UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS CAMPUS JABOTICABAL

UTILIZAÇÃO DE ENZIMAS EXÓGENAS EM DIETAS DE CONFINAMENTO: EFEITO SOBRE CONSUMO, DIGESTIBILIDADE DOS NUTRIENTES, PARÂMETROS RUMINAIS, BALANÇO DE NITROGÊNIO E DESEMPENHO DE BOVINOS NELORE

Igor Machado Ferreira
Zootecnista

2025

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Discente: Igor Machado Ferreira
Orientador: Prof. Dr. Flávio Dutra de Resende

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

2025

F383u

Ferreira, Igor Machado
Utilização de enzimas exógenas em dietas de confinamento: efeito sobre o consumo, digestibilidade dos nutrientes, parâmetros ruminais, balanço de nitrogênio e desempenho de bovinos Nelore / Igor Machado Ferreira. -- Jaboticabal, 2025
85 p.

Tese (doutorado) - Universidade Estadual Paulista (UNESP), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal
Orientador: Flávio Dutra de Resende

1. Zootecnia. 2. Produção Animal. 3. Confinamento de Bovinos. 4. Ciência de dados. 5. Nutrição Animal. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Dados fornecidos pelo autor(a).

IMPACTO POTENCIAL DESTA PESQUISA

Conforme a portaria Unesp nº 117, de 21 de dezembro de 2022

As enzimas são quimicamente definidas como proteínas que catalisam processos biológicos em uma reação bioquímica específica, degradando uma gama restrita de substratos em locais de reação específicos. Está pesquisa estudou o potencial impacto da utilização de enzimas exógenas na nutrição de bovinos de corte confinados, sumarizando os principais efeitos sobre o desempenho, consumo, digestibilidade total dos nutrientes, balanço de nitrogênio, parâmetros fermentativos e microbiota ruminal. Portanto, os dados gerados nessa pesquisa demonstram efetivamente que a suplementação com enzimas exógenas aumenta a digestibilidade dos nutrientes promovendo potencial impacto sobre o ganho de carcaça dos animais, sendo uma ferramenta estratégica para aumentar a eficiência de utilização dos nutrientes dentro do sistema de produção de bovinos confinados, de modo a contribuir para uma pecuária de corte mais eficiente e sustentável.

POTENCIAL IMPACT OF THIS RESEARCH

Acording to Unesp ordinance No. 117, dated December 21, 2022

Enzymes are chemically defined as proteins that catalyze specific biochemical reactions by breaking down a limited range of substrates at defined reaction sites. This study evaluated the potential effects of supplementing exogenous enzyme in the feedlot beef cattle diets, focusing on animal performance, feed intake, total nutrient digestibility, nitrogen balance, fermentation parameters, and ruminal microbiota. The results demonstrate that exogenous enzyme supplementation enhances nutrient digestibility and may contribute to improved carcass gain. This approach represents a strategic tool to increase nutrient use efficiency and promote more sustainable beef cattle production in feedlot systems.

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: UTILIZAÇÃO DE ENZIMAS EXÓGENAS EM DIETAS DE CONFINAMENTO: EFEITO SOBRE CONSUMO, DIGESTIBILIDADE DOS NUTRIENTES, PARÂMETROS RUMINAIS, BALANÇO DE NITROGÊNIO E DESEMPENHO DE BOVINOS NELORE

AUTOR: IGOR MACHADO FERREIRA

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Aprovado como parte das exigências para obtenção do Título de Doutor em Ciência Animal, área: Nutrição Animal pela Comissão Examinadora:

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Jaboticabal, 23 de julho de 2025

DADOS CURRICULARES DO AUTOR

Igor Machado Ferreira, nascido em 08 de novembro de 1995 em Recreio, Minas Gerais. Filho de Eliane Aparecida Machado Ferreira e Amauri da Costa Machado.

Possui curso técnico em Zootecnia (2011-2013) pelo Instituto Federal de Educação, Ciência e Tecnologia do Sudeste de Minas Gerais, Campus Rio Pomba e Graduação em Zootecnia (2014- 2019) na Universidade Federal de Lavras. Durante a graduação, participou do grupo NEFOR como membro efetivo e compôs o quadro de vice coordenador do grupo por dois anos. Sob a orientação do professor Dr. Daniel Rume Casagrande (2015 – 2019) conduziu quatro projetos de iniciações científicas. No início de 2019, o candidato realizou uma atividade acadêmica internacional nos Estado Unidos, onde ficou por 5 meses estagiando no *Range Cattle Research & Education Center* – Ona, Flórida sob a orientação do professor Dr. João Vendramini.

Em agosto de 2019 ingressou como aluno de mestrado no programa de pós-graduação da Faculdade de Ciências Agrárias e Veterinárias – UNESP - Campos Jaboticabal, bolsista FAPESP. Durante o mestrado, atuou em atividades de pesquisa voltada a avaliar a suplementação de bovinos de corte em pastejo, na APTA Colina SP, sob orientação do professor Dr. Flávio Dutra de Resende. Graduou-se mestre em Julho de 2021.

Em agosto de 2021, ainda no PPG em Zootecnia da Faculdade de Ciências Agrárias e Veterinárias – UNESP - Campos Jaboticabal, bolsista FAPESP, ingressou no doutorado, como bolsista FAPESP. Durante o doutorado, trabalhou na condução de projetos de pesquisa voltado à avaliação de aditivos alimentares em bovinos confinados, na APTA Colina SP, sob orientação do professor Dr. Flávio Dutra de Resende. Passou por um período sanduíche de 1 ano na University of Winsconsin – Madison, EUA, sobre a orientação do Prof. Hilário Cuquetto Mantovani, onde teve a oportunidade de desenvolver diversos experimentos que visavam avaliar os efeitos de diversos aditivos alimentares na fermentação ruminal in vitro, na cinética da produção de gases, nas emissões de gases entéricos de efeito estufa e na composição do microbioma ruminal. Graduou-se como doutor em Agosto de 2025.

Dedico...

A Deus, aos grandes amigos e companheiros de estrada
e à minha família!

Aos meus orientadores, pelos valorosos ensinamentos, pelas trocas de experiências, pela inspiração e pela liberdade fornecida para que eu pudesse conduzir os estudos.

... a vocês, **ofereço!**

AGRADECIMENTOS

A Deus, por estar sempre ao me lado, guardando-me de todo o mal e abençoando-me desde o princípio dessa trajetória, abrindo portas e me dando forças para seguir em frente nos meus objetivos.

A minha mãe Eliane Aparecida Machado Ferreira, e meu pai Amauri da Costa Machado pelos valores passados e o esforço para que eu pudesse chegar até aqui. Pai e mãe reconheço tudo o que fizeram por mim, espero enchê-los de orgulho e que nossa família esteja sempre vivendo em abundância.

Aos meus avós, tios e primos que sempre me apoiaram e estavam sempre dispostos a aconselhar e ajudar na realização dos meus sonhos.

Ao meu orientador, professor Flávio Dutra de Resende e ao professor Gustavo Rezende Siqueira, que além de exemplos, me estenderam a mão e ofertaram tudo que fosse necessário para meu desenvolvimento. Gratidão a vocês!

Ao professor Hilário Cuquetto Mantovani por ter me recebido nos EUA durante o meu doutorado sanduíche, além de todo apoio e ensinamentos passados.

Aos amigos, que sempre estiveram ao meu lado, dando suporte. Em especial aos meus companheiros de experimento Mateus e Saulo, pela parceria durante todas as fases desse experimento e dos tantos outros que conduzimos juntos. Foi um grande prazer trabalhar com você.

Aos colegas do Grupo de Estudos em Produção de Ruminantes – GEPROR: Aline, Amanda, Anna Lidia, Ariane, Bianca, Fernanda Rigon, Fernanda Scheeren, Gabriel, Gilyard, Hiam, Iorrano, José Maria, Karla, Luis, Mariana, Mateus Abreu e Saulo, pelas discussões e oportunidade de crescimento profissional e pessoal. Vocês foram essenciais para a condução do experimento. Meu muito obrigado.

Aos estagiários, que passaram pela APTA durante a condução deste estudo, obrigado por toda a ajuda.

Aos funcionários da APTA de Colina: Adenilsom, Alêssandra, Antônio Carlos, Chico, Juninho, Francisco (Sr. Verde), Tio Lore, Luizão, Luizinho, Miltinho, Neia, Roberto, Rogério (Tratador), Tia Sueli e Tio Toizinho, pelos anos de convívio e auxílio no desenvolvimento deste projeto. Sem a ajuda de vocês essa jornada seria muito mais laboriosa.

À Regina, ao Rodolfo (Toga) e à Thais, pelo auxílio nas análises laboratoriais.

À todo time técnico da Alltech pelo apoio financeiro e intelectual das pesquisas. Em especial ao meu amigo Luiz Fernando pelas visitas na APTA e suporte ao experimento.

Um agradecimento especial também ao meu grande amigo/irmão Bruno, você sabe o quanto é responsável por esse momento, agradeço a Deus por ter você como um amigo/irmão. Ao Prof. Daniel Rume Casagrande por todo os ensinamentos durante a orientação nos projetos de iniciação científica na UFLA.

À Universidade Estadual Paulista (UNESP) por me proporcionar condições para o desenvolvimento do doutorado, e a Agência Paulista de Tecnologia dos Agronegócios (APTA), que proporcionou toda estrutura necessária para o desenvolvimento do projeto de pesquisa.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela bolsa concedida durante o período do doutorado (processo 2022/00989-1) e também pelo apoio financeiro durante o período sanduíche em Madison - WI, EUA (processo 2023/11136-2).

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

SUMÁRIO

CERTIFICADO Nº 004/2022 – CEUA

Certificamos que o projeto apresentado dia 18 de novembro de 2022 intitulado **"UTILIZAÇÃO DE ENZIMAS EXÓGENAS EM DIETAS DE CONFINAMENTO: EFEITO SOBRE A DIGESTIBILIDADE, METABOLISMO, DESEMPENHO, CONSUMO, CARACTERÍSTICAS DE CARCAÇA E GENES DIFERENCIALMENTE EXPRESSOS DE BOVINOS NELORE"**, foi registrado com o **protocolo nº 004/2022,** está sob a responsabilidade do Pesquisador Científico **Dr. Flávio Dutra de Resende** 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 13/07/2024
Espécie/linhagem e/ou raça	Bovino Nelore
Nº de animais	136
Sexo/Idade/Peso aprox.	136 M e 24 a 36 meses / 380 Kg
Localização/Origem	Unidade Regional de Pesquisa e Desenvolvimento de Colina
Responsável Técnico	Dr. Gustavo Rezende Siqueira

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SÃO PAULO
GOVERNO DO ESTADO

UTILIZAÇÃO DE ENZIMAS EXÓGENAS EM DIETAS DE CONFINAMENTO: EFEITO SOBRE O CONSUMO, DIGESTIBILIDADE DOS NUTRIENTES, PARÂMETROS RUMINAIS, BALANÇO DE NITROGÊNIO E DESEMPENHO DE BOVINOS NELORE

RESUMO - O objetivo do presente estudo foi avaliar os efeitos da suplementação com enzimas exógenas sobre o desempenho, digestibilidade de nutrientes, metabolismo do nitrogênio, fermentação e microbiota ruminal de bovinos de corte em sistemas de confinamento. Para isso, utilizou-se uma abordagem integrativa composta por uma metanálise (Cap. 2) e um experimento in vivo com animais canulados (Cap. 3). A metanálise estimou os efeitos da inclusão das enzimas exógenas sobre o desempenho, consumo, digestibilidade de nutrientes e parâmetros de fermentação ruminal de bovinos de corte em confinamento, além de identificar fatores que influenciaram esses resultados. Foi selecionado 94 artigos sendo que 23 estudos atenderam aos critérios de elegibilidade. A magnitude do efeito foi determinada utilizando a diferença média ponderada (DMP) entre os grupos tratados com enzimas e o controle (sem inclusão), através do pacote “metafor” no software R. A heterogeneidade das variáveis foi avaliada por meio de meta-regressão e análise de subgrupos. A inclusão das enzimas exógenas não afetou o peso corporal final ($P \geq 0,871$), o consumo de matéria seca (CMS; $P \geq 0,467$), o ganho médio diário (GMD; $P \geq 0,145$) e a eficiência alimentar (EA; $P \geq 0,417$). No entanto, a suplementação com enzimas proporcionou aumento no peso da carcaça quente (PCQ; $P = 0,047$; DMP = 2,21 kg), sem afetar os parâmetros ruminais ($P \geq 0,225$) e a concentração de amônia ruminal ($P = 0,143$). A inclusão das enzimas melhorou a digestibilidade da MS ($P < 0,01$; DMP = 16,9 g/kg), proteína bruta ($P = 0,003$; DMP = 20,2 g/kg) e da fibra em detergente neutro (FDN; $P = 0,003$; DMP = 20,2), sem afetar a digestibilidade da matéria orgânica e do amido ($P \geq 0,388$). Baixa heterogeneidade foi observada ($I^2 < 25\%$) para a maioria dos resultados de desempenho e digestibilidade de nutrientes; enquanto heterogeneidade moderada foi observada ($P \leq 0,057$; $I^2 = 25–50\%$) para a digestibilidade da MS e da FDN, e alta para os parâmetros de fermentação ruminal ($P < 0,01$; $I^2 > 50\%$). A análise do gráfico de funil não revelou viés de publicação para a maioria das variáveis avaliadas ($P \geq 0,10$). No segundo estudo, dez bovinos Nelore canulados no rúmen (543 ± 28,6 kg) foram distribuídos em baias individuais em um quadrado latino duplo (5 × 5). Os seguintes tratamentos foram testados: controle (**CON**, sem suplementação com enzimas exógenas), amilase [**AML**, 0,5 g/kg de matéria seca (MS) da dieta], xilanase (**FBL**, 0,9 g/kg de MS), combinação de meia dose (**MD**, 0,25 g de AML + 0,45 g de FBL/kg de MS) e combinação de dose completa de ambas as enzimas (**DC**, 0,5 g de AML + 0,90 g de FBL/kg de MS). O período experimental foi de 19 dias e incluiu coleta total de urina e fezes (dias 15 a 18) e

amostragem de fluido ruminal (dia 19) para análise de amônia, ácidos graxos voláteis (AGV), pH, diversidade e riqueza microbiana. A suplementação com enzimas exógenas resultou em menor concentração de amônia ruminal (P = 0,040), maior proporção de acetato (P < 0,001), menor excreção de N fecal (P = 0,049) e urinária (P = 0,036), e maior retenção corporal de nitrogênio (P = 0,045). Além disso, apesar de não ter sido observado alterações significativas na diversidade alfa e beta da microbiota, a suplementação com enzimas exógenas provocou alterações em vários táxons microbianos a nível de família e gênero (P ≤ 0,10), o que possivelmente foi associado às alterações observadas na fermentação ruminal. Em conclusão, nossos resultados demonstraram que a suplementação com enzimas exógenas promoveu benefícios consistentes na digestibilidade dos nutrientes e na eficiência de utilização do nitrogênio, o que possivelmente pode ser associado ao aumento do peso de carcaça dos animais, sem que tenha sido observado efeito expressivo sobre os parâmetros ruminais. Sendo assim, a combinação de metodologias (metanálise e estudo in vivo) permitiu uma avaliação abrangente e robusta, contribuindo significativamente para o entendimento dos mecanismos de ação das enzimas exógenas em sistemas de confinamento, fornecendo subsídios para o uso estratégico visando aumentar a eficiência produtiva.

Palavras chaves: Análise de dados**,** Enzimas amilolíticas, Enzimas fibrolíticas, Fermentação ruminal, Metabolismo de nitrogênio, Microbiota ruminal.

USE OF EXOGENOUS FEED ENZYMES IN FEEDLOT DIETS: EFFECT ON THE INTAKE, NUTRIENT DIGESTIBILITY, RUMINAL PARAMETERS, NITROGEN BALANCE AND PERFORMANCE OF NELLORE CATTLE

ABSTRACT - The objective of the present study was to evaluate the effects of exogenous feed enzyme (EFE) supplementation on performance, nutrient digestibility, nitrogen metabolism, ruminal fermentation, and rumen microbiota of beef cattle in feedlot. An integrative approach was adopted, combining a meta-analysis (Chapter 2) with an in vivo experiment using rumen-cannulated animals (Chapter 3). The meta-analysis estimated the effects of EFE inclusion on performance, intake, nutrient digestibility, and ruminal fermentation parameters in beef cattle, as well as identified factors influencing these outcomes. A total of 94 articles were screened, of which 23 studies met the eligibility criteria. The magnitude of the effect was determined using the weighted mean difference (WMD) between enzyme-treated and control (non-supplemented) groups, using the “metafor” package in R software. Heterogeneity among variables was assessed through meta-regression and subgroup analyses. EFE supplementation did not affect final body weight ($P \geq 0.871$), dry matter intake (DMI; $P \geq 0.467$), average daily gain (ADG; $P \geq 0.145$), and feed efficiency (FE; $P \geq 0.417$). However, EFE supplementation increased hot carcass weight (HCW; $P = 0.047$; WMD = 2.21 kg), without affecting ruminal parameters ($P \geq 0.225$) or ruminal ammonia concentration ($P = 0.143$). EFE inclusion improved dry matter digestibility ($P < 0.01$; WMD = 16.9 g/kg), crude protein digestibility ($P = 0.003$; WMD = 20.2 g/kg), and neutral detergent fiber (NDF) digestibility ($P = 0.003$; WMD = 20.2 g/kg), without affecting organic matter and starch digestibility ($P \geq 0.388$). Low heterogeneity was observed ($I^2 < 25\%$) for most performance and nutrient digestibility outcomes, while moderate heterogeneity was found ($P \leq 0.057$; $I^2 = 25–50\%$) for dry matter and NDF digestibility, and high heterogeneity for ruminal fermentation parameters ($P < 0.01$; $I^2 > 50\%$). Funnel plot analysis did not reveal publication bias for most variables assessed ($P \geq 0.10$). In the second study, ten rumen-cannulated Nellore steers (543 ± 28.6 kg) were assigned to a replicated Latin-square design (5 × 5) in individual pens. The animals were assigned to the following treatments: control (CON, no EFE supplementation), amylase (AML, 0.5 g/kg of dietary dry matter [DM]), xylanase (FBL, 0.9 g/kg of DM), combination of half-dose (HD, 0.25 g AML + 0.45 g FBL/kg of DM), and combination of full dose of both enzymes (FD, 0.5 g AML + 0.90 g FBL/kg of DM). The experimental period lasted 19 days and included total urine and feces collection (days 15 to 18) and ruminal fluid sampling (day 19) for analysis of ammonia, volatile fatty acids (VFA), pH, and microbial diversity and richness. EFE supplementation resulted in lower ruminal ammonia concentration ($P = 0.040$), higher acetate proportion ($P < 0.001$), reduced fecal ($P = 0.049$) and urinary ($P = 0.036$) nitrogen excretion, and increased body

nitrogen retention (P = 0.045). Additionally, although no significant changes were observed in alpha and beta diversity of the microbiota, EFE supplementation led to changes in several microbial taxa at the family and genus levels (P ≤ 0.10), which were possibly associated with the observed changes in ruminal fermentation parameters. In conclusion, our results demonstrated that EFE supplementation consistently improved nutrient digestibility and nitrogen utilization efficiency, which may have contributed to increased carcass weight, without significant effects on ruminal parameters. Overall, the combination of methodologies (meta-analysis and in vivo study) enabled a comprehensive and robust evaluation, significantly contributing to the understanding of the mechanisms of action of EFE in feedlot systems and providing support for their strategic use to enhance production efficiency.

Keywords: Amylolytic enzymes, Fibrolytic enzymes, Data analysis, Nitrogen metabolism, Rumen microbiome, Ruminal fermentation.

CAPÍTULO 1 - CONSIDERAÇÕES GERAIS

O Brasil é o maior exportador de carne bovina mundial (FAO, 2019), no qual 14,1% dos animais abatidos são oriundos da terminação em confinamento (IBGE, 2019), aumento de 100% no número de animais confinados de 2009 para 2019 (Silvestre e Millen, 2021). Desse modo, observamos que a cadeia produtiva de carne brasileira está em evolução, justificando a necessidade de tecnologias que visão maximizar a eficiência produtiva no confinamento, haja visto que ocorreu aumento no custo produtivo devido ao aumento da utilização de alimentos concentrados (Silvestre e Millen, 2021).

A eficiência de produção em um sistema de confinamento está relacionada à melhoria do aproveitamento dos alimentos pelos animais. Assim, dentro das diversas tecnologias disponíveis no mercado, a utilização de enzimas exógenas em dietas de ruminantes são alternativas eficazes (Beauchemin et al., 2004b, 2004a; Meale et al., 2014; Tirado-González et al., 2021; Tricarico et al., 2007), uma vez que a eficiência do processo de conversão dos carboidratos estruturais e não estruturais em carne e leite são dependentes da digestibilidade das células e conteúdos vegetais no trato digestório dos ruminantes. Assim, embora existam relatos científicos da utilização de enzimas exógenas para bovinos de corte melhorando o desempenho animal desde a década de 1960 (Burroughs et al., 1960), a adoção dessa tecnologia ao longo dos anos tem sido lenta, principalmente devido aos resultados inconsistentes relacionados à resposta animal. De certa forma, isso pode ser uma justificativa da não utilização das enzimas exógenas em dietas de confinamento observada no último levantamento realizado com nutricionistas de bovinos de corte no Brasil (Silvestre e Millen, 2021) e nos Estados Unidos (Samuelson et al 2016).

Com o intuito de compreender os fatores que levam à inconsistência nas respostas produtivas, revisões sistemáticas e estudos metanalíticos sobre o assunto foram desenvolvidos, mostrando os principais fatores que afetam a atuação das enzimas em dietas para ruminantes, (Arriola et al., 2017; Beauchemin et al., 2003; Meale et al., 2014; Tirado-González et al., 2021, 2018). Esses autores reportaram que as inconsistências nas respostas são atribuídas à diferença na atividade enzimática, taxa de aplicação, composição das enzimas, interações com a composição da dieta e interações com o ambiente ruminal (Adesogan, 2005; Beauchemin et al., 2004). Além

disso, também existem fatores adicionais que incluem o uso de experimentos com poder estatístico insuficiente, desenhos experimentais e/ou durações inadequadas, escolhas enzimáticas erradas, bem como o uso de medidas incorretas da atividade enzimática e designações enzimáticas enganosas (Adesogan et al., 2014). Desse modo, embora esses estudos tenham resumido qualitativamente o impacto da utilização de enzimas na nutrição de bovinos, era necessária uma abordagem quantitativa mais rigorosa, fato que despertou nosso interesse em realizar um estudo metanalítico sobre o efeito da utilização de enzimas exógenas na nutrição de bovinos de corte que compôs o capítulo 2 dessa tese.

O conhecimento dos fatores que afetam a resposta da utilização das enzimas no desempenho e no metabolismo de bovinos possuem devida relevância durante o planejamento experimental e justificam a necessidade de estudos que contemplam ambas as partes. Dessa forma, estudos que têm por objetivo avaliar efeitos de enzimas exógenas no rúmen, através da aplicação de tecnologias como metagenômica em amostras de conteúdo ruminal (Meale et al., 2014), bem como o estudo da digestibilidade dos nutrientes e balanço de nitrogênio no corpo do animal se tornam plausíveis de serem executados e trazem informações enriquecedoras sobre o entendimento mais abrangente dos efeitos da suplementação com enzimas exógenas na nutrição de ruminantes. Portanto, somente assim poderemos identificar os microrganismos presentes e compreender as funções metabólicas exercidas naquele momento e situação experimental testada. Desse modo, foi desenvolvido um estudo in vivo avaliando a utilização de enzimas exógenas em dietas de confinamento, que complementou a atual tese, estando disposto no capítulo 3. Portanto, abaixo está todas as informações e achados pertinentes aos trabalhos desenvolvidos.

CAPÍTULO 2 - IMPACT OF DIETARY EXOGENOUS FEED ENZYMES ON PERFORMANCE, NUTRIENT DIGESTIBILITY, AND RUMINAL FERMENTATION PARAMETERS IN BEEF CATTLE: A META-ANALYSIS

O artigo a seguir está redigido e foi publicado conforme normas de publicação do Periódico: Animal: The International Jornal of Animal Biosciences
https://www.sciencedirect.com/journal/animal/publish/guide-for-authors
https://www.sciencedirect.com/science/article/pii/S1751731125000643

Impact of dietary exogenous feed enzymes on performance, nutrient digestibility, and ruminal fermentation parameters in beef cattle: a meta-analysis

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Abstract: Exogenous feed enzymes **(EFE)** are incorporated into beef cattle diets to improve nutrient utilization and animal performance. This meta-analysis estimates the effects of EFE inclusion on beef cattle performance, feed intake, nutrient digestibility, and ruminal fermentation parameters, while also identifying factors influencing these outcomes. We initially screened 94 articles, and 23 studies met the eligibility criteria, contributing data from up to 83 treatment means. The magnitude of the effect (size effect) was determined using weighted mean differences **(WMD)** between the EFE-treated and control groups (diets without EFE inclusion). Heterogeneity was assessed through meta-regression and subgroup analysis. Results indicated that EFE inclusion did not affect final body weight ($P \geq 0.871$), dry matter intake (**DMI**, $P \geq 0.467$), average daily gain (**ADG**, $P \geq 0.145$), or feed efficiency (**FE**; $P \geq 0.417$). However, a significant increase in hot carcass weight (**HCW**; $P = 0.047$; WMD = 2.21 kg) was observed. The inclusion of EFE in the diet did not affect the profile of ruminal short-chain fat acid (SCFA) ($P \geq 0.225$) or ruminal ammonia nitrogen concentration (**N-NH_3**; $P = 0.143$). However, EFE inclusion improved the digestibility of dry matter ($P < 0.01$; WMD = 16.9 g/kg), crude protein ($P = 0.003$; WMD = 20.2 g/kg), and neutral detergent fiber (**NDF**, $P = 0.003$; WMD = 20.2), with no effect on organic matter or starch digestibility ($P \geq 0.388$). Heterogeneity was low ($I^2 < 25\%$) for most performance and nutrient digestibility outcomes, indicating consistent effect size estimates. Moderate heterogeneity ($P \leq 0.057$; I^2 =25 to 50%) was noted for dry matter and NDF digestibility, with EFE application explaining 28.2% of the variability in dry matter digestibility ($P = 0.032$), and fully accounting for the heterogeneity in NDF digestibility ($P = 0.045$). High heterogeneity was found in ruminal fermentation parameters ($P < 0.01$; $I^2 > 50\%$). Funnel plot analysis

revealed no publication bias for most variables ($P \geq 0.10$). Overall, this meta-analysis demonstrates that EFE inclusion in beef cattle diets increases hot carcass weight, likely due to enhanced digestibility of dry matter, crude protein, and NDF, without affecting rumen fermentation parameters.

Keywords: digestibility, exogenous enzyme, feedlot; rumen fermentation, hot carcass weight

Implications

Exogenous feed enzymes are added to beef cattle diets to enhance nutrient utilization and animal performance. We used a meta-analytical approach to provide a quantitative assessment of the impact of exogenous feed enzymes on performance, intake, nutrient digestibility, and ruminal fermentation parameters. Our findings revealed that exogenous feed enzyme products did not significantly affect final body weight, average daily gain, intake, feed efficiency, organic matter and starch digestibility, ruminal pH, ruminal ammonia nitrogen, and ruminal fermentation parameters. However, products containing exogenous enzymes did lead to increased final hot carcass weight and improved digestibility of dry matter, crude protein, and neutral detergent fiber.

INTRODUCTION

Monensin is the most commonly used feed additive in feedlot diets (Samuelson et al., 2016; Silvestre and Millen, 2021). However, there are growing public and scientific concerns about the use of antibiotics as feed additives in animal production (Jouany and Morgavi, 2007). These concerns are mainly motivated by the threat of selecting antibiotic-resistant human pathogens (Manero et al., 2006; Parveen et al., 2006), releasing antibiotic residues into the environment (Yang and Carlson, 2004), or transferring these drugs into animal-derived food products (Jouany and Morgavi, 2007). This has driven a search for alternative feed additives among animal producers leading to increased interest in exogenous feed enzymes (**EFE**) as a viable option (Bugoni et al., 2023). EFEs are “generally recognized as safe” (Sewalt et al., 2016), and their production costs have decreased, making them an attractive choice for ruminant nutritionists (Zilio et al., 2019; Bugoni et al., 2023).

Initial studies on EFEs in ruminant nutrition focused on enhancing fiber degradation through fibrolytic enzymes, which showed promising results in increasing the average daily gain of cattle fed high-forage diets during the backgrounding phase (Beauchemin et al., 1995). More recently, fibrolytic enzymes have been incorporated into high-grain diets to improve animal performance (Miorin et al., 2022). Additionally, exogenous amylolytic enzymes have been evaluated to optimize starch utilization and increase animal performance in feedlot diets (Tricarico et al., 2007; DiLorenzo et al., 2011). However, amylolytic enzymes do not seem to increase ruminal starch digestion but induce shifts in the molar proportion of butyrate and acetate by exposing oligosaccharides for cross-feeding by rumen microbes (Tricarico et al., 2008).

Some studies have also reported an increase in dry matter (**DM)** and neutral detergent fiber (**NDF)** digestibility (DiLorenzo et al., 2011; Vera et al., 2012) leading to changes in ruminal fermentation parameters (Meschiatti et al., 2019), while others found no effects (He et al., 2014; He et al., 2015).

These inconsistencies have been attributed to variations in dietary composition, enzyme activity, application rate, mode and timing of EFE delivery, ruminal microbial activity, EFE stability in the rumen, EFE-feed specificity, and the diet portion to which EFE are applied. While several literature reviews have qualitatively summarized the impact of EFEs (Beauchemin et al., 2003, 2004; Meale et al., 2014), a more rigorous quantitative approach is needed. Meta-analysis, a statistical method that synthesizes data from multiple studies, enhances the ability to detect treatment effects and identify sources of variation (Higgins, 2008).

Although a previous meta-analysis by Tirado-González et al. (2018) focused on fibrolytic enzymes (xylanase and cellulases), it was limited by the small number of studies included, with most data on nutrient digestibility and short-chain fatty acids (SCFA) production derived from *in vitro* experiments (Tirado-González et al., 2018). Our study aims to fill this gap by evaluating the effects of EFEs *in vivo* on beef cattle performance, feed intake, nutrient digestibility, and ruminal fermentation parameters. We hypothesize that the inclusion of EFE in cattle diets will enhance these outcomes, particularly during the rearing and finishing phase. Our objectives were to conduct a comprehensive meta-analysis of the effects of EFEs on these parameters and to assess the existence of heterogeneity and publication bias among the studies.

MATERIAL AND METHODS

Literature search

A comprehensive bibliographic search was conducted from September 2023 to January 2024 across five electronic databases: PubMed, Scopus, Web of Science, Agricola, and Embase. The search aimed to identify peer-reviewed manuscripts published in English that investigated the effects of exogenous feed enzymes on beef cattle. The search terms utilized were combinations of the following keywords: “beef cattle” OR “feedlot” OR “grazing” OR “pasture” AND “fibrolytic enzymes” OR “amylolytic enzymes” OR “proteolytic enzymes” OR “exogenous enzymes” AND “cellulase” OR “xylanase” OR “beta-glucanase” OR “amylase”.

From an initial pool of 94 references, 36 duplicates were identified and removed using the web tool Rayyan (Ouzzani et al., 2016). The remaining 58 articles were screened for eligibility, leading to the exclusion of 36 articles based on predetermined criteria. The research and eligibility strategy are outlined in a flowchart (Figure 1).

To ensure the quality and relevance of the studies included in this meta-analysis, the following inclusion criteria were applied: (1) published in English (2) utilized concurrent negative control and treatment groups, (3) employed continuous experimental designs, excluding change-over or Latin square designs, (4) included at least one exogenous enzyme as a dietary treatment, (5) described the main enzyme activity(ies), and (6) reported a measure of dispersion [i.e. standard error (**SE**) or standard deviation (**SD**)], along with the number of experimental units in each treatment for animal performance variables, pH, ruminal fermentation, and total tract digestibility.

Studies were excluded if they: (1) did not evaluate EFE supplementation in the backgrounding and finishing phase of beef cattle, (2) lacked detailed information on enzymatic description, (3) did not employ a randomized study design, (4) were conducted *in vitro*, and (5) presented results only in graphical form without accompanying numerical data. Only peer-reviewed publications were included in our study, except for data from Nascimento's doctoral thesis from our research group. The peer-review process served as a proxy for assessing study quality and minimizing heterogeneity (Poppy et al., 2012).

Data extraction

Based on the inclusion criteria, 22 articles were identified and classified by the first author. Additionally, following the eligibility criteria, data from Nascimento's doctoral thesis (Nascimento, 2018) were included in the dataset, bringing the total to 23 studies in quantitative synthesis. Key outcomes parameters extracted included dry matter intake (**DMI**, kg/d), final body weight (**BW**, kg), average daily gain (**ADG**, kg of BW/d), hot carcass weight (**HCW**, kg), feed efficiency (ADG/kg of DMI), total-tract nutrient digestibility (kg/kg) and ruminal fermentation parameters. Additional data extracted included sources of variability such as enzyme type, form of application (liquid vs. solid), microbial source, application rate and method, main enzyme activity, experiment duration, supply period (phase and days), level of concentrate, roughage type and presence of antibiotics (ionophores and/or non-ionophore). For each study, the number of replicates, treatment means, and standard deviations (**SDs**) were recorded. When SDs were not reported, they were calculated using the equation: $SD = SEM \times \sqrt{n}$, where **SEM** = standard error of treatment means, and

n = number of replicates. The complete data set is available in Supplementary File S1.

The types of EFEs identified included xylanase (**XIL**), cellulase-xylanase (**CEL-XIL**), endoglucanase-xylanase (**END-XIL**), and amylase-exogenous proteolytic enzyme (**AMY-PRO**). Application rates were standardized as grams of enzyme per kilogram of total diet DM. The methods of EFE application were categorized as application to the forage, concentrate, ensiling, or total mixed ration (TMR), with analysis conducted for experiments where EFEs were applied through each method. EFEs were also classified according to their application form (liquid or solid).

Statistical analysis and effect sizes

All statistical analyses were performed using the "metafor" package of R Software (Viechtbauer, 2010) version 4.3.2. The effects of dietary EFE inclusion were assessed using weighted mean difference (**WMD**) between control (no EFE) and treatment (EFE-supplemented) diets. WMDs were calculated to allow interpretation of results in their original units, as recommended by Takeshima et al. (2014). Treatment means were weighted by the inverse of the variance, following the random effects model proposed by DerSimonian and Laird (1986).

Heterogeneity and publication bias

Study heterogeneity (i.e. heterogeneity of effect size) was assessed using the chi-square (Q) test and the I^2 statistic, which quantifies the proportion of total variation across studies due to heterogeneity rather than chance. An I^2 value of <25% indicated low heterogeneity, 25 to 50% moderate heterogeneity, and

>50% high heterogeneity (Higgins et al., 2003).

The publication bias was evaluated using funnel plots (Light and Pillemer, 1984) and the Egger regression test, which detects asymmetry in the funnel plot (Egger et al., 1997). A p-value ≤ 0.05 in Egger's test indicated the presence of publication bias. The "trim-and-fill" method was used to estimate the number of studies potentially missing due to publication bias (Shi and Lin, 2019).

Meta-regression and subgroup analysis

For variables with a Q-test p-value ≤ 0.10 or I^2 value > 50% (Borenstein et al., 2017), meta-regression analysis was used to explore sources of heterogeneity using WMD as the dependent variable. Adjusted R^2 values were calculated to represent the proportion of between-study variance (heterogeneity) explained by the covariates (Viechtbauer, 2010). For this, the estimated between-study variance when covariates were included in the model (σ2) was compared with corresponding values when they were excluded (σo^2), according to the equation: adjusted R^2 (%) = (σo^2 - σ^2) / σo^2.

Categorical covariates included EFE type (XIL, CEL-XIL, END-XIL, AMY-PRO), method of EFE application (forage, concentrate, or TMR), form of application (liquid or solid), application rate (≤ 0.50, > 0.50 to 1 and > 1 g of EFE/kg of diet DM), supply period (≤ 80, > 80 to 120, and > 120 days), concentrate level (≤ 700 or > 700 g/kg of concentrate in the diet), and the presence or absence of antibiotics in the diet. Significant categorical covariates (P ≤ 0.05) were further analyzed by subgrouping. Statistical significance was declared at P ≤ 0.05 and tendencies at 0.05 < P ≤ 0.10.

RESULTS

A total of 23 studies, encompassing 86 treatment means, met the inclusion criteria for the meta-analysis. The studies included various EFE products, which were categorized based on enzyme type, application rate (g of EFE per kg of the total diet), application method (concentrate, forage, ensiling, and TMR), form (liquid vs. solid), supply period (days), dietary concentrate level, and presence of antibiotics in the diet (Table 1).

The majority of the studies (69.6%, n = 16) evaluated the effects of EFE products during the feedlot phase. One study assessed the effect of EFE products on grazing cattle, while the remaining studies focused on animals in confined feeding systems. The primary EFE products evaluated were amylase-protease (26.1%; n = 6 studies), endoglucase-xylanase (30.4%, n = 7), xylanase-cellulase (39.1%, n = 9), and xylanase (4.3%, n = 1). EFE inclusion levels were categorized as ≤ 0.5 (39.1%, n = 9), > 0.5 to 1.0 (26.1%, n = 6), and > 1.0 g EFE/kg of diet DM (34.8%, n = 8). The most common method of EFE application was in concentrate (60.9%, n = 14), followed by TMR (21.7%, n = 5), forage (13.0%, n = 3), and ensiling (4.35%, n = 1). EFE products were applied in liquid (56.5%, n = 13) or solid form (43.5%, n = 10). The duration of EFE supplementation varied across studies: ≤ 80 days (17.4%, n = 4), > 80 to 120 days (52.2%, n = 12), and > 120 days (30.4%, n = 7). The levels of concentrate in the diet varied from ≤ 700 g/kg (43.5%, n = 10) to > 700 g/kg of concentrate (56.5%, n = 13). Antibiotics were included in the diet in 52.2% of the studies (n = 12).

Effect sizes

The impact of EFE supplementation on performance, DMI, nutrient digestibility, and ruminal fermentation parameters is summarized in Tables 2 and 3. The inclusion of EFE in the diet did not affect final BW ($P \geq 0.871$), DMI ($P \geq 0.467$), ADG ($P \geq 0.145$), and FE ($P \geq 0.417$). However, EFE supplementation resulted in a significant increase in HCW ($P = 0.047$). Additionally, EFE supplementation improved DM digestibility ($P < 0.01$), CP digestibility ($P = 0.003$), and NDF digestibility ($P = 0.003$) (Table 3). However, no significant effects were observed on starch and OM digestibility ($P \geq 0.388$). The addition of EFE did not alter SCFA production, their proportions ($P \geq 0.225$), or ruminal ammonia nitrogen (NH_3-N) concentration ($P = 0.143$) (Table 3).

Heterogeneity and meta-regression analysis

The heterogeneity of the effect sizes across studies was generally low to moderate for most performance and nutrient digestibility outcomes, with I^2 values ranging from < 25% to 50% (Tables 2 and 3). However, DM and CP digestibility exhibited higher heterogeneity ($P < 0.01$), similar to what was observed for several ruminal fermentation parameters ($P < 0.01$), except for butyrate proportion (moderate heterogeneity, $P < 0.01$) and NH_3-H (low heterogeneity, $P = 0.207$). Heterogeneity was not detected for final BW ($P > 0.891$), DMI ($P > 0.562$; except for DMI during the growing phase), FE during the growing phase ($P = 0,775$), HCW ($P \geq 0.207$), and OM digestibility ($P = 0.922$) (Table 2).

Meta-regression analysis was conducted to explore potential sources of heterogeneity using covariates such as enzyme type, the rate, method, and form of application, supply period, concentrate level, and the presence of antibiotics

(Table 4). These covariates explained a small portion of the heterogeneity observed in ADG, CP digestibility, and Ace:Pro ratio (adjust R^2 = 12.6%, 13.7%, and 26.8%, respectively). Notably, none of these covariates significantly influenced the subgroup analysis ($P \geq 0.239$) and they did not explain the heterogeneity in butyrate proportion (adjusted R^2 = 0%).

DM digestibility was significantly affected by the method of EFE application in the meta-regression ($P = 0.032$), explaining a small proportion of heterogeneity (adjusted R^2 = 28.2%) (Figure 2). Subgroup analysis revealed an increase in DM digestibility when EFE was applied in forage (WMD = 52.5 g/kg; $P = 0.005$) and concentrate (WMD = 11.7 g/kg; $P < 0.004$), but not in TMR ($P = 0.208$). Similarly, the form of EFE application influenced NDF digestibility ($P = 0.018$), explaining virtually all heterogeneity (adjusted R^2 = 100%) (Figure 3). Subgroup assessment indicated an increase in NDF digestibility with EFE products in the solid form (WMD = 49.7 g/kg; $P = 0.001$), while no significant effect was observed for EFE products in liquid form ($P = 0.163$).

The EFE application form ($P > 0.01$), presence of antibiotics in the diet ($P > 0.069$), and concentrate level ($P > 0.061$) showed a tendency to affect ruminal propionate proportion, explaining 36.6% of the heterogeneity (adjusted R^2 = 36.6%). However, no significant differences were found in the subgroup analyses for propionate proportion across these covariates ($P \geq 0.05$, data not shown).

Publication bias

Funnel plots asymmetry tests indicated no significant publication bias for most variables ($P \geq 0.196$, Tables 2 and 3). However, potential publication bias

was detected for the overall effect of EFE on DMI, FE during the growing phase, and Ace:Pro ratio ($P < 0.05$) based on the funnel plot and the Egger test. The trim-and-fill method adjusted the mean estimate of the variables, leading to a change in treatment effects in the random effects meta-analysis for DMI (Fig. 4A; $P = 0.176$), FE during the background phase (Fig. 4B; $P = 0.596$) and Ace:Pro ratio (Fig. 4C; $P = 0.047$).

DISCUSSION

This meta-analysis summarizes the effects of EFE products on the performance and metabolism of beef cattle using an *in vivo* dataset generated under various conditions. These conditions included diverse enzyme types, application rates, methods and forms of enzyme application, supply periods, concentrate levels, and potential interactions (antagonism or synergism) with dietary antibiotics. Our findings demonstrate that EFE products improved animal performance, specifically HCW, without affecting ADG and final BW. This was probably associated with a small increase in HCW (+ 2.21 kg), which was insufficient to promote significant changes in final BW and ADG. These findings were reported by Miorin et al. (2022) in a study evaluating the addition of xylanase to supplements for Nellore cattle during the finishing phase on dry-season pasture. The results showed that animals receiving xylanase had a +9.0 kg increase in HCW compared to the control group, despite having similar final BW. Therefore, although the underlying mechanisms for the increased HCW remain unclear, the results suggest that the EFE products may enhance feed digestion, regardless of the effect size of carcass weight.

Several potential mechanisms could explain the observed effects of EFE products on feed digestion. EFE products may enhance feed utilization by increasing the rate and extent of pre-ingestive, ruminal, and post-ruminal fiber hydrolysis, digestion, and degradation. This could occur through mechanisms such as accelerating the ruminal passage rate, increasing microbial attachment to plant biomass, stimulating the activity of ruminal microbial populations, or decreasing digestive fluid viscosity (Morgavi et al., 2000; Beauchemin et al., 2004; Meale et al., 2014). An increase in passage rate can negatively impact nutrient digestibility due to the rapid flow of digesta through the rumen, reducing gut fill (Meale et al., 2014). For instance, Vera et al. (2012) evaluated the effect of EFE during the backgrounding phase and reported a 14.8% increase in the DMI of steers supplemented with proteolytic enzymes. However, the faster ruminal passage rate reduced NDF digestibility by 4.1%. In this meta-analysis, no differences in DMI were observed between groups, suggesting that the increase in HCW may be linked to improvements in total-tract nutrient digestibility.

The increase in animal performance with EFE supplementation is typically associated with an increase in feed digestion (Beauchemin et al., 2006; Arriola et al., 2017). Previous observations also suggest a change in overall apparent total tract nutrient digestibility when no change in DMI is observed (Kondratovich et al., 2019). Our findings align with this model, showing that EFE products increased the digestibility of DM, CP, and NDF without affecting DMI. Similarly, Arriola et al. (2017) found that exogenous enzymes in dairy cow diets tended to increase DM and NDF digestibility and improve milk yield without affecting DMI. Furthermore, Arriola et al. (2017) reported high heterogeneity for DM and NDF digestibility, indicating that other factors besides the enzymatic effect influenced

these variables. In contrast, in the current study, we observed low to moderate heterogeneity for variables related to nutrient digestibility (except for CP digestibility), indicating consistency in effect sizes across studies.

Most outcomes related to animal performance and nutrient digestibility exhibited low to moderate heterogeneity between studies, indicating low inconsistency in WMD estimates (Arriola et al., 2017). A low heterogeneity (I^2 < 25%) in systematic reviews is indicative of low variability between studies (Higgins and Green, 2011), indicating that the responses were independent of the possible sources of heterogeneity considered in this study, such as enzyme type, application rate, method and form of application, supply period, concentrate level, and antibiotic in the diet. Contrary to our initial hypothesis that heterogeneity would arise from the different types of EFE products, our analysis revealed consistent effects for most variables. However, for those variables exhibiting high heterogeneity, further exploration was conducted through meta-regression and subgroup analyses.

While EFE supplementation in beef cattle diets increased DM, NDF, and CP digestibility, no differences were observed in starch or OM digestibility. According to Takiya et al. (2017), the lack of EFE effect on starch total-tract digestibility can be explained by three possible mechanisms: 1) an increase in post-ruminal compensatory starch digestion for treatments with and without EFE 2) lack of a significant EFE effect on ruminal starch degradation; or 3) starch fermentation occurring in a different digestive compartment when the diet contains high levels of starch or coarsely ground corn. Based on data analyzed in this meta-analysis, the most plausible explanation for the absence of differences in starch digestibility between groups is the lack of enzyme effect. No

increase in starch degradation in the rumen was observed, which is aligned with the absence of changes in ruminal fermentation parameters. Furthermore, the minor effects of EFE on the digestibility of DM, CP, and NDF were insufficient to produce significant changes in OM digestibility.

Meta-regression analysis identified the enzyme application method as a significant factor influencing DM digestibility, explaining 28.2% of the observed heterogeneity. Subgroup analysis revealed that applying EFE products to forage substantially increased DM digestibility by 5.25% compared to other methods. This finding may be attributed to the pre-ingestive effect of enzyme application, where the enzyme alters feed structure, enhancing its degradability (Dean et al., 2013). However, this conclusion should be interpreted carefully due to the limited number of observations included in the analysis (n = 5). The addition of EFE products in the concentrate portion, which had a larger number of observations (n = 24), indicated that exogenous enzymes contributed to improving DM digestibility by 1.17%. Previous studies evaluating the addition of EFE to the concentrate portion have reported enhanced diet digestibility (Rode et al., 1999; Yang et al., 2000), which agrees with the results from this meta-analysis. However, the addition of EFE products to TMR showed no significant benefits. Previously, Yang et al. (2000) reported increased milk production and diet digestibility when EFE were added to the concentrate portion of dairy cow diets, while adding the enzymes directly to TMR did not affect milk production. Although the limited number of studies available for this meta-analysis does not allow us to single out specific factors associated with these effects, the different outcomes could be related to increased enzyme stability in the concentrate, higher specificity targeting more easily fermentable carbohydrates, enhancing their

digestibility, and less interaction with more complex feed components present in the TMR compared to the concentrate. Therefore, further studies should prioritize evaluating the effects of adding EFE to TMR and forage to gain a more comprehensive understanding of their impact on nutrient digestibility.

The form of enzyme application explained 100% of the observed heterogeneity in NDF digestibility. Specifically, applying EFE in solid form increased NDF digestibility by 4.97%, as shown in the subgroup analysis. The solid form of EFE is generally more effective on feeds with higher water activity (e.g., silages), as the increased moisture facilitates enzyme activity. Although it has been claimed that some exogenous enzymes are more effective when applied in a liquid form to dry forage than wet forage (Beauchemin et al., 2006), our results show that products in the liquid form did not confer benefits on cattle diets. Additionally, adding EFE in solid form to beef cattle diet offers practical advantages, including easier mixing with commercially concentrated ingredients (e.g., premix) and reducing possible dosing issues.

The higher CP digestibility among animals that received EFE products suggests an increase in ruminal proteolytic activity. However, this did not translate to an expected increase in the concentration of ruminal ammonia nitrogen (NH_3-N). Additionally, CP digestibility showed high heterogeneity, with the covariables in the meta-regression model explaining only 35.9% of the variability, and no significant covariates influenced the responses in the subgroup analysis. This indicates that the primary effect was driven by the EFE products themselves. A possible explanation is that the nitrogen released in the rumen was used by fibrolytic bacteria, which contributed to increased DM and NDF digestibility. Alternatively, an increase in the nitrogen supply for post-ruminal

absorption could contribute to increasing nitrogen retention by the host. Furthermore, an increase in CP digestibility without changes in nutrient intake contributes to enhanced nitrogen use efficiency (NUE), potentially reducing nitrogen excretion in feces or urine. This mechanism might explain the increased carcass weight without changes in final BW, although this meta-analysis could not assess the effect of EFE on nitrogen metabolism due to a lack of sufficient studies. Nonetheless, positive effects of EFE products on nitrogen metabolism have been reported both in beef (Gouvêa et al., 2019; Nascimento, 2018; Toseti et al., 2020) and dairy cattle (Zilio et al., 2019). Thus, further studies are needed to clarify the potential mechanisms involved.

Although EFE supplementation increased digestibility, it did not affect ruminal pH, total SCFA concentration, or the overall proportion of ruminal fermentation end products. These findings are reported by several experiments (Krause et al., 1998; Vera et al., 2012; He et al., 2014, 2015; Ran et al., 2019), indicating no changes in ruminal fermentation parameters with exogenous enzyme supplementation. However, under certain circumstances, EFE products can influence ruminal fermentation parameters. For example, dietary α-amylase supplementation increased acetate and butyrate and reduced propionate proportions in steers fed a diet with 50:50 forage:concentrate ratio (Tricarico et al., 2005). Miorin et al. (2022) reported an increase in the proportion of acetate and the Ace:Pro ratio in Nellore bulls receiving a supplement containing high levels of xylanase during the dry season. Also, Kondratovich et al. (2019) reported an increase in the molar proportion of ruminal propionate in beef steers fed growing diets supplemented with fibrolytic enzymes. These findings indicate that the effects of EFE on ruminal fermentation parameters are mainly influenced

by enzyme type and diet characteristics. Importantly, most ruminal fermentation outcomes in this meta-analysis exhibited high heterogeneity ($I^2 > 50\%$), indicating substantial variability between studies. Consequently, although factors such as form of application, presence of antibiotics, and level of concentrate in the diet were significant in the meta-analysis, no subgroup differences were observed for propionate proportions.

CONCLUSION

This meta-analysis provides valuable insights into the effects of different exogenous feed enzyme (EFE) products on beef cattle diets. The inclusion of EFE in these diets can result in a modest improvement in hot carcass weight (HCW) without affecting average daily gain (ADG), final body weight (BW), or dry matter intake (DMI). Additionally, EFE supplementation enhanced the digestibility of DMI, crude protein, and neutral detergent fiber, without significantly impacting the total concentration or proportion of short-chain fatty acids. The low to moderate heterogeneity observed between studies indicates consistent effects of EFE products. The observed responses were largely independent of variables such as enzyme type, application rate, method of application, duration of enzyme supplementation, concentrate levels, and presence of antibiotics in the diet. However, caution is warranted when extrapolating these results to conditions outside the study criteria. Factors such as roughage sources, grain types, types and sources of microbial enzymes, and NDF concentration and effectiveness, particularly regarding ruminal fermentation parameters, could influence outcomes.

While the different EFE products evaluated demonstrate similar benefits, the diversity in their mechanisms of action cannot be ruled out. This underscores the need for further research to identify highly efficient enzymes that can be optimized for livestock nutrition. Notably, the method of EFE application influenced DM digestibility, while the form of application was a significant factor affecting NDF digestibility. These findings highlight the potential of EFE supplementation as a viable strategy for enhancing nutrient utilization in beef cattle. Future studies should aim to isolate and characterize specific enzyme activities, explore their underlying mechanisms, and identify the most effective formulations and application methods. This will help optimize the role of EFE in animal performance and nutrient utilization across diverse dietary contexts.

Data and model availability statement

None of the data or models are deposited in an official repository. The data presented in this article will be available upon request to the authors.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

Ethics approval

Not applicable.

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Supplementary data

Supplementary data (articles summary table and metadata) are available in the *Animal Journal* online.

Acknowledgments

We would like to thank the members of Grupo de Estudos em Produção de Ruminantes (Gepror; Colina, Brazil), Agência Paulista de Tecnologia dos Agronegócios (Apta - Polo Regional Alta Mogiana; Colina, Brazil) and University of Wisconsin-Madison (WI, EUA) crew for their help in this study. This study is part of the first author's thesis that will be presented to the São Paulo State

University “Júlio de Mesquita Filho” (UNESP), Jaboticabal in compliance with the requirements for the Doctor’s degree in Animal Science by I. M. Ferreira.

Financial support statement

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; grant number of the first author: “2022/00989-1” and 2023/11136-2) and the PDIP grant “2017/50339-5”, São Paulo, SP, BRASIL). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors also acknowledge support from the Dairy Innovation Hub (Madison, WI).

Conflict of interest statement

The author declares no real or perceived conflict of interest.

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Tables

Table 1: Publications summarized in this meta-analysis on the effects of exogenous enzyme application on beef cattle diets.

Citation	Study ID	Trial	n	Enzyme type	Application rate	Method	Form	Total supply period	Antibiotics in all diets	Level of concentrate	Stage of supply
Beauchemin et al. (1995)	1	1	72	CEL-XIL	0.207	F	L	72	No	3.3	Growing phase
	1	1	72	CEL-XIL	0.414	F	L	72	No	3.3	Growing phase
	1	1	72	CEL-XIL	1.089	F	L	72	No	3.3	Growing phase
	1	1	72	CEL-XIL	1.635	F	L	72	No	3.3	Growing phase
	1	1	72	CEL-XIL	3.269	F	L	72	No	3.3	Growing phase
	1	1	72	CEL-XIL	0.195	F	L	72	No	9	Growing phase
	1	1	72	CEL-XIL	0.390	F	L	72	No	9	Growing phase
	1	1	72	CEL-XIL	0.867	F	L	72	No	9	Growing phase
	1	1	72	CEL-XIL	1.30	F	L	72	No	9	Growing phase
	1	1	72	CEL-XIL	2.60	F	L	72	No	9	Growing phase
	1	1	72	CEL-XIL	0.195	F	L	72	No	9	Growing phase
	1	1	72	CEL-XIL	0.390	F	L	72	No	9	Growing phase
	1	1	72	CEL-XIL	1.03	F	L	72	No	9	Growing phase

	1	1	72	CEL-XIL	1.54	F	L	72	No	9	Growing phase
	1	1	72	CEL-XIL	3.08	F	L	72	No	9	Growing phase
	2	1	5	CEL-XIL	1.386	F	L	90	No	30	Feedlot
Lewis et al. (1996)	2	1	5	CEL-XIL	1.386	F	L	90	No	30	Feedlot
	2	1	5	CEL-XIL	1.386	C	L	90	No	30	Feedlot
	2	1	5	CEL-XIL	1.386	C	L	90	No	30	Feedlot
	3	1	56	CEL-XIL	1.31	C	L	145	No	95.1	Feedlot
Beauchemin et al. (1997)	3	1	56	CEL-XIL	0.524	C	L	145	No	95.1	Feedlot
	3	1	56	CEL-XIL	1.31	C	L	145	No	95.1	Feedlot
	3	1	56	CEL-XIL	0.524	C	L	145	No	95.1	Feedlot
Krause et al. (1998)	4	1	4	CEL-XIL	1.50	C	L	112	No	95	Feedlot
	4	1	4	CEL-XIL	1.50	C	L	112	No	95	Feedlot
	5	2	98	CEL-XIL	0.0013	TMR	L	120	No	17.5	Feedlot
McAllister et al. (1999)	5	2	98	CEL-XIL	0.0035	TMR	L	120	No	17.5	Feedlot
	5	2	98	CEL-XIL	0.0050	TMR	L	120	No	17.5	Feedlot
	5	3	66	CEL-XIL	0.0035	TMR	L	70	No	30	Feedlot
Beauchemin et al. (1999)	6	1	187	CEL-XIL	1.53	C	L	123	No	92.2	Feedlot
	7	1	120	AMY-PRO	1.58	C	S	112	Yes	90	Feedlot
	7	1	120	AMY-PRO	1.58	C	S	152	Yes	90	Feedlot
	7	2	96	AMY-PRO	0.966	C	S	81	Yes	85	Feedlot
Tricarico et al. (2007)	7	2	96	AMY-PRO	1.93	C	S	81	Yes	85	Feedlot
	7	2	96	AMY-PRO	0.966	C	S	81	Yes	85	Feedlot
	7	2	96	AMY-PRO	1.93	C	S	81	Yes	85	Feedlot
	7	3	64	AMY-PRO	1.55	C	S	56	Yes	93	Feedlot

Krueger et al. (2008)	8	1	50	CEL-XIL	0.0009	F	S	86	No	50	Growing phase
	8	1	50	CEL-XIL	0.0009	F	S	86	No	50	Growing phase
	8	1	50	CEL-XIL	0.0009	F	S	86	No	50	Growing phase
Miller et al. (2008)	9	1	16	END-XIL	5.32	C	L	49	No	70.4	Feedlot
	9	1	16	END-XIL	5.32	C	L	49	No	76.4	Feedlot
	9	2	4	END-XIL	5.02	C	L	96	No	75.4	Feedlot
	9	2	4	END-XIL	5.02	C	L	96	No	50.6	Feedlot
	9	3	96	END-XIL	1.30	C	L	81	Yes	93.5	Feedlot
	9	3	96	END-XIL	2.59	C	L	81	Yes	93.5	Feedlot
	9	3	96	END-XIL	5.20	C	L	81	Yes	93.5	Feedlot
Eun et al. (2009)	10	1	60	END-XIL	1.00	TMR	S	84	Yes	42	Growing phase
	10	1	60	END-XIL	2.00	TMR	S	84	Yes	80	Growing phase
	10	2	60	END-XIL	1.00	TMR	S	84	Yes	42	Feedlot
	10	2	60	END-XIL	2.00	TMR	S	84	Yes	80	Feedlot
DiLorenzo et al. (2011)	11	1	32	AMY-PRO	1.91	C	S	42	Yes	90	Feedlot
	11	1	32	AMY-PRO	1.91	C	S	42	Yes	90	Feedlot
Vera et al. (2012)	12	1	48	AMY-PRO	0.520	TMR	L	84	Yes	36.5	Growing phase
	12	2	48	AMY-PRO	0.520	TMR	L	84	Yes	75	Growing phase
He et al. (2014)	13	1	8	END-XIL	0.499	C	L	84	No	90	Feedlot
	13	1	8	END-XIL	0.998	C	L	84	No	90	Feedlot
	13	2	160	END-XIL	0.499	C	L	120	No	90	Feedlot
	13	2	160	END-XIL	0.998	C	L	120	No	90	Feedlot

He et al. (2015)	14	1	6	END-XIL	0.499	C	L	126	No	40	Growing phase
	14	1	6	END-XIL	0.998	C	L	126	No	40	Growing phase
	14	1	6	END-XIL	0.499	C	L	126	No	40	Growing phase
	14	1	6	END-XIL	0.998	C	L	126	No	40	Growing phase
	14	2	120	END-XIL	0.499	C	L	84	No	40	Growing phase
	14	2	120	END-XIL	0.998	C	L	84	No	40	Growing phase
	14	2	120	END-XIL	0.499	C	L	84	No	40	Growing phase
	14	2	120	END-XIL	0.998	C	L	84	No	40	Growing phase
Neumann et al. (2018)	15	1	36	CEL-XIL	1.250	C	S	94	Yes	100	Feedlot
	15	1	36	AMY-PRO	1.24	C	S	94	Yes	100	Feedlot
Elolimy et al 2018	16	1	24	AMY-PRO	0.549	C	S	140	Yes	87	Feedlot
Neumann et al. (2018)	17	1	32	XIL	0.670	C	S	105	Yes	100	Feedlot
Kondratovich et al. (2019)	18	2	5	CEL-XIL	0.375	C	L	84	Yes	55	Growing phase
	18	2	5	CEL-XIL	0.375	C	L	84	Yes	49	Growing phase
Meschiatti et al. (2019)	19	1	300	AMY-PRO	0.560	TMR	S	93	No	91.5	Feedlot
	19	1	300	AMY-PRO	0.560	TMR	S	93	No	91.5	Feedlot
	19	2	5	AMY-PRO	0.560	TMR	S	100	No	91.5	Feedlot
	19	2	5	AMY-PRO	0.560	TMR	S	100	No	91.5	Feedlot

Ran et al. (2019)	20	1	8	END-XIL	0.09	C	L	67	No	10	Feedlot
Salvo et al. (2020)	21	1	468	END-XIL	0.115	E	L	122	Yes	74	Feedlot
	21	1	468	END-XIL	0.078	E	L	122	Yes	74	Feedlot
Miorin et al. (2022)	22	1	76	END-XIL	0.858	C	S	140	Yes	47	Feedlot
	22	1	76	END-XIL	0.800	C	S	140	Yes	47	Feedlot
	22	2	8	END-XIL	0.858	C	S	140	Yes	47	Feedlot
	22	2	8	END-XIL	0.800	C	S	140	Yes	47	Feedlot
Nascimento et al. (2018)	23	1	10	AMY-PRO	0.490	TMR	S	125	Yes	87.6	Feedlot
	23	2	42	AMY-PRO	0.551	TMR	S	96	Yes	87.6	Feedlot

[1]Number of animals in each experiment.
[2]Treatments: xylanase (XIL), cellulase-xylanase (CEL-XIL), endoglucanase-xylanase (END-XIL), and amylase- proteolytic exogenous enzyme (AMY-PRO)
[3]Rate of application: g of exogenous feed enzymes (EFE) / kg of total diet.
[4]Method of EFE application to the forage (F), concentrate (C), ensiling (E), or total mixed ration (TMR).
[5]Form of EFE applied: liquid (L), solid (S).

Table 2: Effects of exogenous enzyme products on animal performance, nutrient digestibility, and ruminal fermentation parameters of beef cattle in feedlot.

		WMD[3] (95% CI)			Heterogeneity[4]			Funnel test[5]
Item	N[1]	Control mean (SD)[2]	Random effect	*P*-value	Q	*P*-value	I^2 (%)	*P*-value
Performance								
Final BW, kg								
Growing phase	10	383 (25.1)	0.054 (-4.40; 4.51)	0.981	1.97	0.992	0.00	0.324
Finishing phase	34	530 (62.1)	-0.307 (-4.00; 3.38)	0.871	22.4	0.891	0.00	0.760
Overall effect	44	497 (53.7)	-0.160 (-3.00; 2.68)	0.912	25.4	0.985	0.00	0.545
DMI, kg/d								
Growing phase	31	8.89 (1.11)	-0.005 (-0.106; 0.096)	0.922	30.1	0.459	0.40	0.165
Finishing phase	49	9.57 (1.43)	-0.030 (-0.110; 0.050)	0.467	45.8	0.562	0.00	0.163
Overall effect	80	9.30 (1.30)	-0.020 (-0.082; 0.042)	0.529	76.1	0.571	0.00	0.050
ADG, kg/d								
Growing phase	25	1.16 (0.213)	0.041 (0.001; 0.081)	0.145	30.2	0.177	20.6	0.196
Finishing phase	36	1.47 (0.321)	0.014 (-0.023; 0.052)	0.449	65.9	< 0.01	46.9	0.932
Overall effect	61	1.34 (0.277)	0.026 (-0.002; 0.053)	0.158	96.7	< 0.01	38.0	0.251
FE, kg/kg								
Growing phase	10	0.151 (0.035)	0.002 (-0.003; 0.008)	0.417	5.64	0.775	0.00	0.050
Finishing phase	22	0.171 (0.038)	0.00 (-0.003; 0.004)	0.980	31.4	0.066	33.2	0.837
Overall effect	32	0.165 (0.037)	0.001 (-0.002; 0.003)	0.685	37.4	0.198	17.2	0.546
HCW, kg	29	310 (43.2)	2.21 (0.032; 4.39)	0.047	15.5	0.972	0.00	0.511

[1] Number of comparisons between exogenous feed enzymes and control treatment.
[2] Mean and standard deviation (SD) for the control treatment.
[3] WMD = weighted mean difference between exogenous feed enzymes and control treatments (and the associated 95% CI).
[4] P-value for χ2 (Q) test of heterogeneity; I^2 = proportion of total variation of size effect estimates that is due to heterogeneity.
[5] Egger´s regression asymmetry test.
Abbreviations: CI = Confidential interval; DMI = DM intake; ADG = Average daily gain; FE = Feed efficiency; HCW = Hot carcass weight.

Table 3: Effects of exogenous enzyme products on nutrient digestibility and ruminal fermentation parameters of beef cattle in feedlot.

Item	N[1]	WMD[3] (95% CI)			Heterogeneity[4]			Funnel test[5]
		Control mean (SD)[2]	Random effect	*P*-value	Q	*P*-value	I^2 (%)	*P*-value
Nutrient digestibility								
DM digestibility, g/kg	33	662 (50)	16.9 (8.73; 25.1)	< 0.01	64.8	< 0.01	50.7	0.408
OM digestibility, g/kg	18	679 (54.3)	3.58 (-4.55; 11.7)	0.388	9.54	0.922	0.00	0.894
CP digestibility, g/kg	21	665 (60.9)	20.2 (6.68; 33.7)	0.003	77.6	< 0.01	74.2	0.138
NDF digestibility, g/kg	31	490 (87.2)	20.2 (-7.09; 33.3)	0.003	43.1	0.057	30.5	0.316
Starch digestibility, g/kg	24	916 (25.9)	-0.53 (-4.41; 3.35)	0.789	26.5	0.279	13.1	0.406
Ruminal fermentation parameters								
Mean ruminal pH	22	6.11 (0.3)	0.004 (-0.07; 0.076)	0.914	77.3	< 0.01	72.8	0.512
Total SCFA, mM	22	121 (16.0)	2.20 (-2.66; 7.06)	0.375	53.6	< 0.01	60.8	0.509
Acetate, mol/100 mol	22	59.8 (2.95)	-0.136 (-0.88; 0.608)	0.719	56.3	< 0.01	62.7	0.424
Propionate, mol/100 mol	22	22.2 (2.90)	-0.10 (-0.904; 0.709)	0.720	57.1	< 0.01	64.9	0.837
Butyrate, mol/100 mol	22	11.2 (2.05)	0.052 (-0.389; 0.491)	0.819	31.4	0.068	33.0	0.829
Ace:Pro ratio	19	2.99 (0.321)	-0.012 (-0.159; 0.136)	0.878	69.0	< 0.01	73.9	0.016
NH_3-H, mg/dL	17	16.0 (6.88)	0.637 (-0.216; 1.49)	0.143	13.3	0.207	24.9	0.968

[1] Number of comparisons between exogenous feed enzymes and control treatment.
[2] Mean and standard deviation (SD), control treatment.
[3] WMD = weighted mean difference between exogenous feed enzymes and control treatments (and the associated 95% CI).
[4] P-value for χ2 (Q) test of heterogeneity; I^2 = proportion of total variation of size effect estimates that is due to heterogeneity.
[5] Egger´s regression asymmetry test
Abbreviations: CI = Confidential interval; OM = Organic matter; SCFA = Ruminal short-chain fat acid; Ace:Pro ratio = Acetate:Propionate ratio; NH_3-H = Ruminal ammonia nitrogen.

Table 4: Meta-regression of the covariate effect of type of enzyme product, application rate, method and form, supply period, presence of antibiotics, and level of concentrate in the beef cattle diet in feedlot on weighted mean differences (WMD) between enzyme products and control treatment.

Dependent variables (Y, WMD)	Meta-regression parameters (*P*-value)								Adjusted R^2 (%)[1]	N[2]
	Intercept	Enzyme type	Rate	Method	Form	Supply period	Antibiotics in diets	Level of concentrate		
ADG, kg/d	0.107 (0.568)	0.018 (0.603)	-0.003 (0.892)	-0.014 (0.659)	-0.042 (0.371)	-0.029 (0.349)	-0.003 (0.949)	0.025 (0.557)	11.1	61
DM digestibility, g/kg	13.2 (0.753)	0.578 (0.896)	-1.49 (0.788)	-13.6 (0.032)	17.3 (0.148)	3.49 (0.665)	3.48 (0.747)	2.82 (0.795)	28.2	34
CP digestibility, g/kg	-50.8 (0.857)	26.6 (0.312)	16.7 (0.394)	-28.8 (0.469)	-2.39 (0.968)	6.65 (0.562)	22.9 (0.458)	11.6 (0.717)	13.7	19
NDF digestibility, g/kg	-62.5 (0.168)	10.4 (0.340)	-16.2 (0.122)	-4.92 (0.640)	37.4 (0.018)	1.07 (0.914)	7.18 (0.703)	28.2 (0.115)	100	31
Mean ruminal pH	-0.326 (0.704)	-0.032 (0.827)	-0.001 (0.995)	0.006 (0.904)	0.189 (0.364)	0.055 (0.356)	0.048 (0.774)	-0.042 (0.692)	51.9	22
Total SCFA, mM	55.8 (0.245)	3.88 (0.621)	-1.60 (0.682)	-7.46 (0.146)	-15.4 (0.169)	-1.73 (0.597)	-12.7 (0.122)	5.07 (0.367)	80.7	22
Acetate, mol/100 mol	-0.742 (0.954)	-2.31 (0.298)	1.61 (0.187)	0.527 (0.404)	1.92 (0.558)	0.048 (0.963)	0.876 (0.731)	-2.17 (0.128)	51.3	22
Propionate, mol/100 mol	10.2 (0.354)	0.727 (0.690)	-0.119 (0.901)	-0.293 (0.552)	-5.06 (0.071)	-0.211 (0.750)	-4.14 (0.069)	2.57 (0.061)	36.6	22
Butyrate, mol/100 mol	9.06 (0.251)	-1.62 (0.221)	0.519 (0.415)	0.092 (0.824)	-1.58 (0.366)	-0.346 (0.554)	-1.05 (0.446)	-1.64 (0.137)	0.0	22
Ace:Pro ratio	-0.833 (0.758)	-0.217 (0.630)	0.054 (0.814)	0.049 (0.670)	0.392 (0.512)	0.092 (0.740)	0.427 (0.371)	-0.310 (0.239)	26.8	19

[1]Adjusted R^2 = proportion of between-study variance (heterogeneity) explained by the covariates.
[2]Number of comparisons between exogenous feed enzymes and control treatment.
Abbreviations: ADG = Average daily gain; SCFA = Ruminal short-chain fat acid; Acetate:Propionate ratio (Ace:Pro ratio)

Figures

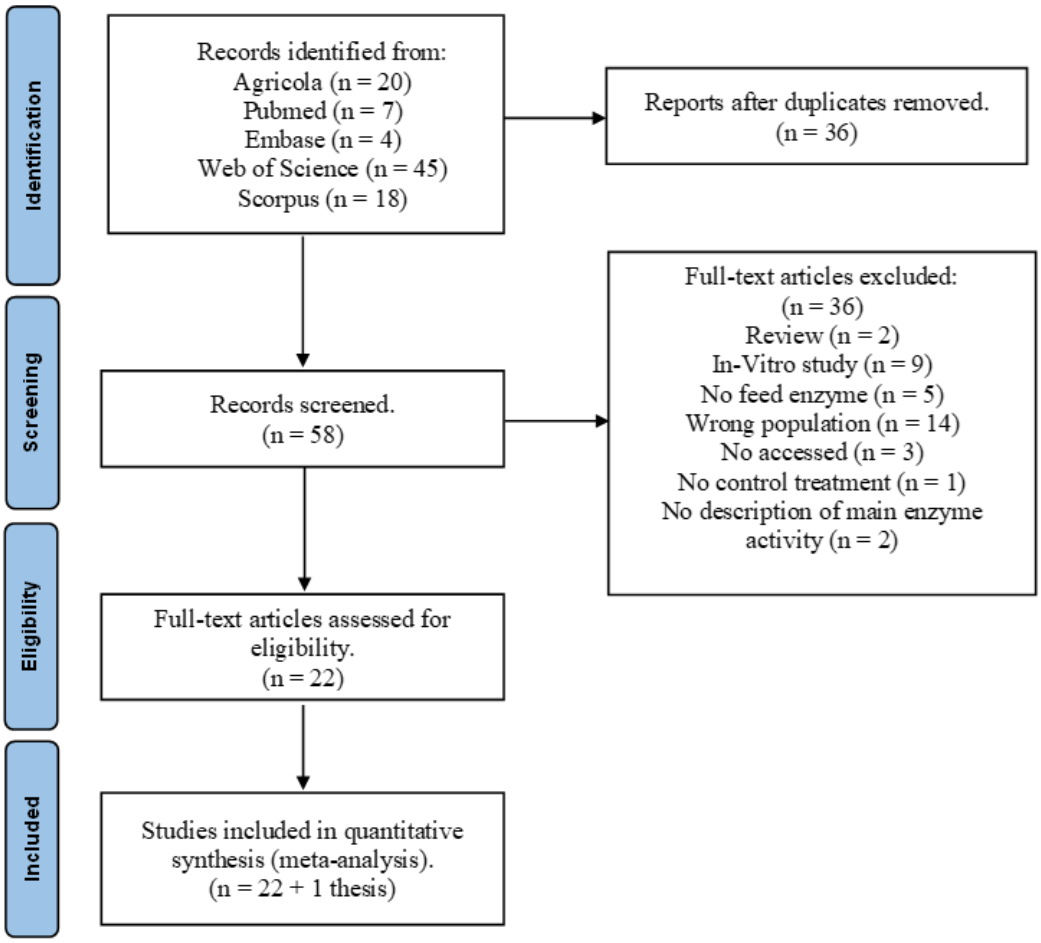

Fig. 1: Flowchart showing the search strategy and eligible studies for the meta-analysis on the effects of exogenous feed enzymes application to beef cattle diets.

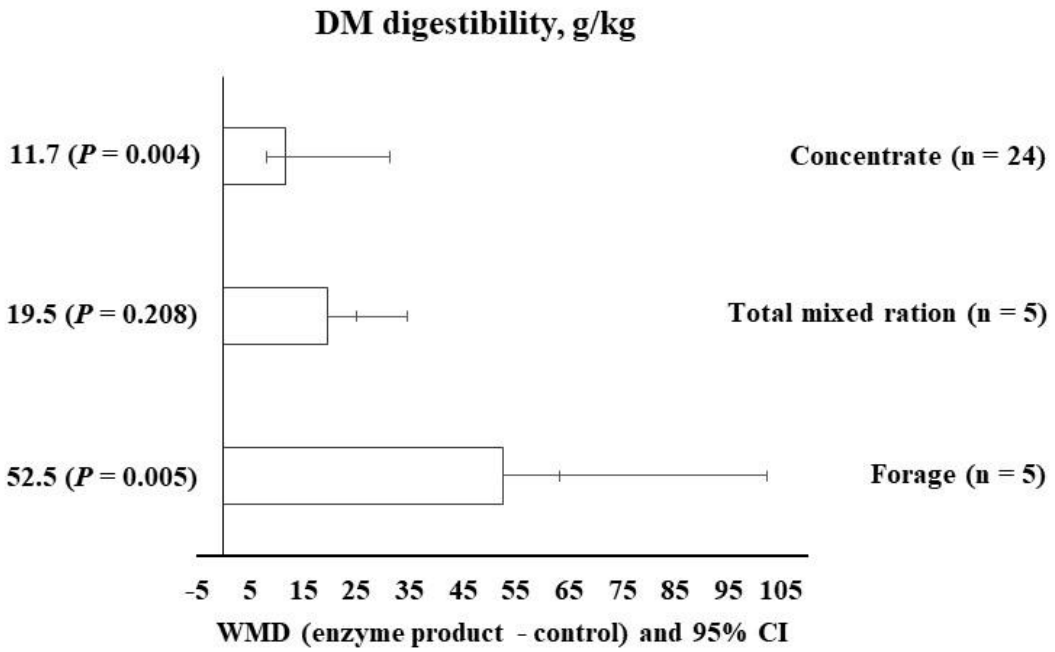

Fig. 2: Effects of different methods of exogenous feed enzyme application on dry matter (DM) digestibility (g/kg) in beef cattle diets. Abbreviations: WMD = Weighted mean difference between exogenous feed enzymes and control treatments (and the associated 95% CI); CI = Confidential interval; n = Number of comparisons between exogenous feed enzymes and control treatment.

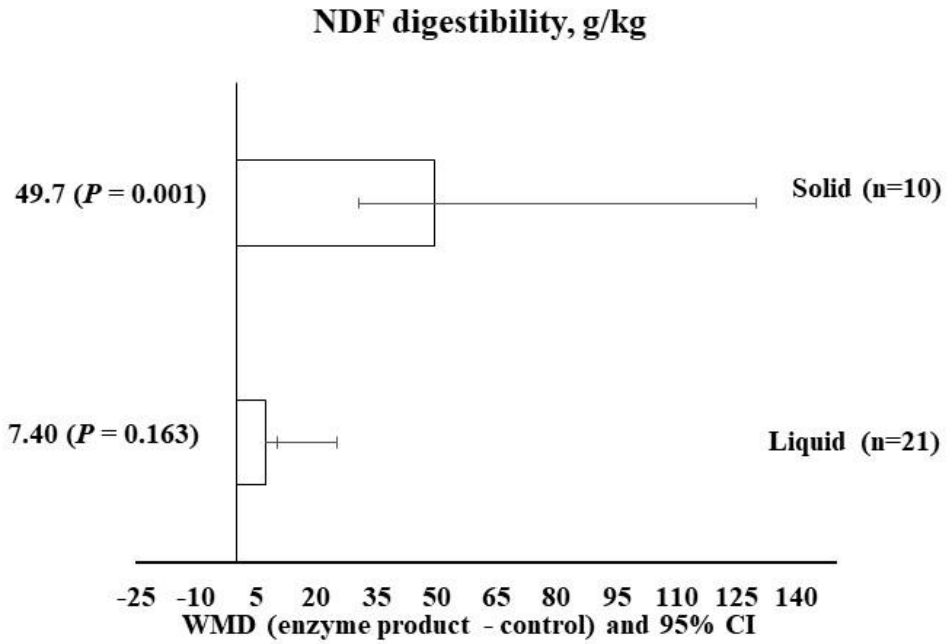

Fig. 3: Effects of different forms of exogenous feed enzyme application on neutral detergent fiber (NDF) digestibility (g/kg) in beef cattle diets. Abbreviations: WMD = Weighted mean difference between exogenous feed enzymes and control treatments (and the associated 95% CI); CI = Confidential interval; n = Number of comparisons between exogenous feed enzymes and control treatment.

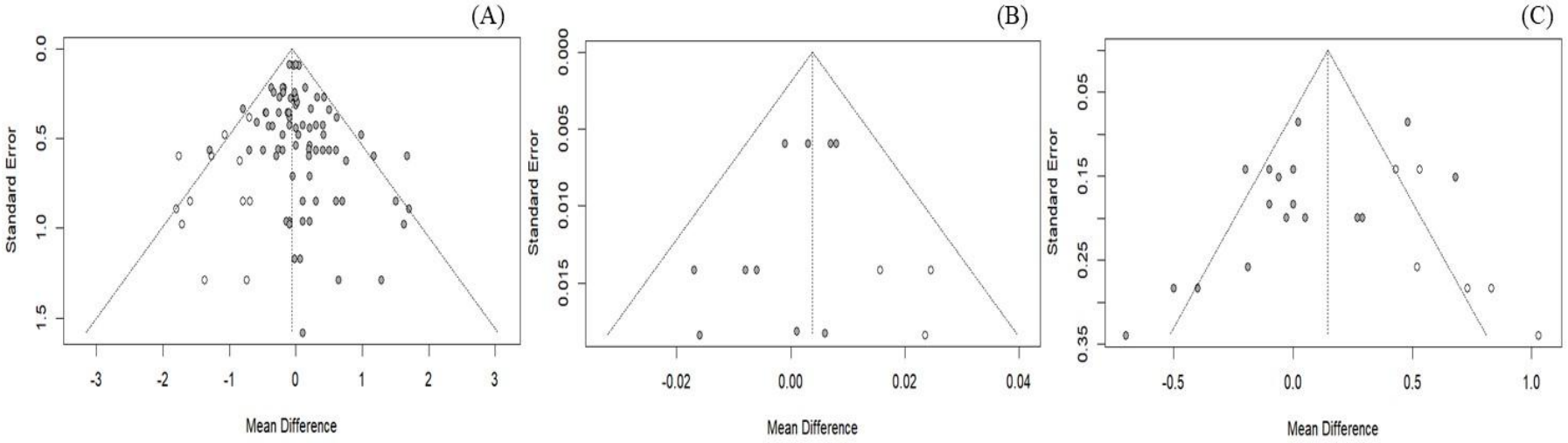

Fig. 4: Funnel plot for analysis of publication bias. (4A) Funnel plot for dry matter intake. (4B) Funnel plot for gain:feed during background phase. (4C) Funnel plot for Acetate:Propionate ratio in rumen. The dashed external lines indicate the triangular region where 95% CI of the studies are expected and the dashed central vertical line is the axis of the fixed effect. The black dots indicate the included studies and the open dots indicate the missing studies imputed by the trim-and-fill method.

CAPÍTULO 3 - FEEDING AMYLOLYTIC AND FIBROLYTIC EXOGENOUS ENZYMES IN FEEDLOT DIETS: EFFECT ON RUMINAL PARAMETERS, NITROGEN BALANCE AND MICROBIAL DIVERSITY OF NELLORE CATTLE

O artigo a seguir está redigido e foi publicado conforme normas de publicação do Periódico: Journal of Animal Science and Biotechnology
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Feeding amylolytic and fibrolytic exogenous enzymes in feedlot diets: Effect on ruminal parameters, nitrogen balance and microbial diversity of Nellore cattle

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Abstract

Background: The environmental impact of feedlot operations is a growing concern, as cattle excrete a significant portion of feed nutrients as waste. Exogenous feed enzymes (EFE) have gained interest for their potential to enhance feed efficiency in ruminants by improving nutrient digestion. However, EFE effects on ruminal parameters have shown inconsistencies, with limited research on nitrogen metabolism and rumen microbiome impacts. Moreover, the synergistic effects of combining different EFEs remain unclear. This study aimed to evaluate the effects of individual and combined EFE products in feedlot diets on ruminal fermentation parameters, nitrogen metabolism, and ruminal microbial communities. Ten rumen-cannulated Nellore steers [543 kg ± 28.6 of body weight (BW)] were distributed in a replicated Latin-square design (5 × 5) in individual pens. Treatments included: control (CON, no EFE supplementation), amylase [AML, 0.5 g/kg of diet dry matter (DM)], xylanase (FBL, 0.9 g of/kg DM), half dose combination (HD, 0.25 g of AML + 0.45g of FBL/kg of DM), and full dose combination (FD, 0.5 g of AML + 0.90 g of FBL/kg of DM). The experimental period lasted 19 days and included total urine and feces collection (days 15 to 18) and rumen fluid sampling (day 19) at 0, 4, 8, 12, and 16h post-feeding for ammonia, volatile fatty acids (VFA), pH and microbiome analysis.

Results: EFE supplemented animals exhibited lower ruminal ammonia concentrations (P = 0.040), and higher acetate proportions ($P < 0.001$) compared to the control group. EFE supplementation resulted in reduced nitrogen (N) excretion in feces ($P = 0.049$) and urine ($P = 0.036$), contributing to improved N retention and efficiency ($P = 0.045$). Additionally, EFE products induced shifts in various microbial taxa at family and genera levels ($P \leq 0.10$), which may be associated with the changes observed in ruminal fermentation.

Conclusions: Our findings demonstrate that EFE supplementation enhances nitrogen retention, reduces ruminal ammonia, and alters ruminal fermentation profiles and microbial populations in feedlot cattle. While the expected synergism between amylase and xylanase did not significantly impact rumen fermentation

parameters, it did induce shifts in the rumen microbiome. These results suggest that EFE supplementation may be a promising strategy for improving nutrient utilization and potentially reducing the environmental impact of feedlot operations.

Keywords: Amylolytic enzymes, Beef cattle, Fibrolytic enzymes, Nitrogen metabolism, Rumen microbiome

BACKGROUND

The environmental impact of feedlot operations is becoming a growing global concern, primarily because feed digestion in beef cattle is incomplete. Cattle retain only a portion of the feed nutrients, with the remainder lost to the environment, mainly as feces and urine [1]. Among the feed additives used in ruminant diets, exogenous feed enzymes (EFE) have gained scientific interest as a strategy to optimize animal feed efficiency by enhancing nutrient digestion [2–4]. EFE may enhance feed utilization by increasing the rate and extent of fiber and starch hydrolysis, digestion, and degradation during pre-ingestive, ruminal, and post-ruminal phases. EFE can catalyze direct hydrolysis of feed substrates, which reduces the viscosity of digestive fluids and helps increase ruminal passage rate, [3,5,6]. Additionally, the enzymatic hydrolysis facilitates access of rumen microbes to complex, non-soluble substrates, therefore contributing to modulating the ruminal microbial population [7,8].

Initial studies on EFEs in ruminant nutrition focused on enhancing the rate and/or extent of fiber degradation through fibrolytic enzymes [4]. Exogenous amylolytic enzymes have also been evaluated to optimize ruminal starch utilization [9,10], aiming to promote changes in ruminal fermentation by increasing total volatile fatty acid concentration. However, the effect of EFE on ruminal parameters has been inconsistent [11,12]. Furthermore, few studies have considered the effect of EFE supplementation in feedlot diets on nitrogen metabolism, and most studies evaluating EFE supplementation on rumen populations have been conducted in vitro [7,13] or using dairy cattle as a research model [14,15]. Thus, to the best of our knowledge, this is the first study to evaluate the effects of combined EFE supplementation on the ruminal microbial community of beef cattle.

Recently, promising results regarding total-tract crude protein digestibility in ruminants were reported when fibrolytic (Fibrozyme®) and amylolytic (Amaize®) enzymes were added to dairy [2] and beef cattle diets [17]. However, the possible synergism between these enzymes in terms of improving nitrogen metabolism in beef cattle has not been explored. The combination of different EFE may allow the breakdown of cross-linkages between structural

polysaccharides of the feed, potentially enhancing nutrient utilization [4,11]. Thus, we hypothesized that feeding fibrolytic and amylolytic enzymes in feedlot diets would alter nitrogen metabolism and total ruminal VFA concentration through modulation of the ruminal microbiome of Nellore cattle. Specifically, the fibrolytic enzymes would target the breakdown of cellulose and hemicellulose, while the amylolytic enzymes would focus on starch degradation. The combination of these enzymes could synergistically enhance nutrient utilization by breaking down complex polysaccharides and cross-linkages between structural carbohydrates, thereby improving overall feed efficiency. Additionally, we hypothesized that feeding both fibrolytic and amylolytic enzymes would have synergistic effects compared to feeding individual enzymes, due to their complementary actions on different substrates within the feed. The objective of this study was to evaluate the effect of both fibrolytic and amylolytic enzymes added to the feedlot diet on ruminal parameters, microbial diversity, and nitrogen balance of Nellore cattle.

MATERIALS AND METHODS

Treatments and experimental design.

The study was conducted at the Experimental Feedlot Cattle facilities of the Agência Paulista de Tecnologia dos Agronegócios, Alta Mogiana regional pole, Colina São Paulo, Brazil. Ten rumen-cannulated Nellore steers (543 kg ± 28.6 BW) were distributed in a replicated (5 x 5) Latin-square design. Initially, the animals were identified, treated for internal and external parasites (Dectomax®, Zoetis Brasil; Topline®, Boehringer Ingelheim, SP, Brazil), housed in individual stalls (2 × 5 m) with concrete flooring, feed bunks, and free access to water. The experiment had five 19-d periods including 14 d of treatment adaptation [18] and the last 5 d for data collection and sampling. Nitrogen balance was assessed through total fecal and urinary sampling carried out over three consecutive days (days 15 to 18), followed by one day for rumen content collection (d19; VFA, ruminal ammonia (N-NH_3), pH, and microbiome).

The animals were assigned to the following treatments: (1) control **(CON)**, basal diet without exogenous enzymes; (2) amylolytic enzyme product added at 0.5 g/kg of diet dry matter (DM; **AML**, Amaize®, Alltech Inc., Nicholasville, KY,

USA); (3) fibrolytic enzyme product added at 0.9 g/kg of diet DM (**FBL**, Fibrozyme®, Alltech Inc.); (4) both amylolytic and fibrolytic enzymes added at half the dose of individual products (Half dose; **HD**); and (5) both amylolytic and fibrolytic enzymes added at the same dose of individual products (Full dose, **FD**). According to the manufacturer, Fibrozyme® is an extract from *Trichoderma longibrachiatum* fermentation (a dry mixture of inactive yeast, dry brewery yeast, and yucca extract) with a minimum of 100 IU (International unit) of xylanase activity per gram of product. Amaize® consists of an *Aspergillus oryzae* culture extract with a minimum α-amylase activity of 600 FAU (Fungal amylase unit)/g of product. One FAU is defined as the amount of enzyme that degrades 1 g of dextrinized soluble starch per hour at 30 °C and pH of 4.8 [19].

Feed management.

The steers were fed daily (8:00 am) ad libitum a total mixed ration (TMR) consisting of 18.5% of whole corn silage as a roughage source, 41.3 % of dry ground corn, 23.6 % citrus pulp, 5.60 % soybean meal, 7.93 % cottonseed and 3.13 % of a feedlot premix (Table 1; DM-basis). Before starting the experimental diets, the steers were adapted to the diet for 21 days without EFE supplementation. The concentrate portion and enzyme products (available in a dry powder form) were weighed individually for each animal, and then manually mixed with whole corn silage at the time of feeding. Thus, the enzyme dose was adjusted according to the daily DMI. Feed bunks were evaluated each day to quantify refusals and adjust daily feed allowance to a maximum of 5 % refusals.

Evaluations

All collections were made according to the experiment workflow (Fig. 1) following the respective methodology described below.

Ruminal fermentation parameters

Ruminal contents were collected at time 0 (before morning feeding) and 4, 8, 12, and 16 hours post-feeding from each cannulated steer on day 19 from the dorsal, central, and ventral regions of the rumen to form a composite sample,

which was immediately filtered through two layers of cheesecloth. Liquor samples were placed into 50 mL falcon tubes and were immediately used for ruminal pH measurement using a pH meter (DM-22, Digimed, Sao Paulo, Brazil). Two aliquots from each time point were then placed into a 15 mL conical centrifuge tube, preserved with 1 mL of H_2SO_4 (sulfuric acid), and stored at -20 °C until analyzed for ruminal ammonia nitrogen (NH_3-N) [20]. Another 15 mL was collected for analysis of the molar proportion of volatile fatty acids (VFA; acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate) and stored at -20°C. A microcentrifuge vial was thawed and processed following standard laboratory procedures. Briefly, a 1.6 mL of ruminal fluid was centrifuged for 30 min at 15,000g at 4 ˚C. The supernatant (0.4 mL) was transferred to a chromatography flask and 0.2 mL of a 3:1 solution of metaphosphoric acid (250 mL/L) and formic acid (908–1000 mL/L) was added. The internal standard was added to each vial (0.1 mL of 100 mM 2-ethyl-butyric acid). Acid separation and analyses were performed using the gas chromatograph (Agilent 7890) equipped with flame ionization detection (7683B) and 25-m long fused silica capillary column (J & W 19091 F-112, Agilent Technologies, Santa Clara, CA, USA), (320 µm internal diameter) following the method described by Erwin et al. [21].

Microbial efficiency, microbial protein synthesis, and nitrogen use

Total feces and urine collection were performed over 24 h on days 15 to 18. Urine output was collected using collecting funnels attached to animals and coupled with flexible hoses into 20 L containers with 500 mL of 20 g/100 mL sulfuric acid. At the end of the first collection day, the total urine volume was measured using a 2 L measuring cylinder, placed in a 20 L bucket, and mixed manually. Then, a 50 mL aliquot was collected from the total urine volume and diluted (1:4 ratio, vol of urine/vol H_2SO_4) into a sulfuric acid solution at 0.018 mol/L, placed in a plastic bottle, and stored at -20 ºC. The previously added acid solution was excluded from all calculations, so the volume measurements and calculations of total urine volume and urine subsample comprised only urine. Urine and feces samples were grouped to obtain a composite sample based on the daily volume.

Allantoin concentrations in urine were assessed by a colorimetric method according to Chen and Gomes [22], with absorbances measured using a microplate reader (Biochrom Asys UVM 340, Biochrom Asys). Uric acid concentration in urine was analyzed using a commercial kit (Uric Acid Stable Liquid, catalog no. K-052, Bioclin). The daily excretion of allantoin and uric acid is used to estimate microbial protein production in the rumen and was estimated by multiplying the urine concentrations by the average urinary volume. Excretion of the purine derivatives in urine was calculated by the sum of the allantoin and uric acid excretions (mmol/d). Absorbed purines were estimated by derivatives of total purines using the formula: Pa (mmol/d) = [PDe - (0.301 × $BW^{0.75}$)] / 0.80, where PDe (mmol/d) is purine derivatives excreted (uric acid and allantoin), BW is body weight, 0.301 × $BW^{0.75}$ represents excretion of endogenous of PDe and 0.80 is the recovery of Pa [23].

Microbial nitrogen synthesis was calculated by absorbed purines using the formula: Nmic (g N/d) = [(70 × Pa) / (0.93 × 1000 × 0.137)], where 70 represents the concentration of N in purines (mg/mmol), 0.93 is the digestibility of purines and 0.137 is the average ratio of RNA-N to total-N for rumen bacteria [23]. The efficiency of microbial synthesis in the rumen [g microbial N / kg of digestible OM intake (DOMI)] was calculated by dividing the production of ruminal microbial N (g microbial N/d) by the DOMI (kg/d). The CP / DOMI ratio was calculated based on the intake of digestible OM and CP (g/kg).

Nitrogen balance was obtained by subtracting the total excreted N (feces and urine) from total N intake, representing the total N that was effectively retained by the animal. For this purpose, diet components, fecal, and urine samples were analyzed for N (920.87) and ash (942.05) according to AOAC method [24]. Organic matter was calculated as organic matter = 100 – ash. To determine the fecal N excretion (FNE), the fecal production was multiplied by the total N concentration in the feces. Urinary N excretion was calculated by multiplying the average of urinary volume by urinary N concentration. The apparent efficiency of N utilization (ENU) by the animal was calculated by dividing the nitrogen balance/nitrogen intake.

Ruminal bacteria and archaea diversity

Ruminal content (solid + liquid) samples of approximately 50 g per animal were collected from the dorsal, central, and ventral regions of the rumen through the ruminal cannula. Collections were made 4 hours after steers were fed the diet supplemented with EFE on day 19 and were immediately stored at -80 °C until further analysis. The samples were processed to obtain a microbial pellet [25], with phosphate saline buffer (pH 7.4). For DNA extraction, 250 mg of the microbial pellet was used. A Quick-DNA™ Fecal/Soil Microbe Miniprep kit according to the manufacturer's instructions (Zymo Research Corporation, CA, United States), and the bead beating method for cell lysis (FastPrep-24, MP, Biomedicals, Illkirch, France) were used. The DNA concentrations were assessed using a spectrophotometer (NanoDropR ND-1000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, United States) and a fluorometer (QubitR 3.0, kit Qubit RdsDNA Broad Range Assay Kit, Life Technologies, Carlsbad, CA, USA). DNA purity was confirmed spectrophotometrically by absorbance ratios at 260/230 and 260/280 nm and DNA integrity (no evidence of degradation) was determined by agarose gel electrophoresis using a 0.8% (w/v) gel and stained with SYBR Safe DNA Gel Stains (Thermo Fisher Scientific, Waltham, MA, EUA).

Libraries were prepared by PCR amplification of the V3/V4 regions of the 16S ribosomal RNA gene (16S rRNA) using barcoded 16S Illumina primers [26]. Each sample was amplified in duplicates, and each PCR reaction mixture (20 µL final volume) contained 20 ng of metagenomic DNA, 10 µmol/L of each forward and reverse primers, 1.25 mM of magnesium chloride, 200 µM of dNTP mix (Invitrogen, Carlsbad, CA, United States), 1.0 U platinum Taq DNA polymerase high fidelity (Invitrogen, Carlsbad, CA, United States), PCR buffer [1X], and milli-Q water. Reactions were conducted at 95 °C for 3 min to denature the DNA, with amplification proceeding for 30 cycles at 95 °C for 30 s, 53.8 °C for 30 s, and 72 °C for 45 s; a final extension at 72 °C for 10 min was added to ensure complete amplification. The fragment length of PCR products was verified by agarose gel (1%) electrophoresis, using a 1 kb plus DNA ladder (1 kb plus DNA ladder, Invitrogen, Carlsbad, CA, USA) for amplicon size estimation. PCR fragments were purified using the Zymoclean TM Gel DNA Recovery kit following the

manufacturer's instructions, and composite samples for sequencing were created by combining equimolar ratios of amplicons from the duplicate samples. Sequencing was performed using the Illumina MiSeq® platform with MiSeq Reagent v2 (2 × 250 pb; Illumina®, USA) kit.

Sequencing data clean-up

Sequence data were demultiplexed and quality-filtered using the q2-demux plugin followed by denoising with DADA2 [27]. The samples were rarefied to 17,405 sequences per sample, and the q2-diversity plugin was used to estimate the diversity metrics as alpha-diversity [28] and beta-diversity [29]. All amplicon sequence variants (ASVs) were aligned with mafft [30] and used to construct a phylogeny with fasttree [31]. The ASVs were assembled by the available reference method in the Quantitative Insights into Microbial Ecology (QIIME) software package version 2. Taxonomy was assigned to ASVs using the q2-feature-classifier classify-consensus-search taxonomy classifier against the Silva 128 database with 97% ASVs reference sequences [32].

Statistical analysis

The effects of enzyme supplementation were performed using contrasts: (1) CON vs. EFEs (2) AML vs. FD, (3) FBL vs. FD, and (4) HD vs. FD. Except for ruminal microbial diversity, all data were analyzed by fitting mixed models [33], using the MIXED procedure of SAS (SAS Institute, Cary, NC). First, the mathematical assumptions of data normality (Shapiro-Wilk test) and homogeneity of variance (Bartlett test) were tested. The data were considered normally distributed (Shapiro-Wilk test, $W \geq 0.80$) using the "Univariate" procedure of SAS. The experimental model considered "treatment" as a fixed effect and, "period", "animal" and "square" as random effects. Variables measured in the same experimental unit over time (rumen pH, NH_3-N, and VFA) were analyzed as repeated measurements using the "repeated" procedure of SAS. The symmetry of the covariance structure of the repeated measure analyses was chosen based on the lowest BIC (Bayesian Information criterion). The Kenward-Rogers approach approximation was used to adjust the denominator degrees of freedom

for the test of fixed effects.

Richness estimators, diversity index, and ruminal microbial relative abundance of bacteria and archaea data were compared between CON vs. EFE using a Kruskal–Wallis test, while the paired Wilcoxon Rank Sum Test was applied to compare (2) AML vs. FD, (3) FBL vs. FD, and (4) HD vs. FD. A permutation test of multivariate homogeneity of group dispersions based on Bray-Curtis as a similarity index was applied using the *vegan* package in R. Spearman's rank correlations were used to investigate the relationship between important microbial ASVs associated with ruminal fermentation parameters. A correlation plot was performed using the corrplot library. All rumen microbiome analyses were performed using R Software version 4.3.3 [34].

Statistical differences were declared at $P \leq 0.05$ and trends toward significance were considered when $0.05 < P \leq 0.10$ for all variables.

RESULTS

Ruminal fermentation parameters

No differences were observed among treatments for ruminal pH and total VFA ($P > 0.177$; Table 2). Additionally, there was no interaction between Treat × Time on ruminal fermentation parameters ($P > 0.221$). Animals fed EFE had 10.2% lower ruminal N-NH_3 concentration ($P = 0.040$) values and lower molar proportion of isovalerate ($P = 0.083$) than CON steers. However, a higher ruminal proportion of acetate ($P < 0.001$), valerate ($P < 0.001$), and Acetate:Propionate ratio ($P = 0.008$) was observed in steers fed EFE. Steers fed AML tended to have a lower molar proportion of propionate ($P = 0.081$) and butyrate ($P = 0.077$) but showed a higher Acetate:Propionate ratio ($P = 0.010$) compared to animals supplemented with both enzymes in FD. A tendency for an increase in ruminal N-NH_3 concentration was observed in steers fed FBL ($P = 0.082$), while they presented a lower valerate than steers fed FD ($P = 0.015$). Therefore, animals in the FD treatment had a higher proportion of propionate in the rumen ($P = 0.034$) than those fed HD of both enzymes.

Nitrogen balance

No difference was observed between treatments for digestible OM intake (DOMI) and total N Intake ($P > 0.324$; Table 3). Animals supplemented with AML had lower CP/DOM values ($P = 0.051$) and tended to have lower fecal N excretion (FNE, $P = 0.059$) compared to animals receiving both enzymes in FD. Animals fed EFE excreted lower amounts of nitrogen in both feces ($P = 0.049$; g of N/d) and urine ($P = 0.036$, g of N/d) than those in the CON group. Additionally, UNE expressed as % of N ingested was lower in animals that received EFE in the diets. Thus, animals fed EFE had higher N retention and ENU values ($P = 0.045$) than animals in the CON group. No differences were observed between FBL vs. FD and HD vs. FD in any of the nitrogen balance variables ($P > 0.218$).

Rumen Microbial Diversity

A total of 5,075,284 raw reads were obtained from 50 samples by sequencing. After trimming, the average number of sequences was 89,248 per sample, of which 66.9% were non-chimeric. The supplementation of EFE did not affect alpha diversity and richness indices ($P > 0.10$; Table S1). In the same way, no difference between treatments in ruminal microbial population beta-diversity Bray-Curtis values was observed ($P > 0.10$; Fig. S1).

A total of 18 phyla were identified, including two from the Archaea domain (Euryarchaeota and Thermoplasmatota) and 16 from bacteria. The Verrumicrobiota phylum tended to have a lower relative ruminal abundance in animals fed FD ($P = 0.066$; Fig. 2) than in animals fed AML. FD also tended to reduce the ruminal abundance of the Verrucomicrobiota phylum compared to animals on the HD treatment ($P = 0.093$). Additionally, animals that received FBL had a lower relative abundance of Actinobacteriota than those fed FD ($P = 0.047$).

A total of 110 families and 222 genera were identified, and the relative abundance of 14 families was influenced by treatments (Table 4). Within the Actinobacteriota phylum, the Atopobiaceae and Bifidobacteriaceae families tended to have higher abundance in the rumen of FD group ($P < 0.10$) compared to FBL, and the Bifidobacteriaceae family was more abundant in the rumen of animals fed the CON diet ($P = 0.007$) than EFE. In contrast, within Bacteroidota, the Bacteroidales UCG-001, Muribaculaceae, and Bacteroidales uncultured

families tended to have lower abundance in animals fed the CON diet compared to those fed EFE ($P < 0.10$). The Bacteroidales RF16 group tended to have higher ruminal abundance in animals fed FD compared to the HD treatment ($P = 0.059$). A lower abundance of the family p-2534-18B5 gut group was observed in animals fed FBL compared to FD ($P = 0.042$).

For the Firmicutes phylum, the FD treatment had lower ruminal abundance of the *Clostridium methylpentosum* group and the Ruminococcaceae family ($P < 0.01$) and tended toward lower relative abundance of Bacillaceae compared to HD ($P = 0.074$), and higher ruminal abundance of Mycoplasmataceae ($P = 0.093$). Additionally, the family UCG-010 was more abundant in the rumen of animals fed AML ($P = 0.037$), compared to FD.

In the Proteobacteria phylum, the Rickettsiales uncultured family ($P < 0.05$) was more abundant in the rumen of animals fed AML and HD than those fed FD. For the Verrucomicrobiota phylum, the family WCHB1-41 had a higher relative abundance in animals fed AML than those fed FD ($P = 0.038$), and FD treatment tended to have lower relative abundance of this family when compared to HD, FBL, and CON ($P < 0.10$).

The FD of both enzymes promoted shifts in 21 genera when compared to the AML treatment. Specifically, the *Prevotellaceae NK3B31 group* and *SP3-e08* were higher in the rumen of FD ($P < 0.05$; Table 5). Conversely, several genera, including *Amnipila,* Lachnospiraceae *XPB1014 group, Hydrogenoanaerobacterium, Incertae_Sedis, Selenomonas, Anaerovibrio, UCG-010,* Christensenellaceae *uncultured, Rhodospirillales uncultured, Rickettsiales uncultured, Sphaerochaeta* and *WCHB1-41* were higher in the rumen of AML. In addition, animals fed FD tended to have a higher ruminal abundance of *Bacteroidales UCG-001,* Muribaculaceae unclassifield*, Kandleria,* and *Roseburia*, and tended to have a lower ruminal abundance of *Anaerovorax,* Ruminococcaceae unclassified*,* and Veillonellaceae*_UCG-001* compared to those fed AML ($P < 0.10$).

The FD of both enzymes resulted in a higher ruminal relative abundance of genera *Bifidobacterium, p-2534-18B5 gut group,* and *Howardella (*$P < 0.05$*),* and tended toward a higher relative abundance of *Olsenella* ($P = 0.059$)

compared to FBL. In contrast, animals fed FBL had a higher relative ruminal abundance of Prevotellaceae *UCG-003, U29-B03, Eubacterium brachy* group*, Papillibacter, Anaerovibrio* (*P* < 0.05)*,* and tended to a lower abundance of *Selenomonas* and *WCHB1-41* (*P* < 0.10) compared to those fed FD.

The FD treatment promoted a higher relative abundance of the genera *Howardella* (*P* < 0.05) and tended to have higher abundance of *Bacteroidales RF16* group, and *Mycoplasma* (*P* < 0.10) compared to both enzymes in HD*.* In contrast, the HD treatment resulted in a higher relative abundance of *Prevotella, Clostridium methylpentosum* group*, Ruminococcus, Selenomonas, Rhodospirillales uncultured, Rickettsiales uncultured* (*P* < 0.05)*,* and tended toward higher abundance of *U29-B03, Mogibacterium, Bacillus*, and Ruminococcaceae unclassified compared to FD.

The ruminal proportion of acetate was positively correlated with *Papillibacter* (0.72), *Incertae Sedis* (0.72), Prevotellaceae *UCG 003* (0.64), *Acidaminococcus* (0.66), *Bradymonadales* (0.71), Rikenellaceae *RC9* gut group (0.72), *Anaerovorax* (0.67), *Sediminispirochaeta* (0.64), and Lachnospiraceae *XPB1014* group (0.61) relative abundance (Fig. 3). In contrast, the abundance of Proteobacteria phylum (-0.59), Actinobacteriota phylum (-0.60), Succinivibrionaceae (-0.61), *Olsenella* (-0.63), and *Pseudoramibacter* (-0.63) were negatively correlated with ruminal acetate.

The ruminal proportion of propionate showed positive correlations with Proteobacteria phylum (0.63), Succinivibrionaceae (0.64), and *Acidaminococcus* (0.59), and was negatively correlated with *Incertae Sedis* (-0.66), *Anaerovorax* (-0.64), *Papillibacter* (-0.63), Prevotellaceae *UCG 003, Rikenellaceae RC9* gut group (-0.61), and *Bradymonadales* (-0.62). Conversely, the ruminal proportion of butyrate was negatively correlated with the phyla Proteobacteria (-0.56) and Verrucomicrobiota (-0.52), and the Succinivibrionaceae family (-0.51).

The ruminal proportion of valerate was positively correlated with *Coprococcus* (0.60), *Shuttleworthia* (0.64), Actinobacteriota phylum (0.65), *Lachnospira* (0.66), *Olsenella* (0.66), Atopobiaceae (0.67), Eubacteriaceae (0.69), *Pseudoramibacter* (0.70), Erysipelotrichaceae *UCG,009* (0.71), *Catenisphaera* (0.75), Erysipelotrichaceae *UCG,006* (0.77) and

Acidaminococcus (0.77). Conversely, negative correlations were observed with *Eubacterium hallii* group (-0.77), *Anaerovorax* (-0.77), *Incertae Sedis* (-0.71), Lachnospiraceae *UCG,008* (-0.69), Prevotellaceae *Ga6A1* group (-0.69), *Papillibacter* (-0.65), Lachnospiraceae *XPB1014 group* (-0.64), and *Sediminispirochaeta* (-0.62).

Finally, rumen pH was associated positively with the relative abundance of *Incertae Sedis* (0.59), Lachnospiraceae *UCG-008* (0.60), and *Papillibacter* (0.64), and negatively with Erysipelotrichaceae *UCG,006* (-0.67), *Acidaminococcus* (-0.66), Atopobiaceae (-0.65), *Olsenella* (-0.64), Actinobacteriota (-0.63), Eubacteriaceae (-0.58), and *Pseudoramibacter* (-0.58).

The ruminal proportion of isovalerate, isobutyrate, Total VFA, and rumen N-NH3 showed weak correlations with the abundance of rumen microorganisms ($r < 0.50$).

DISCUSSION

We investigated the effect of adding EFE products in feedlot diets on fermentation parameters, nitrogen metabolism and microbial communities in the rumen of Nellore cattle. We found no differences in ruminal pH and total VFA production. However, our findings reveal that EFE products resulted in lower ruminal N-NH_3 concentration, shifted VFA profile in the rumen, and reduced nitrogen excretion through feces and urine, consequently leading to greater N retention in the body and improved ENU. Additionally, although we did not observe a significant difference in alpha and beta diversity indices of microbial communities in the rumen, some changes in ruminal fermentation can be associated with shifts in bacterial taxa observed across treatments.

Effect on ruminal fermentation

The main objective when evaluating EFE in ruminant nutrition is related to the primary effects of the molecule on rumen fermentation. Typically, the increase in animal performance observed when animals are fed EFE has been correlated with increased hydrolytic activity in the rumen and total-tract digestibility of nutrients [3], which might contribute to changes in VFA production and their

profiles. Nevertheless, the addition of EFE to feedlot diets has not shown a consistent positive impact on ruminal fermentation [11,12,35]. Some factors like enzyme stability in the rumen [3], enzyme type, and characteristics of the diets being tested [36] have contributed to the inconsistency in the ruminal fermentation of cattle fed EFE. The results of the current study align with the small effects of enzymes on ruminal fermentation reported in the literature [37–40].

Our results related to total VFA production and pH were contrary to our initial hypothesis that the addition of EFE in the diet would impact the ruminal fermentative activity, as demonstrated in in vitro [41] and in vivo studies with dairy [2] and beef cattle on pasture [42] and in feedlots [17]. In the current study, the lack of treatment effect on the DOMI corroborates with the data on the total concentration of VFA. In agreement with our results, Hristov et al. [11] found no effect on the ruminal pH and total VFA production in lactating cows fed both xylanase and α-amylase. Additionally, it is important to highlight that the combined use of EFE (HD or FD treatment) exerted minimal influence on most ruminal parameters (except for propionate and butyrate). This suggests that the increased enzyme dosage may have induced competition with microbial enzyme production, without significantly altering overall ruminal enzymatic activity. Therefore, to maximize the efficacy of EFE supplementation, it is crucial that these exogenous enzymes complement, rather than replace the intrinsic enzymatic capabilities of ruminal microbes.

The addition of EFE promoted changes in the ruminal VFA profile, which may be related to changes in bacterial population (discussed below) due to the hydrolysis of complex carbohydrates by exogenous enzymes and release of different oligosaccharides (malto-, cello-, and xylo-oligosaccharides) that can be used as substrates by multiple species of rumen bacteria. Thus, as postulated by Zilio et al. [2], we expected a synergistic effect between the exogenous enzymes used in the present study because oligosaccharides generated in the rumen can be used by amylolytic and nonamylolytic bacteria in cross-feeding mechanisms [43]. Microbial metabolism of these substrates could lead to changes in ruminal fermentation patterns, including the greater acetate proportion and acetate:propionate ratio observed in the current study.

Considering that acetate is the main product of carbohydrate fermentation by fibrolytic bacteria [44], improved access to the fiber fraction of the diet by rumen microorganisms could potentially drive an increase in the molar proportion of acetate. This is further supported by the enrichment of specific carbohydrate-degrading bacterial genera (discussed below) and a numerical increase of +1.28% in the total tract neutral detergent fiber digestibility coefficient observed in EFE-supplemented animals compared to the control group (unpublished data). It has been reported that exogenous enzymes present synergy with fibrolytic enzymes of rumen microorganisms and promote attachment of rumen microorganisms to plant fiber [5]. Ribeiro et al. [45] reported that an exogenous enzyme induced shifts in rumen microbiota, increased ruminal bacterial diversity (evenness), and decreased protozoa count in lambs.

Animals fed with EFE tended to have a lower isovalerate proportion in the rumen. In contrast, the proportion of isobutyrate was unchanged but the concentration of valerate was higher in animals fed EFE. In addition, the 10.2% lower ruminal N-NH_3 concentration in animals fed with EFE compared to the control group might indicate that EFE contributed to reducing ruminal proteolysis, altering the degraded protein profile, and preserving the main branched-chain amino acids used to form isoacids in the rumen (leucine, isoleucine, and valine) during proteolytic fermentation [46]. These findings suggest that EFE may have contributed to a greater influx of amino acids into the intestine [47].

Ruminal N-NH_3 concentration represents a net balance between ammonia production (degraded dietary protein), ammonia uptake by microbes (that varies with carbohydrate availability), ammonia absorption (which varies with ruminal pH) as well as recycling of urea to the rumen (via saliva and through the ruminal wall). In the current study, since the rumen degradable protein was similar among diets, the ruminal pH did not differ, and EFE did not significantly impact microbial yield or recycling urea, it is speculated that the observed lower ruminal N-NH_3 concentration was attributable to the inhibition of amino acid deamination with EFE. This interpretation may be partially supported by microbial data, which showed a lower relative abundance of members of the Clostridiaceae family and the *Clostridium methylpentosum* group in the EFE treatment. These taxa are

known contributors to proteolytic and deamination processes in the rumen [48]. Their reduction could explain the decreased ammonia levels and altered isoacid profiles, indicating a shift in nitrogen metabolism toward enhanced nitrogen retention.

Our results are in line with the results of He et al. [49], who evaluated the effect of fibrolytic enzymes in barley-based diets containing wheat-dried distillers grain with solubles in feedlot. Moreover, ruminal protein degradation is an inefficient process due to excessive ammonia production relative to microbial demand [50]. Consequently, excess of ruminal N-NH_3 is absorbed through the rumen wall and excreted in urine, significantly reducing ENU by the animal [51,52].

Effect on nitrogen balance

Beef cattle retain only a portion of the nutrient intake (~20%), with the remainder lost mainly in feces and urine [1]. Typically, EFE are added to beef cattle diets to increase nutrient availability, and therefore improve nutrient utilization and feed efficiency [3,53]. Although few studies report lower nutrient excretion with EFE, our results demonstrated that EFE promoted changes in the nitrogen balance of animals, resulting in lower N excretion in feces and urine.

Animals fed diets treated with EFE excreted an average of 12% less fecal nitrogen compared to the control group, likely due to less excretion of indigestible protein, a consequence of numerically greater crude protein (CP) digestibility of the feed (unpublished data). However, due to the potential reduction in ruminal proteolytic capacity in animals fed EFE, it is possible that EFE may have contributed to greater intestinal digestion activity or improved post-ruminal nutrient absorption [3], possibly resulting in a greater flow of amino acids to the intestine. Supporting this, Nascimento [17] observed that animals fed with α-amylase in a high-grain finishing diet had higher CP digestibility and ENU without a change in ruminal N-NH_3. Furthermore, Morgavi et al. [54], noted that some exogenous enzymes survive ruminal fermentation and the abomasal environment, potentially exerting activity in the small intestine. To our knowledge, this study is only the second to investigate the potential influence of EFE on

nitrogen balance of Nellore cattle in feedlot. Consequently, further studies are needed to better understand the post-ruminal impact of EFE and unveil the mechanisms potentially influencing CP digestibility in feedlot diets treated with EFE.

The nitrogen in feces (undigested food, microbial, and endogenous protein) differs substantially from the N in urine (urea, hippuric acid, allantoin, creatinine, ammonia, and uric acid). Considering the main sources of N excreted via urine, our data regarding the lower concentration of $N\text{-}NH_3$ in the rumen align with the lowest values of N excretion in urine, since no change was observed in the concentration of purine derivatives used to estimate microbial protein synthesis. Approximately 35-65% of ammonia is absorbed across the epithelium of the ruminal wall [55], accounting for up to 50% of the total ammonia flow to the liver [56]. Thus, the great amount of ammonia that reaches the portal blood is captured and converted to urea in the liver, with some of this urea being recycled (via saliva and across the epithelium of the rumen and other sections of the gastrointestinal tract) or excreted in the urine. In our study, the lower values of N excretion in urine contribute in part to higher ENU and N retention in animals fed with EFE, which was associated with an increase of 2.8 kg of hot carcass weight (unpublished data) compared to animals in the control group. As reported by Alves et al. [52], urinary excretion of urea synthesized from ammonia lost from the rumen is the main factor resulting in low efficiency of N utilization in Nellore cattle.

Based on a typical 107-day Brazilian feedlot cycle [57], animals fed EFE are estimated to excrete 1.93 kg less nitrogen (fecal and urinary). Focusing solely on nitrous oxide (N_2O) emissions from excreta and applying emission factors for feces (0.27%) and urine (0.63%) established by Cardoso et al. [58] under tropical conditions, this reduction in nitrogen excretion could lead to a possible 10.7% decrease in N_2O emissions per feedlot cycle. While these findings suggest that EFE supplementation may improve nitrogen retention and enhance the efficiency of nitrogen utilization (ENU), the extent to which it directly reduces N_2O emissions remains uncertain and warrants further investigation.

Effect on ruminal microbial population

To manipulate ruminant efficiency, it is important to understand the role that ruminal microbiota plays in feed utilization by the host [52]. However, few studies have demonstrated the effect of EFE on rumen bacteria and their correlations with host biology.

The lack of difference in beta-diversity and alpha-diversity indices with EFE suggests that the overall rumen microbial community structure remains relatively stable. In line with this, Chung et al. [14] reported that exogenous fibrolytic enzyme supplementation did not alter the population richness of total protozoa, bacteria, and methanogens in the rumen of dairy cows. Similarly, Liu et al. [15] found no impact on rumen bacteria richness and diversity indices when dairy cows were fed a combination of fibrolytic and amylolytic enzymes. Interestingly, both studies also did not detect changes in the final products of fermentation, such as VFA and rumen pH.

However, while overall richness and diversity might not change, compositional shifts in key microbial groups can still have important implications for rumen fermentation and animal performance. In this context, the FD treatment with both enzymes promoted shifts in 21 genera compared to the AML treatment. A lower abundance of Verrucomicrobiota phylum and its genera *WCHB1-41* was observed with the FD treatment. This phylum has been linked to fiber degradation, containing groups of bacteria considered specialized degrading complex polysaccharides, such as *WCHB1-41* [59,60]. FD also resulted in lower abundance of several fiber-degrading groups from the Firmicutes phylum, such as Ruminococcaceae unclassified*, Lachnospiraceae* XPB1014 *group, Incertae_Sedis, Christensenellaceae uncultured, Selenomonas, Anaerovibrio,* UCG-010*, Hydrogenoanaerobacterium, Anaerovorax,* and *Veillonellaceae* UCG-001 [61,62]*,* while promoting the enrichment of carbohydrate-degrading bacterial genera, such as Prevotellaceae *NK3B31*, *Bacteroidales UCG-001,* Muribaculaceae unclassifield*, Kandleria,* and *Roseburia* [63–65]. This shift is consistent with the greater ruminal acetate proportions observed in animals receiving the FD treatment, and supports possible modification in the fiber fermentation pathways, diverting fermentation to more readily available

carbohydrates due to improved access to the fiber fraction of the diet. Additionally, the data suggest that differences among treatments in the abundance of the genera Lachnospiraceae *XPB1014 group, Incertae Sedis,* and *Anaerovorax* are associated with the proportions of acetate and propionate in the rumen, including the lower acetate:propionate ratio in FD compared to AML.

In contrast, cattle fed the fibrolytic (FBL) and amylolytic (AML) enzyme treatment had a lower relative abundance of Actinobacteriota phylum compared to FD. This was mainly due to the modulation of the abundance of Atopobiaceae and Bifidobacteriaceae families and their respective genera *Olsenella* and *Bifidobacterium.* Current studies have reported that rumen valerate levels are associated with the *Olsenella* population in ruminants [66,67]. This is consistent with the lower valerate ruminal proportion observed in the FBL group compared to FD, and the association detected between rumen valerate proportion and the *Atopobiaceae* family and its genus *Olsenella* in our study. *Bifidobacterium* can grow on various carbohydrates, mainly dietary carbohydrates and host-derived glycans [68]. The higher abundance of *Bifidobacterium* with the FD treatment compared to FBL may indicate a higher dietary carbohydrate availability promoted by the combination of enzymes.

The observed differences in microbial populations between the FD and HD of both enzymes highlight how variations in enzyme dosing may shift the balance between carbohydrate and fiber degraders during rumen fermentation. The greater relative abundance observed with FD of the genera *Howardella*, *Bacteroidales RF16* group, and *Mycoplasma* is notable. *Howardella* is considered a strong ureolytic genus [69]. The *Bacteroidales RF16* group has been related to fibrolytic isozyme activity [70], and *Mycoplasma* was found in microbial consortia during the digestion of low-quality feeds [71]. In contrast, the HD treatment resulted in greater populations of genera from the Firmicutes phylum linked to fiber degradation, such as the *Ruminococcus*, Ruminococcaceae unclassified*, Bacillus, Mogibacterium, Clostridium methylpentosum* group, and *Selenomonas* [72,73], as well as *Prevotella,* the most abundant genera in the rumen belonging to the Bacteroidetes phylum. *Prevotella* is known as the major pectinolytic bacteria and the most active proteolytic in the rumen [48]. Despite the insights

from the current study, additional research is needed to establish a causal association between exogenous feed enzymes and fiber digestibility in the rumen.

CONCLUSION

This study demonstrated that supplementation with exogenous feed enzymes (EFE) led to lower nitrogen excretion through feces and urine, resulting in greater nitrogen retention in the animal's body. Additionally, EFE supplementation resulted in lower ammonia levels in the rumen and promoted shifts in the ruminal fermentation profile and microbial population. While combining amylase and xylanase did not significantly affect total ruminal fermentation parameters, it did induce shifts in their profile and microbial population. Further studies should further explore the impact of enzyme combinations and dosage rates on ruminal parameters, nitrogen metabolism, and ruminal microbiome populations in a high-starch diet.

Abbreviations

AML	Amylase treatment
ASV	Amplicon sequence variants
BW	Body weight
CON	Control treatment
CP	Crude protein
DM	Dry matter
DMI	Dry matter intake
DOMI	Digestible organic matter intake
EFE	Exogenous feed enzymes
ENU	Efficiency of nitrogen utilization
FAU	Fungal amylase unit
FBL	Xylanase treatment
FD	Full dose combination treatment
FNE	Feces nitrogen excretion
HD	Half dose combination treatment

IU	International unit
ME	Metabolizable energy
MN	Microbial nitrogen
N	Nitrogen
N_2O	Nitrous oxide
NEg	Net energy for gain
NEm	Net energy for maintenance
NI	Not identifield
Nmic	Microbial nitrogen synthesis
N-NH_3	Ruminal ammonia
OM	Organic matter
Pa	Purines absorbed
PDe	Purine derivative excreted
SEM	Standard error of the mean
TDN	Total digestible nutrients
TMR	Total mixed ration
UNE	Urine nitrogen excretion
VFA	Volatile fatty acids

Declarations

Ethics approval

All experimental procedures involving animals were carried out in accordance with the ethical principles established by the Ethics Committee for the Use of Animals of the Department of Development Decentralization (CEUA-DDD), São Paulo, Brazil (Protocol number 004/2022), and followed the relevant guidelines and regulations of the National Council for Animal Experimentation Control (CONCEA).

Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; São Paulo, SP, BRAZIL) for the scholarship of the first authors (2019/24820-3 and 2022/00989-1) and the PDIP grant

(2017/50339-5). Also, we would like to thanks to the Coordenação de Aperfeiçoamento do Pessoal de Nível Superior (CAPES) financial code 001 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarships granted to the other authors.

Acknowledgments

We would like to thank Luiz Fernando Costa e Silva for the support by Alltech®. This study is part of the first author's thesis presented to the São Paulo State University "Júlio de Mesquita Filho" (UNESP), Jaboticabal in compliance with the requirements for the Doctor's degree in Animal Science by I. M. Ferreira (2025).

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Tables:

Table 1: Feed ingredients proportion and chemical composition of the diets.

Ingredient, % DM	Acclimation diet	Experimental diet
Whole corn silage	40.7	18.5
Ground corn	23.3	41.3
Citrus pulp	18.6	23.6
Soybean meal	8.74	5.60
Cottonseed	5.61	7.93
Feedlot premix [a]	3.13	3.13
Nutritional profile, % DM		
Dry matter	63.0	65.8
Organic matter	93.9	93.9
Crude protein	15.1	14.1
Neutral detergent fiber	32.7	26.4
Acid detergent fiber	14.4	13.3
Ether extract	3.53	3.82
Non-fiber carbohydrate	47.9	48.5
Starch	31.2	35.6
Total digestible nutrients	73.3	75.8
ME, mcal/kg [c]	2.65	2.88
NEm, mcal/kg [d]	1.85	2.05
NEg, mcal/kg [d]	1.22	1.29

[a]Content per kilogram of product: 116.7 g of Ca, 19.4 g of P; 38.9 g of Na, 29.2g of S, 1167 mg of Zn, 9722 mg of Mg; 386 mg of Cu, 463 mg of F, 778 mg of Mn, 11.4 mg of Co, 19.4 mg of I, 9.4 mg of Se, 808 mg of monensin sodium, and 323 mg of urea. Manufactured by Alltech®, Parana, Brazil.

[b]Total digestible nutrients (TDN) were estimated using the individual composition of feedstuff according to Valadares Filho et al. [74].

[c] Metabolizable energy (ME) = TDN (g/kg DM) × 4.4 × 0.82 [75].

[d]The net energy for maintenance (NEm) and gain (NEg) were estimated with the equations proposed by NASEM (2016; empirical solution type) with the addition of ionophore and using the TDN values.

Table 2: Effects of EFE on ruminal fermentation parameters in cannulated Nellore finishing steers (*n* = 10).

Itens	Treatments[1]					SEM	*P-value*[2]					
	CON	AML	FBL	HD	FD		C1	C2	C3	C4	Time	Treat×Time
pH ruminal	5.83	5.81	5.85	5.77	5.79	0.055	0.445	0.696	0.177	0.613	< 0.001	0.983
N-NH_3, mg/dL	9.23	7.89	8.79	8.68	7.78	0.739	0.040	0.846	0.082	0.120	< 0.001	0.507
Total VFA, mmol/L	49.2	45.5	48.0	49.8	48.4	2.54	0.469	0.221	0.853	0.558	< 0.001	0.677
VFA profile, mol/100 mol												
Acetate	61.3	62.9	62.6	62.4	62.2	1.19	< 0.001	0.143	0.447	0.699	< 0.001	0.991
Propionate	22.3	21.3	21.5	21.1	22.4	1.59	0.118	0.081	0.158	0.034	0.028	0.999
Isobutyrate	0.598	0.594	0.612	0.565	0.589	0.062	0.681	0.919	0.526	0.509	< 0.001	0.221
Butyrate	12.3	11.8	12.4	12.5	12.4	0.411	0.940	0.077	0.935	0.598	< 0.001	0.998
Isovalerate	1.84	1.76	1.67	1.79	1.70	0.138	0.083	0.485	0.666	0.284	< 0.001	0.998
Valerate	1.29	1.45	1.37	1.49	1.47	0.174	< 0.001	0.736	0.015	0.547	< 0.001	0.999
Acetate to propionate ratio	2.94	3.25	3.17	3.07	3.08	0.256	0.008	0.010	0.534	0.881	0.003	0.996

[1]Control (CON) = no feed additives; Amylase (AML) = amylolytic enzyme (Amaize, Alltech) added at 0.5 g/kg diet DM; Xylanase (FBL) = fibrolytic enzyme (Fibrozyme, Alltech) added at 0.9 g/kg diet DM; Half dose of Amylase and Xylanase (HD) = amylolytic enzyme added at 0.25 g/kg diet DM and fibrolytic enzyme (Fibrozyme, Alltech) added at 0.45 g/kg diet DM; and Full dose of Amylase and Xylanase (FD) = amylolytic enzyme added at 0.5 g/kg diet DM and fibrolytic enzyme added at 0.90 g/kg diet DM;
[2]Contrast: C1 (CON vs. EFE), C2 (AML vs. FD), C3 (FBL vs. FD), and C4 (HD vs. FD)
SEM = standard error of the mean

Table 3: Effect of EFE on nitrogen balance in cannulated Nellore finishing steers (*n* = 10).

Itens	Treatments[1]					SEM	*P-value*[2]			
	CON	AML	FBL	HD	FD		C1	C2	C3	C4
DOMI, kg/d	7.92	7.83	7.71	7.97	7.80	0.329	0.619	0.901	0.704	0.497
CP/DOMI, g/kg	174	166	173	172	173	6.55	0.400	0.035	0.919	0.984
N utilization										
Total N intake, g/d	219	210	212	220	216	12.1	0.324	0.416	0.613	0.531
UNE, g of N/d	99.4	92.9	81.1	91.5	84.6	8.24	0.036	0.174	0.558	0.257
UNE, % N ingested	46.2	44.9	38.3	42.1	39.6	3.48	0.072	0.134	0.694	0.471
FNE, g of N/d	87.3	77.0	79.9	82.9	84.6	6.24	0.054	0.059	0.233	0.662
FNE, % N ingested	40.1	37.3	37.7	38.0	39.8	3.84	0.155	0.149	0.218	0.303
N retention, g of N/d	40.7	44.8	50.8	51.5	50.6	6.32	0.036	0.251	0.960	0.900
ENU, g/g	0.187	0.212	0.241	0.234	0.216	0.020	0.045	0.870	0.282	0.425
Microbial efficiency										
MN, g of microbial nitrogen/d	165	199	164	184	185	18.6	0.334	0.561	0.359	0.959
Emic, g of microbial nitrogen/kg DOMI	20.9	25.4	21.0	23.2	24.1	2.34	0.262	0.646	0.285	0.776

[1]Control (CON) = no feed additives; Amylase (AML) = amylolytic enzyme (Amaize, Alltech) added at 0.5 g/kg diet DM; Xylanase (FBL) = fibrolytic enzyme (Fibrozyme, Alltech) added at 0.9 g/kg diet DM; Half dose of Amylase and Xylanase (HD) = amylolytic enzyme added at 0.25 g/kg diet DM and fibrolytic enzyme (Fibrozyme, Alltech) added at 0.45 g/kg diet DM; and Full dose of Amylase and Xylanase (FD) = amylolytic enzyme added at 0.5 g/kg diet DM and fibrolytic enzyme added at 0.90 g/kg diet DM.

[2]Contrast: C1 (CON vs. EFE), C2 (AML vs. FD), C3 (FBL vs. FD), and C4 (HD vs. FD)

CP/DOMI, crude protein/digestible organic matter intake ratio; DOMI, digestible organic matter intake; Emic, efficiency of microbial nitrogen synthesis; ENU, apparent efficiency of nitrogen utilization in the animals' body; FNE, feces nitrogen excretion; MN, microbial nitrogen synthesis; UNE, urine nitrogen excretion; SEM = standard error of the mean

Table 4: Differential bacteria family abundance in cannulated Nellore finishing steers (n = 10) feeding EFE.

Phylum	Family	Treatments[1]					*P-value*[2]			
		CON	AML	FBL	HD	FD	C1	C2	C3	C4
Actinobacteriota	Atopobiaceae	0.859±1.20	0.522±1.40	0.558±0.384	0.621±0.925	0.807±1.18	0.716	0.799	0.059	0.878
	Bifidobacteriaceae	0.035±0.031	0.009±0.010	NI	0.011±0.022	0.025±0.136	0.007	0.139	0.028	0.214
Bacteroidota	Bacteroidales UCG-001	0.181±0.221	0.113±0.216	0.400±0.164	0.134±0.158	0.224±0.331	0.144	0.093	0.203	0.285
	Bacteroidales RF16 group	0.075±0.176	0.110±0.238	0.041±0.123	0.078±0.068	0.264±0.282	0.706	0.575	0.173	0.059
	Muribaculaceae	6.23±3.98	1.45±3.06	4.61±5.36	3.72±4.14	3.87±7.85	0.099	0.059	0.508	0.721
	p-2534-18B5 gut group	0.033±0.088	0.301±0.655	0.001±0.022	0.009±0.055	0.032±0.422	0.038	0.241	0.043	0.779
	Bacteroidales uncultured	0.391±0.143	0.265±0.173	0.472±0.135	0.420±0.287	0.384±0.221	0.058	0.074	0.333	0.241
Firmicutes	Bacillaceae	0.006±0.012	0.010±0.014	0.002±0.011	0.012±0.034	0.006±0.010	0.641	0.889	0.414	0.074
	Clostridiaceae	0.026±0.072	0.003±0.013	0.014±0.021	0.032±0.025	0.007±0.010	0.008	0.767	0.327	0.021
	Mycoplasmataceae	0.021±0.041	0.029±0.083	0.033±0.144	0.016±0.032	0.021±0.053	0.868	0.799	0.959	0.091
	Ruminococcaceae	2.12±1.25	1.48±1.31	1.52±1.37	2.93±2.45	1.29±0.609	0.188	0.333	0.799	0.009
	UCG-010	0.163±0.116	0.287±0.190	0.186±0.194	0.127±0.180	0.144±0.056	0.386	0.037	0.203	0.721
Proteobacteria	Rickettsiales uncultured	0.021±0.061	0.033±0.076	0.024±0.056	0.046±0.175	0.010±0.016	0.364	0.021	0.114	0.038
Verrucomicrobiota	WCHB1-41	0.032±0.036	0.023±0.052	0.012±0.079	0.018±0.017	0.007±0.012	0.088	0.038	0.074	0.093

[1]Control (CON) = no feed additives; Amylase (AML) = amylolytic enzyme (Amaize, Alltech) added at 0.5 g/kg diet DM; Xylanase (FBL) = fibrolytic enzyme (Fibrozyme, Alltech) added at 0.9 g/kg diet DM; Half dose of Amylase and Xylanase (HD) = amylolytic enzyme added at 0.25 g/kg diet DM and fibrolitic enzyme (Fibrozyme, Alltech) added at 0.45 g/kg diet DM; and Full dose of Amylase and Xylanase (FD) = amylolytic enzyme added at 0.5 g/kg diet DM and fibrolitic enzyme added at 0.90 g/kg diet DM.

[2] C1 (CON vs. EFE) using the Kruskal-Wallis test. Paired Wilcoxon Rank Sum Test was applied to compare C2 (AML vs. FD), C3 (FBL vs. FD), and C4 (HD vs. FD)

NI = Not identifield

Table 5: Differential bacteria genera abundance in cannulated Nellore finishing steers (n = 10) feeding EFE.

Phylum	Genera	Treatments[1]					*P-value*[2]			
		CON	AML	FBL	HD	FD	C1	C2	C3	C4
Actinobacteriota	*Olsenella*	0.782±1.01	0.398±1.38	0.410±0.311	0.541±0.863	0.675±1.24	0.660	0.878	0.059	0.878
	Bifidobacterium	0.030±0.039	0.009±0.012	NI	0.011±0.015	0.022±0.109	0.015	0.139	0.028	0.214
Bacteroidota	*Bacteroidales RF16 group*	0.075±0.176	0.110±0.238	0.041±0.123	0.078±0.068	0.264±0.282	0.706	0.575	0.173	0.059
	Bacteroidales UCG-001	0.181±0.221	0.113±0.216	0.400±0.164	0.134±0.158	0.224±0.331	0.144	0.093	0.203	0.285
	Muribaculaceae unclassified	6.23±3.98	1.447±3.06	4.61±5.36	3.72±4.14	3.87±7.85	0.099	0.059	0.508	0.721
	p-2534-18B5 gut group	0.033±0.088	0.301±0.655	0.001±0.022	0.009±0.055	0.032±0.422	0.038	0.241	0.043	0.779
	Prevotella	31.8±10.9	35.6±13.2	27.8±8.94	35.4±15.3	24.7±13.4	0.368	0.241	0.508	0.047
	Prevotellaceae *UCG-003*	0.830±0.862	0.508±0.862	1.294±1.33	0.877±0.492	0.950±0.773	0.498	0.333	0.037	0.646
	Prevotellaceae *YAB2003 group*	0.201±0.240	0.075±0.077	0.149±0.083	0.097±0.126	0.142±0.117	0.077	0.241	0.508	0.646
	Prevotellaceae *NK3B31 group*	0.425±0.879	0.043±0.083	0.353±1.400	0.132±0.623	0.712±3.07	0.069	0.007	0.445	0.203
	SP3-e08	0.687±1.41	0.068±0.611	0.082±0.871	0.349±1.12	0.453±1.18	0.612	0.050	0.735	0.779
	U29-B03	0.102±0.117	0.055±0.086	0.105±0.149	0.119±0.078	0.037±0.049	0.059	0.203	0.007	0.059
	Uncultured	0.391±0.143	0.265±0.173	0.472±0.135	0.420±0.287	0.384±0.221	0.058	0.074	0.333	0.241
Firmicutes	*Mogibacterium*	0.016±0.015	0.006±0.014	0.010±0.010	0.014±0.010	0.013±0.014	0.556	0.515	0.953	0.093
	Eubacterium brachy group	0.030±0.041	0.026±0.036	0.020±0.021	0.036±0.039	0.016±0.017	0.402	0.173	0.028	0.114
	Anaerovorax	0.084±0.044	0.132±0.083	0.089±0.077	0.064±0.115	0.077±0.079	0.570	0.086	0.203	0.508
	Amnipila	NI	0.006±0.008	0.001±0.007	NI	NI	0.326	0.027	0.244	0.892
	Bacillus	0.006±0.012	0.010±0.012	0.002±0.011	0.012±0.034	0.006±0.010	0.658	0.889	0.414	0.074
	Clostridium methylpentosum group	0.026±0.072	0.003±0.013	0.014±0.021	0.032±0.025	0.007±0.010	0.008	0.767	0.327	0.021
	Kandleria	0.006±0.022	0.004±0.017	0.009±0.016	0.010±0.015	0.018±0.011	0.803	0.093	0.208	0.203
	Roseburia	0.134±0.172	0.083±0.057	0.109±0.141	0.102±0.216	0.161±0.459	0.592	0.093	0.169	0.241
	Lachnospiraceae *XPB1014 group*	NI	0.037±0.053	0.005±0.033	NI	0.014±0.021	0.300	0.038	0.612	0.889
	Lachnospiraceae *FE2018 group*	NI	0.016±0.061	0.009±0.065	0.016±0.033	0.013±0.073	0.093	0.859	0.889	0.721

	Howardella	0.003±0.010	0.010±0.014	0.010±0.012	NI	0.011±0.005	0.054	0.386	0.047	0.020
	Mycoplasma	0.021±0.041	0.029±0.083	0.033±0.144	0.016±0.032	0.021±0.053	0.868	0.799	0.959	0.091
	Papillibacter	0.144±0.255	0.110±0.203	0.265±0.267	0.068±0.164	0.081±0.067	0.624	0.214	0.037	0.959
	Hydrogenoanaerobacterium	NI	NI	NI	NI	NI	0.229	0.049	0.518	0.516
	Ruminococcus	1.52±0.889	1.04±0.766	1.10±1.02	1.43±1.68	0.988±0.595	0.401	0.959	0.959	0.017
	Ruminococcaceae unclassified	0.033±0.058	0.049±0.109	0.022±0.036	0.033±0.044	0.021±0.024	0.335	0.051	0.374	0.086
	Incertae_Sedis	0.023±0.040	0.029±0.032	0.023±0.026	0.007±0.033	0.008±0.018	0.705	0.035	0.110	0.333
	Selenomonas	0.418±0.571	0.301±0.242	0.400±0.530	0.198±0.134	0.126±0.149	0.197	0.037	0.074	0.037
	Veillonellaceae *UCG-001*	0.292±0.165	0.257±0.281	0.164±0.257	0.246±0.238	0.159±0.117	0.430	0.074	0.139	0.203
	Anaerovibrio	0.070±0.146	0.100±0.100	0.105±0.192	0.037±0.094	0.026±0.055	0.294	0.008	0.021	0.241
	UCG-010	0.163±0.116	0.287±0.190	0.186±0.194	0.127±0.180	0.144±0.056	0.386	0.037	0.203	0.721
	Christensenellaceae uncultured	0.025±0.013	0.023±0.037	0.031±0.043	0.010±0.030	0.013±0.027	0.511	0.028	0.011	0.959
Proteobacteria	*Rhodospirillales uncultured*	0.021±0.061	0.033±0.076	0.024±0.056	0.046±0.175	0.010±0.016	0.364	0.021	0.114	0.038
	Rickettsiales uncultured	NI	0.001±0.004	NI	0.003±0.005	NI	0.040	0.042	0.180	0.017
Spirochaetota	*Sphaerochaeta*	0.156±0.317	0.554±0.370	0.199±0.223	0.128±0.301	0.211±0.294	0.023	0.017	0.721	0.878
Verrucomicrobiota	*WCHB1-41*	0.032±0.036	0.023±0.052	0.012±0.079	0.018±0.017	0.007±0.012	0.088	0.038	0.074	0.093

[1]Control (CON) = no feed additives; Amylase (AML) = amylolytic enzyme (Amaize, Alltech) added at 0.5 g/kg diet DM; Xylanase (FBL) = fibrolytic enzyme (Fibrozyme, Alltech) added at 0.9 g/kg diet DM; Half dose of Amylase and Xylanase (HD) = amylolytic enzyme added at 0.25 g/kg diet DM and fibrolitic enzyme (Fibrozyme, Alltech) added at 0.45 g/kg diet DM; and Full dose of Amylase and Xylanase (FD) = amylolytic enzyme added at 0.5 g/kg diet DM and fibrolitic enzyme added at 0.90 g/kg diet DM.

[2] C1 (CON vs. EFE) using the Kruskal-Wallis test. Paired Wilcoxon Rank Sum Test was applied to compare C2 (AML vs. FD), C3 (FBL vs. FD), and C4 (HD vs. FD).

NI = Not identifiel

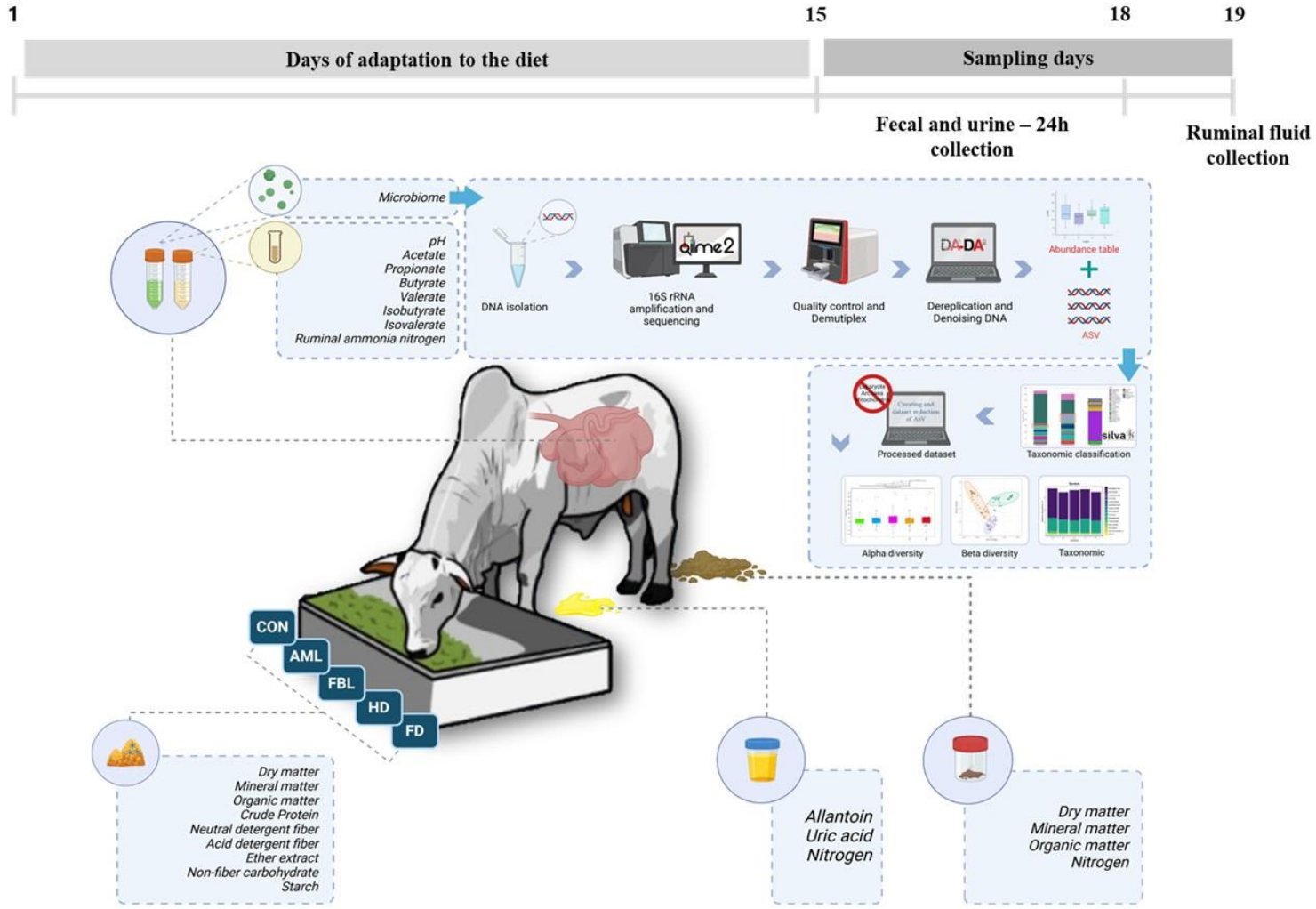

Figure. 1: Experimental workflow. Created in https://BioRender.com.

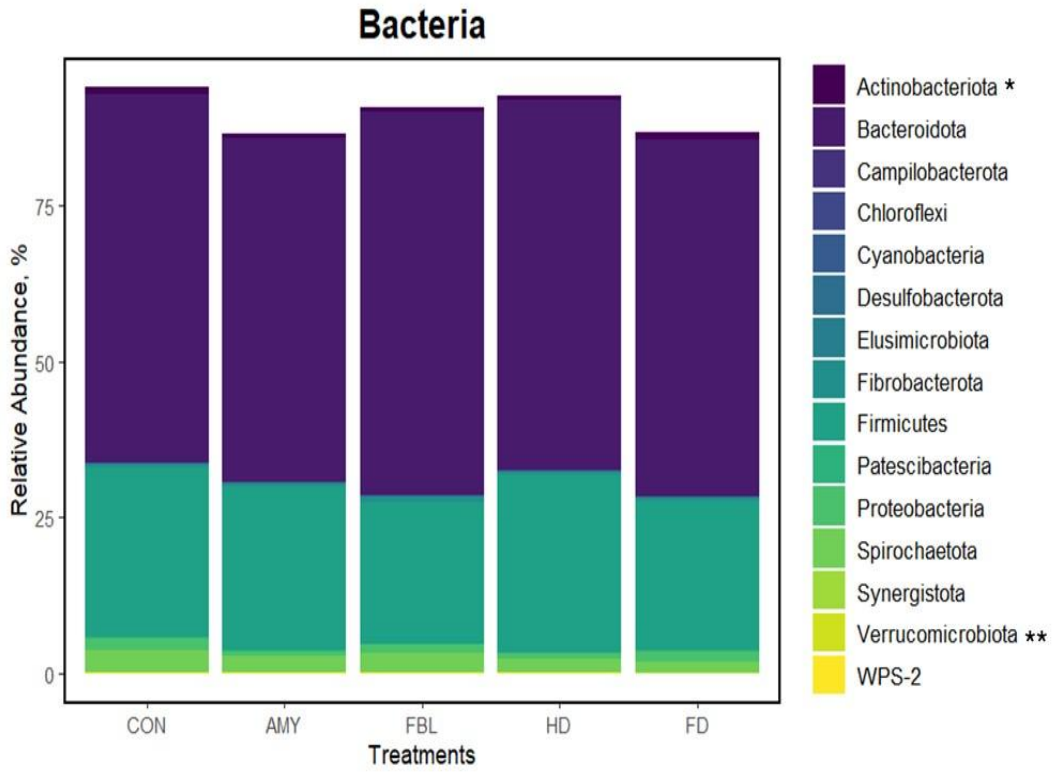

Figure 2: Effect of EFE on the relative abundance of ruminal bacteria at phylum level of cannulated Nellore finishing steers (*n* = 10). * = C3 (FBL vs. FD) = *P* = 0.047; ** = C2 (AML vs. FD) = *P* = 0.066 and C4 (HD vs. FD) = *P* = 0.093.

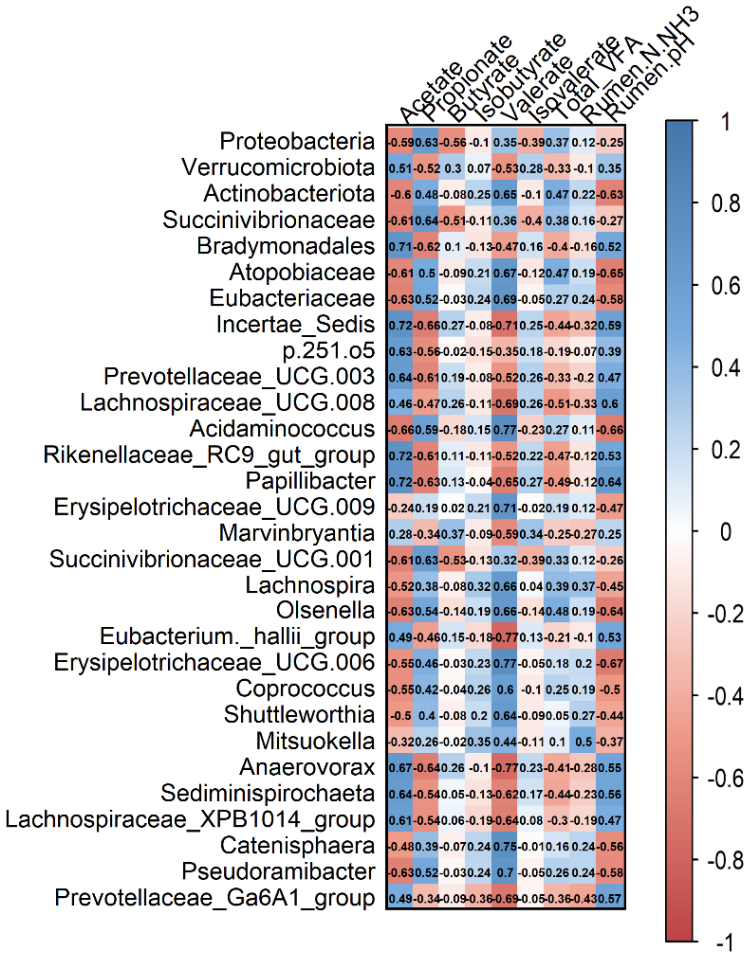

Figure 3: Correlation between rumen fermentation parameters and relative abundances of rumen bacteria in cannulated Nellore finishing steers (*n* = 10).

CAPÍTULO 4 - CONSIDERAÇÕES FINAIS

As enzimas exógenas são aditivos alimentares com potencial para otimizar o aproveitamento de nutrientes e a produtividade de bovinos de corte. Embora seu uso venha sendo estudado há décadas, ainda há inconsistências nos resultados quanto aos seus reais efeitos sobre a digestibilidade, desempenho animal e fermentação ruminal. Assim, no atual trabalho buscou-se avançar no entendimento sobre os impactos da suplementação com enzimas exógenas, por meio de uma abordagem abrangente que integrou dados de literatura científica e avaliação experimental com bovinos canulados.

A metanálise desenvolvida forneceu evidências robustas de que a inclusão de enzimas exógenas nas dietas de confinamento promoveu ganhos significativos na digestibilidade da matéria seca, proteína bruta e fibra em detergente neutro, sem, no entanto, alterar os principais parâmetros de fermentação ruminal, como pH, ácidos graxos voláteis e concentração de amônia ruminal. Além disso, foi observada uma melhoria no peso de carcaça quente, mesmo sem impacto sobre o ganho médio diário, ingestão e peso corporal final dos animais, sugerindo que a maior eficiência digestiva possa explicar os benefícios zootécnicos observados.

Complementarmente, o estudo experimental realizado com bovinos da raça Nelore demonstrou que a suplementação com enzimas amilolíticas e fibrolíticas, isoladas ou em combinação, promoveu alterações significativas na microbiota ruminal, além de reduzir a concentração de amônia no rúmen e as excreções de nitrogênio nas fezes e urina. Esses efeitos resultaram em maior retenção de nitrogênio e melhor eficiência de utilização de nitrogênio pelos animais. Ainda que o sinergismo esperado entre as enzimas não tenha se refletido de forma clara nos parâmetros fermentativos, mudanças na abundância relativa de famílias microbianas chaves indicam que diferentes combinações de enzimas modulam de maneira distinta a comunidade microbiana ruminal.

Apesar dos avanços proporcionados por este trabalho, permanecem lacunas importantes no conhecimento sobre os efeitos a longo prazo da suplementação com enzimas exógenas, especialmente sob diferentes condições dietéticas e fases de produção. Além disso, a ausência de medições diretas dos

efeitos pós rúmen ainda dificulta o entendimento do real efeito da suplementação com enzimas exógenas no trato digestivo dos animais.

Portanto, estudos futuros devem priorizar:

1. Isolar e caracterizar atividades enzimáticas específicas, além de explorar seus mecanismos subjacentes, de modo a identificar formulações enzimáticas e métodos de aplicação mais eficazes;
2. Avaliações em escala comercial e com duração prolongada;
3. Avaliação dos efeitos pós ruminais utilizando metodologias robustas;
4. Estudos com abordagens multi-ômicas para aprofundar a compreensão das interações entre dieta, microbiota e metabolismo animal;

Dessa forma, nossos achados contribuem para consolidar o uso de enzimas exógenas como ferramenta viável na intensificação sustentável da bovinocultura de corte, ao mesmo tempo que apontam caminhos para pesquisas futuras que possibilitem uma aplicação mais eficaz desses aditivos na nutrição de ruminantes.