NIVERSIDADE ESTADUAL PAULISTA – UNESP
CÂMPUS DE JABOTICABAL

INCLUSÃO DE ADITIVOS À BASE DE MICRORGANISMOS DE ALIMENTAÇÃO DIRETA NA DIETA DE BOVINOS NELORE RECRIADOS A PASTO EM CONDIÇÕES TROPICAIS

Fernanda Rigon
Zootecnista

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Discente: Fernanda Rigon

Orientador: Prof. Dr. Gustavo Rezende Siqueira

Coorientador: Dr. Eduardo Marostegan de Paula

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutora em Ciência Animal.

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IMPACTO POTENCIAL DESTA PESQUISA

A presente pesquisa demonstra um potencial impacto significativo para sistemas tropicais de produção de bovinos, ao evidenciar que microrganismos de alimentação direta à base de *Bacillus*, utilizados isoladamente ou em combinação com levedura hidrolisada, podem atuar como ferramentas nutricionais eficazes para mitigar os desafios da estação seca — período marcado por queda na qualidade da forragem e limitações no desempenho animal. Ao revelar melhorias na digestibilidade da proteína, na dinâmica do metabolismo ruminal e, principalmente, no ganho de peso e no desenvolvimento muscular de bovinos jovens em pastejo, os resultados sugerem que tais aditivos podem elevar a eficiência biológica e produtiva dos rebanhos Nelore, com potencial para reduzir custos associados à suplementação convencional e aumentar a sustentabilidade dos sistemas de produção. Os achados desta tese podem fornecer subsídios para a adoção estratégica de DFM em condições tropicais, contribuindo para o avanço tecnológico da pecuária de corte e para a intensificação sustentável da produção de carne.

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: INCLUSÃO DE ADITIVOS À BASE DE MICRORGANISMOS DE ALIMENTAÇÃO DIRETA NA DIETA DE BOVINOS NELORE RECRIADOS A PASTO EM CONDIÇÕES TROPICAIS

AUTORA: FERNANDA RIGON
ORIENTADOR: GUSTAVO REZENDE SIQUEIRA
COORIENTADOR: EDUARDO MAROSTEGAN DE PAULA

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GUSTAVO REZENDE SIQUEIRA
Data: 13/11/2025 12:34:18-0300
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Pesquisador Dr. GUSTAVO REZENDE SIQUEIRA (Participaçao Virtual)
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Universidade do Estado de Santa Catarina (UDESC) / Chapecó/SC

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OTAVIO RODRIGUES MACHADO NETO
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Prof. Assoc. OTAVIO RODRIGUES MACHADO NETO (Participaçao Virtual)
Departamento de Produção Animal e Medicina Veterinária Preventiva / FMVZ UNESP Botucatu

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Pesquisadora Dra. ELAINE MAGNANI (Participaçao Virtual)
IZ - Instituto de Zootecnia / São José do Rio Preto/SP

Pesquisadora Dra. KARLA ALVES OLIVEIRA (Participaçao Virtual)
Instituto Federal do Triângulo Mineiro (IFTM) / Uberaba/MG

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DADOS CURRICULARES DA AUTORA

Fernanda Rigon, nascida em 09 de janeiro de 1996, Concórdia, Santa Catarina. Filha de Cerli Cimadon Rigon e Airton Rigon. Possui graduação em Zootecnia (2014 – 2018), pela Universidade do Estado de Santa Catarina – Centro de Educação Superior do Oeste (UDESC – CEO). Durante a graduação, participou do Programa de Educação Tutorial (PET Zootecnia – 2015 a 2016) sob tutoria do professor Dr. Diovani Paiano. Durante o período de participação no PET, realizou atividades de pesquisa e extensão relacionadas à bovinocultura leiteira, sob orientação da professora Dra. Ana Luiza Bachman Schogor; e de atividades de ensino (monitoria) relacionadas à área de fisiologia animal, sob orientação do professor Dr. Rogerio Ferreira. Foi monitora das disciplinas de nutrição de ruminantes e nutrição de não-ruminantes (2016 – 2018), sob orientação dos professores Dra. Ana Luiza Bachman Schogor e Dr. Tiago Goulart Petrolli. Participou de atividades de pesquisa junto ao Laboratório de Nutrição Animal (LANA – UDESC). Em novembro de 2018 ingressou como analista de garantia de qualidade Jr em uma das unidades da JBS responsáveis pelo abate de aves na região Oeste de Santa Catarina, cargo que ocupou até abril de 2019. Em agosto de 2019, ingressou no Programa de Pós-Graduação em Zootecnia no curso de mestrado da universidade pela qual formou-se. Realizou o experimento de mestrado em parceria com o Instituto de Zootecnia de Sertãozinho, São Paulo, na área de nutrição e produção de bovinos de corte, sob orientação do professor Dr. Pedro Del Bianco Benedeti. Em janeiro de 2022 ingressou no Programa de Pós-Graduação em Ciência Animal no curso de Doutorado da UNESP de Jaboticabal, sob orientação do professor Dr. Gustavo Rezende Siqueira. Foi membro do Grupo de Estudos em Produção de Ruminantes (GEPROR), durante o período de doutorado.

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"*Aprendia que tudo estava em movimento – bem diferente das coisas sem vida que a professora mostrava em suas aulas. Meu pai olhava para mim e dizia: "O vento não sopra, ele é a própria viração". E tudo aquilo fazia sentido. "Se o ar não se movimenta, não tem vento, se a gente não se movimenta não tem vida", ele tentava me ensinar*." (Itamar Vieira Junior, *in* Torto Arado). Agradeço com todo o meu coração à minha família, meus pais Airton e Cerli, pela minha vida, pelo amor, pelo zelo, carinho e cuidado. Agradeço profundamente a estes dois corações que movem, me guiam, me inspiram e me movimentam. Graças a eles, cheguei até aqui.

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SUMÁRIO

CERTIFICADO Nº 006/2022 – CEUA

Certificamos que o projeto apresentado dia 18 de novembro de 2022 intitulado "**AVALIAÇÃO DE ADITIVOS NATURAIS NA RECRIA DE BOVINOS NELORE EM PASTEJO EM CONDIÇÕES TROPICAIS**", foi registrado com o **protocolo nº 006/2022**, está sob a responsabilidade do Pesquisador Científico **Dr. Gustavo Rezende Siqueira** e envolve a produção, a manutenção e a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica e encontra-se de acordo com os preceitos da lei nº 11.794, de oito de outubro de 2008; do decreto nº 6.899, de 15 de julho de 2009; e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), sendo então **APROVADO** pela **Comissão de Ética no Uso de Animais da Apta Regional**, na X Reunião Extraordinária realizada no dia **30 de novembro de 2022**.

Finalidade: (X) Pesquisa científica – ( ) Ensino	
Vigência da autorização	01/12/2022 a 30/03/2024
Espécie/linhagem e/ou raça	Bovino Nelore
Nº de animais	96 M + 12 M Fistulados (CEUA 0013/2021) – Total: 108
Sexo/Idade/Peso aprox.	96 M / 8 a 12 meses / 200 a 300 Kg
Localização/Origem	Unidade Regional de Pesquisa e Desenvolvimento de Colina
Responsável Técnico	Dr. Gustavo Rezende Siqueira

Maria Izabel Merino de Medeiros
Coordenadora CEUA-DDD

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Comissão de Ética no Uso de Animais
Departamento de Descentralização do Desenvolvimento CEUA - DDD
Av. Rodrigues Alves, 40-40
CEP: 17030-000, Bauru, SP
Telefone: (14) 3203-3257- Ramal 24
E-mail: maria.medeiros@sp.gov.br

Agência Paulista de Tecnologia dos Agronegócios | Secretaria de Agricultura e Abastecimento

SÃO PAULO
GOVERNO DO ESTADO

Inclusão de aditivos a base de microrganismos de alimentação direta na dieta de bovinos Nelore recriados a pasto em condições tropicais

RESUMO: a presente tese avaliou a inclusão de aditivos à base de microrganismos de alimentação direta (DFM) nas dietas de bovinos Nelore recriados em pastagens sob condições tropicais, com o objetivo de melhorar a fermentação ruminal, a digestibilidade dos nutrientes e o desempenho animal, especialmente durante a estação seca. O projeto foi desenvolvido em duas etapas complementares. Na primeira etapa, conduzimos um estudo *in vitro* para compreender como diferentes combinações de *Bacillus* spp. e leveduras hidrolisadas ou inativadas modulam a fermentação de forragens de baixa e média qualidade. Observamos que a associação entre *Bacillus* spp. e levedura hidrolisada elevou a produção total de ácidos graxos de cadeia curta (AGCC) e as concentrações de acetato e propionato, além de aumentar a disponibilidade de nitrogênio amoniacal (N-NH_3). Esses resultados indicam que a combinação de *Bacillus* e levedura hidrolisada pode favorecer o ambiente ruminal, estimular o crescimento microbiano e potencializar a utilização de nutrientes provenientes de forragens tropicais — justificando, assim, sua seleção para avaliação em experimentos *in vivo*. A segunda etapa foi composta por dois experimentos in vivo. No Experimento 1, avaliamos o metabolismo ruminal de 30 bovinos canulados recebendo suplemento com *Bacillus* ou com *Bacillus* associado à levedura hidrolisada. O tratamento com *Bacillus* + levedura aumentou a digestibilidade da proteína bruta, sem alterar ingestão de matéria seca, pH ou perfil de AGCC, embora a dinâmica do N-NH_3 tenha sido influenciada pelo tempo e tratamento. No Experimento 2, avaliamos 84 bovinos Nelore jovens em pastejo ao longo de 132 dias e verificamos que o tratamento *Bacillus* + levedura resultou em maior ganho médio diário quando comparado ao controle. Ambos os tratamentos microbianos (*Bacillus* e *Bacillus* + levedura) promoveram maior área de olho de lombo, indicando efeitos positivos sobre o desenvolvimento muscular. De forma integrada, os resultados mostram que microrganismos de alimentação direta à base de Bacillus, isoladamente ou combinados com levedura hidrolisada, podem melhorar a digestibilidade da proteína, modular positivamente aspectos do metabolismo ruminal e favorecer o desempenho de bovinos em pastejo durante a estação seca. Esses achados reforçam o potencial

do uso estratégico de DFM como alternativa viável em sistemas tropicais de produção de carne bovina.

Palavras-chave: *Bacillus* spp.; digestibilidade de forragens tropicais; fermentação ruminal.

ABSTRACT: this thesis evaluated the inclusion of direct-fed microbial (DFM) additives in the diets of grazing Nellore cattle under tropical conditions, aiming to improve ruminal fermentation, nutrient digestibility, and animal performance, particularly during the dry season. The project was developed in two complementary phases. The first phase consisted of an *in vitro* study designed to understand how different combinations of *Bacillus* spp. and hydrolyzed or inactive yeast modulate the fermentation of low- and medium-quality tropical forages. The combination of *Bacillus* spp. with hydrolyzed yeast increased total short-chain fatty acids (SCFA) and the concentrations of acetate and propionate, in addition to raising ammonia nitrogen (NH_3-N). These findings indicate that this combination can enhance the ruminal environment, stimulate microbial growth, and improve nutrient utilization from tropical forages—supporting its selection for *in vivo* evaluation. The second phase comprised two in vivo experiments. In Experiment 1, we assessed ruminal metabolism in 30 cannulated cattle supplemented with *Bacillus* or with *Bacillus* plus hydrolyzed yeast. The *Bacillus* + yeast treatment increased crude protein digestibility without altering dry matter intake, ruminal pH, or SCFA profile, although NH_3-N dynamics were affected by treatment and time. In Experiment 2, we evaluated 84 young Nellore bulls grazing for 132 days and observed that the *Bacillus* + yeast treatment increased average daily gain compared with the control. Both microbial treatments (*Bacillus* and *Bacillus* + yeast) increased longissimus muscle area, indicating improvements in muscle development. Overall, the results demonstrate that *Bacillus*-based DFMs, alone or combined with hydrolyzed yeast, can enhance protein digestibility, beneficially modulate ruminal metabolism, and improve the performance of grazing cattle during the dry season. These findings reinforce the potential of strategic DFM supplementation in tropical beef production systems.

Key-words: Bacillus spp.; ruminal fermentation; tropical forages digestibility.

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

Introdução

A busca por sistemas de produção de carne bovina sustentáveis tem sido o principal desafio para os profissionais da cadeia produtiva da carne. Desta forma, a intensificação da criação de bovinos a pasto, bem como a melhora no aproveitamento das forragens disponíveis deve ser levado em consideração, uma vez que as forragens são o alimento mais barato para a alimentação de animais ruminantes.

Todavia, os problemas de sazonalidade no Brasil central, além da variação no valor nutritivo das plantas têm sido grandes entraves na produção de carne (Costa et al., 2021a). Neste sentido, a utilização de suplementos dietéticos para suprir as deficiências nutricionais dos animais provocadas pela alteração da composição química das plantas têm sido amplamente difundidos nas fazendas de corte (Reis et al., 2010). Além da utilização da suplementação, a inclusão de aditivos alimentares antimicrobianos também tem sido pauta na nutrição de ruminantes. O uso destes aditivos pode promover melhora no aproveitamento da forragem disponível aos animais, por atuar diretamente na modulação da fermentação ruminal (Castagnino et al., 2018).

A inclusão de aditivos microbianos na dieta de bovinos de corte pode melhorar o desempenho animal e o peso de carcaça (Lopez et al., 2024). Além disso, podem promover a melhora do ambiente ruminal, favorecer o crescimento de microrganismos fibrolíticos e aumentar a degradabilidade da fração fibrosa dos carboidratos (Cappellozza et al., 2023; Oyebade et al., 2023). O emprego de aditivos microbianos e de cepas de leveduras como moduladores da fermentação ruminal têm crescido nos últimos anos (Carvalho et al., 2017; Mombach et al., 2021). Entretanto, os efeitos destes aditivos sobre o desempenho de bovinos de corte criados em pastagens tropicais ainda são bastante inconsistentes na literatura, conforme os dados reportados por Gubbles et al. (2023), o que abre caminho para mais estudos envolvendo diferentes microrganismos e culturas de leveduras.

REVISÃO DE LITERATURA

Produção de bovinos a pasto: principais limitantes

Sistemas de produção de carne baseados no uso de pastagens tropicais podem permitir elevados ganhos por área, quando bem manejados (Sakamoto et al., 2020). Devido à sua anatomia, as plantas de origem tropical, de maneira geral, apresentam baixos valores de digestibilidade e proteína e alto teor de lignina (Carvalho et al., 2017). Além do mais, a sazonalidade climática afeta a qualidade e produtividade das forrageiras, o que impacta de forma direta a produção de carne. Durante a estação seca do ano, no Brasil Central, há queda significativa na qualidade nutricional das pastagens devido aos baixos teores de proteína bruta (PB) (Detmann et al., 2014). Neste período, os níveis de PB em forragens tropicais tendem a ser inferiores a 7%, o que limita a degradação da fibra pelos microrganismos estabelecidos no ambiente ruminal (Batista et al., 2016; Russell et al., 1992) em contrapartida, durante a estação chuvosa a qualidade das plantas melhora em função da maior disponibilidade de energia (Detmann et al., 2014).

Apesar da melhora na qualidade das pastagens na estação chuvosa, é nessa época que ocorre desbalanço da relação entre energia e proteína na forragem, o que não permite que pode limitar os ganhos quando não há utilização de suplmentos (Detmann et al., 2014). Desta forma, o uso de suplementos na dieta de bovinos de corte criados à pasto, pode ser uma alternativa para melhorar o desempenho animal e contornar os entraves encontrados no sistema de produção (Batista et al., 2016; Carvalho et al., 2017; Detmann et al., 2014).

Fermentação ruminal de bovinos a pasto: mecanismos e possibilidades de alteração

As forragens são a base da alimentação de ruminantes, e podem contemplar o atendimento de exigências nutricionais destes animais em sua totalidade, em alguns casos. Entretanto, apresentam menor valor energético quando comparadas aos alimentos concentrados (Adesogan et al., 2019). A

natureza da dieta consumida pelos animais determina os produtos da fermentação, o que influencia a atividade metabólica dos microrganismos ruminais, e os produtos de absorção pelo animal hospedeiro (Owens e Basalan, 2016; Valadares Filho e Pina, 2011). Animais alimentados com dietas que possuem as forragens como base tendem a apresentar maiores valores de pH ruminal (6,2 a 6,9), maior número de microrganismos responsáveis pela degradação da fibra, quando comparados a animais alimentados com dietas com maior proporção de alimentos concentrados; e têm como principal produto da fermentação o ácido acético (Kozloski, 2016; Russell et al., 1992; Valadares Filho e Pina, 2011).

Os ácidos graxos de cadeia curta (AGCC), acético, propiônico e butírico são as fontes primárias de energia para os ruminantes (Russell et al., 1992). Estas características determinam a proporção molar de cada ácido no ambiente ruminal, e caracterizam o tipo de fermentação. Em dietas à base de forragem, a proporção molar dos ácidos acético, propiônico e butírico são geralmente de 65:15:10 moles (Nussio e Campos, 2011). Entretanto, o ácido propiônico é o principal precursor da síntese de glicose para ruminantes (Russell et al., 1992). Desta forma, dietas que propiciam baixas concentrações de ácido propiônico podem ser ponto limitante do ponto de vista produtivo, uma vez que a maioria dos tecidos é dependente de glicose como fonte de energia (Kozloski, 2016; Valadares Filho e Pina, 2011). Desta forma, estratégias nutricionais para manipular a fermentação ruminal podem ser adotadas, com vistas a melhorar a produção animal.

Dentre as estratégias nutricionais disponíveis para manipular a fermentação ruminal estão o uso de suplementos proteicos e energéticos, os quais podem favorecer a sincronicidade de degradação entre carboidratos e compostos nitrogenados (Reis et al., 2010). Além disso, o uso de aditivos moduladores da fermentação, como antibióticos e aditivos naturais como cepas bacterianas e de leveduras também pode contribuir para alterações no metabolismo e fermentação ruminal (Costa et al., 2018; McAllister et al., 2011; Monteiro et al., 2022) e melhorar a resposta animal em condições de pastejo. Estes aditivos têm potencial para alterar as proporções molares dos AGCC, controlar o pH ruminal e modificar as colônias de microrgasnismos, por meio de

mecanismos de inibição ou favorecimento de determinadas cepas (Berchielli e Bertipaglia, 2010).

Efeito de antimicrobianos em bovinos suplementados com baixo concentrado

A suplementação de bovinos em pastagens tropicais é utilizada para corrigir as deficiências nutricionais das plantas, tanto na estação chuvosa como seca do ano, para atender às exigências nutricionais dos animais (Detmann et al., 2014; Silva et al., 2021). Consumos de suplemento de até 0,3% do peso corporal representam efeito de adição em relação ao consumo de pasto, com ausência do efeito substitutivo (Almeida et al., 2018; Silva et al., 2021) . Neste sentido, o uso de suplementos dietéticos associados à antibióticos pode contribuir para aumento no consumo de forragem (Castagnino et al., 2018; Costa et al., 2018).

Antibióticos utilizados como moduladores da fermentação ruminal apresentam efeito positivo no desempenho animal, devido à melhora na eficiência energética (Berchielli e Bertipaglia, 2010), redução da relação acetato:propionato no rúmen (Carvalho et al., 2017), melhora no ganho de peso, melhora do metabolismo ruminal de nitrogênio (Costa et al., 2018). Entretanto, estes efeitos positivos sobre o desempenho de bovinos de corte estão elucidados principalmente em animais confinados (Carvalho et al., 2017), sendo o efeito dos antibióticos sobre o desempenho de animais recebendo suplementação a pasto ainda controverso.

Em estudo realizado por Maciel et al. (2019), os autores verificaram que não houve melhora no desempenho de novilhas Nelore criadas à pasto, e que receberam suplemento dietético com a presença de virginiamicina, monensina ou associação entre ambas. Segundo os autores, houve redução na ingestão do suplemento apenas dos animais que receberam monensina, desta forma, os autores classificaram os resultados como inconsistentes (Maciel et al., 2019). Neste sentido, o estudo realizado por Carvalho et al. (2017) também demonstrou que a suplementação de novilhas Nelore em pastagem tropical com adição de monensina não melhorou o desempenho dos animais em relação ao grupo controle (sem antibiótico).

Em contrapartida, o estudo de Mombach et al. (2021), demonstrou que a suplementação dietética de 1,5% do peso corporal de machos Nelore em pastagem de *Brachiaria brizantha* cv. BRS Piatã melhorou as características de fermentação ruminal, e elevou o número de colônias de bactérias fibrolíticas. De acordo com os autores, os resultados encontrados podem estar relacionados ao nível de suplementação e ao uso dos aditivos avaliados no estudo. Contudo, devido às divergências nos resultados encontrados na literatura, faz-se necessário buscar aditivos alternativos aos antibióticos melhoradores de desempenho, a fim de alcançar resultados concisos.

Uso de aditivos naturais: bactérias e leveduras como microrganismos de alimentação direta, seus efeitos na alimentação de ruminantes e modo de ação

Além das divergências encontradas na comunidade científica acerca dos resultados do uso de aditivos antimicrobianos para bovinos em pastejo, a preocupação da comunidade global quanto aos riscos do uso destes produtos tornou-se um entrave para a indústria da proteína animal (Ban e Guan, 2021). Desta forma, houve avanço no campo do uso dos microrganismos de alimentação direta, do inglês "*direct fed microbials"* (DFM), que são utilizados como melhoradores de desempenho e são fornecidos diretamente aos ruminantes (McAllister et al., 2011).

Dentre os microrganismos mais estudados estão os *Bacillus* sp, como *B. subtilis* e *B. licheniformis, B. amyloliquefaciens e B. coagulans* (Ban e Guan, 2021; Bennett et al., 2021; McAllister et al., 2011). O emprego de bactérias como probióticos ou microrgasnismos de alimentação direta promove benefícios na saúde do hospedeiro, que como consequência melhora o desempenho animal (Ban e Guan, 2021). Além disso, o uso de bactérias não produz resíduos nos produtos de origem animal e não desenvolvem resistência às drogas, uma vez que são essencialmente naturais (Berchielli e Bertipaglia, 2010). O emprego de bactérias como aditivos alimentares para bovinos de corte é pautado no equilíbrio da microbiota ao longo de todo trato gastrintestinal, o que limita o desenvolvimento de espécies patogênicas; além de atuar na fermentação ruminal e na síntese de proteína microbiana (Ban e Guan, 2021).

De acordo com Bennett et al. (2021), os efeitos positivos do uso de bactérias como aditivos possivelmente, estão associados a melhora no sistema imune do animal hospedeiro e na absorção intestinal de nutrientes. Segundo Sun et al. (2013), a adição de *B. subtilis* na dieta de vacas leiteiras aumentou a produção de leite, teor de gordura e rendimento de proteína e lactose do leite. Os autores recomendam o uso de cepas de *Bacillus* como potenciais probióticos para ruminantes (Sun et al., 2013).

Além do emprego de bactérias como microrganismos de alimentação direta para ruminantes, outras culturas microbiológicas que merecem destaque são as leveduras, vivas ou inativadas. Os princípios de atuação das leveduras são o consumo de oxigênio e de ácido lático no ambiente ruminal, e a promoção do crescimento de bactérias celulolíticas (McAllister et al., 2011; Monteiro et al., 2022). Para bovinos mantidos em pastagens, o uso de leveduras pode atuar na melhora da degradabilidade da fibra, produção de fatores de crescimento para os microrganismos, e aumento na produção total dos AGCCs (Berchielli e Bertipaglia, 2010). Outros efeitos positivos do emprego de leveduras vivas no desempenho de bovinos de corte estão associados a melhora no ganho de peso, ingestão de matéria seca e degradabilidade da fibra insolúvel em detergente neutro (FDN) das pastagens (Ban e Guan, 2021). Entretanto, ainda há poucos relatos consistentes na comunidade científica em relação ao uso de bactérias e leveduras vivas ou inativadas como aditivos alimentares para bovinos de corte em pastejo.

Os microrganismos de alimentação direta (DFM) têm sido amplamente estudados como estratégias nutricionais capazes de modular a fermentação ruminal, melhorar a utilização de forragens e favorecer o desempenho de ruminantes em sistemas tropicais. Seu modo de ação é multifatorial e envolve interações diretas com a microbiota ruminal, efeitos enzimáticos sobre a degradação dos nutrientes, modulação do ambiente fermentativo e alterações no metabolismo do nitrogênio, podendo ainda influenciar a saúde intestinal e a estabilidade do ecossistema ruminal. Estudos recentes reforçam que esses mecanismos atuam de forma integrada, resultando em respostas produtivas especialmente evidentes quando as dietas são baseadas em forragens tropicais de baixa ou média qualidade.

A modulação da microbiota ruminal é um dos mecanismos centrais dos DFM. Pesquisas recentes mostram que combinações de Bacillus subtilis, B. licheniformis e outras espécies podem alterar a estrutura microbiana, estimulando populações fibrolíticas e rotas fermentativas mais eficientes, elevando as concentrações totais de ácidos graxos de cadeia curta (AGCC) e, em vários casos, aumentando a proporção de acetato e propionato (Lopez et al., 2024; Cappellozza et al., 2023). Esses efeitos sugerem maior degradação da fibra e melhor disponibilização de energia fermentável. Resultados observados em estudos com dietas tropicais, como os apresentados por Gubbels et al. (2023), indicam que o uso prolongado de DFM pode melhorar o desempenho animal e a eficiência de utilização da energia líquida, reforçando o impacto positivo dessa modulação microbiana em sistemas produtivos tropicais.

Outro eixo fundamental envolve a ação enzimática dos DFM. Cepas de Bacillus são reconhecidas por sua capacidade de produzir um conjunto diverso de enzimas — incluindo celulases, hemicelulases, amilases e proteases — que complementam a atividade dos microrganismos ruminais nativos e aceleram a degradação de carboidratos estruturais e não estruturais (Luise et al., 2022; Shi et al., 2017). Paralelamente, derivados de leveduras, como leveduras hidrolisadas, atuam como fontes de aminoácidos, peptídeos, nucleotídeos e vitaminas do complexo B, fornecendo fatores de crescimento essenciais para o aumento da taxa de colonização microbiana da partícula vegetal (Baker et al., 2022). Esse conjunto de mecanismos explica os incrementos consistentes em digestibilidade relatados por Cappellozza et al. (2023), que observaram melhor degradabilidade de diversos substratos quando DFMs foram associados a produtos derivados de leveduras.

Os efeitos sobre o ambiente ruminal também desempenham um papel determinante. Leveduras vivas e hidrolisadas têm a capacidade de remover oxigênio introduzido durante a ingestão, criando um ambiente anaeróbio mais favorável ao desenvolvimento de bactérias celulolíticas (Jouany, 2006; Firkins & Mitchell, 2023). Além disso, tais produtos ajudam a estabilizar o pH ruminal ao competir com microrganismos lactato-produtores ou ao estimular populações lactato-utilizadoras, reduzindo a probabilidade de flutuações bruscas no ambiente fermentativo. Esse efeito é especialmente relevante em sistemas

tropicais, nos quais a qualidade da forragem varia sazonalmente e pode prejudicar a homeostase ruminal.

Outro mecanismo amplamente relatado na literatura recente diz respeito ao metabolismo do nitrogênio. A inclusão de DFM frequentemente resulta em maiores concentrações de nitrogênio amoniacal (NH_3-N), como demonstrado por Pan et al. (2022) e Cappellozza et al. (2023). Esse aumento pode refletir maior degradação proteica ou maior liberação de compostos nitrogenados pela ação enzimática das bactérias, o que, quando bem sincronizado com a disponibilidade de carboidratos fermentáveis, favorece a síntese de proteína microbiana — uma das principais fontes de aminoácidos metabolizáveis para ruminantes. Silva et al. (2024) também observaram que a associação entre *Bacillus* spp. e produtos de levedura estimulam comunidades microbianas benéficas e otimiza a assimilação de nitrogênio, reforçando o potencial dessas combinações em sistemas baseados em pastagens de baixa proteína.

Adicionalmente, alguns DFMs podem exercer efeitos positivos sobre a saúde intestinal ao inibir patógenos, seja por competição por nichos, seja pela produção de compostos antimicrobianos, como bacteriocinas. Estudos recentes mostraram que determinadas cepas de Bacillus são capazes de reduzir a carga de microrganismos indesejáveis no trato digestivo, potencialmente diminuindo perdas subclínicas associadas a desafios sanitários e favorecendo melhor desempenho produtivo (Bennett et al., 2021). Esse tipo de resposta tende a ser mais evidente em animais submetidos a estresse nutricional ou ambiental, como ocorre durante a estação seca.

Há ainda evidências de que certos DFMs podem contribuir para mitigar emissões de metano entérico. Sarmikasoglou et al. (2024) e Jia et al. (2023) reportaram reduções moderadas em metanogênese associadas ao uso de Bacillus subtilis, possivelmente devido ao desvio do hidrogênio para rotas de produção de propionato ou por interações com arqueias metanogênicas. Contudo, esses efeitos são altamente variáveis entre estudos e dietas, sugerindo que o impacto sobre o metano depende fortemente do contexto nutricional.

Apesar dos benefícios observados, meta-análises recentes indicam grande variabilidade nas respostas aos DFM, e que tais efeitos dependem da cepa utilizada, dose, tipo de produto (vivo, inativado, hidrolisado), via de fornecimento e qualidade da dieta (Baker et al., 2022; Firkins & Mitchell, 2023).

Por isso, recomenda-se que a seleção de produtos seja baseada em evidências específicas e em avaliações prévias, incluindo ensaios in vitro que permitam triagem mais precisa antes da sua aplicação em experimentos in vivo.

Em síntese, os microrganismos de alimentação direta exercem seus efeitos por meio de vias complementares que envolvem modulação da microbiota, estímulo enzimático, melhora do ambiente ruminal e interação com o metabolismo do nitrogênio, além de potenciais efeitos sobre saúde intestinal e sustentabilidade ambiental. Quando adequadamente selecionados e alinhados às características da dieta, esses aditivos têm potencial para melhorar a digestibilidade da fibra, aumentar a eficiência fermentativa e favorecer o desempenho produtivo de bovinos em sistemas tropicais, especialmente aqueles baseados em pastagens com variação sazonal de qualidade.

Referências

ABOAGYE, Isaac A. *et al.* Environmental performance of commercial beef production systems utilizing conventional productivity-enhancing technologies. **Translational Animal Science**, v. 6, n. 3, 1 jul. 2022.

ADESOGAN, A. T. *et al.* Symposium review: Technologies for improving fiber utilization. **Journal of Dairy Science**, v. 102, n. 6, p. 5726–5755, 1 jun. 2019.

ALLEN, M. S. Carbohydrate nutrition. **The Veterinary clinics of North America. Food animal practice**, v. 7, n. 2, p. 327–340, 1991.

ASSOCIATION OFFICIAL OF ANALYTICAL CHEMISTS. **Official Methods of Analysis of AOAC International**. 18a. ed. Gaithersburg M.D: AOAC, 2006.

BACH, A.; CALSAMIGLIA, S.; STERN, M. D. Nitrogen metabolism in the rumen. **Journal of Dairy Science**, v. 88, n. S, p. E9–E21, 2005.

BAKER, L. M. *et al.* **Review: The effects of dietary yeast and yeast-derived extracts on rumen microbiota and their function**. **Animal Feed Science and Technology**Elsevier B.V., , 1 dez. 2022.

BAN, Yajing; GUAN, Le Luo. **Implication and challenges of direct-fed microbial supplementation to improve ruminant production and health**. **Journal of Animal Science and Biotechnology**BioMed Central Ltd, , 1 dez. 2021.

BATISTA, E. D. *et al.* Effects of varying ruminally undegradable protein supplementation on forage digestion, nitrogen metabolism, and urea kinetics in nellore cattle fed low-quality tropical forage. **Journal of Animal Science**, v. 94, n. 1, p. 201–216, 2016.

BAYER, Edward A. *et al.* **Cellulose, cellulases and cellulosomesCurrent Opinion in Structural Biology**. *[S.l.: S.n.]*. Disponível em: <http://biomednet.com/elecref/O959440XO0800548>.

BENNETT, S. L. *et al.* Effects of bacterial cultures, enzymes, and yeast-based feed additive combinations on ruminal fermentation in a dual-flow continuous culture system. **Translational Animal Science**, v. 5, n. 2, 1 abr. 2021.

BERCHIELLI, Telma Teresinha; BERTIPAGLIA, Liandra Maria Abaker. Utilização de aditivos na produção de bovinos de corte. *In*: VAZ PIRES, Alexandre (Org.). **Bovinocultura de Corte**. Piracibaca: FEALQ, 2010. v. I p. 295–330.

CALSAMIGLIA, S.; FERRET, A.; DEVANT, M. Effects of pH and pH fluctuations on microbial fermentation and nutrient flow from a dual-flow continuous culture system. **Journal of Dairy Science**, v. 85, n. 3, p. 574–579, 2002.

CAPPELLOZZA, Bruno I. *et al.* Evaluation of a Bacillus-based direct-fed microbial probiotic on in vitro rumen gas production and nutrient digestibility of different feedstuffs and total mixed rations. **Translational Animal Science**, v. 7, n. 1, 1 jan. 2023.

CARVALHO, V. V. *et al.* Effects of supplements containing different additives on nutritional and productive performance of beef cattle grazing tropical grass. **Tropical Animal Health and Production**, v. 49, n. 5, p. 983–988, 1 jun. 2017.

CASTAGNINO, Pablo S. *et al.* Changes in ruminal fermentation and microbial population of feedlot Nellore cattle fed crude glycerin and virginiamycin. **Animal Feed Science and Technology**, v. 242, p. 69–76, 1 ago. 2018.

CECONI, Irene *et al.* Combined use of monensin and virginiamycin to improve rumen and liver health and performance of feedlot-finished steers. **Translational Animal Science**, v. 6, n. 4, 1 out. 2022.

CHANEY, A. L.; MARBACH, E. P. Modified reagents for determination of urea and ammonia. **Clinical chemistry**, v. 8, p. 130–132, 1962.

COOKE, Reinaldo F. *et al.* Board Invited Review-Cattle adapted to tropical and subtropical environments (I): social, nutritional, and carcass quality considerations. **Journal of Animal Science**, v. 98, n. 2, 2020.

COSTA, C. M. *et al.* Grazing intensity as a management strategy in tropical grasses for beef cattle production: a meta-analysis. **Animal**, v. 15, n. 4, 1 abr. 2021a.

COSTA, C. M. *et al.* Grazing intensity as a management strategy in tropical grasses for beef cattle production: a meta-analysis. **Animal**, v. 15, n. 4, 1 abr. 2021b.

COSTA, João Paulo R. *et al.* Does virginiamycin supplementation affect the metabolism and performance of Nellore bulls grazing under low and high gain rates? **Animal Science Journal**, v. 89, n. 10, p. 1432–1441, 1 out. 2018.

DE ALMEIDA, Daniel Mageste *et al.* Supplementation strategies for Nellore female calves in creep feeding to improve the performance: nutritional and metabolic responses. **Tropical Animal Health and Production**, v. 50, n. 8, p. 1779–1785, 1 dez. 2018.

DETMANN, Edenio *et al.* An evaluation of the performance and efficiency of nitrogen utilization in cattle fed tropical grass pastures with supplementation. **Livestock Science**, v. 162, n. 1, p. 141–153, 2014.

E SILVA, Yasmim Rodrigues Vilas Boas *et al.* Supplementation with different protein profiles for grazing beef cattle supplemented in tropical grass during the rainy-dry transition season. **Tropical Animal Health and Production**, v. 53, n. 1, 1 dez. 2021.

FIRKINS, J. L.; MITCHELL, K. E. **Invited review: Rumen modifiers in today's dairy rations**. **Journal of Dairy Science**Elsevier Inc., , 1 maio 2023.

GUBBELS, Erin R. *et al.* Evaluation of long-Term supplementation of a direct-fed microbial and enzymatically hydrolyzed yeast cell culture product on feedlot growth performance, efficiency of dietary net energy utilization, heat stress measures, and carcass characteristics in beef steers. **Translational Animal Science**, v. 7, n. 1, 1 jan. 2023.

HIGGS, R. J. *et al.* Updating the Cornell Net Carbohydrate and Protein System feed library and analyzing model sensitivity to feed inputs. **Journal of Dairy Science**, v. 98, n. 9, p. 6340–6360, 2015.

HRISTOV, A. N. *et al.* A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. **Journal of Dairy Science**, v. 95, n. 9, p. 5299–5307, 2012.

JIA, T.; CHEN, L.; ZHAO, G**. Effects of Bacillus subtilis supplementation on enteric methane emissions and rumen microbial ecology in cattle.** Science of the Total Environment, v. 883, p. 163628, 2023. DOI: 10.1016/j.scitotenv.2023.163628.

JOUANY, J. P. Optimizing rumen functions in the close-up transition period and early lactation to drive dry matter intake and energy balance in cows. **Animal Reproduction Science**, v. 96, n. 3–4, p. 250–264, dez. 2006.

KOZLOSKI, G. B. **Bioquímica dos Ruminantes**. 3a. ed. Santa Maria: Editora UFSM, 2016.

LEVENTINI, M. W. *et al.* **EFFECT OF DIETARY LEVEL OF BARLEY-BASED SUPPLEMENTS AND RUMINAL BUFFER ON DIGESTION AND GROWTH BY BEEF CAITLE'**. *[S.l.: S.n.]*. Disponível em: <https://academic.oup.com/jas/article-abstract/68/12/4334/4705516>.

LOPEZ, Alejandra M. *et al.* Effects of bacterial direct-fed microbial combinations on beef cattle growth performance, feeding behavior, nutrient digestibility, ruminal morphology, and carcass characteristics. **Journal of Animal Science**, v. 102, 2024.

LUISE, Diana *et al.* **Bacillus spp. Probiotic Strains as a Potential Tool for Limiting the Use of Antibiotics, and Improving the Growth and Health of Pigs and Chickens**. **Frontiers in Microbiology**Frontiers Media S.A., , 7 fev. 2022.

LUIZ GUSTAVO NUSSIO, FÁBIO PRUDÊNCIO DE CAMPOS, Milto Luiz Moreira de Lima. Metabolismo de Carboidratos Estuturais. *In*: TELMATERESINHA BERCHIELLI, ALEXANDRE VAZ PIRES, Simone Gisele de Oliveira (Org.). **Nutrição de Ruminantes**. 2a. ed. Jaboticabal: FUNEP, 2011. p. 616.

MACIEL, I. C. F. *et al.* Virginiamycin and sodium monensin supplementation for beef cattle on pasture. **Arquivo Brasileiro de Medicina Veterinaria e Zootecnia**, v. 71, n. 6, p. 1999–2008, 1 nov. 2019.

MARQUES, Rodrigo da Silva; COOKE, Reinaldo Fernandes. **Effects of ionophores on ruminal function of beef cattle**. **Animals**MDPI, , 1 out. 2021.

MCALLISTER, T. A. *et al.* **Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle**. **Canadian Journal of Animal Science**, jun. 2011.

MENKE, K. H. ;. Steingass, H. The Estimation of The Energetic Feed Value Obtained from Chemical Analysis and In Vitro Gas Production Using Rumen Fluid. p. 49, 1988.

MOMBACH, Mircéia Angele *et al.* Association of ionophores, yeast, and bacterial probiotics alters the abundance of ruminal microbial species of pasture intensively finished beef cattle. **Tropical Animal Health and Production**, v. 53, n. 1, 1 dez. 2021.

MONTEIRO, H. F. *et al.* Effects of lactic acid-producing bacteria as direct-fed microbials on the ruminal microbiome. **Journal of Dairy Science**, v. 105, n. 3, p. 2242–2255, 1 mar. 2022.

NASEM. **Nutrient requirements of beef cattle.** Washington, DC: National Academy Press, 2016.

NRC, 2001. **Requirements of Dairy Cattle Seventh Revised Edition , 2001**. *[S.l.: S.n.]*.

OWENS, F.; GOETSCH, A. Ruminal Fermentation. *In*: CHURCH, D. C. (Org.). **The ruminant animal digestive physioly and metabolism.** Englewood Cliffs, NJ: Prentice-Hall, 1988. p. 145–171.

OWENS, F. N.; BASALAN, M. Ruminal Fermentation. *In*: **Rumenology**. *[S.l.: S.n.]*. p. 1–314.

OYEBADE, A. O. *et al.* Effects of direct-fed microbial supplement on ruminal and plasma metabolome of early-lactation dairy cows: Untargeted metabolomics approach. **Journal of Dairy Science**, nov. 2023.

PAN, Liyi *et al.* Effects of a Bacillus-based direct-fed microbial on in vitro nutrient digestibility of forage and high-starch concentrate substrates. **Translational Animal Science**, v. 6, n. 2, 1 abr. 2022.

PARRA, Mariano C. *et al.* The use of live yeast to increase intake and performance of cattle receiving low-quality tropical forages. **Journal of animal science**, v. 99, n. 2, 1 fev. 2021.

REIS, Ricardo Andradade; SIQUEIRA, Gustavo Resende; CASAGRANDE, Daniel Rume. Suplementação alimentar de bovinos em pastagens. *In*: VAZ PIRESE, Alexandre (Org.). **Bovinocultura de Corte**. Piracicaba: FEALQ, 2010. v. I p. 219–255.

RIGON, Fernanda *et al.* Employing direct-fed microbial and hydrolysed yeast to modulate in vitro ruminal fermentation parameters of medium- and low-quality forages. **Journal of Agricultural Science**, 2025.

RUSSELL, J. B. *et al.* A Net Carbohydrate and Protein System for Evaluating Cattle Diets: I. Ruminal Fermentation. **Journal of Animal Science**, n. 70, p. 3551–6561, 1992.

SAKAMOTO, Leandro S. *et al.* Pasture Intensification in Beef Cattle Production can affect Methane Emission Intensity. **Journal of Animal Science**, 2020.

SARMIKASOGLOU, E.; HOLTSHAUSEN, L.; BEAUCHEMIN, K. A. **Ruminal pathways associated with methane reduction in cattle supplemented with Bacillus subtilis.** Journal of Dairy Science, v. 107, n. 3, p. 1856-1870, 2024. DOI: 10.3168/jds.2023-23812.

SHI, Changyou *et al.* Solid-state fermentation of corn-soybean meal mixed feed with Bacillus subtilis and Enterococcus faecium for degrading antinutritional

factors and enhancing nutritional value. **Journal of Animal Science and Biotechnology**, v. 8, n. 1, p. 1–9, 2017.

SILVA, S. C. da; PEDREIRA, C. G. S. Fatores condicionantes e predisponentes da produção animal em pastagens. *In*: VAZ PIRES, A. (Org.). **Bovinocultura de Corte**. 1. ed. Piracicaba: FEALQ, 2010. v. I p. 419–431.

SILVA, Thiago H. *et al.* Evaluation of direct-fed microbials on in vitro ruminal fermentation, gas production kinetic, and greenhouse gas emissions in different ruminants' diet. **Frontiers in Animal Science**, v. 5, 23 jan. 2024.

SHI, Y.; HUANG, H.; ZHAO, L. **Enzymatic activity and functional properties of Bacillus strains used as feed additives in ruminants.** BMC Microbiology, v. 17, p. 199, 2017. DOI: 10.1186/s12866-017-1127-z.

SNIFFEN, C. J. *et al.* A net carbohydrate and protein system for evaluating cattle diets: IV. Predicting amino acid adequacy. **Journal of animal science**, v. 71, n. 5, p. 1298–1311, 1992.

SUN, P.; WANG, J. Q.; DENG, L. F. Effects of Bacillus subtilis natto on milk production, rumen fermentation and ruminal microbiome of dairy cows. **Animal**, v. 7, n. 2, p. 216–222, fev. 2013.

TAGLIAPIETRA, Franco *et al.* Metabolizable energy content of feeds based on 24 or 48h in situ NDF digestibility and on in vitro 24h gas production methods. **Animal Feed Science and Technology**, v. 170, n. 3–4, p. 182–191, 2011.

VALADARES FILHO, SEBASTIÃO DE CAMPOS, PINA, Douglas dos Santos. Fermentação Ruminal. *In*: BERCHIELLI, T.T., PIRES, A.V., OLIVEIRA, S. G. (Org.). **Nutrição de Ruminantes**. 2a. ed. Joboticabal: FUNEP, 2011. p. 616.

VAN SOEST, P. J.; ROBERTSON, J. B.; LEWIS, B. A. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. **Journal of Dairy Science**, v. 74, n. 10, p. 3583–3597, 1991.

YÁÑEZ-RUIZ, D. R. *et al.* Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants-a review. **Animal Feed Science and Technology**, v. 216, p. 1–18, 2016.

CAPÍTULO 2 – Employing Direct-fed microbial and hydrolyzed yeast to modulate *in vitro* ruminal fermentation parameters of medium and low-quality forages

Fernanda Rigon[1], Amanda R. Cagliari[2], Elaine Magnani[3], Marcos I. Marcondes[4,5], Danilo Grandini[6], Brooke Humphrey[7], Pedro D. B. Benedeti[2], Flavio D. Resende[*,2,8], Gustavo R. Siqueira[2,8], Renata H. Branco[3], Eduardo M. Paula[3]

[1]Department of Animal Science, São Paulo State University Júlio de Mesquita Filho, UNESP, Jaboticabal, SP, Brazil, [2]Department of Animal Science, Santa Catarina State University, Chapecó, SC, Brazil, [3]Beef Cattle Research Center, Instituto de Zootecnia, Sertãozinho, SP, Brazil,[4]Department of Animal Sciences, Washington State University, Pullman, D.C., United States, [5]William H. Miner Agricultural Research Institute. Chazy – NY, USA, [6]Phibro Animal Health Corporation, Campinas, SP, Brazil,[7]Phibro Animal Health Corporation, Teaneck, NJ, USA,[8]Department of Animal Science, APTA – Agência Paulista de Tecnologia dos Agronegócios, Colina, SP, Brazil

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Abstract

Nutritional strategies, such as supplementation and additives that modulate ruminal fermentation, can enhance nutrient utilization and animal performance. Thus, this study conducted three experiments to evaluate the effects of combining *Bacillus* species with hydrolyzed and inactive Torula yeast on rumen fermentation and the degradability of tropical forages using the i*n vitro* gas production (GP) technique. The first experiment aimed to assess and screen different combinations of direct-fed microbials (DFM) and hydrolyzed/inactive yeast, and their effects on the ruminal fermentation of *Urochloa brizantha* hay. Exp. 1 was designed in a randomized block design in a 3x3 factorial scheme with the following treatments: 1) Negative control, no DFM inclusion (**NC**); 2) *B. subtilis* and *B. licheniformis* (**SL**); 3) *B. subtilis, B. licheniformis, B. amyloliquefaciens,* and *B. coagulans* (**SLAC**); 4) Hydrolyzed Torula yeast (**HY**); 5) Dry inactive Torula yeast (**IY**); 6) *B. subtilis, B. licheniformis,* and dry inactive Torula yeast (**SL+IY**); 7) *B. subtilis*, *B. licheniformis,* and hydrolyzed Torula yeast (**SL+HY**); 8) *B. subtilis*, *B. licheniformis, B. amyloliquefaciens*, *B. coagulans* and dry inactive Torula yeast (**SLAC+IY**); 9) *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. coagulans* and hydrolyzed Torula yeast (**SLAC+HY**). In **Exp. 1**, hydrolyzed yeast increased Total short-chain fatty acids (SCFA) and acetate in treatments with *Bacillus* species. Additionally, *B. subtilis* increased ammonia nitrogen (NH_3-N), butyrate, and isobutyrate levels, whereas yeast treatments resulted in higher NH_3-N and propionate levels compared to the control. Based on these results, **Exp.2** and **Exp.3** evaluated low and medium-quality forage simultaneously with selected treatments (**NC**, **SL**, **SLAC**, **SL+HY**, and the combination of **SLAC+IY** plus **SLAC+HY**) to assess ruminal fermentation parameters. For low-quality forage, the **SLAC+IY** plus **SLAC+HY** treatment had the highest NH_3-N concentration, while the **SL+HY** treatment had the highest concentrations of total SCFA, acetate, and propionate, as well as the highest GP and *in vitro* digestible organic matter.

For medium-quality forage, the **SL+HY** treatment had the highest concentration of total SCFA. The inclusion of a combination of *Bacillus* species and hydrolyzed Torula yeast may increase the total concentration of SCFA and NH_3-N in the rumen, benefiting microbial protein synthesis and nutrient utilization in grazing beef cattle.

Keywords: *Bacillus* spp.; fiber degradability; *in vitro* digestibility; tropical forages

Introduction

The use of forages for beef animals in tropical regions is crucial for meat production systems. In Brazil, for example, approximately 80% of the animals are finished in 100% grass-fed systems (Cooke et al., 2020; Costa et al., 2021b). However, these regions experience two well-defined seasons: the rainy season, from October to March (spring and summer), during which 70 to 90% of the forage mass is produced; and the dry season, from April to September (fall and winter). This seasonal variation leads to fluctuations in forage mass production and decrease in its nutritional value. During the dry season, forages are of lower quality compared to the rainy season, negatively impacting animal performance, the environmental ecosystem, and system profitability (Detmann et al. 2014; Casagrande et al. 2023; Hubbart et al. 2023). Therefore, improving forage fiber digestibility, especially during the dry season, is an important strategy to maximize animal performance and enhance economic and environmental sustainability.

Supplementing concentrated feed to address nutritional deficiencies in animals resulting from shifts in plant chemical composition has been widely adopted in grass-fed meat production systems, potentially improving nutrient utilization by grazing cattle (Detmann *et al*. 2014; Batista *et al*. 2016, 2017b; Scholz *et al*. 2022). Additionally, the inclusion of direct-fed microbials (DFM) has been extensively investigated in ruminant nutrition. These additives have the potential to enhance the availability of forage nutrients

to animals by directly modulating ruminal fermentation (McAllister et al. 2011; Carvalho et al. 2017; Mombach et al. 2021; Lopez et al. 2024).

Studies have indicated that DFMs might enhance dry matter (DM) and fiber digestibility (Cappellozza et al., 2023), and stimulate the growth of fiber-degrading microorganisms (Adesogan et al., 2019; Firkins; Mitchell, 2023). These growth factors (e.g., B vitamins amino acids and organic acids) are beneficial for rumen microbial communities (Baker et al., 2022; Parra et al., 2021), which can enhance the performance of pasture-raised animals. Moreover, incorporating *Bacillus subtilis* into ruminant diets can stimulate rumen fermentation, promote the growth of beneficial microorganisms, and decrease methane (CH_4) emissions (Sun et al. 2013; Jia et al. 2023; Sarmikasoglou et al. 2024). Additionally, the combination of *B. subtilis* and *B. licheniformis* has the potential to produce fibrolytic enzymes, improve fiber digestibility (Cappellozza et al., 2023; Oyebade et al., 2023), and enhance starch degradation (Cappellozza et al., 2023; Shi et al., 2017; Sun; Wang; Deng, 2013).

Recently, the inclusion of DFM in concentrated supplements to improve the utilization of forages substrates, such as *B. subtilis*, the combination of *B. subtilis* and *B. licheniformis*, *Enterococcus faecium*, and *Saccharomyces cerevisiae*, has been evaluated as a strategy to enhance forage fermentability in the rumen (Cappellozza et al., 2023; Pan et al., 2022; Silva et al., 2024). Most fibrolytic bacteria depend on anaerobic conditions for the development of cellulosomes and the release of their cellulases (Bayer et al., 1998). Yeast products may contribute to enhanced fiber degradation by scavenging oxygen from the newly ingested feed, thereby facilitating the colonization of fibrolytic bacteria (Firkins; Mitchell, 2023; Jouany, 2006). Furthermore, the incorporation of hydrolyzed yeast as a prebiotic may effectively stimulate the growth of cellulolytic bacteria, fiber degradation, and increase the concentration of short-chain fatty acids

(SCFA) in the rumen, such as acetate (Baker *et al*., 2022). However, despite the well-known beneficial effects of inactive Torula yeast in modulating digestion in non-ruminantes (Leeper *et al*. 2022; Verstrepen *et al*. 2023; Timlin *et al*. 2024), there are no studies evaluating this inactive yeast in ruminants. Moreover, to our knowledge, few studies have explored the possible synergistic effects of different *Bacillus* species (e.g., *B. subtilis, licheniformis, amyloliquefaciens, coagulans),* dry inactivated and hydrolyzed Torula yeast on the ruminal fermentation of tropical forages.

Therefore, the objective of our study was to evaluate and screen different combinations of DFMs for future *in vivo* trials, with the potential to enhance ruminal fermentation of tropical forages using the *in vitro* gas production (GP) technique. We hypothesized that the inclusion of DFMs and/or DFMs plus hydrolyzed/inactive Torula yeast on a forage-based diet would alter rumen fermentation parameters (e.g., increase SCFA and ammonia nitrogen (NH_3-N) and improve nutrient availability.

Material and methods

Experiment location and ethic committee approval

The study was conducted at the *Instituto de Zootecnia*, Beef Cattle Research Center, located in Sertãozinho, São Paulo, Brazil. The care and handling of the experimental animals were approved by the Animal Use Ethics Committee of the *Instituto de Zootecnia* under the protocol nº 249-19.

Experiment 1 (Exp. 1)

Experimental design

The first trial (**Exp. 1**) was conducted to evaluate and screen the effects of different DFMs and hydrolyzed Torula yeast combinations on the ruminal fermentation of *Urochloa brizantha* (cv. Marandu) hay. The experiment was designed in a randomized block design

in a 3x3 factorial scheme that included nine treatments (Table 1): 1) negative control (**NC**); 2) *B. subtilis* and *B. licheniformis* (**SL**); 3) *B. subtilis, B. licheniformis, B. amyloliquefaciens,* and *B. coagulans* (**SLAC**); 4) hydrolyzed Torula yeast (**HY**); 5) dry inactive Torula yeast (**IY**); 6) *B. subtilis, B. licheniformis,* and dry inactive Torula yeast (**SL + IY**); 7) *Bacillus subtilis, B. licheniformis,* and hydrolyzed Torula yeast (**SL + HY**); 8) *B. subtilis, B. licheniformis*, *B. amyloliquefaciens*, *B. coagulans*, and dry inactive Torula yeast (**SLAC + IY**); and 9) *B. subtilis, B. licheniformis*, *B. amyloliquefaciens, B. coagulans,* and hydrolyzed Torula yeast (**SLAC + HY**).

These additives were incorporated into a premix composed of corn and corn gluten in varying amounts to ensure all test products had similar crude protein (CP) content. All treatments received an equal amount of these ingredients (0.1 g/0.001 kg of forage). The additive levels were determined in previous unpublished *in vitro* studies. The quantity of DFM used was equivalent to 1^{e9} CFU/head/d, while hydrolyzed Torula yeast and dry inactive Torula yeast inclusion were equivalent to 10 g/head/d and 680 ppm, respectively. All dosage calculations assumed an 80 L rumen capacity. All test products were provided by Phibro Animal Health, Teaneck, NJ, USA.

In this study, we used a 30-bottle automated *in vitro* GP system (Ankom Technology, Macedon, NY, USA) equipped with wireless pressure sensors connected to a computer to evaluate the ruminal fermentation patterns of the tested ingredients. Treatments were assessed over three consecutive 48-h fermentation incubations to measure SCFA and NH_3-N concentrations in the rumen. Each treatment was individually incubated in 250 mL bottles, randomly arranged in the incubator. Each fermentation batch included 3 replicates of each treatment, plus 3 blanks (containing only rumen/mineral/buffer solution), totaling 81 observations.

Experiments 2 and 3 (Exp. 2 and Exp. 3)

Experimental design

The selection of DFMs for these trials was based on the ruminal parameters observed in **Exp.1**. Consequently, the second and third assays evaluated two types of forage: *Urochloa brizantha* cv. Marandu hay (**low-quality forage**, **Exp. 2**, the same used in **Exp. 1**) and *Brachiaria decumbens* grass (**medium-quality forage**, **Exp. 3**). These substrates were chosen to simulate an entire dry season, encompassing two distinct phases: the severe dry season characterized by low-quality forage, and the transitional phase between the end of the rainy season and the beginning of dry season, featuring medium-quality forage. Thus, the five selected treatments evaluated in both forages were: 1) **NC**; 2) **SL**; 3) **SLAC**; 4) **SL + HY**; and 5) **SLAC + IY** plus **SLAC + HY** at 1:1 concentration. The premix inclusions and dosages were the same as in **Exp. 1**.

The experimental design and operational procedures were consistent for both forage types. Thus, a 40-bottle automated *in vitro* GP system (Ankom Technology, Macedon, NY, USA) equipped with wireless pressure sensors connected to a computer was used to evaluate the ruminal fermentation patterns of the tested ingredients. Treatments were assessed in three consecutive 72-hours fermentation incubations to determine the GP profiles and ruminal fermentation parameters. In each incubation, ingredients were individually placed in 250 mL bottles, which were randomly arranged in the incubator. Thus, each fermentation batch included 4 replicates of each treatment, plus 3 blanks (containing only rumen/mineral/buffer solution), totaling 129 observations. Both low and medium-quality forages assays were performed simultaneously.

Operational procedures

All experiments followed the same operational procedures.

Ruminal fluid collection and buffer solutions preparation

Rumen fluid was collected from three Nellore steers (average body weight of 550 kg) that were cannulated in the rumen. The steers were maintained on *Brachiaria decumbens* pasture with mineral salt supplementation. Rumen fluid collections were performed two-h before the morning feeding (Yáñez-Ruiz et al., 2016). A total of 2000 mL of rumen fluid was collected from each animal, immediately filtered through four layers of cheesecloth, and kept in pre-warmed (39^{o}C) thermal bottles. The samples were then promptly transported to the laboratory.

The buffer mineral solution was adapted from the methodology described by Menke and Steingass (1988). The final solution was composed by 50 mL of rumen fluid and 100 mL of buffer mineral solution (1:2 v/v, 150 mL). The rumen fluid was mixed with the buffer mineral solutions in a water bath at 39°C under anaerobic conditions, maintained by continuous N_2 infusion.

In vitro gas production

The trial followed the methodologies proposed by Benedeti *et al*. (2018). Samples were introduced into the bottles and hydrated with 5 mL of deionized water to prevent particle dispersion. Each bottle received 1.00 ± 0.05 g (DM basis) of forage and 0.1 g of premix containing the additive. The headspace was flushed with N_2 to maintain anaerobic conditions. After incubation, the bottles were sealed and placed in a ventilated incubator (EI-450T, ENGCO, Piracicaba, SP, BR) at 39°C with constant agitation (83 rpm) for 72 h. The system recorded cumulative pressure every 5 minutes, with data logged every 60 minutes.

The cumulative pressure data at 24, 48 and 72 hours were converted to mL according to Tagliapietra et al. (2011), using the formula: GP (mL) = (P_c/P_o) x V_o, where P_c is the

cumulated pressure change (kPa) in the bottle headspace; V_o is the bottle headspace volume (95 mL), P_o is the atmospheric pressure read by the equipment at the beginning of the measurement. The final GP volumes were corrected for inoculum contribution by subtracting the final GP of the blank bottles (Pell and Schofield, 1994). The *in vitro* digestible organic matter (IVDOM) was calculated according to Menke & Steingass (1988) as: IVDOM (g/kg DM) = 31.55 + 0.8343GP_{200}, where GP_{200} is the net GP (ml/200 mg DM) at 72 h.

Ammonia-nitrogen, pH, and short-chain fatty acids

The solution pH was measured at the beginning and end of each 72-h incubation. Concurrently, a subsample was collected from each bottle to determine SCFA and NH_3-N concentrations. The collected material was filtered through four layers of cheesecloth. To preserve the samples for NH_3-N analysis, 0.2 mL of a 500 mL/L H_2SO_4 solution was added. The NH_3-N concentration was determined using the colorimetric method described by Chaney & Marbach (1962).

The total SCFA concentrations and profiles were determined using gas chromatography (Nexis CG-2030AF, Shimadzu, Montevideo, Uruguay) equipped with a fused silica capillary column (30m x 0.53nm x 0.5µm film thickness, Sulpelco, Darmstadt, Germany) and a flame ionization detector (FID). Nitrogen was used as the carrier gas at a flow rate of 34.5mL/min^{-1} (Leventini et al. 1990; Timm et al. 2024). The initial temperature was set at 100 ^oC for 2 minutes, then increased to 130 ^cC at a rate of 10 ^oC/minute. Subsequently, the temperature was raised to reach 170 ^oC, at a rate of 15 ^oC/minute and held for 11 minutes. The detector and inlet temperatures were 230 and 250 ^oC, respectively, and 0.5 µL samples were injected in split mode.

Enteric methane production

As the Ankom RF is a vented system, a second closed system consisting of 33 serum bottles (3 bottles per treatment plus 3 blanks) was used to investigate *vitro* enteric CH_4. Each 100 mL serum bottle was filled with 0.2 g of each ingredient and 0.02 g of each supplement containing the additive. Bottles were inoculated with 20 mL of rumen/buffer solution, with the headspace continuously flushed with N_2. After sample incubation, the bottles were sealed and placed in a ventilated incubator (EI-450T, ENGCO, Piracicaba, SP, BR) at 39°C with constant agitation (83 rpm) for 72 h. At the end of each fermentation batch, CH_4 concentrations were measured using high-performance gas chromatography. A gas chromatograph (GC-Nexis 2030AF, Shimadzu, Montevideo, Uruguay) equipped with a capillary column (GS Carbonplot, Agilent Technologies, Santa Clara, CA, USA 30m x 0.32mm) and a dielectric barrier discharge (BID) ionization detector was used. The temperature was programmed to start at 35°C and remain there for 3 minutes, then increase to 140°C at a rate of 25°C/minute and hold for 4 minutes. The detector and inlet temperatures were 140 and 170 ^{o}C, respectively. Samples were injected in split mode, using Helium gas as a carrier.

Chemical analysis

All feeds were ground through a 1 mm screen using a Wiley mill (Thomson Scientific Inc., Philadelphia, PA). Chemical analyses were conducted following AOAC methodology (Association Official of Analytical Chemists, 2006). Samples were analyzed for DM (method 934.01), ash (method 938.08), and CP (method 990.13). Organic matter (OM) was calculated as the difference between DM content and ash. Neutral detergent fiber (NDF) was determined using the methodology proposed by Van Soest et al. (1991) and adapted for the fiber determiner (*TE-149*, Tecnal, Piracicaba, SP,

BRA). The chemical composition of the ingredients, including *Urochloa brizantha* hay (low-quality forage), *Brachiaria decumbens* grass (medium-quality forage), and premix, is presented in Table 2.

Statistical analysis

All statistical analyses were performed in SAS version 9.4 (SAS Institute Inc.). All results were assessed for residual normality and variance homogeneity. For **Exp. 1**, data were collected and analyzed in a randomized block design in a 3x3 factorial scheme by fitting the data using generalized linear mixed model (GLIMMIX procedure) and considering DFMs inclusion as fixed factors and fermentation batch as a random factor. Factor one was considered the levels of *Bacillus*, and factor 2 was considered the Yeast types.

For **Exp. 2 and 3**, data were collected and analyzed in a randomized block design by fitting the data using generalized linear mixed model (GLIMMIX procedure) and considering DFMs inclusion as fixed factors and fermentation batch as a random factor. For all the analyses, differences detected at $P \leq 0.10$ were considered significant.

Results

Experiment 1

There was a significant interaction between *Bacillus* and Yeast affecting Total SCFA ($P = 0.03$), acetate ($P = 0.02$), valerate ($P = 0.03$), isovalerate ($P = 0.03$), and total branched-chain fatty acids (BCFA; $P = 0.07$) in terms of molar concentration (Figure 1). Additionally, there was an interaction between Bacillus and Yeast affecting isovalerate ($P = 0.01$), and valerate ($P = 0.01$) in terms of proportion (Figure 2). In treatments without *Bacillus* (NC, HY, IY), inactivated yeast (IY) resulted in higher isovalerate molar concentration (mM/dL) compared to treatments without yeast (NC; $P = 0.01$). Regarding

proportion comparison (mol/100mol), the no yeast treatment (NC) also had lower isovalerate concentration than other treatments (HY, IY) ($P = 0.01$).

For treatments with *B. subtilis* + *B. lincheniformis* (SL, SL+IY, SL+HY), hydrolyzed yeast (SL+HY) led to higher Total SCFA (S$P < 0.01$), and acetate ($P < 0.01$) molar concentrations compared to inactivated yeast (SL+HY; mM/dL). Conversely, the no yeast treatment (SL) showed higher isovalerate ($P = 0.04$) and BCFA ($P = 0.02$) levels than inactivated yeast (SL+IY; mM/dL). Additionally, regarding proportion comparison (mol/100mol), the no yeast treatment (SL) had a higher isovalerate concentration compared to other treatments (SL+IY, SL+HY; $P = 0.07$).

For combined *Bacillus species* treatments (SLAC, SLAC+IY, SLAC+HY), the no yeast treatment (SLAC) exhibited lower total SCFA (mM/dL), acetate (mM/dL), isovalerate (mM/dL), BCFA (mM/dL), and valerate (mol/100mol) than hydrolyzed yeast (SLAC+HY; $P < 0.01$). The no yeast treatment (SLAC) also had lower SCFA (mM/dL), acetate (mM/dL) compared to inactivated yeast treatment (SLAC+IY; $P = 0.09$).

Since no interaction was observed for the remaining variables, we analyzed the results by considering *Bacillus* and Yeast comparisons separately. For *Bacillus*, the combination of *Bacillus* (SLAC, SLAC+IY, SLAC+HY) resulted in higher NH_3-N levels than the control treatment (NC, $P = 0.09$; Table 3). Additionally, *B. subtilis*+ *B. lincheniformis* (SL, SL+IY, SL+HY) led to higher concentrations of butyrate and isobutyrate (mM) compared to the other treatments (NC, SLAC, SLAC+IY, SLAC+HY; $P < 0.03$). In terms of Yeast comparison, the control treatment (NC) exhibited lower NH_3-N concentration ($P < 0.01$), and propionate (mM; $P = 0.04$) compared to hydrolyzed yeast (HY, SL+HY, SLAC+HY). However, for propionate, the control was similar to inactivated yeast (IY, SL+IY, SLAC+IY). No significant differences were found among treatments for the remaining ruminal fermentation variables ($P > 0.10$).

Experiment 2 (Low-quality forage)

For total GP at 24, 48 and 72-h (mL/g DM), the combinations of **SLAC + IY** and **SLAC + HY** had the lowest values ($P < 0.01$; **Table 4**). The estimated IVOMD for the combination of **SLAC + IY** and **SLAC + HY** exhibited the lowest values ($P = 0.08$). No significant differences were found among treatments for CH_4 concentrations ($P = 0.96$).

The **SLAC + HY** and the combination **SLAC+IY + SLAC+HY** had higher NH_3-N concentrations (mg/dL) compared to **NC** and **SL** ($P < 0.001$), but did not differ from SLAC. The **SL + HY** treatment exhibited a higher concentration of total SCFA compared to the combinations of **SLAC + IY** and **SLAC + HY** ($P = 0.08$). Regarding acetate concentration, **SL + HY** had a higher value compared to **NC** and the combinations of **SLAC + IY** and **SLAC + HY** ($P = 0.07$). Additionally, **SL + HY** had the highest propionate concentration ($P = 0.03$). No significant differences were found among treatments for pH, butyrate, isobutyrate, isovalerate, valerate, and total BCFA ($P > 0.20$).

For the SCFA profile (mol/100 mol), **SL, SL + HY,** and the combination of **SLAC + IY** and **SLAC + HY** had higher proportion of acetate compared to **NC** ($P = 0.01$). The **NC** treatment had a higher proportion of butyrate than the combinations of **SLAC + IY** and **SLAC + HY** ($P = 0.044$). The proportions of propionate, isovalerate, and valerate, as well as acetate: propionate ratio, were similar among all treatments **(**$P = 0.42$, 0.74, 0.86, and 0.20, respectively).

Experiment 3 (Medium-quality forage)

We found no differences among treatments for total GP at 24, 48 and 72-h, IVOMD, CH_4, propionate concentration, and pH ($P > 0.10$; **Table 5**). The combination of **SLAC + IY** and **SLAC+ HY** had higher NH_3-N concentrations compared to **NC, SL,** and **SLAC** ($P < 0.001$). The **SL + HY** treatment had higher values for total SCFA and acetate

concentrations compared to **NC**, **SL** and **SLAC** ($P < 0.02$). For butyrate concentrations, **SL + HY** had higher values than **SL** ($P = 0.01$). Additionally, **SL + HY** had higher isobutyrate concentration compared to **SL** ($P = 0.004$). The **SL + HY** also had higher values of isovalerate, valerate and total BCFA concentrations compared to **NC**, **SL** and **SLAC** ($P < 0.02$).

For the SCFA profile (mol/100 mol), we found no difference among treatments for the acetate proportion ($P = 0.60$). The **SL** had a higher propionate proportion compared to **SL + HY** ($P = 0.01$). Both **NC** and **SL+ HY** had higher butyrate proportions compared to **SL** ($P = 0.052$). For isovalerate proportions, **SL + HY** and the combination of **SLAC + IY** and **SLAC + HY** had higher values compared to **NC**, **SL** and **BSLAC** ($P = 0.002$). Regarding valerate, **SL + HY** had a higher proportion than **NC**, **SL** and **SLAC** ($P = 0.08$). The acetate: propionate ratio was lower for **NC** and **SL** compared to **SL + HY** and the combination of **SLAC + IY** and **SLAC + HY** ($P = 0.016$). Furthermore, **SL + HY** had a higher acetate: propionate ratio than all treatments except the combination of **SLAC + IY** and **SLAC + HY** ($P = 0.016$).

Discussion

Experiment 1

In our study, we hypothesized that combinations of DFMs and yeast products could modulate ruminal fermentation to enhance the degradability of medium and low-quality forage.

In **Exp.1**, we observed that treatments with these additives resulted in higher NH_3-N concentrations compared to the control in both *Bacillus* and Yeast comparisons. As reported by Cappellozza *et al*. (2023), the inclusion of DFMs based on *B. licheniformis* and *B. subtilis* in rumen fermentation might create a synergism between proteins and

fibrolytic enzymes, contributing to the increased NH_3-N concentrations observed. The use of DFMs enhances rumen nutrient digestibility (Cappelloza *et al*., 2023).

The mode of action of *Bacillus* spp. includes a diverse range and quantity of enzymes that improve the degradability of fiber, starch, protein, and lipids (Luise et al., 2022). We speculate that the higher NH_3-N concentrations in these treatments may increase microbial protein synthesis due to more ammonia being available in the medium (Cappellozza et al., 2023; Pan et al., 2022; Sun; Wang; Deng, 2013). According to Silva *et al*. (2024), the inclusion of DFM based on *Bacillus* strains and yeast may benefits microbial communities, stimulating better bioassimilation and increasing microbial protein synthesis. Furthermore, Sun *et al*. (2013) observed that the inclusion of *B. Subtilis* in lactating dairy cow diets, increased ruminal NH_3-N concentrations and milk protein, which aligns with our findings.

The optimal concentration of NH_3-N in ruminal fluid for efficient microbial protein synthesis ranges from 15 to 25 mg/dL throughout the day (NASEM, 2016; NRC, 2001). In **Exp.1**, we observed values below this range. We believe these results may be due to the low CP content in the forage used in the trial. The absence of additional protein and energy sources likely contributed to the low NH_3-N values, as only one premix was incubated with the forage. Additionally, *in vitro* systems do not allow the entry or absorption of substrates, which might alter the normal results of ruminal parameters (Hristov et al., 2012).

Hydrolyzed yeast had the highest concentrations of propionate and isovalerate, while *B. subtilus* plus *lincheniforms* exhibited the greatest concentrations of butyrate and isobutyrate. Additionally, the interaction between Yeast and *Bacillus* showed that both hydrolyzed and inactivated yeast combined with *Bacillus* species increased the total SCFA, acetate, and BCFA concentrations. These results may be attributed to the probiotic

effects of hydrolyzed yeast, which stimulate the growth of cellulolytic bacteria, enhance fiber degradation, and increase the proportion of SCFA (Baker et al., 2022). The presence of *Bacillus* species likely stimulates the production of fibrolytic enzymes and enhances fiber digestion, as reported by Cappellozza *et al*. (2023). This effect, combined with the characteristics of the forage, which contains a high amount of fibrous carbohydrates for degradation in the ruminal environment, likely contributed to the greater SCFA concentrations observed. Furthermore, *Bacillus spp*. can produce a large number of enzymes that enhance nutrient digestibility in the rumen (Luise et al., 2022). In summary, the combination of factors related to the forage substrate characteristics and the feed additives likely contributed to the greater SCFA concentrations observed in treatments with yeast plus *Bacillus*.

Cappellozza *et al*. (2023) suggested that the improvement in fiber breakdown contributes to elevated concentrations of SCFA and BCFA in the ruminal environment. This insight potentially explains the observed higher concentrations of isobutyrate and total BCFA for this treatment. The higher valerate and isovalerate concentrations (mol/100 mol) for the combination of *Bacillus* without yeast could be attributed to the diverse microbiome involved in fiber degradation with the different *Bacillus* strains. Forage-based diets promote greater microbial diversity, which can lead to unpredictable changes in the proportions of SCFA (Adesogan et al., 2019).

Based on the overall results of NH_3-N and SCFA concentration and profiles, the following treatments were selected for evaluation in **Exp. 2**: **SL, SLAC, SL + HY,** and a combination of **SL + IY** and **SL + HY**. These treatments were tested on two different substrates: *Urochloa brizantha* hay (low quality) and *Urochloa decumbens* grass (medium quality). These substrates were chosen to represent pasture conditions during the dry season in Brazil, as previously mentioned.

Experiment 2 and 3

In **Exp.2**, our findings revealed NH_3-N concentrations within the normal range. However, in **Exp.3**, we observed NH_3-N concentrations that exceeded the normal range for medium-quality forage. Since we work in a closed *in vitro* system, where there is no entry of substrates and no removal of final fermentation products (Hristov et al., 2012), we can infer that there was an accumulation of NH_3-N for the **SLAC + IY plus SLAC + HY** treatment with medium-quality forage.

These results may be a consequence of improved ruminal protein degradation of medium-quality fiber. As reported by Silva *et al*. (2024), including DFM on forage-based diets may be improving microbial protein synthesis because it promotes better N bioassimilation, as we mentioned before. This fact might be linked to the increased diet fermentability, which enhances NH_3-N absorption and may improve N utilization on rumen environment (NASEM, 2016). Though not measured in this study, the inoculation of *Bacillus* strains and yeast may have improved *in vitro* DM and NDF digestibility, as concluded by Cappellozza *et al*. (2023) on a study that evaluated different forage sources.

We observed that for both low and medium-quality forage, the **SL + HY** and **SLAC + IY plus SLAC + HY** treatments had the highest concentrations of total SCFA and acetate, compared to **NC**. The use of yeast products may enhance fiber degradation by scavenging oxygen from newly ingested feed, which helps fibrolytic bacteria colonize the feed (Firkins; Mitchell, 2023; Jouany, 2006). This process can improve fermentation rate and SCFA synthesis (Cappellozza et al., 2023; Pan et al., 2022). Additionally, the inclusion of *B. subtilis* in ruminant diets promotes the growth of anaerobic microorganisms in the ruminal environment (Sun; Wang; Deng, 2013).

In symbiosis with the hydrolyzed yeast, which acts as a prebiotic for *B. subtilis*, the *in vitro* environment may have become more favorable for the growth of microbial

species involved in fibrous carbohydrate degradation (Baker et al., 2022; McAllister et al., 2011; Parra et al., 2021). For medium-quality forage (**Exp.3**), **SL + HY** had higher concentrations of total SCFA, acetate, butyrate, *iso*-butyrate, *iso*-valerate, valerate and total BCFA concentrations (m*M*). These results may be related to the probiotic effects of hydrolyzed yeast in combination with the *Bacillus*, as discussed in the results from **Exp. 1**. These BCFA are end products of branched-chain amino acids, which are important for the growth of fibrolytic and cellulolytic bacteria (Adesogan et al., 2019; Calsamiglia; Ferret; Devant, 2002). The effects of **SL + HY** mentioned above may have contributed to the higher BCFA proportions observed for medium-quality forage.

For the SCFA profile (mol/100 mol), we observed that **SL** had the highest acetate proportion and the highest acetate: propionate ratio for low-quality forage. This result may be linked to the characteristics of the microorganisms in the feed additive, since *B. subtilis* and *licheniformis* have the potential to produce fibrolytic enzymes and enhance fiber digestibility (Cappellozza et al., 2023; Oyebade et al., 2023). This improvement could be attributed to the diverse microbiome involved in fiber degradation, as discussed in **Exp. 1**.

For the medium-quality forage, **SL** had the highest proportion of propionate, likely due to the ability of *B. subtilis* and *B. licheniformis* to enhance starch degradation (Cappellozza et al., 2023; Shi et al., 2017; Sun; Wang; Deng, 2013). Propionic acid is the main product of non-fibrous carbohydrate fermentation (Owens; Goetsch, 1988; Owens; Basalan, 2016). In this process, pyruvate conversion pathways, such as acrylate and succinate, produce propionic acid as the final product (Higgs et al., 2015; Owens; Basalan, 2016). This suggests an enhancement in the degradation of the starch contained in the premix used in incubation. However, **SL + HY** had the highest proportions of butyrate, isovalerate, and valerate, as well as the highest acetate: propionate ratio. We

believe these results are directly linked to the effects of yeast-based products as prebiotics and the use of *Bacillus* strains in the ruminal environment. Furthermore, recent studies have shown that these additives may also modulate rumen microbiome composition and reduce methane emissions (Ban & Guan 2021; Cheng *et al*. 2023; Sarmikasoglou *et al*. 2024). While our study observed higher NH_3-N and SCFA concentrations, some results such as the variations in specific SCFA profiles, suggest there may be additional underlying mechanisms at play. Therefore, further research could explore these unexpected outcomes, potentially uncovering new benefits of Bacillus and yeast products.

The GP parameters of low-quality forage indicated that the combination of **SLAC + IY** and **SLAC + HY** resulted in lower GP at 24, 48 and 72-h. According to Allen (1991), a lower fiber fermentation rate can be linked to lower starch content and higher indigestible fractions. These findings likely influenced the total SCFA production for **SLAC + IY plus SLAC + HY** in low-quality forage, as this treatment exhibited the lowest total SCFA (m*M*)compared to other treatments. However, no significant differences were observed in the GP production parameters during the experiment with medium-quality forage. This could be due to the substrate's characteristics, as forages typically contain a higher concentration of carbohydrate fraction B (Allen, 1991; Higgs et al., 2015; Sniffen et al., 1992), which degrades after more than 24 hours in the rumen environment (NASEM, 2016). These characteristics may have facilitated a more uniform fermentation process, potentially mitigating the effects of additives in this context. Our study demonstrated that incorporating yeast-based products alongside *Bacillus* strains can enhance forage degradation and optimize the fermentation environment for the tropical forages evaluated.

Conclusion

Combining *Bacillus* strains with dry inactive and hydrolyzed Torula yeast on low and medium-quality forage can enhance the concentration and profile of total SCFA, including acetate and BCFA. These combinations also increase NH_3-N concentration in the rumen, potentially boosting microbial protein synthesis and nutrient utilization. Therefore, using DFMs, especially *B. subtilis* and *B. licheniformis*, in combination with hydrolyzed yeast, may improve feed utilization for low-quality forage in grazing beef cattle. Future *in vivo* studies are needed to evaluate these combinations on beef cattle performance during the dry season in tropical regions.

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References

ADESOGAN, A.T., ARRIOLA, K.G., JIANG, Y., OYEBADE, A., PAULA, E.M., PECH-CERVANTES, A.A., ROMERO, J.J., FERRARETTO, L.F. & VYAS, D. (2019). Symposium review: Technologies for improving fiber utilization. *Journal of Dairy Science*, **102**, 5726–5755.

ALLEN, M.S. (1991). Carbohydrate nutrition. *The Veterinary clinics of North America. Food animal practice*, **7**, 327–340.

ASSOCIATION OFFICIAL OF ANALYTICAL CHEMISTS. (2006). *Official Methods of Analysis of AOAC International*, 18a ed (Ed AOAC). Gaithersburg M.D: AOAC.

BAKER, L.M., KRAFT, J., KARNEZOS, T.P. & GREENWOOD, S.L. (2022). Review: The effects of dietary yeast and yeast-derived extracts on rumen microbiota and their function. *Animal Feed Science and Technology*, **294**.

BAN, Y. & GUAN, L.L. (2021). Implication and challenges of direct-fed microbial supplementation to improve ruminant production and health. JOURNAL OF ANIMAL SCIENCE AND BIOTECHNOLOGY, 12, 109.

BAYER, E.A., CHANZYT, H., LAMED¢-, R. & SHOHAM, Y. (1998). *Cellulose, Cellulases and Cellulosomes*.

BENEDETI, P.D.B., FONSECA, M.A., SHENKORU, T., MARCONDES, M.I., PAULA, E.M. DE, SILVA, L.G. DA & FACIOLA, A.P. (2018). DOES PARTIAL REPLACEMENT OF CORN WITH GLYCERIN IN BEEF CATTLE DIETS AFFECT IN VITRO RUMINAL FERMENTATION, GAS PRODUCTION KINETIC, AND ENTERIC GREENHOUSE GAS EMISSIONS? PLOS ONE, 13, E0199577.

CALSAMIGLIA, S., FERRET, A. & DEVANT, M. (2002). Effects of pH and pH fluctuations on microbial fermentation and nutrient flow from a dual-flow continuous culture system. *Journal of Dairy Science*, **85**, 574–579.

CAPPELLOZZA, B.I., JOERGENSEN, J.N., COPANI, G., BRYAN, K.A., FANTINATI, P., BODIN, J.C., KHAHI, M.M., NINODEGUZMAN, C., ARRIOLA, K.G., LIMA, L.O., FAROOQ, S. & VYAS, D. (2023). Evaluation of a Bacillus-based direct-fed microbial probiotic on in vitro rumen gas production and nutrient digestibility of different feedstuffs and total mixed rations. *Translational Animal Science*, **7**.

CARVALHO, V. V., PAULINO, M.F., DETMANN, E., CHIZZOTTI, M.L., MARTINS, L.S., SILVA, A.G., LOPES, S.A. & MOURA, F.H. (2017). Effects of supplements containing different additives on nutritional and productive performance of beef cattle grazing tropical grass. *Tropical Animal Health and Production*, **49**, 983–988.

CASAGRANDA, Y.G., WIŚNIEWSKA-PALUSZAK, J., PALUSZAK, G., MORES, G. DE V., MORO, L.D., MALAFAIA, G.C., AZEVEDO, D.B. DE & ZHANG, D. (2023). EMERGENT RESEARCH THEMES ON SUSTAINABILITY IN THE BEEF CATTLE INDUSTRY IN BRAZIL: AN INTEGRATIVE LITERATURE REVIEW. SUSTAINABILITY, 15.

CHANEY, A.L. & MARBACH, E.P. (1962). Modified reagents for determination of urea and ammonia. *Clinical chemistry*, **8**, 130–132.

CHENG, Y., ZHANG, J., REN, W., ZHANG, L. & XU, X. (2023). RESPONSE OF A NEW RUMEN-DERIVED BACILLUS LICHENIFORMIS TO DIFFERENT CARBON SOURCES. FRONTIERS IN MICROBIOLOGY, 14.

COOKE, R.F., DAIGLE, C.L., MORIEL, P., SMITH, S.B., TEDESCHI, L.O. & VENDRAMINI, J.M.B. (2020). Board Invited Review-Cattle adapted to tropical and subtropical environments (I): social, nutritional, and carcass quality considerations. *Journal of Animal Science*, **98**.

COSTA, C.M., DIFANTE, G.S., COSTA, A.B.G., GURGEL, A.L.C., FERREIRA, M.A. & SANTOS, G.T. (2021). Grazing intensity as a management strategy in tropical grasses for beef cattle production: a meta-analysis. *Animal*, **15**.

DETMANN, E., VALENTE, É.E.L., BATISTA, E.D. & HUHTANEN, P. (2014). An evaluation of the performance and efficiency of nitrogen utilization in cattle fed tropical grass pastures with supplementation. *Livestock Science*, **162**, 141–153.

FIRKINS, J.L. & MITCHELL, K.E. (2023). Invited review: Rumen modifiers in today's dairy rations. *Journal of Dairy Science*, **106**, 3053–3071.

GETACHEW, G., CROVETTO, G.M., FONDEVILA, M., KRISHNAMOORTHY, U., SINGH, B., SPANGHERO, M., STEINGASS, H., ROBINSON, P.H. & KAILAS, M.M. (2002). Laboratory

variation of 24 h in vitro gas production and estimated metabolizable energy values of ruminant feeds. *Animal Feed Science and Technology*, **102**, 169–180.

HIGGS, R.J., CHASE, L.E., ROSS, D.A. & VAN AMBURGH, M.E. (2015). Updating the Cornell Net Carbohydrate and Protein System feed library and analyzing model sensitivity to feed inputs. *Journal of Dairy Science*, **98**, 6340–6360.

HRISTOV, A.N., LEE, C., HRISTOVA, R., HUHTANEN, P. & FIRKINS, J.L. (2012). A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. *Journal of Dairy Science*, **95**, 5299–5307.

HUBBART, J.A., BLAKE, N., HOLÁSKOVÁ, I., MATA PADRINO, D., WALKER, M. & WILSON, M. (2023). CHALLENGES IN SUSTAINABLE BEEF CATTLE PRODUCTION: A SUBSET OF NEEDED ADVANCEMENTS. CHALLENGES, 14, 14.

HUHTANEN, P., SEPPÄLÄ, A., AHVENJÄRVI, S. & RINNE, M. (2008). Prediction of in vivo neutral detergent fiber digestibility and digestion rate of potentially digestible neutral detergent fiber: Comparison of models. *Journal of Animal Science*, **86**, 2657–2669.

JIA, P., DONG, L., TU, Y. & DIAO, Q. (2023). BACILLUS SUBTILIS AND MACLEAYA CORDATA EXTRACT REGULATE THE RUMEN MICROBIOTA ASSOCIATED WITH ENTERIC METHANE EMISSION IN DAIRY COWS. MICROBIOME, 11, 229.

JOUANY, J.P. (2006). Optimizing rumen functions in the close-up transition period and early lactation to drive dry matter intake and energy balance in cows. *Animal Reproduction Science*, **96**, 250–264.

KEIM, J.P., ALVARADO-GILIS, C., ARIAS, R.A., GANDARILLAS, M. & CABANILLA, J. (2017). Evaluation of sources of variation on in vitro fermentation kinetics of feedstuffs in a gas production system. *Animal Science Journal*, **88**, 1547–1555.

LEEPER, A., EKMAY, R., KNOBLOCH, S., SKÍRNISDÓTTIR, S., VARUNJIKAR, M., DUBOIS, M., SMÁRASON, B.Ö., ÁRNASON, J., KOPPE, W. & BENHAÏM, D. (2022). TORULA YEAST IN THE DIET OF ATLANTIC SALMON SALMO SALAR AND THE IMPACT ON GROWTH PERFORMANCE AND GUT MICROBIOME. SCIENTIFIC REPORTS, 12, 567.

LEVENTINI, M.W., HUNT2, C.W., ROFFLER, R.E. & CASEBOLT, D.G. (1990). *EFFECT OF DIETARY LEVEL OF BARLEY-BASED SUPPLEMENTS AND RUMINAL BUFFER ON DIGESTION AND GROWTH BY BEEF CAITLE'*.

LOBO, R.R. & FACIOLA, A.P. (2021). Ruminal Phages – A Review. *Frontiers in Microbiology*, **12**.

LOPEZ, A.M., SARTURI, J.O., JOHNSON, B.J., WOERNER, D.R., HENRY, D.D., CIRIACO, F.M., SILVA, K.G.S. & RUSH, C.J. (2024). EFFECTS OF BACTERIAL DIRECT-FED MICROBIAL COMBINATIONS ON BEEF CATTLE GROWTH PERFORMANCE, FEEDING BEHAVIOR, NUTRIENT DIGESTIBILITY, RUMINAL MORPHOLOGY, AND CARCASS CHARACTERISTICS. JOURNAL OF ANIMAL SCIENCE, 102.

LUISE, D., BOSI, P., RAFF, L., AMATUCCI, L., VIRDIS, S. & TREVISI, P. (2022). Bacillus spp. Probiotic Strains as a Potential Tool for Limiting the Use of Antibiotics, and Improving the Growth and Health of Pigs and Chickens. *Frontiers in Microbiology*, **13**.

MCALLISTER, T.A., BEAUCHEMIN, K.A., ALAZZEH, A.Y., BAAH, J., TEATHER, R.M. & STANFORD, K. (2011). Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle. *Canadian Journal of Animal Science*, **91**, 193–211.

MENKE, K.H.; S.H. (1988). The Estimation of The Energetic Feed Value Obtained from Chemical Analysis and In Vitro Gas Production Using Rumen Fluid. , 49.

MOMBACH, M.A., DA SILVA CABRAL, L., LIMA, L.R., FERREIRA, D.C., PEDREIRA, B.C. & PEREIRA, D.H. (2021). Association of ionophores, yeast, and bacterial probiotics alters

the abundance of ruminal microbial species of pasture intensively finished beef cattle. *Tropical Animal Health and Production*, **53**.

NASEM. (2016). *Nutrient Requirements of Beef Cattle.* (Ed NASEM). Washington, DC: National Academy Press.

NRC, 2001. (2001). *Requirements of Dairy Cattle Seventh Revised Edition , 2001*.

OWENS, F.N. & BASALAN, M. (2016). Ruminal Fermentation. In *Rumenology*, pp. 1–314.

OWENS, F. & GOETSCH, A. (1988). Ruminal Fermentation. In *The ruminant animal digestive physioly and metabolism.* (Ed D.C. Church), pp. 145–171. Englewood Cliffs, NJ: Prentice-Hall.

OYEBADE, A.O., TAIWO, G.A., IDOWU, M., TAYLOR, S., QUEIROZ, O., ADESOGAN, A.T., VYAS, D. & OGUNADE, I.M. (2023). Effects of direct-fed microbial supplement on ruminal and plasma metabolome of early-lactation dairy cows: Untargeted metabolomics approach. *Journal of Dairy Science*.

PAN, L., HARPER, K., QUEIROZ, O., COPANI, G. & CAPPELLOZZA, B.I. (2022). Effects of a Bacillus-based direct-fed microbial on in vitro nutrient digestibility of forage and high-starch concentrate substrates. *Translational Animal Science*, **6**.

PARRA, M.C., COSTA, D.F.A., PALMA, A.S.V., CAMARGO, K.D.V., LIMA, L.O., HARPER, K.J., MEALE, S.J. & SILVA, L.F.P. (2021). The use of live yeast to increase intake and performance of cattle receiving low-quality tropical forages. *Journal of animal science*, **99**.

PELL, A.N. & SCHOFIELD, P. (1993). Computerized Monitoring of Gas Production to Measure Forage Digestion In Vitro. *Journal of Dairy Science*, **76**, 1063–1073.

SARMIKASOGLOU, E., SUMADONG, P., DAGAEW, G., JOHNSON, M.L., VINYARD, J.R., SALAS-SOLIS, G., SIREGAR, M. & FACIOLA, A.P. (2024). Effects of Bacillus subtilis on in vitro ruminal fermentation and methane production. Translational Animal Science, 8.

SHI, C., ZHANG, Y., LU, Z. & WANG, Y. (2017). Solid-state fermentation of corn-soybean meal mixed feed with Bacillus subtilis and Enterococcus faecium for degrading antinutritional factors and enhancing nutritional value. *Journal of Animal Science and Biotechnology*, **8**, 1–9.

SILVA, T.H., AMÂNCIO, B.R., MAGNANI, E., MEURER, G.W., REOLON, H.G., TIMM, T.G., CAPPELLOZZA, B.I., BRANCO, R.H. & PAULA, E.M. (2024). Evaluation of direct-fed microbials on in vitro ruminal fermentation, gas production kinetic, and greenhouse gas emissions in different ruminants' diet. *Frontiers in Animal Science*, **5**.

SNIFFEN, C.J., O'CONNOR, D., FOX, D.G. & CHALUPA, W. (1992). A net carbohydrate and protein system for evaluating cattle diets: IV. Predicting amino acid adequacy. *Journal of animal science*, **71**, 1298–1311.

SCHOLZ BERÇA, A., PRATES ROMANZINI, E., DA SILVA CARDOSO, A., EDUARDO FERREIRA, L., PASTORI D'AUREA, A., BERTELLI FERNANDES, L. & ANDRADE REIS, R. (2022). ADVANCES IN PASTURE MANAGEMENT AND ANIMAL NUTRITION TO OPTIMIZE BEEF CATTLE PRODUCTION IN GRAZING SYSTEMS.

VERSTREPEN, L., CALATAYUD-ARROYO, M., DUYSBURGH, C., DE MEDTS, J., EKMAY, R.D. & MARZORATI, M. (2023). AMINO ACID DIGESTIBILITY OF DIFFERENT FORMULATIONS OF TORULA YEAST IN AN IN VITRO PORCINE GASTROINTESTINAL DIGESTION MODEL AND THEIR PROTECTIVE EFFECTS ON BARRIER FUNCTION AND INFLAMMATION IN A CACO-2/THP1CO-CULTURE MODEL. ANIMALS, 13, 2812.

VAN SOEST, P.J., ROBERTSON, J.B. & LEWIS, B.A. (1991). Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science*, **74**, 3583–3597.

SUN, P., WANG, J.Q. & DENG, L.F. (2013). Effects of Bacillus subtilis natto on milk production, rumen fermentation and ruminal microbiome of dairy cows. *Animal*, **7**, 216–222.

TAGLIAPIETRA, F., CATTANI, M., HANSEN, H.H., HINDRICHSEN, I.K., BAILONI, L. & SCHIAVON, S. (2011). Metabolizable energy content of feeds based on 24 or 48h in situ NDF digestibility and on in vitro 24h gas production methods. *Animal Feed Science and Technology*, **170**, 182–191.

TIMM, T.G., AMÂNCIO, B.R., LOREGIAN, K.E., MAGNANI, E., HELM, C.V., DE LIMA, E.A., MARCONDES, M.I., BRANCO, R.H., DE PAULA, E.M., BENEDETI, P.D.B. & TAVARES, L.B.B. (2024). PEACH PALM SHELLS (BACTRIS GASIPAES KUNTH) BIOCONVERSION BY LENTINULA EDODES: POTENTIAL AS NEW BIOPRODUCTS FOR BEEF CATTLE FEEDING. BIORESOURCE TECHNOLOGY, 394, 130292.

TIMLIN, C.L., DICKERSON, S.M., FOWLER, J.W., MCCRACKEN, F.B., SKAGGS, P.M., EKMAY, R. & COON, C.N. (2024). THE EFFECTS OF TORULA YEAST AS A PROTEIN SOURCE ON APPARENT TOTAL TRACT DIGESTIBILITY, INFLAMMATORY MARKERS, AND FECAL MICROBIOTA DYSBIOSIS INDEX IN LABRADOR RETRIEVERS WITH CHRONICALLY POOR STOOL QUALITY. JOURNAL OF ANIMAL SCIENCE.

YÁÑEZ-RUIZ, D.R., BANNINK, A., DIJKSTRA, J., KEBREAB, E., MORGAVI, D.P., O'KIELY, P., REYNOLDS, C.K., SCHWARM, A., SHINGFIELD, K.J., YU, Z. & HRISTOV, A.N. (2016). Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants-a review. *Animal Feed Science and Technology*, **216**, 1–18.

Tables

Table 1. Composition of direct-fed microbial additives for each treatment

Acronyms	Bacillus specie	Yeast type
1 - NC[1]	-	-
2 - SL	*B. subtilis*, *B. licheniformis*	-
3 - SLAC	*B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. coagulans*	-
4 - HY	-	Hydrolyzed yeast
5 - IY	-	Inactive Torula yeast
6 – SL + IY	*B. subtilis*, *B. licheniformis*	Inactive Torula yeast
7 – SL + HY	*B. subtilis*, *B. licheniformis*	Hydrolyzed yeast
8 – SLAC + IY	*B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. coagulans*	Inactive Torula yeast
9 – SLAC + HY	*B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. coagulans*	Hydrolyzed yeast

[1]NC = control

Table 2. Chemical composition of *Urochloa brizantha* (cv. Marandu) hay (low-quality forage), *B. decumbens* grass (medium-quality forage) and diluting vehicle (vehicle*)* used in **Exp. 1** and **2**.

Item	*B. brizantha hay* (Low-quality)	*B. decumbens* grass (Medium-quality)	*Vehicle*[1]
Dry matter, g/kg	910	971	943
Ash, g/kg	45.0	77.1	13.7
Organic matter, g/kg	865	894	929
Crude protein, g/kg	30.0	113	133
Neutral detergente fiber, g/kg	830	667	98,7

[1] Vehicle was composed by a mixture of corn and corn gluten.

Table 3. Effects of direct-fed microbial (DFM) on ruminal fermentation variables within a gas production system in **Exp. 1** (*Urochloa brizantha* cv. Marandu hay).

Item[1]	*Bacillus*			Yeast			S.E.M.	*P* - value[2]		
	Control	*Subtilis* + *Lincheniformis*	Combination[3]	Control	Hydrolized	Inactivated		Bacillus	Yeast	Bac. x Yeast
NH_3-N, mg/dL	4.77^{b}	5.63^{ab}	6.00^{a}	4.39^{B}	5.56^{A}	6.46^{A}	1.15	0.09	<0.01	0.61
Total SCFA, m*M*	41.7^{ab}	44.9^{a}	41.0^{b}	40.3^{B}	44.5^{A}	42.9^{AB}	4.33	0.05	0.05	0.03
Acetate, m*M*	33.7^{b}	36.2^{a}	33.2^{b}	32.7^{B}	35.8^{A}	34.7^{AB}	3.65	0.04	0.05	0.02
Propionate, m*M*	5.94	6.37	5.67	5.43^{B}	6.47^{A}	6.09^{AB}	0.73	0.22	0.04	0.17
Butyrate, m*M*	0.90^{b}	1.01^{a}	0.88^{b}	0.93	0.95	0.92	0.14	0.03	0.91	0.55
Isobutyrate, m*M*	0.51^{b}	0.56^{a}	0.52^{b}	0.52	0.54	0.52	0.07	0.01	0.37	0.27
Isovalerate, m*M*	0.29	0.30	0.29	0.28	0.30	0.29	0.02	0.18	0.13	0.03
Valerate, m*M*	0.43^{ab}	0.46^{a}	0.41^{b}	0.41^{B}	0.45^{A}	0.42^{AB}	0.03	0.02	0.13	0.03
Total BCFA, m*M*	0.79^{b}	0.87^{a}	0.81^{b}	0.82	0.84	0.82	0.05	0.01	0.50	0.07
SCFA profile, mol/100mol										
Acetate	80.7	80.7	80.8	81.0	80.4	80.8	0.93	0.92	0.32	0.59
Propionate	14.2	14.0	13.9	13.5	14.5	14.0	1.05	0.80	0.15	0.79
Butyrate	2.17	2.28	2.24	2.31	2.21	2.16	0.18	0.46	0.24	0.12
Isobutyrate	1.22	1.26	1.26	1.28	1.23	1.23	0.08	0.46	0.30	0.15
Isovalerate	1.04	1.06	1.03	1.04	1.06	1.03	0.12	0.63	0.73	0.01
Valerate	0.72	0.71	0.75	0.75	0.71	0.72	0.11	0.28	0.26	0.01
Acetate: propionate	5.70	5.80	5.80	5.81	5.68	5.81	0.40	0.63	0.45	0.97

[1]NH_3-N = ammonia nitrogen; SCFA = short-chain fatty acids; BCFA = branched-chain fatty acids;

[2]Different superscripts indicate significant differences among means within rows ($P \leq 0.10$). Lower-case letters denote comparisons for *Bacillus*, while upper-case letters denote comparisons for Yeast.

[3]Combination = combination of *B. subtilis, B. licheniformis, B. amyloliquefaciens, B. Coagulans.*

Table 4. Effects of direct-fed microbial (DFM) on ruminal fermentation variables within a gas production system in **Exp. 2** (low-quality forage, *Urochloa brizantha* cv. Marandu hay).

Item[1]	Treatment[2]					S.E.M.	*P* – value[3]
	NC	*SL*	*SLAC*	*SL+HY*	*SLAC+IY +HY*		
Gas production 24h, mL/g DM	71.7[a]	76.4[a]	74.1[a]	64.5[a]	44.5[b]	7.72	0.01
Gas production 48h, mL/g DM	102[a]	108[a]	107[a]	98.4[a]	63.6[b]	10.2	<0.01
Gas production 72h, mL/g DM	113[a]	117[a]	119[a]	110[a]	69.0[b]	10.3	<0.01
IVOMD[4], g/kg	477[a]	461[a]	469[a]	472[a]	429[b]	19.7	0.08
CH_4, m*M*	2.89	2.79	2.89	2.71	2.71	0.51	0.96
pH	6.62	6.62	6.60	6.64	6.56	0.02	0.27
NH_3-N, mg/dL	12.7[c]	17.6b[c]	22.9[ab]	25.9[a]	26.5[a]	2.62	<0.01
Total SCFA, mM	75.5[ab]	77.6[ab]	82.2[a]	83.1[a]	74.0[b]	3.16	0.08
Acetate, mM	55.6[bc]	57.8[abc]	60.7[ab]	61.4[a]	54.9[c]	2.53	0.07
Propionate, mM	12.0[ab]	11.9[b]	12.9[a]	12.9[a]	11.8[b]	0.34	0.03
Butyrate, mM	4.93	4.69	5.23	5.35	4.54	0.30	0.21
Isobutyrate, mM	0.793	0.816	0.869	0.884	0.746	0.06	0.38
Isovalerate, mM	1.54	1.65	1.78	1.84	1.46	0.15	0.36
Valerate, mM	0.64	0.67	0.72	0.73	0.62	0.04	0.28
Total BCFA, mM	1.43	1.48	1.59	1.61	1.37	0.09	0.34
SCFA profile, mol/100mol							
Acetate	73.6[c]	74.4[a]	73.9[abc]	74.0[ab]	74.1[ab]	0.59	0.01
Propionate	15.9	15.6	15.8	15.6	15.9	0.43	0.42
Butyrate	6.51[a]	6.02[ab]	6.31[ab]	6.33[ab]	6.14[b]	0.20	0.04
Isovalerate	2.03	2.09	2.12	2.16	1.98	0.11	0.74
Valerate	0.84	0.86	0.87	0.86	0.83	0.02	0.86
Acetate:propionate	4.62[b]	4.80[a]	4.70[ab]	4.75[ab]	4.67[ab]	0.16	0.20

[1]BCFA = branched-chain fatty acids; NH_3-N = ammonia nitrogen; SCFA = short-chain fatty acids.

[2]**NC** = control diet no additive inclusion; **SL**: *Bacillus subtilis* and *B. licheniformis*; **SLAC**: *B. subtilis, B. licheniformis, B. amyloliquefaciens* and *B. coagulans*; **Sl+HY***: B. subtilis* and hydrolyzed yeast; **SLAC+IY + SLAC+HY**: *B. subtilis, B. licheniformis, B. amyloliquefaciens*, *B. coagulans* and dry inactive Torula yeast and hydrolyzed yeast;

[3]*P - value*. Means within row followed by different superscripts (a,b,c) differ at $*P \leq 0.10$.

[4]The in vitro organic matter digestibility (IVOMD) was calculated according to Menke & Steingass (1988) as IVOMD (g/kg DM) = 31.55 + 0.8343GP.

Table 5. Effects of direct-fed microbial (DFM) on ruminal fermentation variables within a gas production system in **Exp. 3** (medium-quality forage, *Brachiaria decumbens* grass).

Item	Treatment[1]					S.E.M.	*P* - value[2]
	NC	*SL*	*SLAC*	*SL+HY*	*SLAC+IY + SLAC+HY*		
Gas production 24h, mL/g DM	91.1	77.8	91.4	101	97.1	12.4	0.51
Gas production 48h, mL/g DM	123	100	124	121	130.9	12.4	0.12
Gas production 72h, mL/g DM	129	108	132	137	136.0	12.8	0.17
IVOMD, g/kg	507	473	513	501	523	21.9	0.56
CH_4, mmol	3.52	3.87	3.52	3.43	3.51	0.52	0.71
pH	6.53	6.51	6.51	6.49	6.53	0.03	0.82
NH_3-N, mg/dL	27.6^{c}	34.6^{bc}	34.4^{bc}	42.2^{ab}	50.2^{a}	7.33	<0.01
Total SCFA, mM	89.2^{b}	86.1^{b}	89.2^{b}	95.8^{a}	91.0^{ab}	4.86	0.02
Acetate, mM	65.3^{b}	63.3^{b}	65.7^{b}	70.5^{a}	67.0^{ab}	3.74	0.02
Propionate, mM	14.5	14.1	14.4	14.9	14.3	0.83	0.15
Butyrate, mM	5.84^{ab}	5.33^{c}	5.64^{bc}	6.25^{a}	5.80^{abc}	0.33	0.01
Isobutyrate, mM	0.90^{bc}	0.87^{c}	0.91^{bc}	1.06^{a}	0.99^{ab}	0.044	<0.01
Isovalerate, mM	1.81^{b}	1.76^{b}	1.82^{a}	2.26^{a}	2.03^{ab}	0.106	<0.01
Valerate, mM	0.77^{b}	0.73^{b}	0.77^{b}	0.88^{a}	0.79^{ab}	0.044	0.02
Total BCFA, mM	1.66^{b}	1.60^{b}	1.67^{b}	1.94^{a}	1.78^{ab}	0.086	<0.01
SCFA profile, mol/100mol							
Acetate	73.3	73.5	73.6	73.6	73.6	0.476	0.60
Propionate	16.3^{ab}	16.4^{a}	16.1^{ab}	15.6^{c}	15.8^{bc}	0.52	0.01
Butyrate	6.56^{a}	6.19^{b}	6.33^{ab}	6.51^{a}	6.36^{ab}	0.09	0.05
Isovalerate	2.03^{b}	2.05^{b}	2.06^{b}	2.32^{a}	2.24^{a}	0.097	<0.01
Valerate	0.86^{b}	0.85^{b}	0.86^{b}	0.91^{a}	0.87^{ab}	0.088	0.08
Acetate:propionate	4.50^{c}	4.49^{c}	4.56^{bc}	4.74^{a}	4.68^{ab}	0.17	0.02

[1]BCFA = branched-chain fatty acids; NH_3-N = ammonia nitrogen; SCFA = short-chain fatty acids.

[2]**NC** = control diet no additive inclusion; **SL**: *Bacillus subtilis* and *B. licheniformis*; **SLAC**: *B. subtilis, B. licheniformis, B. amyloliquefaciens* and *B. coagulans*; **Sl+HY***: B. subtilis* and hydrolyzed yeast; **SLAC+IY + SLAC+HY**: *B. subtilis, B. licheniformis, B. amyloliquefaciens*, *B. coagulans* and dry inactive Torula yeast and hydrolyzed yeast;

[3]*P - value*. Means within row followed by different superscripts (a,b,c) differ at $*P \leq 0.10$.

[4]The in vitro organic matter digestibility (IVOMD) was calculated according to Menke & Steingass (1988) as IVOMD (g/kg DM) = 31.55 + 0.8343GP.

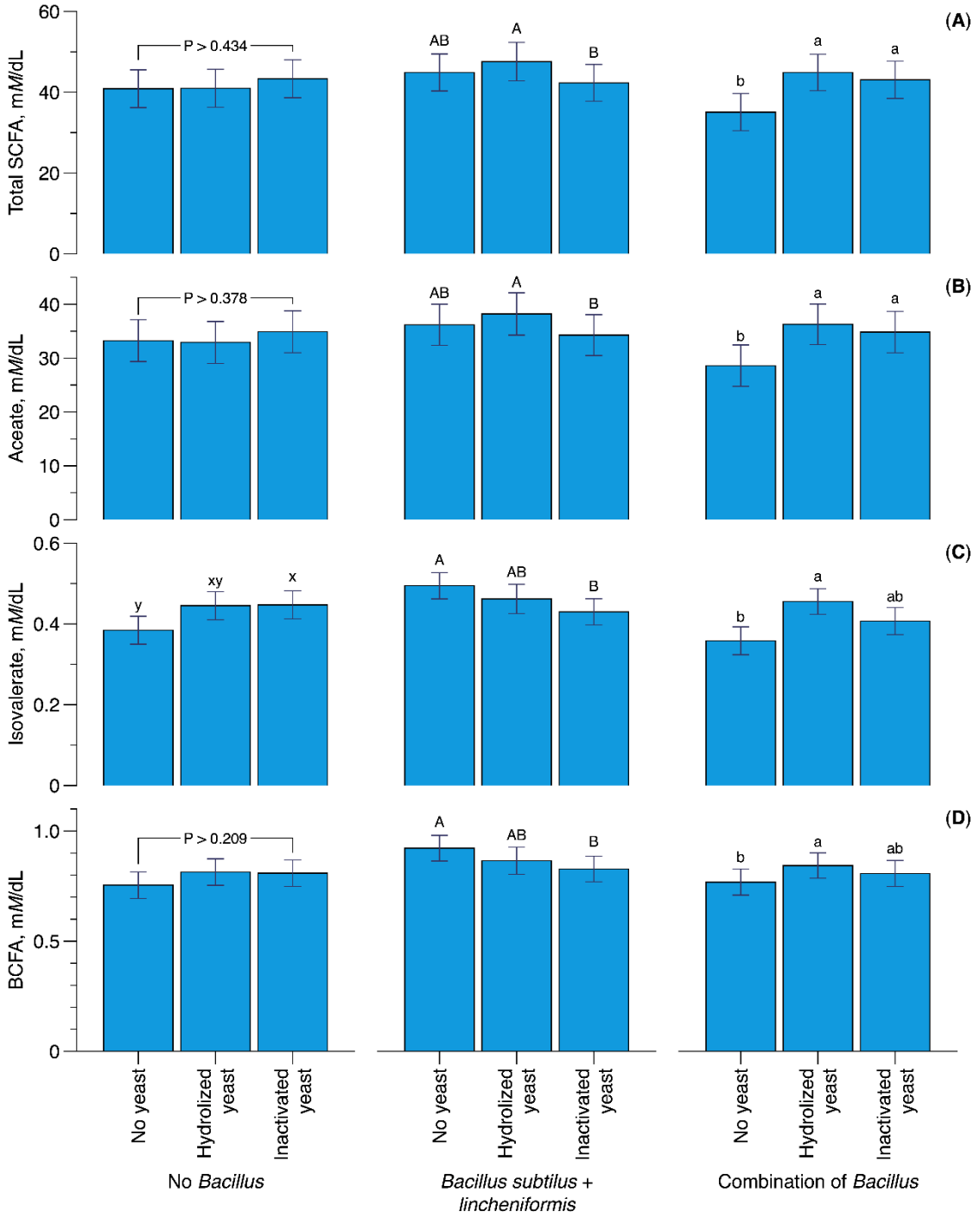

Figure 1. Effects of direct-fed microbial (DFM) on short chain fatty acids variables (in mM/dL) within a gas production system in **Exp. 1** (*Urochloa brizantha* cv. Marandu hay). BCFA = branched-chain fatty acids; SCFA = short-chain fatty acids. Different superscripts indicate significant differences among means ($P \leq 0.10$). The letters x and y denote comparisons for No Bacillus. Lowercase letters a and b denote comparisons for Bacillus subtilis + licheniformis, while uppercase letters A and B denote comparisons for the Combination of Bacillus. Combination of Bacillus = B. subtilis, B. licheniformis, B. amyloliquefaciens, B. coagulans.

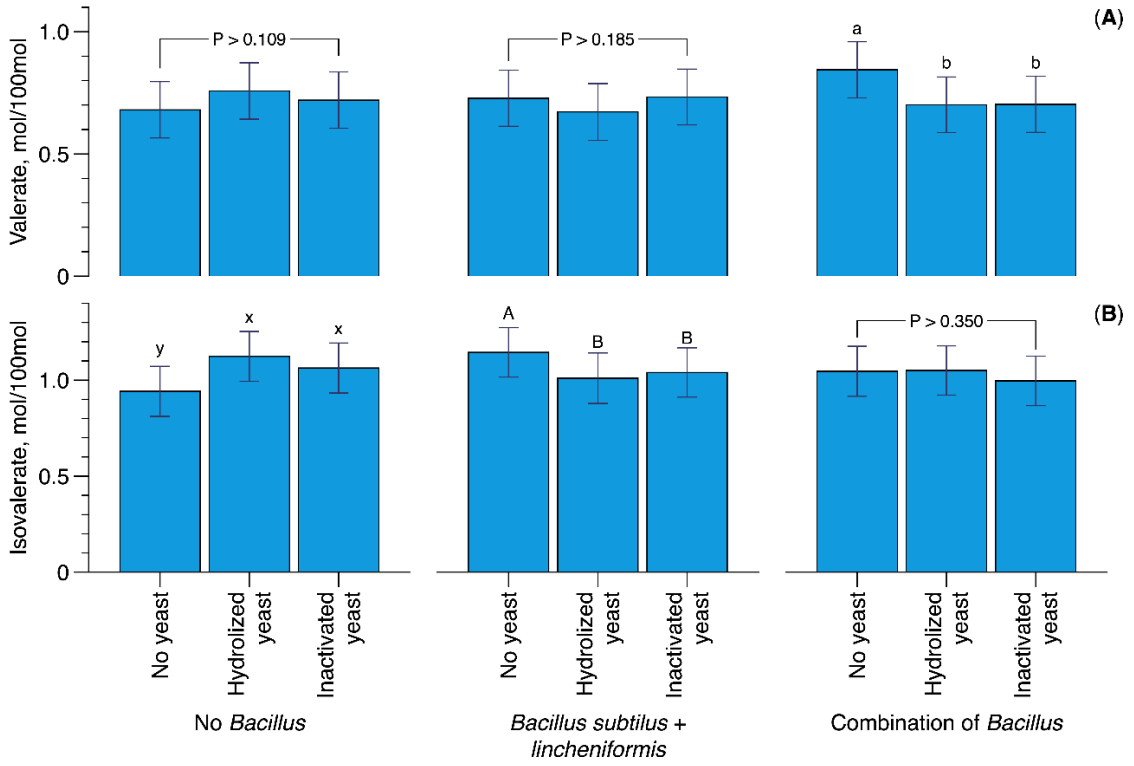

Figure 2. Effects of direct-fed microbial (DFM) on short chain fatty acids variables (in mol/100mol) within a gas production system in **Exp. 1** (*Urochloa brizantha* cv. Marandu hay).

[1]Different superscripts indicate significant differences among means ($P \leq 0.10$). The letters x and y denote comparisons for No Bacillus. Lowercase letters a and b denote comparisons for Bacillus subtilis + licheniformis, while uppercase letters A and B denote comparisons for the Combination of Bacillus. Combination of Bacillus = B. subtilis, B. licheniformis, B. amyloliquefaciens, B. coagulans.

CAPÍTULO 3 – Effect of direct-fed microbials on growth performance and ruminal metabolism parameters of grazing Nellore young bulls at dry season

RUNNING TITTLE: DIRECT-FED MICROBIAL AND PERFORMANCE

Effect of direct-fed microbials on ruminal fermentation and growth performance of Nellore bulls grazing during the dry season

Fernanda Rigon[*], Luciana Melo Sousa[*], Yury Tatiana Granja-Salcedo[§], Danilo Grandini[#], Pedro D. B. Benedeti[†], Renata H. Branco[††], Eduardo M. Paula[††,1], Flavio D. Resende[*,¶], Gustavo R. Siqueira[*,¶],

[*]*Department of Animal Science, São Paulo State University Júlio de Mesquita Filho, UNESP, Jaboticabal, SP, 16050-680, Brazil*

[‡] *Department of Veterinary Sciences, São Paulo State University Júlio de Mesquita Filho, UNESP, Jaboticabal, SP, 16050-680, Brazil*

[§]*Corporación Colombiana de Investigación Agropecuaria, AGROSAVIA, Antioquia, SR, 05303, Colombia*

[#]*Phibro Animal Health Corporation, Campinas, SP, 13025-170, Brazil*

[†]*Department of Animal Science, Santa Catarina State University, Chapecó, Santa Catarina, 89815-630, Brazil*

[††]*Beef Cattle Research Center, Instituto de Zootecnia, Sertãozinho, São Paulo, 14160-900, Brazil*

[¶]*Department of Animal Science, APTA – Agência Paulista de Tecnologia dos Agronegócios, Colina, SP, 14770-000, Brazil*

Abstract: this study investigated the effects of dietary supplementation with *Bacillus subtilis*, *B. licheniformis*, and autolyzed sugar-cane yeast on the performance and nutrient utilization of grazing Nellore bulls under tropical conditions. The research was conducted in two experiments: **Exp. 1** evaluated ruminal metabolism using 30 cannulated bulls allocated in individual pens. In **Exp. 1**, treatments included a negative control (**NC**), a *Bacillus* blend (**SLAC –** *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *B. coagulans*), and *Bacillus* plus yeast (**SL+Y -** *Bacillus subtilis*, *B. licheniformis* plus autolyzed sugar-cane yeast). We found no differences among treatments for dry matter intake. The **SL+Y** had higher crude protein digestibility (CP – $P = 0.005$). No significant treatment effect was observed on ruminal pH or short-chain fatty acids (SCFA), though NH_3-N concentration was affected by the interaction time and treatment. The **Exp. 2** assessed the performance of 84 young Nellore bulls over 132 days. We performed Exp. 2 using 12 paddocks with an average size of 4 ha. The paddocks were considered experimental units. The experimental treatments were: negative control (**NC**), a *Bacillus* blend (**SL –** *B. subtilis* and *B. licheniformis*), and *Bacillus* plus yeast (**SL+Y -** *Bacillus subtilis*, *B. licheniformis* plus autolyzed sugar-cane yeast). In **Exp. 2**, **SL + Y** treatment led to the highest average daily gain (AGD – $P = 0.018$) in comparison to **NC**. However, **SL** and **SL+Y** had similar ADG. Regarding carcass traits, **SL** and **SL + Y** had higher longissimus muscle area in comparison to **NC** ($P = 0.015$). The findings support that *Bacillus*-based direct-fed microbials (DFM), can enhance protein digestibility and growth performance in grazing cattle during the dry season. This suggests potential for strategic supplementation in tropical beef production systems.

Keywords: average daily gain; autolyzed sugar-cane yeast; Bacillus sp.; Tropical conditions.

INTRODUCTION

Meat production in grazing-based systems faces major challenges, especially in regions with a tropical climate. These challenges are mainly related to production seasonality and the characteristics of tropical plants (Costa et al., 2021b; Detmann et al., 2014). In this sense, nutritional strategies, such as protein supplementation, have been used to improve animal performance and the use of forage, especially during the dry season (Batista et al., 2016; Detmann et al., 2014).

However, the use of protein supplements in the dry season is frequently accompanied by the inclusion of feed additives that modulate ruminal fermentation (Maciel et al., 2019). In this case, feed additives can modulate the ruminal environment to favor the degradation of forages (Ceconi et al., 2022; Marques; Cooke, 2021). Among the feed additives used as an alternative to synthetic molecules to modulate ruminal fermentation are the direct-fed microbials (DFM) (Lopez et al., 2024; Pan et al., 2022).

The inclusion of *Bacillus* strains like *B. subtilis* and *B. licheniformis* as yeast-based products has been widely studied in ruminant nutrition (Baker et al., 2022; Pan et al., 2022; Cappellozza et al., 2023; Rigon et al., 2025). The main effects of including these additives in grazing-systems reported in the literature are improvement in ruminal fiber degradability (Mombach et al., 2021; Parra et al., 2021), and improve animal performance by stimulating the growth of fiber degradation microorganisms (Adesogan et al., 2019; Firkins; Mitchell, 2023), growth factors important to the rumen microbial communities (Baker et al., 2022; Parra et al., 2021). Also, according Rigon et al. (2025) the combination of Bacillus strains with dry inactive and hydrolyzed Torula yeast on low and medium-quality forage can enhance the concentration and profile of total SCFA, acetate and BCFA. The NH_3-N concentration in the rumen can be increased too, potentially enhancing microbial protein synthesis and nutrient utilization. The N-NH_3 in the rumen

is captured by ruminal bacteria to synthesize its own proteins, which will serve as a source of nitrogen for the host animal (Bach; Calsamiglia; Stern, 2005).

Furthermore, the combination *B*. *subtilis* and *B*. *licheniformis* can improve and starch degradation (Cappellozza et al., 2023; Shi et al., 2017; Sun; Wang; Deng, 2013) and has the potential to produce fibrolytic enzymes and improve fiber digestibility (Cappellozza et al., 2023; Oyebade et al., 2023). Furthermore, the use of autolyzed sugar-cane yeast as prebiotic may enhance short chain fatty acid (SCFA) concentration in the rumen, such as acetate (Baker et al., 2022). However, to our knowledge, few studies have explored the possible synergistic effect of different bacillus and yeast-based products (e.g., *subtilis* and *licheniformis* and autolyzed sugar-cane yeast), on performance and metabolism of grazing cattle.

Therefore, we hypothesized that the inclusion of a blend of DFM or the combination of DFM and plus autolyzed sugar-cane yeast of Nellore young bulls grazing would improve the performance and nutrient utilization. Thus, our objective was to investigate the effects of the use of *Bacillus amyloliquefaciens*, *B. coagulans* and autolyzed sugar-cane yeast on performance, blood parameters and ruminal metabolism, of Nellore young bulls grazing under tropical conditions.

MATERIAL AND METHODS

Experiment location and ethic committee approval

This study was conducted at the Agência Paulista de Tecnologia dos Agronegócios (APTA), Alta Mogiana regional pole, Colina, São Paulo, Brazil. The unit is located at 20º42’50” S and 48º33’52.7” W. The study was conducted from May 2023 to October 2023, during the dry season. The climate is subtropical humid, characterized by dry winters and rainy summers, classified as aW according to Köppen classification. During

the experiment, the total precipitation was 179 mm (Figure 1). The study was conducted in accordance with animal welfare guidelines and the protocol was approved by the APTA Regional Animal Use Ethics Committee (CEUA), Bauru, São Paulo, Brazil (Protocol number 006/2022).

Animals, grazing area, experimental design and periods

The study was divided into two experiments. Experiment 1 (**Exp. 1**) was conducted to evaluate ruminal metabolism parameters, and Experiment 2 (**Exp. 2**), was conducted to evaluate animal performance. The choice of distinct treatments among the trials is due to the differences in objectives: while the metabolism trial sought to understand physiological and digestive mechanisms, justifying the use of the *Bacillus* + yeast (**SL+Y**) treatment, the performance trial prioritized evaluating productive effects under field conditions, using a more robust microbial supplement (**SLAC**). This strategy allowed for complementary exploration of the functional and practical effects of the additives, optimizing the applicability of the results to tropical production systems.

Metabolism trial – Exp. 1

For this trial, 30 rumen-cannulated male Nellore bulls were used, with an initial body weight (BW) of 470 kg ± 45 kg. The animals were arranged in a randomized block design, based on animals' BW. The animals were allocated in 30 individual pens (2 × 5 m). The pens were equipped with individual drinking fountains, and individual concrete feeders to the offer of *Brachiaria brizantha* hay, and individual plastic feeders for the supplement offer. The animals had free access to water, and the forage intake was *ad libitum*. The supplement was offered at a proportion of 3 g/kg of BW (Table 1), being a protein supplement with approximately 22% of crude protein (CP). The additives were

included in the supplement at the dose recommended by the manufacturer. The additives were added to the supplement manually. The additives were weighed on a precision analytical balance and stored in plastic packaging. During the weighing of the supplement for feeding the animals, the additives were mixed into the supplement, and the container holding both the additive and the supplement was shaken to promote the incorporation of the additives into the supplement. The study lasted 32 days, 28 days for animals' adaptation to the facilities and diet and 5 days for sample collection (Figure 2).

Experimental treatments

In **Exp. 1** the treatments were:

(a) **NC**: negative control – only supplement without any feed additive;

(b) **SLAC**: a blend of *Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *B. coagulans* (≥ 2.0 x 10^6 CFU/g) was included in the supplement at 10 g/animal/day;

(c) **SL + Y**: a blend of *Bacillus subtilis*, *B. licheniformis* (≥ 2.0 x 10^5 CFU/g) plus autolyzed sugar-cane yeast. The Bacillus were included in the supplement at 5 g/animal/day, while yeast was included in the proportion of 10 g/animal/day.

Sample collection

a) Supplement and forage intake

Daily, the supplement and the hay provided to the animals were weighed, and the day after the offering, the orts collected from the feeders were also weighed, in order to measure feed intake (as fed). The animals were fed once a day, in the morning, at 7:00 AM.

b) Nutrient digestibility

From day 29 to day 31, the total fecal collection was performed. After 24 hours, the feces were weighed and homogenized, and a sample was collected and stored at -20 C. This procedure was repeated during the 3 days of fecal collection (Ferreira et al., 2009). After 3 days, a representative stool sample from the three days was collected and stored for nutrient analysis. During the 3 days of collection a sample of hay and supplement offered was collected, as well as the leftover samples of both in each pen. Samples of feed offered, as well as leftovers and feces were used to determine the apparent nutrient digestibility of nutrients. The digestibility was calculated using the following equation:

$$\textbf{DMD}\ (\%) = \frac{(\text{DMI x \% nutrient}) - (\text{DME x \% nutrient})}{(\text{DMI x \% nutrient})}\ \text{x}\ 100$$

Where DMI is dry matter intake, % nutrient is the proportion of the nutrient on the feed, leftovers, and feces and DME is dry matter excreted.

Chemical analysis

The samples of forage and supplement were ground through a 1 mm screen (Wiley mill; Thomson Scientific Inc., Philadelphia, PA). The chemical analyses were performed following the AOAC methodology (Association Official of Analytical Chemists, 2006). Samples were analyzed for dry matter (DM, method 934.01), ash (method 938.08), crude protein (CP, method 990.13). Organic matter (OM) was calculated by the difference between DM content and ash. The neutral detergent fiber (NDF) followed the methodology proposed by Van Soest et al (1991) and adapted for the fiber determiner (*TE-149*, Tecnal, Piracicaba, SP, BRA). The chemical composition of the feed is presented in Table 1.

c) Ruminal parameters

The rumen fluid collection was performed on day 33, at 0, 6, 12, and 18-hours post-feeding. Samples were collected from the dorsal, central, and ventral regions of the rumen of each cannulated animal, and a composite sample was formed. The samples were filtered with two layers of cheesecloth to determine pH, ammonia nitrogen (NH_3-N), and short-chain fatty acids (SCFA). Samples for NH_3-N analysis were preserved with 1 mL of H_2SO_4 (sulfuric acid) and stored at -20 ºC until analysis.

Ammonia nitrogen (NH^3-N), short chain fatty acids (SCFA) and ruminal pH

The NH_3-N concentration was determined by the colorimetric method described by Chaney and Marbach (1962). The pH was analyzed shortly after collection, using an electric pH meter (DM-22, Digimed, São Paulo, Brazil). Samples for SCFA analysis (15 mL) were stored at -20 ºC for further analysis. The total SCFA concentrations and profiles were determined using gas chromatography (Nexis CG-2030AF, Shimadzu, Montevideo, Uruguay equipped with a fused silica capillary column [30m x 0.53nm x 0.5µm film thickness, Sulpelco, Darmstadt, Germany]) coupled to a flame ionization detector (FID), with Nitrogen as a carrier gas (flow rate of 34.5mL/min^{-1}). The initial temperature was 100 ºC for 2 minutes, then was elevated for 130 ºC at a rate of 10 ºC/minute. After, the temperature was elevated until reach 170 ºC, at a rate of 15 ºC/minute, and was kept for 11 minutes. The detector and inlet temperatures were 230 and 250 ºC, respectively, and 0.5 µL samples were injected in split mode.

d) Blood parameters

Blood collections were performed by jugular venipuncture with vacuum tubes without anticoagulant (BD Vacutainer®) at 0, and 6 h after supplementation on the last day of the trial. The samples were centrifuged at 3080 x g for 15 min at 4 ^{o}C. Serum was harvested and stored at -20 ^{o}C until later analysis. The serum was analyzed for urea, creatinine, albumin, total protein, glucose and cholesterol. For the analysis commercial kits from the company (Bioclin®) according to the manufacturer's specifications, (urea, code K-056), (albumin, code K-040), (creatinine, code K-222), (total protein, code K-031), (glucose, code K-082) and (cholesterol, code K-088-27). The readings were performed in an automatic biochemistry analyzer (Sistema de Bioquímica Automático SBA-200; CELM®).

Statistical analysis

All data analyses were performed in SAS version 9.4 (SAS Institute Inc.). All results were assessed for residual normality and variance homogeneity. For all experiments, data were collected and analyzed in a completely randomized design by fitting the data using the PROC MIXED of SAS (SAS Inst. Inc., Cary, NC, United States). For nutrient intake and diet digestibility treatment was considered as fixed factors and animal within block as a random factor. The variables conducted on the same animal, but at different times, were analyzed as a repeated measure over time (pH, NH_3-N, SCFA and blood parameters) considering treatment, block, hour, and the interaction treatment x hour as fixed factors and animal within block as a random factor. The lowest value of BIC (Bayesian information criterion) was used to determine the matrices chosen for each variable, in the parameters analyzed over time. Significance was considered when $p < 0.05$, while trend was considered when $0.05 < p > 0.10$ for all tests.

Performance trial – Exp. 2

In **Exp. 2**, 84 Nellore bulls were used, blocked according to the BW. A randomized block design was used to evaluate the performance of the animals, with an average of initial BW of 404 ± 14 kg and an average age of 20 months. The experiment lasted 132 days, with 20 days for adaptation, followed by four experimental periods of 28 days each. The experiment was carried out in an area of 44.7 hectares composed of *Urochloa brizantha* cv. Marandu and divided into 12 paddocks (3.4 - 4 hectares each), equipped with drinkers to supply water and a trough to supply supplement. The animals were distributed in 12 paddocks, divided into four blocks, according to the initial weight of the animals in a light one, medium one, intermediary one and heavy block, with four paddocks per treatment, and 7 animals each, where they were kept in a continuous stocking system, with a variable stocking rate. All animals received the supplement daily in the morning, offered at 3 g/kg of body weight.

Experimental treatments

(a) **NC**: negative control – the animals receiving this treatment consumed only supplement without any DFM;

(b) **SL**: a blend of *Bacillus subtilis* and *B. licheniformis* (≥ 2.0 x 10^5 CFU/g) was included in the supplement at 25 g/animal/day;

(c) **SL + Y**: a blend of *Bacillus subtilis*, *B. licheniformis* (≥ 2.0 x 10^5 CFU/g) plus autolyzed sugar-cane yeast. The Bacillus were included in the supplement at 5 g/animal/day, while yeast was included in the proportion of 10 g/animal/day.

Forage evaluation

Quantitative and qualitative assessments of the pasture conditions, measuring herbage mass, structural components, every 28 days. The forage mass was estimated using the double sampling method (Sollenberger e Cherney, 1995). The quantitative and structural components of the forage sward were evaluated at medium heights, the forage components were weighed and oven-dried at 55°C for 72 h to obtain the partial DM. Hand-plucked samples were used to estimate the dietary nutritional value (De Vries, 1995). Samples were oven-dried at 55°C for 72 h and then ground in a Wiley mill (Thomas Model 4, Thomas Scientific, Swedesboro, NJ, USA) to pass a 1-mm mesh sieve, for further analysis. Samples were analyzed for dry matter (DM), ash, crude protein (CP), ethereal extract (EE), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin and indigestible neutral detergent fiber (iNDF). All analyses were described by spectroscopy in the near-infrared light region, NIRS (Near Infrared Spectrometer), using FT-NIR modular spectrometer, model NIRFlex N500, brand Büchi Labortechnik AG (Flawil, Switzerland), the equipment was calibrated using the Spectralon® standard every 120 minutes. Spectral measurements were obtained using the NIRWare Operator 1.2 Software (Büchi Labortechnik, Flawil, Switzerland), in diffuse reflectance mode. The multivariate calibration model was developed at the Colina Regional Development and Research Unit (APTA), based on the chemical composition results obtained by reference methods (laboratory) and spectral measurements (NIRS) of the samples.

Animal performance evaluation

To determine animal performance, the average daily gain (ADG, kg/day) was determined by the difference between the final fasting body weight of each experimental

period and the initial fasting body weight. The animals were weighed at the beginning of the experiment, after fasting for 16 h from solids and liquids, and every 28 days.

Carcass traits

To evaluate the carcass traits by real-time ultrasound, we used ultrasound equipment, brand ALOKA 500, with a 12 cm acoustic probe and a frequency of 3.5 Mhz and a silicone coupler, allowing perfect coupling of the transducer with the body of the animal. The carcass traits were evaluated on day 0 and day 112 of the trial. Before capturing the images, the region between the 12th and 13th thoracic vertebrae on the animal's left side was trichotomized. The probe equipped with an acoustic guide was placed perpendicular to the length of the *Longissimus dorsi* muscle, between the 12th and 13th thoracic vertebrae to measure the *Longissimus* muscle area and subcutaneous fat thickness (12^{th}-rib fat), obtained from ¾ distance from the medial side of the longissimus dorsi muscle to the lateral side of the *dorsolumbar* line, and parallel, to obtain the marbling of the longissimus dorsi muscle (scale from 0 to 10) using carcass evaluation software BIA PRO PLUS, from company Designer Genes Technologies.

Statistical analysis

The data were analyzed using a randomized block design with three treatments and four replications, with each paddock considered an experimental unit. A mixed model was used and included treatment, period, and per x treatment interaction as a fixed effect and the block and animal within paddock as a random effect. Herbage characteristics were analyzed as repeated measures and tested for the fixed effects of treatment, day of the study, and treatment×day of the study, using the pasture as the subject. For carcass traits the following model was applied: included treatment was considered as fixed effect and

the block and animal within paddock as a random effect. The first measurements at d0 were used in the model as covariate. The data were analyzed as repeated measures, using the PROC MIXED of SAS (SAS Inst. Inc., Cary, NC, United States), with a previous normal distribution test (Shapiro–Wilk test) and homoscedasticity of variances (Bartlett test). The lowest value of BIC (Bayesian information criterion) was used to determine the matrices chosen for each variable, in the parameters analyzed over time. Significance was considered when $p < 0.05$, while trend was considered when $p > 0.05 \leq 0.10$ for all tests.

RESULTS

Experiment 1

Nutrient intake and digestibility - EXP. 1

The results of nutrient intake and digestibility are presented in Table 2. We found no differences among treatments for DM ($P = 0.39$), CP ($P = 0.70$) and NDF ($P = 0.34$) intake. Also, we found no differences among treatments for DM and NDF digestibility ($P = 0.12$ and $P = 0.162$, respectively). Regarding CP digestibility (CPD), **SL + Y** had the greatest value ($P = 0.005$), in comparison to **NC** and **SL**.

Ruminal parameters – EXP. 1

The results of ruminal parameters are showed on Table 3. For ruminal pH, short chain fatty acid (SCFA) and propionate concentration (Mol/100 mol) we found effect for collection time ($P < 0.001$) but there was no effect of treatment. For ammonia nitrogen (NH_3-N, mg/dL) we found time effect ($P < 0.001$) and an interaction for treatment and time collection ($P = 0.07$ – Figure 3). The **NC** had the highest concentration of NH_3-N at 06 hours after feeding, in comparison to **SL** and **SL + Y** ($P < 0.05$). For 12 h after feeding,

SL had the lowest NH_3-N concentration in comparison to **NC** and **SL + Y** ($P \leq 0.1$). For SCFA (mmol), acetate, butyrate, isobutyrate, isovalerate, valerate and branched chain short fatty acid (BCSFA) and acetate to propionate ratio we found no significant difference among treatments.

Blood parameters – EXP. 1

The results of blood parameters are showed on Table 4. We did not find any significant difference between treatments or collection times for glucose ($P = 0.452$) and cholesterol ($P = 0.452$). We observed a difference between collection times for plasma urea concentration ($P < 0.001$), with urea values being 20.9 and 29.7 mg/mL at 0 and 6 hours after feeding, respectively. We found a statistical difference between treatments for blood creatinine concentration ($P = 0.0006$), with **SL + Y** presenting the highest concentration, compared to the other treatments. For total protein values, we found a difference between collection times ($P < 0.0001$). We observed a similar behavior to that of urea. Regarding albumin concentration, we observed a treatment effect ($P = 0.095$) and an effect of collection time ($P = 0.003$).

Experiment 2

Forage evaluation

Forage quantitative and qualitative characteristics are presented on Table 5. Effects of day of the study, but not treatment × day or treatment ($P \geq 0.16$), were detected for pasture height, forage mass, forage density, morphological components (green leaf, senescent leaf, and senescent stem), and chemical composition (dry matter, ash, crude protein, ether extract, NDF, ADF, lignin, and iNDF) ($P \leq 0.04$).

Pasture height increased with advancing days ($P = 0.003$), whereas forage density decreased ($P = 0.040$). The proportion of green leaves increased over time ($P < 0.001$), while senescent leaves and stems were greatest at day 56 ($P \leq 0.003$). Forage mass ($P = 0.285$), green stem ($P = 0.499$), and forage offer ($P = 0.158$) were not affected by day of study.

Qualitative characteristics as dry matter, ash, NDF, ADF, lignin, and iNDF were greatest on day 56 ($P < 0.001$), whereas crude protein and ether extract concentrations decreased from day 28 to day 56 and increased from day 56 to day 112 ($P < 0.001$). Stocking rate increased across sampling days ($P = 0.012$).

Performance results - EXP. 2

Regarding the performance results, **SL** had the highest body weight (BW, kg) in comparison to **NC** at day 112, at the end of the trial ($P < 0.001$ – Table 6). Also, **SL + Y** was similar to **SL** and **NC** for BW. For the average daily gain (ADG, kg/d) **NC** had the lowest ADG, when compared to **SL** and **SL+Y**, which were similar to each other ($P <0.001$).

Carcass traits - EXP. 2

The results for carcass traits are presented on Table 7. Regarding the Longissimus muscle area (LMA, cm^2), **SL** and **SL+Y** showed higher values in comparison to **NC** ($P = 0.015$). For LMA ($cm^2/100$), we found no difference among treatments, ($P = 0.995$). We found no difference among treatments for ratio ($P = 0.635$), marbling score ($P = 0.903$), backfat thickness (BFT, mm and BFT, mm/100 – $P = 0.651$ and $P = 0.882$, respectively). We had a trend for rump fat thickness, **NC** was lower in comparison to **SL** and **SL+Y** ($P = 0.087$).

DISCUSSION

The use of direct-fed microbials (DFM), alone or in combination with yeast derivatives, has been proposed as a strategy to enhance ruminal fermentation, improve nitrogen use efficiency, and support animal performance in grazing systems, particularly those based on tropical forages. The rationale behind this approach is grounded in the capacity of *Bacillus* species and yeast products to modify rumen microbial ecology and optimize the synchrony between nutrient release and microbial requirements. Because these additives supply enzymes, cofactors, and growth-promoting substrates to rumen microorganisms, their inclusion in grazing beef cattle diets has the potential to improve digestion and metabolic efficiency under tropical conditions.

Yeast derivatives play a central role in modulating rumen fermentation. They supply peptides, B vitamins, amino acids, and nucleotides, which act as growth factors for fibrolytic and proteolytic bacteria (Baker et al., 2022). In turn, Bacillus spp. complement this activity by producing extracellular enzymes such as cellulases, hemicellulases, and proteases, which increase the accessibility of structural carbohydrates and enhance substrate availability for fermentation (Lopez et al., 2024; Pan et al., 2022). When used in combination, these additives create a ruminal environment that favors microbial growth, improves fermentation efficiency, and stimulates bacterial populations responsible for fiber and protein degradation (Cappellozza et al., 2025; Silva et al., 2024).

In grazing systems, these mechanisms become particularly relevant due to the nutritional limitations imposed by tropical pastures. Tropical grasses typically exhibit rapid structural maturation, accumulating lignin and reducing soluble protein content as the grazing season progresses. As a result, cattle commonly face a systemic protein deficit, which directly restricts rumen microbial growth. It is well established that crude

protein concentrations below 7% limit ammonia supply, slow microbial proliferation, and reduce the degradation rates of fiber fractions, especially neutral detergent fiber (NDF) (Detmann et al., 2014). In such scenarios, nitrogen recycling and amino acid catabolism become essential physiological strategies for maintaining rumen function, but these mechanisms alone are insufficient to fully support microbial requirements (Silva et al., 2021). Thus, any additive capable of improving nitrogen utilization or enhancing microbial protein synthesis has the potential to mitigate the biological constraints imposed by low-protein forages.

The combined use of *Bacillus* and yeast derivatives is particularly promising in this context. Beyond supplying growth factors, these additives may improve the capture of ruminal ammonia by stimulating the synthesis of microbial protein, thereby increasing the flow of metabolizable protein to the small intestine (Silva et al., 2024). Improvements in ruminal nitrogen assimilation may also reduce nitrogen losses through ammonia accumulation and subsequent conversion to urea, which is energetically costly for the animal. A more synchronized nitrogen–energy environment supports greater microbial biomass production, improves the turnover of rumen microorganisms, and increases the efficiency of fiber digestion (Baker et al., 2022).

The importance of improving nitrogen metabolism in grazing systems goes beyond ruminal function. Protein deficiency also influences systemic indicators such as plasma urea, albumin, and creatinine. These biomarkers reflect nitrogen recycling, hepatic metabolism, muscle protein turnover, and the overall adequacy of dietary protein supply (Prahl et al., 2022; Pereira et al., 2022; Erickson et al., 2024). In contexts where dietary protein is limiting, supplementation strategies that enhance ruminal microbial activity may indirectly improve the efficiency of nitrogen utilization at the animal level, contributing to better physiological status.

Another aspect to consider is the interaction between supplementation and pasture dynamics. Tropical forage systems typically experience significant seasonal fluctuations in structure and nutritive value, including reductions in leaf proportion and increases in fiber and lignin content during maturity. These changes naturally reduce voluntary intake and NDF digestibility because animals become limited both by the physical constraints of fiber digestion and the metabolic constraints imposed by low nitrogen availability (Wróbel et al., 2025). Additives that enhance microbial activity can partially compensate for the decline in forage quality by improving the efficiency of rumen fermentation. Furthermore, supplementation practices may improve grazing behavior, stabilize rumen pH, and support more consistent nutrient intake, mitigating performance losses commonly observed as pastures mature (Womack et al., 2024; Naves et al., 2024).

Given these considerations, the strategic use of DFM and yeast derivatives in tropical grazing systems represents a biologically relevant tool to alleviate the constraints of low-protein forages. Their modes of action—enzymatic enhancement, stimulation of fibrolytic communities, improved nitrogen assimilation, and stabilization of ruminal fermentation—align with the major limiting factors faced by beef cattle in tropical pastures. Although their efficacy is context-dependent, especially under systems where nitrogen availability is the primary limiting factor, these additives offer a promising complementary strategy to support nutrient utilization and maintain productive efficiency throughout seasonal variations in forage quality.

CONCLUSION

Direct-fed microbials based on *Bacillus* spp., alone or combined with autolyzed yeast, improved nutrient use and supported greater growth performance of grazing cattle during the dry season. By enhancing ruminal microbial activity and nitrogen utilization,

these additives help mitigate the nutritional limitations of tropical forages, contributing to more efficient grass-fed beef production under tropical conditions.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

Aboagye, I. A., M. R. C. Cordeiro, T. A. McAllister, M. L. May, S. J. Hannon, C. W. Booker, S. L. Parr, O. C. Schunicht, L. O. Burciaga-Robles, T. M. Grimson, E. Boonstra, G. F. Mengistu, D. L. Fulawka, and K. H. Ominski. 2022. Environmental performance of commercial beef production systems utilizing conventional productivity-enhancing technologies. **Transl. Anim.** Sci. 6. doi:10.1093/tas/txac074.

Adesogan, A. T., K. G. Arriola, Y. Jiang, A. Oyebade, E. M. Paula, A. A. Pech-Cervantes, J. J. Romero, L. F. Ferraretto, and D. Vyas. 2019. Symposium review: Technologies for improving fiber utilization. **J. Dairy Sci.** 102:5726–5755. doi:10.3168/jds.2018-15334.

Association of Official Analytical Chemists. 2006. **Official Methods of Analysis of AOAC International.** 18th ed. AOAC International, Gaithersburg, MD.

Bach, A., S. Calsamiglia, and M. D. Stern. 2005. Nitrogen metabolism in the rumen. **J. Dairy Sci.** 88:E9–E21. doi:10.3168/jds.S0022-0302(05)73133-7.

Baker, L. M., J. Kraft, T. P. Karnezos, and S. L. Greenwood. 2022. Review: The effects of dietary yeast and yeast-derived extracts on rumen microbiota and their function. **Anim. Feed Sci.** Technol. 294. doi:10.1016/j.anifeedsci.2022.115476.

Batista, E. D., E. Detmann, E. C. Titgemeyer, S. C. V. Filho, R. F. D. Valadares, L. L. Prates, L. N. Rennó, and M. F. Paulino. 2016. Effects of varying ruminally undegradable protein supplementation on forage digestion, nitrogen metabolism, and urea kinetics in Nellore cattle fed low-quality tropical forage. **J. Anim. Sci.** 94:201–216. doi:10.2527/jas.2015-9493.

Cappellozza, B. I., R. F. Cooke, R. C. Amaral, A. Bach, T. E. Engle, L. F. Ferraretto, R. S. Marques, A. C. Miller, P. Moriel, and J. M. B. Vendramini. 2025. Ruminant nutrition symposium: Novel microbial solutions to optimize production efficiency in beef and dairy systems. **J. Anim. Sci.** 103. doi:10.1093/jas/skaf165.

Cappellozza, B. I., J. N. Joergensen, G. Copani, K. A. Bryan, P. Fantinati, J. C. Bodin, M. M. Khahi, C. Ninodeguzman, K. G. Arriola, L. O. Lima, S. Farooq, and D. Vyas. 2023. Evaluation of a Bacillus-based direct-fed microbial probiotic on in vitro rumen gas production and nutrient digestibility of different feedstuffs and total mixed rations. **Transl. Anim. Sci.** 7. doi:10.1093/tas/txad044.

Ceconi, I., S. A. Viano, D. G. Mendez, L. González, P. Davies, J. C. Elizalde, E. Bressan, D. Grandini, T. G. Nagaraja, and L. O. Tedeschi. 2022. Combined use of monensin and virginiamycin to improve rumen and liver health and performance of feedlot-finished steers. **Transl. Anim.** Sci. 6. doi:10.1093/tas/txac154.

Chaney, A. L., and E. P. Marbach. 1962. Modified reagents for determination of urea and ammonia. **Clin. Chem.** 8:130–132.

Costa, C. M., G. S. Difante, A. B. G. Costa, A. L. C. Gurgel, M. A. Ferreira, and G. T. Santos. 2021. Grazing intensity as a management strategy in tropical grasses for beef cattle production: A meta-analysis. **Anim.** 15. doi:10.1016/j.animal.2021.100192.

Detmann, E., É. E. L. Valente, E. D. Batista, and P. Huhtanen. 2014. An evaluation of the performance and efficiency of nitrogen utilization in cattle fed tropical grass pastures with supplementation. **Livest. Sci.** 162:141–153. doi:10.1016/j.livsci.2014.01.029.

De Vries, M.F.W. 1995. Estimating forage intake and quality in grazing cattle: a reconsideration of the hand-plucking method. **Journal of Range Management**. 48:370-375. doi: 10.2307/4002491.

e Silva, Y. R. V. B., J. T. Zervoudakis, L. K. Hatamoto-Zervoudakis, M. L. C. Abreu, L. S. Cabral, L. B. Freiria, P. I. J. L. R. e Silva, and A. J. Possamai. 2021. Supplementation with different protein profiles for grazing beef cattle during the rainy-dry transition season. **Trop. Anim. Health Prod.** 53. doi:10.1007/s11250-020-02467-4.

Erickson, M. G., D. L. Harmon, A. M. Salim, and K. J. Harvatine. 2024. Reducing dietary crude protein: Effects on nutrient digestibility, rumen function, nitrogen balance, and serum amino acids. **J. Dairy Sci.** 107:1842–1855. doi:10.3168/jds.2023-23041.

Ferreira, M.A.; Valadares Filho, S.C.; Marcondes, M.I.; Paixão, M.L.; Paulino, M.F.; Valadares, R.F.D. 2009. Avaliação de indicadores em estudos com ruminantes: digestibilidade. **Revista Brasileira de Zootecnia**. 38: 1568-1573. doi: 10.1590/S1516-35982009000800022.

Firkins, J. L., and K. E. Mitchell. 2023. Invited review: Rumen modifiers in today's dairy rations. **J. Dairy Sci.** 106:3053–3071. doi:10.3168/jds.2022-22644.

Gubbels, E. R., W. C. Rusche, E. Block, T. Rehberger, J. S. Thomson, and Z. K. Smith. 2023. Evaluation of long-term supplementation of a direct-fed microbial and enzymatically hydrolyzed yeast cell culture product on feedlot growth performance, efficiency of dietary net energy utilization, heat stress measures, and carcass characteristics in beef steers. **Transl. Anim. Sci.** 7. doi:10.1093/tas/txad016.

Lopez, A. M., J. O. Sarturi, B. J. Johnson, D. R. Woerner, D. D. Henry, F. M. Ciriaco, K. G. S. Silva, and C. J. Rush. 2024. Effects of bacterial direct-fed microbial combinations on beef cattle growth performance, feeding behavior, nutrient digestibility, ruminal morphology, and carcass characteristics. **J. Anim. Sci.** 102. doi:10.1093/jas/skae004.

Maciel, I. C. F., H. M. Saturnino, F. A. Barbosa, V. M. R. Malacco, J. M. C. Andrade Júnior, G. H. B. Maia Filho, and P. M. Costa. 2019. Virginiamycin and sodium monensin supplementation for beef cattle on pasture. **Arq. Bras. Med. Vet. Zootec.** 71:1999–2008. doi:10.1590/1678-4162-10659.

Marques, R. S., and R. F. Cooke. 2021. Effects of ionophores on ruminal function of beef cattle. **Anim.** 11. doi:10.3390/ani11102871.

Mombach, M. A., L. S. Cabral, L. R. Lima, D. C. Ferreira, B. C. Pedreira, and D. H. Pereira. 2021. Association of ionophores, yeast, and bacterial probiotics alters the

abundance of ruminal microbial species of pasture-finished beef cattle. **Trop. Anim. Health Prod.** 53. doi:10.1007/s11250-021-02617-2.

Naves, K. R. S., K. A. K. Moraes, L. O. Cunha, N. B. Petrenko, J. C. Ortelam, J. N. Sousa, C. F. Covatti, D. Nunes, C. S. Chaves, F. L. Menezes, A. S. Oliveira, and E. H. B. K. Moraes. 2024. Additives in supplements for grazing beef cattle. **Anim.** 14. doi:10.3390/ani14243688.

Oyebade, A. O., G. A. Taiwo, M. Idowu, S. Taylor, O. Queiroz, A. T. Adesogan, D. Vyas, and I. M. Ogunade. 2023. Effects of direct-fed microbial supplement on ruminal and plasma metabolome of early-lactation dairy cows: Untargeted metabolomics approach. **J. Dairy Sci.** 106:5574–5587. doi:10.3168/jds.2023-23876.

Pan, L., K. Harper, O. Queiroz, G. Copani, and B. I. Cappellozza. 2022. Effects of a Bacillus-based direct-fed microbial on in vitro nutrient digestibility of forage and high-starch concentrate substrates. **Transl. Anim. Sci.** 6. doi:10.1093/tas/txac067.

Parra, M. C., D. F. A. Costa, A. S. V. Palma, K. D. V. Camargo, L. O. Lima, K. J. Harper, S. J. Meale, and L. F. P. Silva. 2021. The use of live yeast to increase intake and performance of cattle receiving low-quality tropical forages. **J. Anim. Sci.** 99. doi:10.1093/jas/skab017.

Pereira, T. C. J., M. C. Prahl, E. M. Paula, and R. F. Cooke. 2022. Creatinine as a urinary marker of purine derivatives and factors that influence creatinine in ruminants. **Anim.** 12. doi:10.3390/ani12080988.

Prahl, M. C., E. M. Paula, R. F. Cooke, and T. C. J. Pereira. 2022. Hepatic urea, creatinine, and uric acid metabolism in dairy cows. **Front. Physiol.** 13. doi:10.3389/fphys.2022.1002456.

Rigon, F., A. R. Cagliari, E. Magnani, M. I. Marcondes, D. Grandini, B. Humphrey, P. D. B. Benedeti, F. D. Resende, G. R. Siqueira, R. H. Branco, and E. M. Paula. 2025. Employing direct-fed microbial and hydrolyzed yeast to modulate in vitro ruminal fermentation parameters of medium- and low-quality forages. **J. Agric. Sci.** doi:10.1017/S0021859625100099.

Shi, C., Y. Zhang, Z. Lu, and Y. Wang. 2017. Solid-state fermentation of corn–soybean meal mixed feed with Bacillus subtilis and Enterococcus faecium for degrading antinutritional factors and enhancing nutritional value. **J. Anim. Sci. Biotechnol.** 8:1–9. doi:10.1186/s40104-017-0184-2.

Silva, T. H., B. R. Amâncio, E. Magnani, G. W. Meurer, H. G. Reolon, T. G. Timm, B. I. Cappellozza, R. H. Branco, and E. M. Paula. 2024. Evaluation of direct-fed microbials on in vitro ruminal fermentation, gas production kinetics, and greenhouse gas emissions in different ruminant diets. **Front. Anim. Sci.** 5. doi:10.3389/fanim.2024.1320075.

Silva, T. P., Moraes, E. H. B. K., & Barbero, R. P. (2024). Combined supplementation of Bacillus spp. and yeast improves nitrogen metabolism in cattle fed tropical forages. **Animal Feed Science and Technology**, 307, 115822.

Silva, J. F. C., et al. (2021). Nitrogen deficiency constraints on rumen fiber degradation in tropical grasses. **Grass and Forage Science**, 76(3), 412–422.

Sollenberger, L. E., and D. Cherney. 2015. **Evaluating forage production and quality.** In: Optimizing ecosystem services of grassland–livestock systems. USDA-ARS, Gainesville, FL.

Sun, P., J. Q. Wang, and L. F. Deng. 2013. Effects of Bacillus subtilis natto on milk production, rumen fermentation, and ruminal microbiome of dairy cows. **Anim.** 7:216–222. doi:10.1017/S1751731112001188.

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. doi:10.3168/jds.S0022-0302(91)78551-2.

Womack, A. M., E. B. Kegley, S. Gadberry, T. Hess, D. Hubbell, and P. A. Beck. 2024. Evaluating supplementation programs for growing calves grazing bermudagrass pastures. **Appl. Anim. Sci.** 40:91–102. doi:10.15232/aas.2023-02494.

Wróbel, B., W. Zielewicz, and A. Paszkiewicz-Jasińska. 2025. Improving forage quality from permanent grasslands to enhance ruminant productivity. **Agric.** 15. doi:10.3390/agriculture15131438.

FIGURES AND TABLES

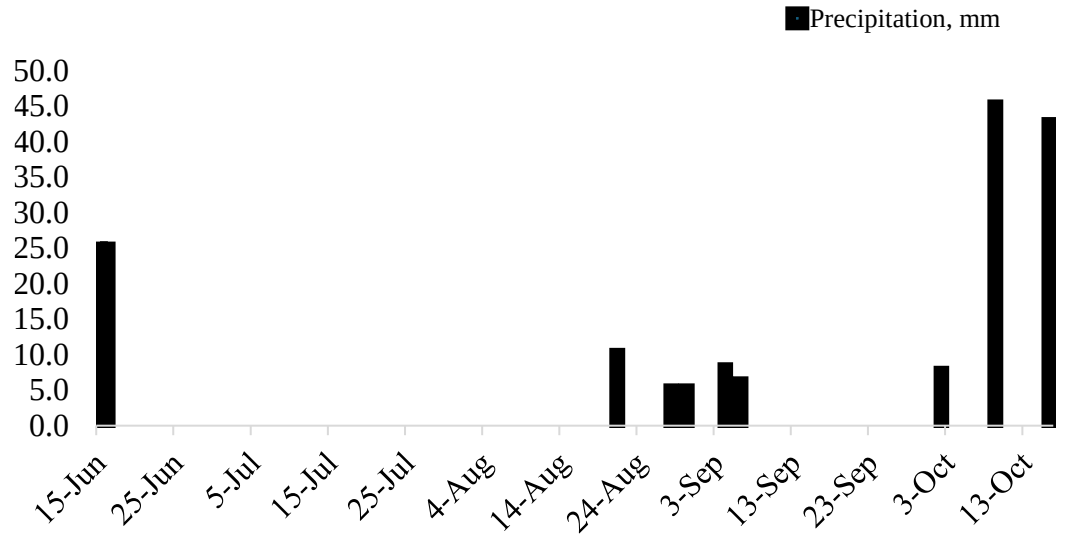

Figure 1. Precipitation throughout the trial, from June to October 2023

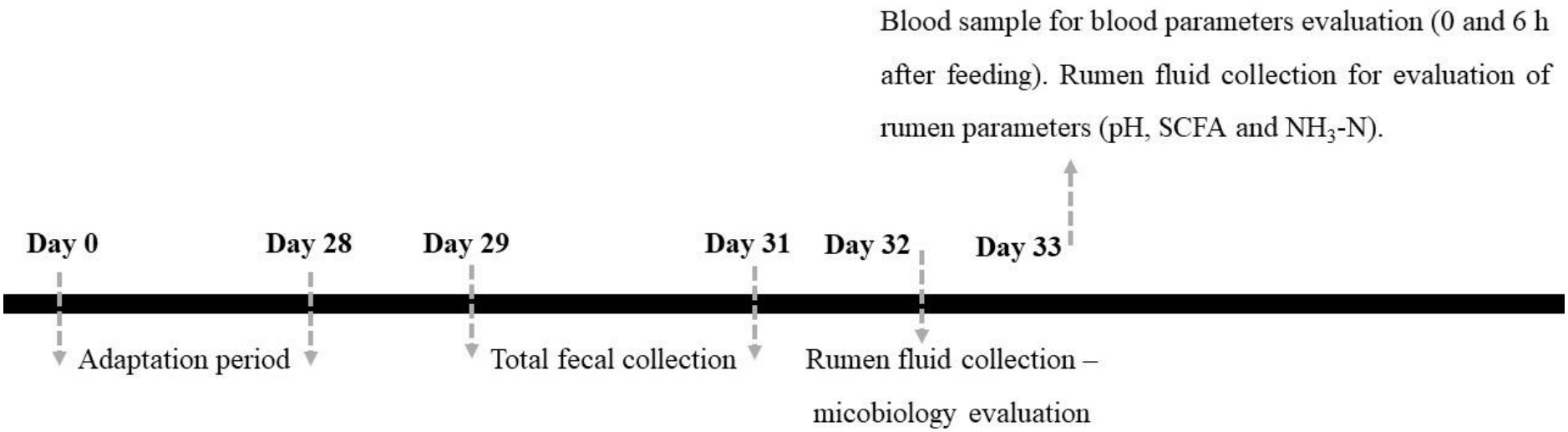

Figure 2. Timeline of sample collection from Exp. 1 (metabolism trial).

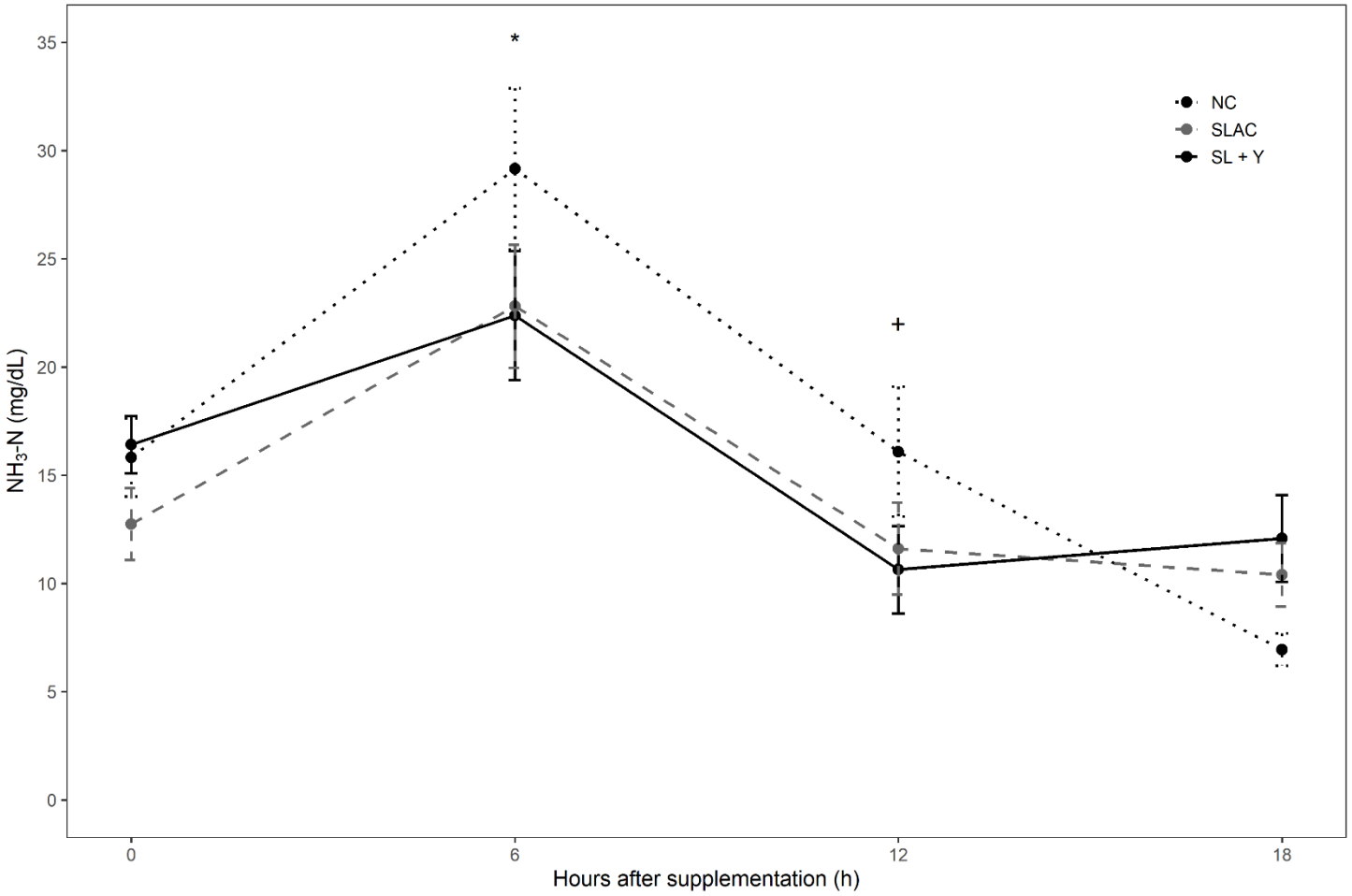

Figure 3. Ruminal ammonia nitrogen concentration (NH_3-N, mg/dL) over the of Nellore young bulls grazing under tropical conditions (**Exp. 1**).

Table 1. Chemical composition of *Urochloa brizantha* (cv. Marandu) hay used in the metabolism trial and commercial supplement used in metabolism and performance trial (**Exp. 1** and **2).**

Item	*B. brizantha hay*	*Commercial supplement*[1]
Dry matter, g/kg	830	964
Ash, g/kg	41.3	342
Organic matter, g/kg	959	622
Crude protein, g/kg	27.8	221
Neutral detergent fiber, g/kg	849	114

[1]**Supplement composition:** livestock urea, zinc oxide, manganese monoxide, ground dried corn, limestone, peanut meal, dicalcium phosphate, copper sulfate, ventilated sulfur, iron sulfate, cobalt sulfate, calcium iodide, sodium selenite and sodium chloride.

Table 2. Effects of different natural feed additives on nutrient intake and total tract apparent digestibility of Nellore young bulls grazing under tropical conditions (Exp. 1).

	Treatment[1]				
Item	**NC**	**SLAC**	**SL + Y**	**SEM[2]**	***P*-value**
Hay + supplement intake, kg/d					
Dry matter	6.70	6.22	5.83	0.441	0.39
Crude protein	0.445	0.428	0.416	0.024	0.70
Neutral detergent fiber	4.49	4.05	3.74	0.357	0.34
Total tract apparent digestibility, %					
Dry matter	58.5	56.8	62.8	2.103	0.12
Crude protein	55.3[b]	56.0[b]	64.1[a]	2.075	**0.005**
Neutral detergent fiber	56.3	50.8	57.6	2.651	0.162

[1]NC: negative control; SLAC: blend of *Bacillus subtilis*, *B. licheniformis, B. amyloliquefaciens* and *B. coagulans*; SL+Y: blend of *Bacillus subtilis* and *B. licheniformis* plus autolyzed sugar-cane yeast; [2]Standard error mean.

Table 3. Effects of different natural feed additives on ruminal parameters (pH, ammonia-N, and short chain fatty acid profile) of Nellore young bulls grazing under tropical conditions (Exp. 1).

	Treatment[1]				***P*-value[3]**		
Item	**NC**	**SLAC**	**SL + Y**	**SEM[2]**	**TRT**	**Hour**	**TRT X Hour**
pH	6.72	6.81	6.69	0.046	0.17	<0.001	0.59
NH_3-N, mg/dL	17.0	14.4	15.4	1.530	0.67	<0.001	0.07
SCFA, mmol	90.5	95.8	95.5	3.185	0.43	0.04	0.83
SCFA, Mol/100 mol	4.12	4.11	4.17	0.049	0.63	<0.001	0.66
Acetate	71.5	71.7	72.2	0.404	0.49	0.69	0.84
Propionate	14.7	14.9	14.6	0.151	0.27	<0.001	0.90
Butyrate	7.06	6.99	6.77	0.144	0.33	0.17	0.87
Isobutyrate	2.36	2.25	2.26	0.095	0.64	0.26	0.64
Isovalerate	2.00	1.90	1.90	0.089	0.59	0.06	0.60
Valerate	2.37	2.23	2.27	0.088	0.54	0.84	0.59
BCSFA, mmol							
Ace:prop	4.89	4.81	4.96	0.061	0.25	0.001	0.96

[1]NC: negative control; SLAC: blend of *Bacillus subtilis*, *B. licheniformis, B. amyloliquefaciens* and *B. coagulans*; SL+Y: blend of *Bacillus subtilis* and *B. licheniformis* plus autolyzed sugar-cane yeast; NH_3-N: ammonia-nitrogen; SCFA: short chain fatty acid; BCSFA: branched chain short fatty acid; Ace:prop: acetate to propionate ratio; [2]Standard error mean; [3]*P*-value treatment, hour, and treatment x hour, respectively.

Table 4. Effects of different natural feed additives on blood parameters (glucose, cholesterol, urea, creatinine, total protein and albumin concentrations) of Nellore young bulls grazing under tropical conditions (**Exp. 1).**

Item	Treatment[1]			SEM[2]	Hour [3]		SEM[2]	*P*-value[4]		
	NC	SLAC	SL + Y		0	6		T	H	T x H
Glucose	79.0	75.7	75.7	2.99	75.8	77.7	2.44	0.452	0.441	0.782
Cholesterol	108	107	108	4.68	111	104	3.82	0.995	0.101	0.864
Urea	25.1	23.8	27.0	1.64	20.9^{b}	29.7^{a}	1.34	0.159	<0.001	0.751
Creatinin	2.29^{b}	2.22^{b}	2.40^{a}	0.05	2.27	2.34	0.04	0.006	0.111	0.328
Total protein	7.32	7.26	7.22	0.14	7.47^{a}	7.07^{b}	0.11	0.764	<0.001	0.111
Albumin	3.16^{x}	3.20^{x}	3.05^{y}	0.07	3.23^{a}	3.05^{b}	0.06	0.095	0.003	0.721

[1]NC: negative control; SLAC: blend of *Bacillus subtilis*, *B. licheniformis, B. amyloliquefaciens* and *B. coagulans*; SL+Y: blend of *Bacillus subtilis* and *B. licheniformis* plus autolyzed sugar-cane yeast; [2]Standard error mean; [3]Hour after feeding. [4]T: Treatment effect, H: effect of collection time and interaction; Averages followed by letters "a or b" when $P < 0.05$ and "x or y" when $0.05 < P \leq 0.10$. mean.

Table 5. Quantitative and qualitative characteristics of *Urochloa brizantha* cv. Marandu during the growing phase of Nellore young bulls grazing under tropical conditions (**Exp. 2**).

Item[1]	**Treatment**[2]			**SEM**[3]	***P*-value**[4]	**Day of study**[5]			**SEM**[3]	***P*-value**[4]
	NC	*SL*	*SL+Y*			*28*	*56*	*112*		
Quantitative characteristics										
Height, cm	29.1	28.9	27.9	0.690	0.493	28.1^{a}	27.6^{a}	30.2^{b}	0.509	0.003
Forage mass, kg DM/ha	6072	5262	5405	466.5	0.465	5156	5341	6272	467.6	0.285
Density, kg DM/m^{3}	1.73	1.55	1.77	0.140	0.516	1.81^{a}	1.67^{ab}	1.56^{b}	0.156	0.040
Green leaf, %	20.5	19.5	22.5	5.01	0.911	10.5^{a}	11.2^{a}	40.7^{b}	4.60	< 0.001
Green stem, %	19.6	17.5	24.3	5.08	0.639	16.7	21.6	23.2	5.51	0.499
Senescent leaf, %	9.45	13.2	9.81	3.44	0.69	9.53^{a}	16.3^{ab}	6.59^{a}	4.11	0.003
Senescent stem, %	34.1	33.4	20.8	17.9	0.75	13.7^{a}	50.8^{b}	23.6^{a}	17.5	< 0.001
Forage offer, kg DM/kg BW	7.86	6.25	7.02	1.38	0.601	6.59	6.24	8.31	1.39	0.158
Stocking rate, (AU ha-^{1})	2.01	1.82	1.83	0.299	0.605	1.75^{a}	1.82^{ab}	1.83^{b}	0.367	0.012
Qualitative characteristics, % of DM										
Dry matter	38.1	38.0	38.1	1.39	0.998	27.3^{a}	58.8^{b}	28.1^{a}	1.59	< 0.001
Ash	7.68	7.50	7.64	0.137	0.649	8.57^{a}	6.7^{b}	7.55^{c}	0.192	< 0.001
Crude protein	10.1	9.79	9.87	0.268	0.736	12^{a}	5.86^{b}	11.8^{a}	0.427	< 0.001
Ethereal extract	1.23	1.23	1.25	0.035	0.878	1.44^{a}	0.98^{b}	1.29^{c}	0.047	< 0.001
Neutral detergent fiber	71.5	72.1	72.4	0.615	0.586	67.6^{a}	78.6^{b}	69.7^{a}	0.823	< 0.001
Acid detergent fiber	34.9	34.9	35.2	0.608	0.956	30.5^{a}	39.9^{b}	34.8^{c}	0.911	< 0.001

Lignin	4.88	5.07	4.95	0.163	0.576	4.53[a]	6.39[b]	3.99[a]	0.182	< 0.001
iNDF	23.4	24.2	24.3	0.826	0.202	21.1[a]	28.2[b]	22.6[a]	1.04	< 0.001

[1]Indigestible neutral detergent fiber; [2]NC: negative control; SL: blend of *Bacillus subtilis* and *B. licheniformis*; SL+Y: blend of *Bacillus subtilis* and *B. licheniformis* plus autolyzed sugar-cane yeast; [3]Standard error mean of treatment and day of study, respectively; [4]*P*-value of treatment and day of study, respectively; [5]Effects of day of the study were not detected ($P > 0.05$) for all variables. [a,b]For each variable, within a row, means without a common superscript letter are significantly different.

Table 6. Effects of different natural feed additives on body weight (BW) and average daily gain (ADG) of Nellore bulls grazing under tropical conditions (**Exp. 2**).

	Treatment[1]				***P*-value[3]**		
Item	**NC**	**SL**	**SL + Y**	**SEM[2]**	**TRT**	**Per**	**TRT X Per**
Body weight, kg							
Overall	439.1	449.6	442.0	1.754	0.09	<0.001	**0.006**
d 28	437.9	443.8	438.4				
d 56	448.5	456.0	449.7				
d 84	436.7	447.9	438.6				
d 112	433.2[b]	450.8[a]	441.2[ab]				
ADG, kg[2]							
Overall	0.121[b]	0.251[a]	0.200[ab]	0.043	**0.018**	<0.001	0.50
d 28	0.577	0.701	0.642				
d 56	0.399	0.456	0.411				
d 84	-0.370	-0.258	-0.351				
d 112	-0.123	0.104	0.100				

[1]NC: negative control; SL: blend of *Bacillus subtilis* and *B. licheniformis*; SL+Y: blend of *Bacillus subtilis* and *B. licheniformis* plus autolyzed sugar-cane yeast;[2]Average daily gain; [3]Standard error mean; [4]*P*-value treatment, period, and treatment x period, respectively.

Table 7. Effects of different natural feed additives on carcass traits of Nellore young bulls grazing under tropical conditions obtained by *B*-mode ultrasound (**Exp. 2**).

	Treatment[2]				
Item[1]	**NC**	**SL**	**SL + Y**	**SEM[3]**	***P*-value[4]**
LMA, cm^2	68.4[b]	71.4[a]	70.1[a]	0.72	0.015
LMA, cm2/100	15.1	15.1	15.1	0.95	0.995
Ratio	0.46	0.47	0.46	0.005	0.635
Marbling	2.88	2.90	2.90	0.48	0.903
BFT, mm	2.44	2.52	2.47	0.06	0.651
BFT, mm/100	0.55	0.54	0.54	0.02	0.802
RFT, mm	3.59	3.69	3.89	0.16	0.087

[1]LMA: Longissimus muscle area; BFT: backfat thickness; RFT: rumpfat thickness; [2]NC: negative control; SL: blend of *Bacillus subtilis* and *B. licheniformis*; SL+Y: blend of *Bacillus subtilis* and *B. licheniformis* plus autolyzed sugar-cane yeast; [3]tandard error mean; [4]*P*-value treatment.