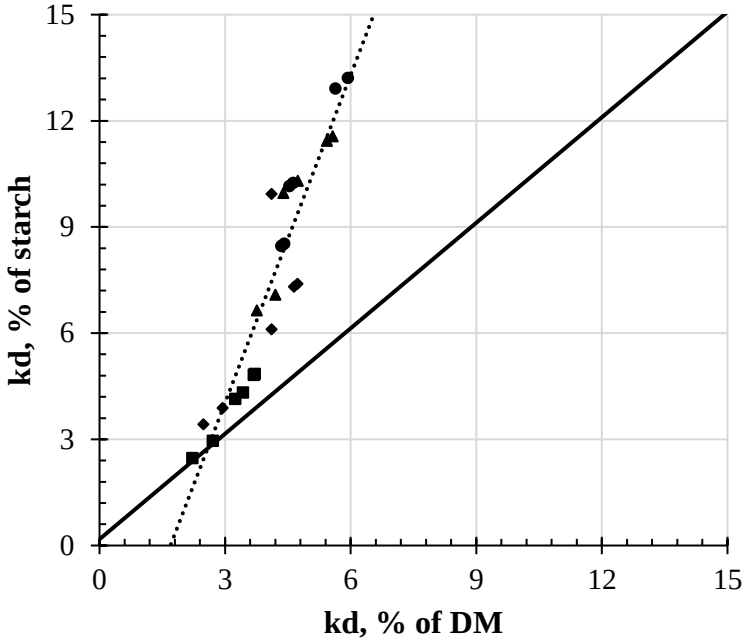

Figure 7. Relationship between the fractional disappearance rate of Fraction B (kd) estimated with a 2-pool model (Fractions A and B) of DM and starch of dry corn ground at different grinding sizes (1-mm ●, 2-mm ▲, 4-mm ◆, and 6-mm ■). Prediction equation: Y = - 5.2317 + 3.0882 X; n = 24, root mean square error = 8.15, $R^2 = 0.88$, $P < 0.001$.

Table 3. The linear correlation coefficients between the fractional disappearance rate (kd) of starch estimated with all ruminal incubation time-points (Times. 0, 3, 6, 12, 18, 24, and 48-h) and the kd estimated with 3 or 2 incubation time-points using a 2-pool model (Fractions A and B) [1]

Times	kd, % h^{-1}	r	*P*-value	Linear regression[2]	Times	kd, % h^{-1}	r	*P*-value	Linear regression[1]
0, 3, 6	3.31	0.350	0.261	Y = 5.167 + 0.712 X	0, 18	4.15	0.882	< 0.001	Y = - 0.414 + 1.912 X
0, 3, 12	3.89	0.422	0.166	Y = 4.327 + 0.820 X	0, 24	4.47	0.749	0.004	Y = 2.313 + 1.166 X
0, 3, 18	3.94	0.903	< 0.001	Y = 0.490 + 1.783 X	0, 48	7.73	0.997	< 0.001	Y = - 0.229 + 1.003 X
0, 3, 24	4.32	0.749	0.004	Y = 2.909 + 1.069 X	3, 6	0.73	0.355	0.253	Y = 7.650 + 0.177 X
0, 3, 48	7.74	0.998	< 0.001	Y = 0.030 + 0.968 X	3, 12	3.07	0.468	0.119	Y = 5.634 + 0.614 X
0, 6, 12	4.14	0.347	0.265	Y = 4.682 + 0.686 X	3, 18	3.51	0.834	< 0.001	Y = 3.180 + 1.237 X
0, 6, 18	4.21	0.884	< 0.001	Y = - 0.242 + 1.844 X	3, 24	4.06	0.727	0.006	Y = 3.966 + 0.877 X
0, 6, 24	4.56	0.739	0.005	Y = 2.589 + 1.082 X	3, 48	7.75	0.997	< 0.001	Y = 0.395 + 0.919 X
0, 6, 48	7.96	0.997	< 0.001	Y = - 0.134 + 0.962 X	6, 12	4.97	0.152	0.636	Y = 6.882 + 0.129 X
0, 12, 18	4.15	0.876	< 0.001	Y = - 0.980 + 2.049 X	6, 18	4.57	0.846	< 0.001	Y = 1.289 + 1.363 X
0, 12, 24	4.47	0.749	0.004	Y = 2.313 + 1.166 X	6, 24	4.85	0.704	0.009	Y = 3.398 + 0.850 X
0, 12, 48	8.00	0.997	< 0.001	Y = - 0.037 + 0.945 X	6, 48	8.36	0.996	< 0.001	Y = 0.029 + 0.897 X
0, 18, 24	4.39	0.814	0.001	Y = 1.247 + 1.428 X	12, 18	4.17	0.629	0.025	Y = 5.630 + 0.454 X
0, 18, 48	7.94	0.997	< 0.001	Y = - 0.172 + 0.968 X	12, 24	4.79	0.611	0.031	Y = 5.246 + 0.475 X
0, 24, 48	7.73	0.997	< 0.001	Y = - 0.229 + 1.003 X	12, 48	8.92	0.993	< 0.001	Y = 0.547 + 0.782 X
0, 3	7.35	- 0.191	0.055	Y = 8.247 - 0.099 X	18, 24	5.42	0.359	0.248	Y = 6.527 + 0.184 X
0, 6	3.31	0.350	0.261	Y = 5.167 + 0.712 X	18, 48	9.87	0.993	< 0.001	Y = 0.257 + 0.736 X
0, 12	4.14	0.347	0.265	Y = 4.682 + 0.686 X	24, 48	10.98	0.938	< 0.001	Y = 1.075 + 0.587 X

[1]Twelve samples of dry corn per incubation time were ground through sieves varying in mesh diameter and incubated in 3 rumen cannulated cows. Time 0 degradation was the disappearance measured by wash with a washing machine. The kd estimated with all incubation times was 7.52 % h^{-1}

[2]Y = kd estimated with all incubation times. X = kd estimated with 2 or 3 incubation times.

Table 4. Feed starch concentration, fractions A, B, and C[1], fractional disappearance rate of Fraction B (kd), effective ruminal disappearance (ERD[2]) estimated with differing ruminal fractional passage rates (kp). All samples were ground at 2-mm

Feed	Starch % of DM	Fraction A % of starch	Fraction B % of starch	kd % h^{-1}	ERD 1[3] % of starch	ERD 2[4] % of starch	ERD 3[5] % of starch	Residue 120h % of starch
Barley, grain 1	71.46	35.71	64.29	10.98	73.97	75.30	61.87	0.004
Barley, grain 2	71.77	33.70	66.30	11.49	73.88	74.84	61.41	0.003
Cassava, root	79.87	94.06	5.94	7.59	97.05	97.31	95.97	0.003
Cassava, starch waste 1	48.17	22.15	77.85	7.73	61.74	63.16	47.51	0.060
Cassava, starch waste 2	51.44	23.97	76.03	7.46	61.97	63.73	48.16	0.068
Corn, grain from silage[6] 1	72.15	30.81	69.19	6.84	63.13	69.88	51.14	0.011
Corn, grain from silage 2	72.18	13.83	86.17	5.75	50.80	57.72	36.37	0.004
Corn, grain from silage 3	74.23	84.84	15.16	6.50	91.89	92.05	89.22	0.007
Corn, grain from silage 4	77.87	74.00	26.00	8.63	87.93	88.31	83.11	0.001
Corn, grain from silage 5	79.42	82.45	17.55	6.74	90.77	91.13	87.65	0.003
Corn, grain ground BR 1	66.19	13.50	86.50	3.52	40.79	47.48	28.94	0.007
Corn, grain ground BR 2	67.02	13.36	86.64	6.44	52.32	61.56	37.64	0.004
Corn, grain ground BR 3	68.72	24.78	75.22	4.83	53.80	60.32	42.00	0.015
Corn, grain ground BR 4	69.29	21.03	78.97	5.44	53.24	62.00	40.57	0.002
Corn, grain ground BR 5	71.17	20.19	79.81	5.67	54.03	61.02	40.79	0.003
Corn, grain ground BR 6	72.35	26.05	73.95	4.53	53.33	60.30	42.10	0.024
Corn, grain ground US 1	56.76	29.35	70.65	6.87	63.01	67.11	50.47	0.026
Corn, grain ground US 2	57.67	15.69	84.31	6.89	55.82	61.49	40.90	0.002
Corn, ground grain US 3	69.88	56.37	43.63	8.60	79.61	82.05	71.56	0.003
Corn, rehydrated and ensiled grain 1	62.65	36.25	63.75	4.65	41.45	50.18	27.03	0.002
Corn, rehydrated and ensiled grain 2	66.75	95.41	4.59	5.00	97.20	97.67	96.48	0.004
Corn, rehydrated and ensiled grain 3	66.88	87.47	12.53	5.34	92.69	93.02	90.61	0.004
Corn, rehydrated and ensiled grain 4	69.11	33.97	66.03	4.75	40.16	50.55	25.47	0.005
Corn, rehydrated and ensiled grain 5	69.59	49.32	50.68	6.25	72.31	74.98	63.50	0.004
Corn, rehydrated and ensiled grain 6	70.44	83.82	16.18	5.27	90.44	89.91	87.79	0.004
Corn, steam-flaked grain BR 1	82.89	18.80	81.20	8.93	63.02	64.87	47.89	0.004
Corn, steam-flaked grain BR 2	85.61	27.15	72.85	9.41	67.57	71.62	54.01	0.004

Corn, steam-flaked grain BR 3	86.81	30.99	69.01	10.73	71.66	72.88	58.68	0.002
Corn, steam-flaked grain US	70.78	42.47	57.53	8.63	73.22	75.91	62.58	0.033
Corn, whole-plant silage BR 1	19.03	80.53	19.47	2.82	85.83	85.38	83.44	0.232
Corn, whole-plant silage BR 2	19.85	31.38	68.62	5.28	59.69	58.96	48.36	0.148
Corn, whole-plant silage BR 3	22.29	61.33	38.67	4.81	76.45	78.12	70.26	0.125
Corn, whole-plant silage BR 4	34.89	88.28	11.72	3.93	92.31	92.19	90.59	0.073
Corn, whole-plant silage US	33.15	69.93	30.07	5.51	82.67	83.90	77.62	0.006
Oats, rolled grain 1	59.08	81.81	18.19	6.51	90.28	90.52	87.07	0.006
Oats, rolled grain 2	60.54	92.50	7.50	5.19	95.57	95.64	94.34	0.007
Oats, rolled grain 3	65.30	96.14	3.86	3.96	97.48	97.58	96.90	0.009
Potato, root 1	65.78	76.45	23.55	8.51	88.81	90.74	84.52	0.003
Potato, root 2	67.39	71.67	28.33	11.18	88.61	88.49	83.30	0.004
Potato, root 3	69.83	81.09	18.91	10.63	92.19	92.53	88.63	0.003
Sorghum, ground grain 1	71.18	27.05	72.95	4.73	54.16	63.04	43.19	0.456
Sorghum, ground grain 2	71.61	31.87	68.13	3.35	52.26	59.55	43.41	1.444
Sorghum, rehydrated and ensiled grain 1	64.21	26.29	73.71	3.56	48.35	59.44	39.07	0.485
Sorghum, rehydrated and ensiled grain 2	65.86	49.81	50.19	4.04	67.06	71.63	59.79	1.100
Sorghum, rehydrated and ensiled grain 3	66.36	23.30	76.70	4.00	47.97	60.00	37.82	0.290
Sorghum, rehydrated and ensiled grain 4	72.59	45.45	54.55	3.51	62.23	68.38	55.02	0.939
Sweet potato, root 1	62.78	82.00	18.00	10.28	92.38	92.22	89.01	0.006
Sweet potato, root 2	64.78	84.32	15.68	9.28	93.00	93.37	90.07	0.016
Sweet potato, root 3	64.79	89.17	10.83	7.88	94.73	94.95	92.74	0.000
Wheat, bran 1	18.08	78.96	21.04	3.26	85.36	85.86	82.52	0.315
Wheat, bran 2	18.63	81.18	18.82	2.41	85.76	86.37	83.64	0.308
Wheat, bran 3	19.51	52.27	47.73	2.11	62.72	64.79	57.81	0.560
Wheat, flour 1	68.04	91.30	8.70	5.26	94.85	94.80	93.43	0.271
Wheat, flour 2	69.91	86.93	13.07	6.51	93.02	93.28	90.71	0.020
Wheat, ground grain	75.15	50.45	49.55	9.36	78.00	79.36	68.74	0.010
Wheat, rolled grain	63.18	60.05	39.95	8.43	81.20	82.78	73.82	0.003
Mean	62.19	53.80	46.20	6.39	73.42	76.47	65.66	0.128
Maximum	86.81	96.14	86.64	11.49	97.48	97.67	96.90	1.44

Minimum	18.08	13.36	3.86	2.11	40.16	47.48	25.47	0.00
Standard deviation	17.68	27.97	27.97	2.44	17.63	14.69	21.98	0.285
Coefficient of variation	28.43	51.98	60.55	38.17	24.01	19.22	33.47	223.363

[1]A = soluble fraction, B = slowly degradable fraction, C = undigested fraction; Fraction C was the residue of a 120-h incubation.
[2]ERD = Fraction A + Fraction B × [kd / (kd + kp)]
[3]ERD 1 = kp estimated by the Cornel Net Carbohydrate and Protein System, 7.47 % h^{-1} (Tylutki et al., 2008)
[4]ERD 2 = kp estimated by the Nordic Feed Evaluation System, 6.85 % h^{-1} (Volden and Larsen, 2011)
[5]ERD 3 = kp of 16.0 % h^{-1} (Hoffman et al., 2012).
[6]Corn grain hand-picked from whole-plant corn silage.