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Three studies on ruminant nutrition

Luiz Carlos Oliveira de Sousa
Doctor Scientiae

VIÇOSA - MINAS GERAIS
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LUIZ CARLOS OLIVEIRA DE SOUSA

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Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Edenio Detmann

Co-adviser: Marcio de Souza Duarte

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Assent:

Luiz Carlos Oliveira de Sousa
Author

Edenio Detmann
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To my beloved parents,
Gleyson Sousa and Andrelina Mendes.

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To God, in the name of His Son Jesus Christ, for His unmerited grace and for being with me in every moment of my life.

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“Theory without data is fantasy,
but data without theory is chaos”.
(Edward Lawler)

ABSTRACT

SOUSA, Luiz Carlos Oliveira de, D.Sc., Universidade Federal de Viçosa, March, 2026. **Three studies on ruminant nutrition**. Adviser: Edenio Detmann. Co-adviser: Marcio de Souza Duarte.

This thesis comprised three independent experiments on ruminant nutrition. In the first experiment, our objective was to evaluate whether guanidinoacetic acid (GAA) affects the performance, metabolism, and placental vascularization in pregnant beef cows during late gestation as well as its impact on the offspring's performance. Twenty-eight pregnant Brahman cows (body weight = 532±15.1 kg) carrying male (n=15) and female (n=13) fetuses, were used. On day 170 of gestation, cows were housed in individual pens and fed a corn silage-based diet. Two treatments were evaluated: 1) control (no addition of GAA) or 2) addition of 0.2% GAA to the total diet (on a dry matter basis). Maternal treatments started on day 180 and extended until day 270 of gestation. Our results showed that providing GAA decreased skeletal muscle mobilization and enhanced placental vascularization in beef cows during late gestation. However, we did not observe any impact of GAA on performance of the offspring. Notably, dietary GAA addition induced a deficiency of methyl groups, leading to decreased plasma methionine. In the second experiment, we investigated methodological variations in in vitro gas production techniques. Our objective was to evaluate the influence of substrate dispersion method and particle size on in vitro digestibility, gas production kinetics and composition, and fermentation characteristics of different feed types. Alfalfa hay, tall fescue hay, ground corn, soybean meal, and a total mixed ration (TMR) were used as substrates. Treatments were designed according to a 2 × 2 × 5 factorial arrangement, consisting of two substrate dispersion methods (loose substrate and in filter bags), two particle sizes (1 mm and 2 mm), and five feeds. The experiment was conducted over four incubations, with two bottles per treatment in each incubation. Cumulative gas pressure was recorded at 5-minute intervals throughout a 72-h incubation period. Our findings showed that incubating substrates in filter bags compromised in vitro digestibility, gas production, and fermentation characteristics regardless of particle size. However, the magnitude of responses was greater for forage-based feeds than concentrate feeds. In the third experiment, we aimed to investigate the effects of soybean-based feeds on feeding behavior, arterial hemodynamics, heat stress responses, and metabolic and inflammatory indicators in beef cattle experiencing fescue toxicosis. Ten ruminally-cannulated Angus × Holstein steers were used in a 5 × 10 Latin rectangle

design. Five treatments were evaluated: 1) non-toxic endophyte-infected tall fescue seed (NTE), 2) toxic endophyte-infected tall fescue seed (TE), 3) TE + soybean hulls (SBH), 4) TE + whole soybean (WSB), or 5) TE + soybean meal (SBM). Treatment periods lasted 7 d, followed by a 14-d washout. Soybean meal supplementation mitigated ergot alkaloid-induced vasoconstriction, promoted vasodilation, and restored normal feeding behavior in beef cattle experiencing fescue toxicosis. Additionally, soybean meal supplementation increased peripheral serotonin concentration, which may be associated with the beneficial physiological responses observed in beef cattle during fescue toxicosis.

Keywords: cattle; metabolism; in vitro

RESUMO

SOUSA, Luiz Carlos Oliveira de, D.Sc., Universidade Federal de Viçosa, março de 2026. **Três estudos sobre nutrição de ruminantes**. Orientador: Edenio Detmann. Coorientador: Marcio de Souza Duarte.

Esta tese compreendeu três experimentos independentes em nutrição de ruminantes. No primeiro experimento, nosso objetivo foi avaliar se a suplementação com ácido guanidinoacético (GAA) afeta o desempenho, o metabolismo e a vascularização placentária em vacas de corte gestantes durante o terço final da gestação, bem como seu impacto sobre o desempenho da progênie. Foram utilizadas vinte e oito vacas Brahman gestantes (peso corporal = 532 ± 15,1 kg), gestando fetos machos (n = 15) e fêmeas (n = 13). No dia 170 de gestação, as vacas foram alojadas em baias individuais e alimentadas com uma dieta à base de silagem de milho. Os tratamentos maternos tiveram início no dia 180 e se estenderam até o dia 270 de gestação. Dois tratamentos foram avaliados: 1) controle (sem adição de GAA) e 2) adição de 0,2% de GAA na dieta total (com base na matéria seca). Nossos resultados demonstraram que o fornecimento de GAA reduziu a mobilização do músculo esquelético e aumentou a vascularização placentária em vacas de corte durante o terço final da gestação. Contudo, nós não observamos qualquer efeito do GAA sobre o desempenho da progênie. De forma relevante, a adição dietética de GAA induziu deficiência de grupos metil, resultando em redução da concentração plasmática de metionina. No segundo experimento, nós investigamos variações metodológicas em técnicas in vitro de produção de gás. Nosso objetivo foi avaliar a influência do método de dispersão do substrato e do tamanho de partícula sobre a digestibilidade in vitro, a cinética e produção de gás e as características de fermentação em diferentes tipos de alimentos. Foram utilizados como substratos feno de alfafa, feno de festuca, milho moído, farelo de soja e uma ração total misturada (TMR). Os tratamentos foram arranjados em esquema fatorial 2 × 2 × 5, composto por dois métodos de dispersão do substrato (substrato livre ou acondicionado em filter bags), dois tamanhos de partícula (1 mm e 2 mm) e cinco alimentos. O experimento foi conduzido em quatro incubações, com dois frascos por tratamento em cada incubação. A pressão cumulativa de gás foi registrada a cada 5 minutos ao longo de um período de incubação de 72 horas. Nossos resultados indicaram que a incubação de substratos em filter bags comprometeu a digestibilidade in vitro, a produção de gás e as características de fermentação, independentemente do tamanho de partícula. Contudo, a magnitude dessas respostas foi maior para alimentos volumosos do que para

alimentos concentrados. No terceiro experimento, nós objetivamos investigar os efeitos de alimentos à base de soja sobre o comportamento alimentar, a hemodinâmica arterial, as respostas ao estresse térmico e os indicadores metabólicos e inflamatórios em bovinos de corte experienciando intoxicação por festuca. Foram utilizados dez novilhos Angus × Holandês, canulados no rúmen, em um delineamento em retângulo latino 5 × 10. Cinco tratamentos foram avaliados: 1) semente de festuca não infectada por endófito toxico, 2) semente de festuca infectada por endófito tóxico (TE), 3) TE + casca de soja, 4) TE + grão de soja e 5) TE + farelo de soja. Os períodos experimentais tiveram duração de 7 dias, seguidos por um período de 14 dias para eliminação do efeito do tratamento previamente aplicado. A suplementação com farelo de soja atenuou a vasoconstrição induzida por alcaloides do ergot, promoveu vasodilatação e restabeleceu o comportamento alimentar normal em bovinos de corte experienciando intoxicação por festuca. Adicionalmente, o aumento da concentração periférica de serotonina pode estar associado às respostas fisiológicas benéficas observadas com a suplementação de farelo de soja em bovinos de corte durante a intoxicação por festuca.

Palavras-chave: bovinos; metabolismo; in vitro

SUMMARY

General Introduction

The implementation of strategic nutritional interventions plays an essential role in ensuring animal health and productivity, as well as economic and environmental efficiency in ruminant production systems. In this context, understanding how different components of the production system influence animal physiology and health is critical for the development of targeted nutritional strategies. Physiological and environmental challenges, such as gestation, exposure to bioactive compounds in forages, and heat stress, can alter nutrient utilization and thereby animal performance. Accordingly, research on feeds, additives, and feed evaluation methods used in ruminant nutrition is necessary to elucidate their effects on the modulation of digestion and metabolism in ruminants. Within this framework, this thesis is based on three independent experiments investigating nutritional strategies and feed evaluation methodologies on ruminant nutrition.

Guanidinoacetic acid (GAA) is a naturally occurring amino acid derivative that serves as the immediate precursor of creatine, a compound that plays a central role in cellular energy metabolism (Ostojic, 2015). Even though the biological effects of GAA are primarily mediated through creatine synthesis, GAA may also function as an arginine-sparing agent, thereby supporting various physiological processes dependent on arginine availability (Baker et al., 2009; Ostojic, 2015).

Arginine is a conditionally essential amino acid that plays crucial roles in protein synthesis, cell signaling, and placental angiogenesis and blood flow during gestation (Reynolds and Redmer, 2001; Wu et al., 2013; Gilbreath et al., 2021). Endogenous GAA synthesis accounts for approximately one-third of arginine's amidino groups (Brosnan et al., 2011; Wu et al., 2022). Research in pregnant sows indicated that the *de novo* creatine synthesis from GAA represents the primary pathway of arginine utilization and increases progressively throughout gestation (Wu et al., 2013). Under these circumstances, it is feasible that a considerable portion of available arginine may be directed toward GAA synthesis, particularly in late gestation. In this light, providing dietary GAA could decrease the utilization of arginine for GAA synthesis so that arginine may be used for other functions such as placental vasculogenesis and angiogenesis. To date, most research on GAA has focused on growing and finishing cattle, whereas its effects on cow-calf systems remain poorly understood.

Most studies with *in vitro* gas production systems have reported incubating substrates directly in bottles (Schofield et al., 1994; Huhtanen et al., 2008; Christodoulou et al., 2025). However, some researchers have used filter bags due to their operational simplicity and the

elimination of quantitative residue transfer for digestibility estimates (Eun and Beauchemin, 2007; Yang et al., 2014; Trotta et al., 2024). There are limited studies available comparing the incubation of substrates in bottles versus filter bags and these often yield mixed results. Substrates incubated directly in bottles may adhere to bottle walls, potentially reducing *in vitro* dry matter (DM) digestibility and gas production, particularly for forages (He et al., 2016; Sampaio et al., 2024). Conversely, incubating samples into filter bags raises concerns about restricted microbial access to substrates and potentially alter the diffusion of fermentation end-products out of the filter bag, potentially impairing microbial activity (Marinucci et al., 1992; Krizsan et al., 2013). Several studies have shown that the use of filter bags decreases *in vitro* DM digestibility, methane production, and total gas output (Ramin et al., 2013; Schlau et al., 2021; García et al., 2024). Therefore, the overall impact of using filter bags in *in vitro* gas production systems remains uncertain, particularly across different feed types.

An additional methodological factor influencing *in vitro* incubation systems is substrate particle size. Particle size should be sufficiently large to resemble *in vivo* conditions, yet small enough to ensure adequate microbial access, minimize sampling bias, and reduce experimental variability (Lowman et al., 2002; Nocek, 1988). As microbial access to substrates within the filter bags may be restricted, and larger particles have a reduced specific surface area, the use of filter bags could have a greater impact on digestibility and gas production in substrates processed at larger particle sizes. To our knowledge, no studies have investigated the combined effects of substrate dispersion method and particle size on in vitro digestibility and gas production kinetics across different feed types.

In North America, most tall fescue (*Lolium arundinaceum*) pastures are infected with the endophyte, *Epichlöe coenophialia*, that enhances strand persistence, forage productivity, and drought tolerance (Dinkins et al., 2017). However, consumption of ergot alkaloids produced by the endophyte can induce fescue toxicosis, negatively affecting growth, reproduction, and lactation in ruminant livestock (Strickland et al., 2011; Klotz, 2015). Clinical signs of fescue toxicosis include decreased feed intake, body weight gain, milk production and reproductive performance, as well as increased respiration rate, body temperature, and vasoconstriction (Strickland et al., 2011).

Several studies have reported positive effects when supplementing soy-based feeds to ruminants grazing toxic tall fescue or exposed to ergot alkaloids (Aiken et al., 2008; Carter et al., 2010; Aiken et al., 2016; Harlow et al., 2022). However, interpretation of these responses is complicated by potential confounding effects from decreased feed intake associated with ergot alkaloid ingestion, and the effects of supplements on forage and ergot alkaloids intake.

Differences in nutrient intake, dilution of ergot alkaloid intake, and uncontrolled environmental conditions have made it difficult to delineate factors associated with the onset and amelioration of fescue toxicosis in grazing ruminants (Koontz et al., 2012; Nicol and Klotz, 2016). To our knowledge, no studies have directly compared use of soybean feeds to mitigate symptoms of fescue toxicosis in beef cattle with controlled environmental heat loads and equalized feed intakes.

Therefore, this thesis was based on three independent experiments in which our general objectives were:

1) to evaluate whether guanidinoacetic acid affects the performance, metabolism, and placental vascularization in pregnant beef cows during late gestation as well as its impact on the offspring's performance;

2) to evaluate whether the method of substrate dispersion and particle size affects in vitro digestibility, gas production kinetics and composition, and fermentation characteristics in different feed types; and

3) to evaluate the effects of soybean-based feeds on feeding behavior, arterial hemodynamics, heat stress responses, and metabolic and inflammatory indicators in beef cattle experiencing fescue toxicosis.

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Dietary guanidinoacetic acid as arginine spare molecule for beef cows at late gestation: Effects on cow's performance and metabolism, and offspring growth and development

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Maternal nutrition

ABSTRACT

We aimed to assess whether guanidinoacetic acid (GAA) affects the performance, metabolism, and placental vascularization of pregnant beef cows during late gestation as well as its impact on the offspring's performance. Twenty-eight pregnant Brahman cows, averaging 532±15.1 kg and carrying male (n=15) and female (n=13) fetuses, were used. The basal diet consisted of 688 g/kg corn silage, 147 g/kg sugarcane bagasse, 47.7 g/kg corn, 89.6 g/kg soybean meal, 6.86 g/kg urea, and 21.2 g/kg mineral mixture (DM basis). Cows were fed the experimental diets from 180 to 270 days of gestation. The following treatments were evaluated: control (no addition of GAA) or addition of 0.2 % GAA to the total diet (DM basis). There was no effect ($P \geq 0.37$) of GAA on voluntary intake. Similarly, GAA addition did not affect ($P \geq 0.54$) cows performance variables, except for ribeye area (REA), which had a lower ($P < 0.01$) variation compared to the initial REA in cows fed dietary GAA compared to the control group. Dietary GAA increased ($P \leq 0.02$) both serum nitric oxide and placental vascularization compared to cows fed the control diet. There was no effect ($P \geq 0.43$) of GAA on urine and serum creatine concentrations. In contrast, dietary GAA increased ($P \leq 0.03$) plasma concentration of arginine, ornithine, citrulline, and tyrosine compared to the control. Conversely, dietary GAA decreased ($P < 0.02$) plasma methionine concentration. Dietary GAA increased AGAT activity ($P < 0.03$) in the liver, with no differences observed ($P > 0.68$) on GAMT activity. There was no effect ($P \geq 0.15$) of GAA on performance of the offspring. Addition of GAA in maternal diet did not affect skeletal muscle fiber number ($P > 0.09$) and diameter ($P > 0.23$) of the offspring. Guanidinoacetic acid decreases skeletal muscle

Abbreviations: ADP, adenosine diphosphate; ADG, average daily gain; AGAT, L-arginine:glycine aminotransferase; Akt, protein kinase B; ATP, adenosine triphosphate; BW, body weight; CV, coefficient of variation; DM, dry matter; FoxO1, forkhead box O1; GAMT, guanidinoacetate N-methyltransferase; MTOR, mechanistic target of rapamycin; NEFA, non-esterified fatty acid; NaCl, sodium chloride; $NaNO_2$, sodium nitrite; REA, ribeye area; SFT, subcutaneous fat thickness; VBW, variation of body weight; VREA, variation of ribeye area; VSFT, variation of subcutaneous fat thickness.

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mobilization and enhances placental vascularization of beef cows during late gestation. However, providing GAA seems to not affect the performance of the offspring.

1. Introduction

Guanidinoacetic acid (GAA) is a naturally occurring amino acid derivative that serves as an immediate precursor of creatine, a crucial compound involved in cellular energy metabolism (Ostojic, 2015). *De novo* creatine synthesis involves a 2-step process. First, glycine receives a guanidine group from L-arginine in a reaction catalyzed by L-arginine:glycine aminotransferase (AGAT), producing GAA and L-ornithine. This reaction takes place mainly in the kidneys, but can also occur in the pancreas, liver, and muscle (Wyss and Kaddurah-Daouk, 2000; Edison et al., 2007). Thereafter, GAA receives a methyl group from S-adenosylmethionine, which is synthesized from methionine, producing creatine and adenosylhomocysteine. This reaction takes place mainly in the liver and is catalyzed by guanidinoacetate N-methyltransferase (GAMT). Creatine is then transported from the liver to tissues with high energy demands such as skeletal muscle, brain, and reproductive organs in order to ensure the intracellular ATP turnover and an adequate ATP/ADP ratio (Wyss and Kaddurah-Daouk, 2000).

Creatine has not been widely adopted as a feed additive in the livestock industry due to its high cost and thermal instability during manufacturing processes (Baker et al., 2009). Accordingly, GAA has been investigated as an alternative to creatine in the animal nutrition industry, including poultry (Khalil et al., 2021; Pirgozliev et al., 2022), swine (Li et al., 2018; Valini et al., 2020) and cattle (Ardalan et al., 2020; Liu et al., 2021). Even though GAA is recognized for its effects mediated through creatine, it can also act through several other metabolic pathways. For instance, providing GAA may serve as an arginine-sparing agent for various physiological functions (Baker et al., 2009; Ostojic, 2015). In poultry, GAA demonstrates to have an effective arginine-sparing effect (Khajali et al., 2020; Sharma et al., 2022).

Arginine is a conditionally essential amino acid that plays crucial roles in protein synthesis, cell signaling, placental angiogenesis and blood flow during gestation (Reynolds and Redmer, 2001; Wu et al., 2013; Gilbreath et al., 2021). Indeed, endogenous GAA synthesis consumes about one-third of arginine's amidino groups (Brosnan et al., 2011; Wu et al., 2022). Studies carried out with pregnant sows have shown that the *de novo* synthesis of creatine from GAA represents the primary pathway of arginine utilization and progressively intensifies with pregnancy (Wu et al., 2013). Moreover, a recent study has revealed a gradual increase in the amount of creatine in fetal fluids as pregnancy advances in sheep (Sah et al., 2023). Under these circumstances, it is feasible that a considerable portion of available arginine may be directed toward GAA synthesis, particularly in late gestation.

Numerous studies have demonstrated that alterations in maternal nutrition throughout gestation can induce modifications in the development, metabolism, and physiology of the offspring (Duarte et al., 2014; Marquez et al., 2017; Costa et al., 2021; Gu et al., 2021). Several authors have reported that enhancing placental blood flow through maternal nutrition could serve as an effective approach to augment the delivery of nutrients to the fetus, thereby promoting adequate fetal development and subsequently, favorable postnatal performance (Vonnahme et al., 2015; Reynolds et al., 2023). In this light, providing dietary GAA could decrease the utilization of arginine for GAA synthesis so that arginine may be used for other functions such as placental vasculogenesis and

Table 1
Ingredients and chemical composition of the basal diet.

Item	g/kg DM
Ingredients	
Corn silage	688
Sugarcane bagasse	147
Ground corn	47.7
Soybean meal	89.6
Urea	6.86
Mineral mixture[a]	21.2
Chemical composition	
Dry matter, g/kg as fed	387
Organic matter	929
Crude protein	125
Ether extract	34.5
Neutral detergent fiber	462
Neutral detergent insoluble protein	14.4
Lignin	41.4
Metabolizable energy, Mcal/kg	2.29

[a] Probeef Reprodução Seca (Cargill Nutrição Animal, Itapira, SP, Brazil) – assurance concentrations per kg of product: 108 g Ca (mín); 170 g Ca (máx); 8000 mg S (mín); 550 mg F (máx); 50 g P (mín); 120 g Na (mín); 38.3 mg Co (mín); 681,8 mg Cu (mín); 9.0 mg Cr (mín); 34 mg I (mín); 2954 mg Mg (mín); 8.40 mg Se (mín); 2045 mg Zn (mín); 365 g NPN protein eq. (max); 900 mg Lasalocid; 15600 UI Vit. A (mín); 1500 UI Vit. D3 (mín); 333 UI Vit. E (mín).

angiogenesis.

To our knowledge, most research on GAA has primarily focused on its effects on growing and finishing cattle, with limited investigation regarding its impact on cow-calf performance. Thus, the effects of providing GAA on pregnant cows remain to be understood. We hypothesized that GAA enhances both performance and placental vascularization of pregnant beef cows during late gestation, thereby potentially benefiting fetal development and postnatal performance of the offspring. Our objective was to evaluate whether GAA affects the performance, metabolism, and placental vascularization of pregnant beef cows during late gestation as well as its impact on the offspring's performance.

2. Material and methods

2.1. Animals, treatments, and experimental design

All animal care and handling procedures were previously approved by the Animal Care and Use Committee of the Department of Animal Science at the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil (protocol 04/2022).

Twenty-eight pregnant Brahman cows, averaging 532±15.1 kg and carrying male (n=15) and female (n=13) fetuses, were used. Cows were submitted to a fixed-time artificial insemination protocol with four attempts using semen from the same sire. Therefore, four groups of cows were established based on the days of gestation. Following insemination and subsequent pregnancy diagnosis, all cows were managed as a single herd and maintained in a Marandu palisade grass (*Urochloa brizantha*) grazing system under the same conditions. On day 170 of gestation, cows were housed in individual pens (12 m^2) equipped with individual feeders and water dispensers for an adaptation period of 10 days. The basal diet consisted of 688 g/kg corn silage, 147 g/kg sugarcane bagasse, 47.7 g/kg corn, 89.6 g/kg soybean meal, 6.86 g/kg urea, and 21.2 g/kg mineral mixture (DM basis; Table 1). All cows were fed *ad libitum* twice daily at 0700 and 1800 h, allowing up to 5 % in orts (as fed).

Maternal treatments started on day 180 and extended until day 270 of gestation. The following treatments were evaluated: control (no addition of GAA) or addition of 0.2 % GAA to the total diet (DM basis). The GAA (GuanAMINO®, Evonik Operations GmbH, Hanau-Wolfgang, Germany) was added into the mineral mixture (Probeef Reprodução Seca, Cargill Nutrição Animal, Itapira, SP, Brazil).

At 270 days of gestation, cows from both experimental treatments were allocated to a Marandu palisade grass (*Urochloa brizantha*) pasture area to avoid calving issues in individual pens. After parturition, the cow-calf pair from both experimental treatments was managed as a single herd under grazing conditions until weaning (210 days of age). At 90 days, all calves were provided access to a supplement through a creep-feeding system (5.0 g/kg BW).

2.2. Sampling and measurements

Cow's voluntary intake was individually monitored daily throughout the 90 days of the experiment period. Forage and orts samples were collected daily, stored in plastic bags, and frozen at −20ºC. Weekly, orts samples were manually blended to form pooled samples per cow. Similarly, orts samples were manually blended to create pooled samples per week. Concentrate samples were obtained for each batch separately. On day 225 of gestation, urine spot samples were collected 4 h after the morning feeding (Valadares et al., 1999). An aliquot of approximately 40 mL was filtered through four layers of cheesecloth and then frozen (-80ºC) for subsequent analysis.

On day 227 of gestation, blood samples were taken via jugular venipuncture into vacuum tubes (Vacuplast® Collect Time, Cotia, SP, Brazil) containing either sodium heparin or coagulation accelerator gel for analysis of the plasma amino acids profile and other metabolic characteristics in serum, respectively. Blood samples collected at parturition and five days after parturition were used to quantify serum NEFA. After collection, samples were centrifuged at ~1600 × g for 15 min. Plasma and serum were immediately frozen (-20ºC) for subsequent analysis.

On day 227 of gestation, concomitantly with blood sampling, placental vascularization was assessed using an ultrasound Z5VET (Mindray Medical International Technology, Shenzhen, Guangdong, China) equipped with B and color Doppler modes linear probe (6–8 MHz), as described by Oliveira et al. (2023). Briefly, images from five placentomes per animal were acquired at a frequency of 7.5 MHz with 94 % gain (grayscale) and 5.7 MHz with 60 % gain (color mode), and a pulse repetition frequency of 1.7 kHz. Images were processed and analyzed by Adobe Premiere Pro CC 2019 (Adobe Systems, San Jose, CA, USA) in order to obtain the average total placentome area (grayscale pixels plus color pixels) and the vascularized area (color pixels). Placental vascularization was calculated by dividing vascularized area and the total area of the placentome.

On day 229 of gestation, liver biopsy was performed via needle biopsy (Tru-Cut biopsy needle, Care Fusion Corporation, San Diego, CA, USA) according to the procedures described by Mølgaard et al. (2012). Briefly, liver sampling took place between the 11th and 12th ribs. Initially, the area was cleaned with 70 % ethanol and then the incision was made 10 minutes after local anesthesia (2 % Lidocaine). Liver samples were obtained from the right lobe. Immediately after collection, the samples were washed with sterile saline solution (0.9 % NaCl), stored in cryotubes, and then snap-frozen in liquid nitrogen. Afterward, the samples were stored at −80ºC until further analysis.

The cows were weighed at the beginning and the end of the experiment after 16 h of fasting in order to calculate final BW, average daily gain (ADG), and voluntary intake relative to average BW. Carcass measurements of the cows were evaluated at the beginning and the end of the experiment by ultrasound (Aloka SSD500II, Mitaka, Tokyo, Japan) with a 3.5 MHz 18 cm linear array probe. Images were taken at *Longissimus lumborum* muscle between the 12th and 13th ribs in order to measure ribeye area (REA) and subcutaneous fat thickness (SFT). A second image for subcutaneous fat thickness was taken in the pelvic region, between the ischium and the pubis

(Rump). Images were analyzed using the BioSoft Toolbox® II for Beef software (Biotronics, Ames, IA, USA). SFT was obtained from the average of the rib and the rump fat thickness. The variation in BW, REA, and SFT was obtained relative to the measurements at the beginning of the experiment as follows:

$$\text{VBW} = \frac{\text{Final BW} - \text{Initial BW}}{\text{Initial BW}} \quad (1)$$

$$\text{VREA} = \frac{\text{Final REA} - \text{Initial REA}}{\text{Initial REA}} \quad (2)$$

$$\text{VSFT} = \frac{\text{Final SFT} - \text{Initial SFT}}{\text{Initial SFT}} \quad (3)$$

Where VBW is a variation of BW scaled to initial BW, initial BW is a measurement of BW at the beginning of the experiment (kg), final BW is a measurement of BW at the end of the experiment (kg), VREA is a variation of REA scaled to initial REA, initial REA is a measurement of REA at the beginning of the experiment (cm^2), final REA is a measurement of REA at the end of the experiment (cm^2), VSFT is a variation of SFT scaled to initial REA, initial SFT is a measurement of SFT at the beginning of the experiment (mm), final SFT is a measurement of SFT at the end of the experiment (mm).

Calves were weighed at birth, every 28 days (to adjust supplement intake), and at weaning. On the day of weaning, carcass ultrasound measurements were taken from the calves using the same method previously described for dams.

2.3. Offspring's skeletal muscle sampling

At 45 days of age, *Longissimus lumborum* sampling was performed via biopsy between the 12th and 13th ribs. Briefly, the area was cleaned with 70 % ethanol, and then the incision was made 10 minutes after local anesthesia (2 % Lidocaine). The muscle sample was washed with sterile saline solution (0.9 % NaCl) and subsequently fixed in 10 % buffered formalin solution for histological analyses. After 24 hours, the sample fixed in 10 % buffered formalin solution was transferred to a new tube containing 70 % ethanol and stored until further analysis.

2.4. Skeletal muscle's histology

Skeletal muscle samples, previously fixed in 10 % buffered formalin solution, were dehydrated with ethanol in increasing concentrations (70 %, 80 %, 90 %, and 100 %) for 1 hour in each solution, dewaxed in HistoChoice® solution for 2 hours, and subsequently embedded in Paraplast® (Sigma-Aldrich, Saint Louis, MO, USA). Sections of 5 μm thickness were obtained and placed in glass slides, which were then used for hematoxylin and eosin staining procedure. To evaluate the number and diameter of muscle fibers, digital images (6 per animal) were obtained utilizing an inverted microscope (Evos® FL, ThermoScientific, Waltham, MA, USA). The images were analyzed for muscle cell number and size by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.5. Laboratory analysis

Weekly samples of feeds and orts were oven-dried (55ºC) and processed to pass through a 1-mm screen sieve. After grinding, orts (per cow) and feed samples were pooled encompassing 30-d periods. Subsequently, the pooled samples were sent to a commercial laboratory (EsalqLab, Piracicaba, SP, Brazil). Feed samples were analyzed for dry matter, ash, crude protein, ether extract, neutral detergent fiber, lignin, acid detergent fiber, neutral detergent insoluble protein, and acid detergent insoluble protein contents by using the near-infrared spectroscopy (NIRS). Metabolizable energy content of the diet was estimated according to equations proposed by (Detmann et al., 2023).

Serum samples were analyzed for creatine (colorimetric method), creatinine (colorimetric method), homocysteine (chemiluminescence method, Atellica® Kit IM HCY), and non-esterified fatty acid (NEFA, enzymatic spectrometry method) in a commercial laboratory (Hermes Pardini Laboratory, Belo Horizonte, MG, Brazil). Urine samples were analyzed for creatine (colorimetric method) and creatinine (enzymatic method, Atellica® Kit CH ECre3). Plasma amino acids were quantified by high-performance liquid chromatography (Xevo™ TQD, Waters Corporation, Milford, MA, USA).

Serum nitric oxide was measured using a standard Griess reaction. Briefly, 50 μl of serum was gently mixed with an equal volume of sulfanilamide solution (1 % sulfanilamide in 5 % phosphoric acid) and incubated in the dark at room temperature for 10 min. After the incubation, 50 μl of 0.1 % naphthylene diamine dihydrochloride was added to the reaction and incubated in the dark at room temperature for another 10 min. The final solution (i.e., sample plus Griess reagent) was read at 540 nm using a microplate reader. Nitrite concentration, an indicator of nitric oxide production, was calculated from a $NaNO_2$ standard curve.

2.6. Liver enzyme activity

Liver enzymatic activity of arginine: glycine amidinotransferase (AGAT: #MBS2614477–96, sensibility: 1.0 ng/mL, precision: intra-assay CV = 4.4–5.6 %, inter-assay CV = 6.6–7.9 %) and guanidinoacetate methyltransferase (GAMT: #MBS2614477, sensibility: 0.06 ng/mL, precision: intra-assay CV = ≤8.0 %, inter-assay CV = ≤12.0 %) in the supernatants of the liver homogenate was assessed

through immunoassay techniques using commercially available kits following manufacturer's instructions (MyBioSource, San Diego, CA, EUA). Standard curves were generated using serial twofold dilutions of a reference standard to determine the enzymatic activity. Absorbance readings were obtained at 450 nm and analyzed to quantify the liver enzymatic activity of AGAT and GAMT.

2.7. Statistical analyses

The data were analyzed according to the following model:

$$Y_{ijlk} = \mu + T_j + OS_i + GG_l + \varepsilon_{ijlk}$$

Where Y_{ijlk} is the response measured on the experimental unit k from gestation group l and offspring sex j submitted to maternal treatment i, μ is the overall constant, T_j is the fixed effect of maternal treatment (control or GAA), OS_i is the fixed effect of the offspring sex (male or female), GG_l is the random effect of gestation group, assumed to be NIID (0, σ^2_{GG}), and $\varepsilon_{(ij)k}$ is the random error, assumed to be NIID (0, σ^2_{ε}). We emphasize that incorporating offspring sex into the model aimed to account for any potential influence it may have had, but it was not the focus of discussion. When pertinent, initial BW, initial REA, and initial SFT were used as covariates. If the effect of these variables was found to be non-significant, the model was reparameterized by excluding them.

All variables were tested for assumptions of normal error distribution and homoscedasticity of error variances between treatments using the Shapiro-Wilk and Levene tests, respectively. Outliers were identified and removed from the dataset when the externally studentized residuals exceeded ±2.0. All analyses were performed using the MIXED procedure of SAS 9.4 considering 0.05 as the critical level of probability for the occurrence of type I error.

3. Results

3.1. Cow's performance

There was no effect (P≥0.37) of GAA on voluntary intake (Table 2). Similarly, dietary GAA addition did not affect (P≥0.54) final BW, variation of BW scaled to initial BW, and ADG (Table 3). Despite the lack of changes in cow's BW, cows receiving GAA exhibited a greater (P<0.01) final REA than control. Notably, cows fed GAA showed a decreased (P<0.01) variation of REA relative to the initial REA compared to the cows from the control group. No effect (P≥0.87) of GAA was verified either on the final SFT or the variation of SFT relative to the initial SFT.

3.2. Placental vascularization, blood and urinary characteristics

Cows fed diets containing GAA had enhanced (P<0.01) placental vascularization compared to cows from the control group (Fig. 1 A). Indeed, serum nitric oxide increased (P<0.02) with the addition of GAA to the diet (Fig. 1B). On the other hand, there was no effect (P≥0.38) of GAA on serum creatine and creatinine concentrations (Table 4). Similarly, the addition of GAA to the diet showed no effect (P≥0.07) on urinary creatine and creatine excretion. In contrast, cows fed diet containing GAA had increased (P<0.01) serum homocysteine concentration compared to the control. There was no effect (P>0.85) of GAA on serum NEFA concentration.

Dietary GAA increased (P≤0.03) plasma concentration of arginine, ornithine, citrulline, and tyrosine (Table 5). Conversely, dietary GAA addition led to a decrease in plasma methionine concentration (P<0.02). No effect (P≥0.14) of GAA was observed on plasma concentration of the remaining amino acids.

3.3. Liver enzyme activity

Dietary GAA improved (P<0.03) AGAT activity in the liver but had no effect (P>0.68) on GAMT activity (Fig. 2).

Table 2
Effects of guanidinoacetic acid on voluntary intake of pregnant beef cows during late gestation.

Item	Maternal treatment[a]		P-value
	Control	GAA	
Intake, kg/day			
Dry matter	8.12±0.62	8.24± 0.62	0.77
Organic matter	7.52± 0.60	7.63± 0.59	0.77
Crude protein	0.99±0.08	1.01±0.08	0.83
Neutral detergent fiber	3.84±0.23	4.01±0.23	0.42
Intake, g/kg BW			
Dry matter	14.3±1.05	14.7±1.04	0.65
Organic matter	13.4±1.01	13.6±0.99	0.65
Crude protein	1.77±0.14	1.81±0.14	0.64
Neutral detergent fiber	6.85±0.35	7.12±0.35	0.37

[a] Control, no addition of GAA; GAA, addition of 0.2 % GAA to the total diet (DM basis).

Table 3
Effects of guanidinoacetic acid on performance of pregnant beef cows during late gestation.

Item[a]	Maternal treatment[b]		P-value
	Control	GAA	
Initial BW, kg	528±22.6	537±22.5	0.78
Final BW, kg	599±7.9	603±7.9	0.69
VBW	0.103±0.019	0.116±0.019	0.54
ADG, kg/day	0.670± 0.084	0.698± 0.084	0.79
Initial REA, mm	65.4±2.75	61.2±2.78	0.20
Final REA, mm	58.1±1.75	62.9±1.70	0.01
VREA	-0.063±0.012	0.031±0.012	<0.001
Initial SFT, mm	8.62±0.99	5.96±1.01	0.04
Final SFT, mm	9.20±0.81	9.37±0.79	0.87
VSFT	0.344±0.110	0.354±0.123	0.95

[a] Initial BW, initial body weight; Final BW, final body weight; VBW, variation of BW scaled to initial BW; ADG, average daily gain; Initial REA, initial ribeye area; Final REA, final ribeye area; VREA, variation of REA scaled to initial REA; Initial SFT, initial subcutaneous fat thickness; Final SFT, final subcutaneous fat thickness; VSFT, variation of SFT scaled to initial SFT.
[b] Control, no addition of GAA; GAA, addition of 0.2 % GAA to the total diet (DM basis).

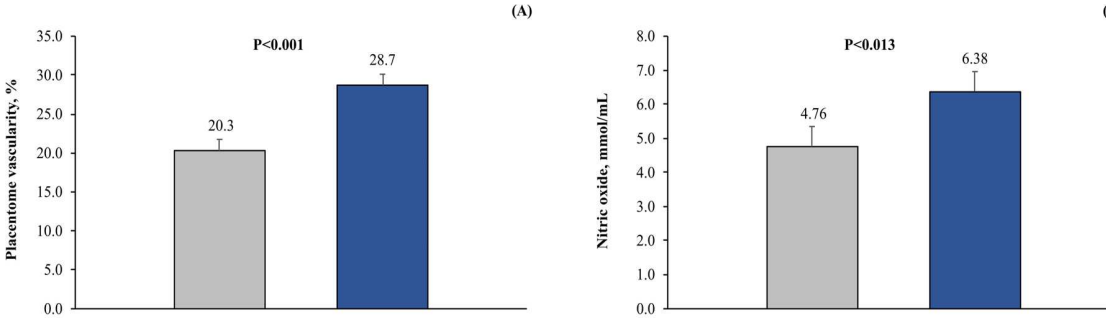

Fig. 1. Effects of guanidinoacetic acid on placentome vascularity (A) and serum nitric oxide (B) in pregnant beef cows during late gestation [Control, no addition of GAA; GAA, addition of 0.2 % GAA to the total diet (DM basis)].

Table 4
Effects of guanidinoacetic acid on serum and urinary concentrations of metabolites in pregnant beef cows during late gestation.

Item[a]	Maternal treatment[b]		P-value
	Control	GAA	
Serum			
Creatine, μmol/L	266±11.9	256±12.1	0.48
Creatinine, mg/dL	1.53±0.09	1.60±0.09	0.38
Homocysteine, μmol/L	8.20±0.26	10.8±0.32	<0.001
NEFA, μmol/L	0.485±0.060	0.499±0.056	0.85
Urine			
Creatine, μmol/L	1289±150.1	1213±148.4	0.43
Creatinine, mg/dL	252±11.51	230±11.7	0.07

[a] Control, no addition of GAA; GAA, addition of 0.2 % GAA to the total diet (DM basis).

3.4. Offspring's performance and skeletal muscle histology

There was no effect of GAA on birth weight (P>0.29) and weaning weight (P>0.38) of the offspring (Table 6). Similarly, dietary GAA addition did not impact ribeye area (P>0.15) and subcutaneous fat thickness (P>0.19). No effects of maternal dietary GAA were detected on both skeletal muscle fiber number (P>0.09) and diameter (P>0.23) of the offspring.

4. Discussion

To the best of our knowledge, most research on GAA has primarily focused on its effects on growing and finishing cattle, with a limited investigation regarding beef cow-calf performance. In the current study, we aimed to evaluate the potential of GAA as a feed additive for beef cows during late gestation. Our main findings indicate that GAA decreases skeletal muscle mobilization and enhances

Table 5
Effects of guanidinoacetic acid on plasma amino acids concentrations in pregnant beef cows during late gestation.

Item	Maternal treatment[a]		P-value
	Control	GAA	
μmol/L			
Aspartate	15.4±2.22	16.1±2.18	0.70
Alanine	85.0±7.14	83.2±7.15	0.81
Arginine	26.1±1.88	33.7±1.64	0.01
Citrulline	26.9±0.79	29.9±0.84	0.02
Glutamate	63.1±4.25	68.9±4.02	0.24
Glycine	90.1±4.94	95.6±5.45	0.45
Histidine	40.4±4.40	39.4±4.40	0.87
Methionine	11.4±0.42	9.64±0.46	0.02
Ornithine	18.8±0.69	21.5±0.68	0.01
Phenylalanine	19.8±1.37	20.5±1.31	0.63
Serine	21.3±0.14	21.2±0.15	0.54
Tyrosine	22.5±1.82	28.4±1.77	0.01
Threonine	11.3±0.59	11.2±0.56	0.83
Tryptophan	17.6±1.25	17.2±1.29	0.80
Valine	56.7±3.51	61.9±3.39	0.14

[a] Control, no addition of GAA; GAA, addition of 0.2 % GAA to the total diet (DM basis).

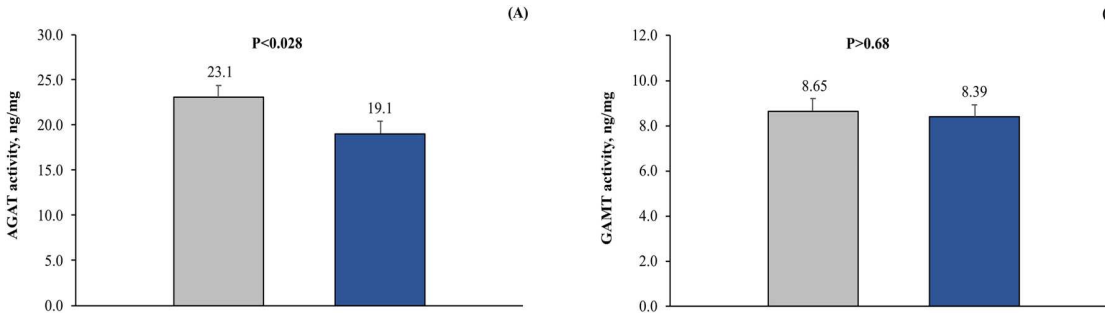

Fig. 2. Effects of guanidinoacetic acid on liver enzymatic activity of arginine:glycine amidinotransferase (AGAT) (A) and guanidinoacetate N-methyltransferase (GAMT) (B) in pregnant beef cows during late gestation [Control, no addition of GAA; GAA, addition of 0.2 % GAA to the total diet (DM basis)].

Table 6
Effects of guanidinoacetic acid on performance, muscle fiber number and diameter of the offspring.

Item	Maternal treatment[a]		P-value
	Control	GAA	
Birth weight	37.5±0.82	36.3±0.74	0.29
Weaning weight	203±0.09	208±5.8	0.38
Muscle fiber diameter, μm	27.2±1.39	23.6±1.42	0.09
Muscle fiber number	143±17.2	173±17.5	0.23
REA, cm^2	35.3±1.64	38.1±1.77	0.15
SFT, mm	2.32±0.26	2.62±0.26	0.19

bControl, no addition of GAA; GAA, addition of 0.2 % GAA to the total diet (DM basis)
[a] REA, ribeye area; SFT, subcutaneous fat thickness

the placental vascularization of beef cows during late gestation. Conversely, despite these effects on cows, we did not detect any impact of GAA on offspring's performance.

Overall, we found no effect of GAA on voluntary intake of the cows. This result aligns with most of studies, which indicate that GAA does not influence the voluntary intake in cattle (Liu et al., 2021; Liu et al., 2021; Speer et al., 2022). In contrast, some authors have reported that GAA can increase voluntary intake by improving both microbial growth and ruminal fiber digestibility (Li et al., 2020). It is noteworthy that we designed the diets in the current study to ensure adequate conditions for optimal microbial growth. Therefore, any potential influence of GAA on intake by improving digestion characteristics would be unlikely. Hence, the differences found in this study between treatments refer to the direct effects of GAA on metabolism without any effect arising from differences in nutrient intake.

Our primary objective in the current study was to investigate the potential of GAA as an arginine-sparing agent. In this regard, providing GAA led to a notable increase in plasma arginine compared to the control. Such observation was supported by increased AGAT activity in the liver of cows that received GAA. AGAT is a key enzyme in endogenous GAA synthesis, utilizing L-arginine and glycine as substrates to produce GAA and L-ornithine (Ostojic, 2021). The synthesis of GAA from L-arginine and glycine seems to be regulated by both serum creatine and ornithine concentration effects on AGAT activity (Wyss and Kaddurah-Daouk, 2000). In fact, a greater plasma ornithine was observed for cows receiving dietary GAA. Both arginine and L- ornithine are amino acids that play a key role in the urea cycle. Additionally, L-ornithine may be produced from arginine in a reaction catalyzed by arginase (Morris Jr, 2016). This could explain, at least partially, the reduced AGAT activity observed for cows receiving GAA. Furthermore, some authors have reported that providing both GAA and creatine leads to a downregulation of AGAT activity in rats (Edison et al., 2007) and piglets (Dinesh et al., 2021). In such scenarios, the conversion rate of arginine to GAA could be notably reduced. In summary, our data indicate that GAA effectively decreased the arginine utilization for GAA synthesis pathway, thereby increasing the availability of arginine for other physiological functions.

In agreement with the arguments above, cows receiving GAA exhibited higher placental vascularization compared to control cows. This finding directly aligns with the increased availability of arginine facilitated by GAA dietary addition. Arginine is a conditionally essential amino acid that plays a central role in placental angiogenesis and growth in mammals (Reynolds and Redmer, 2001; Huang et al., 2021; Wu et al., 2022). In addition to its direct effects on placental development and growth, arginine serves as the precursor of nitric oxide synthesis, a crucial vasodilator and angiogenic factor, intimately associated with placental enhancing blood flow (Bird et al., 2003; Wu et al., 2013). In fact, we observed an increase in serum nitric oxide for cows receiving GAA. Moreover, GAA addition led to greater plasma citrulline concentration compared to the control, directly reflecting increased nitric oxide production from arginine, as both citrulline and nitric oxide are the final products of this pathway (Morris Jr, 2016). Collectively, our results demonstrate that GAA enhanced blood flux from the cow to the placental-fetal interface during late gestation, primarily by augmenting the availability of arginine.

Regarding cow's performance, our results suggest that GAA avoided protein mobilization from the cows' skeletal muscle despite not affecting total weight gain. Such a statement is primarily supported by evaluating the variation in ribeye area between treatments. Cows receiving GAA exhibited a 0.031-fold increase in ribeye area compared to their initial ribeye area, whereas cows without GAA addition displayed a corresponding −0.061-fold decrease in ribeye area relative to their initial ribeye area. Additionally, we did not detect effect of GAA on subcutaneous fat thickness. This appears to indicate that GAA directly decreased protein mobilization without any effect on fat, which is corroborated by the similarity in serum NEFA between treatments.

According to our understanding, two main reasons may elucidate the effects of GAA in preventing protein mobilization from cows' skeletal muscle. First, cows exhibit a remarkable capacity to adapt their metabolism to meet the escalating demands for both utero and fetal-placental growth and development. For instance, several authors have reported increased protein mobilization from cows' skeletal muscle during gestation (Bell et al., 2005; Kennedy et al., 2016; Croft et al., 2022). Furthermore, research carried out in tropical conditions has demonstrated that even in cows receiving protein supplements, some degree of body mobilization still occurs, indicating that protein supplementation has reduced but not completely ceased body reserve mobilization during late gestation (Lopes et al., 2020; Santos et al., 2023). Such results represent a cow's adaptive mechanism to meet the high amino acid demands for both fetal-placental growth and development during late gestation (Bell et al., 2005). In the current study, providing GAA led to an increase in plasma arginine compared to control cows. In addition to its impacts on placental vasculogenesis and angiogenesis, arginine can be either utilized by the dam for protein synthesis or directed towards the placental-fetal interface, where it may also participate in the conceptus protein turnover (Wu et al., 2022). Several authors have reported that arginine regulates the activation of the mechanistic target of rapamycin (mTOR) signaling pathway to both increase protein synthesis and inhibit protein degradation in ruminants (Ma et al., 2017; Zhang et al., 2019; Gu et al., 2021). Hence, it is plausible that the higher plasma arginine observed in cows receiving GAA may have enhanced protein turnover in both the cow and the fetus, thereby reducing the need for protein mobilization from the cow's skeletal muscle.

Second, GAA may reduce protein mobilization from cow's skeletal muscle by improving creatine availability. It is widely recognized that creatine plays a significant role in energy metabolism and protein synthesis by storing energy as creatine phosphate in excitable tissue such as skeletal muscle, brain, and the reproductive tract (Wyss and Kaddurah-Daouk, 2000). Indeed, dietary GAA addition has been shown to activate the mTOR/Akt signaling, enhancing protein synthesis in both cattle (Liu et al., 2021) and sheep (Li et al., 2022). This effect has been coupled with a boost in the availability of both creatine and insulin-like growth factor-1 (IGF-1) for animals receiving GAA. Furthermore, it has been reported that GAA reduces the skeletal muscle myostatin and phosphor-FoxO1 abundance in sheep (Li et al., 2022). At last, this could aid in minimizing the body's protein breakdown. Even though we did not detect effect of GAA on serum creatine between treatments, our data suggest that the addition of GAA somehow enhanced the creatine availability in metabolism.

Creatine synthesis from GAA primarily occurs in the liver through a reaction catalyzed by GAMT. In this process, GAA receives a methyl group from S-adenosylmethionine, which is derived from methionine, resulting in the production of creatine and adenosylhomocysteine. Creatine is then released into the circulation, where it can be taken up by various tissues. S-adenosylmethionine can undergo hydrolysis to produce homocysteine and adenosine. Homocysteine further can be metabolized to cysteine, methylated to methionine, or exported into the circulation (Wyss and Kaddurah-Daouk, 2000; Edison et al., 2007). At first glance, one might assume that GAA was not converted to creatine, given that we observed no difference in liver GAMT activity between treatments. Nevertheless, it is noteworthy that feedback regulation of creatine synthesis primarily occurs at AGAT, with minimal evidence of feedback regulation at GAMT (Wyss and Kaddurah-Daouk, 2000). Furthermore, it has been suggested that the availability of substrates, rather than the enzyme activity itself, determines GAA and creatine biosynthesis (Dinesh et al., 2021).

In this regard, we observed higher serum homocysteine in cows receiving GAA. Additionally, providing GAA led to a decrease in plasma methionine concentration. Such result leads us to two reasonings. First, considering the increase in serum homocysteine, it is plausible to assume that the provided GAA was utilized in the creatine synthesis pathway despite no detectable change in serum creatine concentration. It should be noted that alterations in metabolite concentrations in body fluids result from a balance between the rates of production and removal of these metabolites (Sah et al., 2023). Hence, it is feasible that the rise in the rate of creatine synthesis was accompanied by an increase in the uptake of creatine by the tissues, thereby maintaining the concentration of creatine in the blood unchanged. Put together, our results suggest that the increase in both arginine and creatine may have contributed to lower protein mobilization from cow's skeletal muscle receiving GAA.

Second, it is notable that providing GAA led to a methyl group deficiency in cows. This is primarily evidenced by the increased serum homocysteine and decreased plasma methionine. Under adequate availability of methyl groups, homocysteine is remethylated to methionine by either the action of methionine synthase or betaine-homocysteine methyltransferase (Speer et al., 2022). If there is a deficiency of methyl groups, homocysteine may not be converted to methionine, resulting in an accumulation of plasma homocysteine (Ostojic, 2014). Thus, elevation of plasma homocysteine has been utilized as a useful marker for methyl group deficiency in cattle (Ardalan et al., 2020; 2021). As GAA methylation is not a regulated process, creatine synthesis will occur as GAA becomes available. In this context, providing GAA exceeding endogenous renal GAA synthesis may deplete significant amounts of methyl groups and, under certain conditions, lead to methyl group deficiency (Wyss and Kaddurah-Daouk, 2000; Stead et al., 2001; Brosnan et al., 2011).

In the current study, cows received 0.2 % GAA in the total diet (approximately 16 g GAA/cow). Our data suggest that this dosage was able to induce a deficiency in methyl groups and reduce the availability of plasma methionine. Indeed, several studies have indicated that dietary providing GAA at dosages similar to those used in the present study result in a deficiency of methyl groups in growing cattle. However, this effect can be alleviated by providing a methyl group donor such as methionine (Ardalan et al., 2021; Speer et al., 2022). Consequently, further research investigating the combined use of GAA and methyl group donors for pregnant beef cows is warranted.

Despite the effects on cows, we did not detect any impact of GAA on offspring's performance. We anticipated that the increased availability of arginine and improved placental blood flow resulting from providing GAA could positively influence the fetal development and postnatal performance of the offspring. Indeed, several studies have shown that supplementation with either rumen-protected L-arginine or N-carbamoylglutamate, a structural analog of N-acetylglutamate essential for endogenous arginine synthesis, enhances fetal development and postnatal performance of offspring in sheep (Peine et al., 2018; Prezotto et al., 2018; Zhang et al., 2023) and cattle (Gu et al., 2021).

Conversely, it is noteworthy that the provision of GAA led to a deficiency of methyl groups, resulting in a decrease in plasma methionine. Methionine is an essential amino acid with a range of physiological functions including protein synthesis, placental development, and methyl group donor in one-carbon metabolism pathway (Batistel et al., 2017; Coleman et al., 2020). Additionally, it is also noteworthy that a methyl deficiency group may impair placental metabolism and function and fetal development (Bergen et al., 2011; Tsitsiou et al., 2011). Taking this into account, it seems plausible that the advantages of elevated plasma arginine and improved placental blood flow have been offset by the methyl group deficiency resulting from providing GAA. Ultimately, this could potentially account for the absence of effects of GAA on progeny postnatal performance.

In summary, our findings suggest that providing GAA decreases skeletal muscle mobilization and enhances the placental vascularization of beef cows during late gestation. However, we did not observe any impact of GAA on performance of the offspring. Importantly, dietary GAA addition resulted in a deficiency of methyl groups, leading to decreased plasma methionine. These effects may have attenuated the potential benefits of elevated plasma arginine and improved placental blood flow, thereby explaining the lack of effects on progeny performance. Therefore, future research on GAA in cow-calf studies should consider providing methyl group donors simultaneously to enhance its beneficial effects.

5. Conclusion

Guanidinoacetic acid decreases skeletal muscle mobilization and enhances the placental vascularization of beef cows during late gestation. However, providing dietary GAA to beef cows at late gestation does not affect the performance of their offspring.

Declaration of Competing Interest

None.

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Effect of substrate dispersion method and particle size on *in vitro* digestibility, gas production kinetics and composition, and fermentation characteristics in different feed types

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Our objective was to evaluate the influence of substrate dispersion method and particle size on *in vitro* digestibility, gas production kinetics and composition, and fermentation characteristics of different feed types. Alfalfa hay, tall fescue hay, ground corn, soybean meal, and a total mixed ration (TMR) were used as substrates. Treatments were designed according to a 2 × 2 × 5 factorial arrangement: two substrate dispersion methods (loose substrate and in filter bags), two particle sizes (1 mm and 2 mm), and five feeds. Filter bags decreased ($P \leq 0.001$) digestibility and gas production compared with loose samples. Filter bags decreased digestibility and total gas production to a greater extent for forages, whereas the effects on concentrate feeds were less pronounced. Using filter bags decreased ($P \leq 0.015$) both methane production and methane concentration in headspace gas across all feeds. Substrates incubated in filter bags showed a lower molar proportion of acetate and a greater molar proportion of propionate than loose substrates ($P < 0.001$). In general, energy-rich feeds incubated using filter bags had a decreased ($P \leq 0.036$) acetate-to-propionate ratio, while substrate dispersion method did not influence ($P \geq 0.16$) the VFA profile of protein-rich feeds. Incubating substrates in filter bags alters *in vitro* digestibility, gas production, and fermentation characteristics regardless of particle size. However, the magnitude of responses is greater for forage-based feeds than concentrate feeds.

KEYWORDS

digestion, fiber, filter bags, *in vitro*, methane

1 Introduction

In vitro gas production systems are widely employed for evaluating diets and feed additives globally (Dijkstra et al., 2005; Yáñez-Ruiz et al., 2016). However, there are many variations in the application of the method, including differences in inoculum source, medium composition, pressure measurement systems, substrate dispersion method inside the bottles, substrate mass, and processing methods (Rymer et al., 2005). Differences in *in vitro* gas production system methodologies may affect measurements of digestibility and gas production and potentially compromise the ability to accurately and precisely compare feeds and diets both within and across laboratories.

Most studies with *in vitro* gas production systems have reported incubating substrates directly in bottles (Schofield et al., 1994; Huhtanen et al., 2008; Christodoulou et al., 2025). However, there are alternative methodologies for substrate inclusion that have incubated the substrates inside filter bags (Ankom Technology, Macedon, NY, USA), rather than directly into the bottles (Eun and Beauchemin, 2007; Yang et al., 2014; Trotta et al., 2024). The main appeal of using filter bags lies in their operational simplicity, and in the elimination of the need for quantitative transfer of residues after incubation to measure digestibility (Krizsan et al., 2013). In addition, some authors (Castro-Montoya and Dickhoefer, 2019; Camacho et al., 2023; Ajayi et al., 2025) have employed filter bags to evaluate digestion characteristics of individual feeds and investigate potential associative effects among feeds.

There are limited studies available comparing the incubation of substrates in bottles versus filter bags and these often yield mixed results (Krizsan et al., 2013; He et al., 2016; Sampaio et al., 2024). Adhesion of loose substrates to the surface of the flask decreased *in vitro* dry matter (DM) digestibility and total gas production (He et al., 2016; Sampaio et al., 2024). Conversely, potential residue losses during the filtration process of loose substrates could lead to artificially inflated digestibility estimates. In contrast, there is concern that incubating substrates within filter bags could decrease the surface area available for microbial contact and potentially alter the diffusion of fermentation end-products out of the filter bag. Accumulation of fermentation end-products within the filter bag microenvironment impaired microbial activity and degradation within the bag (Marinucci et al., 1992; Valente et al., 2011b). Indeed, several studies (Ramin et al., 2013; Schlau et al., 2021; García et al., 2024) have shown that the use of filter bags decreased *in vitro* DM digestibility, methane production, and total gas output. However, the effect of filter bags appears to depend on the type of substrate used. For instance, some studies (Krizsan et al., 2013; Castro-Montoya and Dickhoefer, 2019) report that the use of filter bags decreases digestibility and gas production to a greater extent in forages than in concentrate feeds, while others (Sampaio et al., 2024) have shown the opposite trend, with a greater decrease observed in concentrate feeds. Therefore, the overall impact of using filter bags in *in vitro* gas production systems remains uncertain, particularly across different feed types.

An additional consideration in *in vitro* incubation systems for feed evaluation is the substrate particle size. Substrate particle size of feeds being evaluated, whether *in situ* or *in vitro*, should be large enough to resemble particle size in *in vivo* conditions, but small enough to ensure proper microbial access, minimize sampling bias, and reduce experimental variation (Lowman et al., 2002; Nocek, 1988). Currently, it is recommended that the samples should be ground to pass through 1-mm screen to measure *in vitro* digestibility and gas production characteristics (Yáñez-Ruiz et al., 2016; Camacho et al., 2022). However, some studies (Earing et al., 2010; Mesgaran et al., 2010; Altman et al., 2022) using *in vitro* gas production systems have employed a 2-mm particle size. There are a few studies evaluating the impact of changing particle size on *in vitro* digestibility and gas production characteristics. Larger particle sizes generally reduce *in vitro* digestibility and gas production, although this effect may be feed-dependent (Lowman et al., 2002; Damiran et al., 2008; He et al., 2016). As microbial access to substrates within the filter bags may be restricted, and larger particles have a reduced specific surface area, the use of filter bags could have a greater impact on digestibility and gas production in substrates processed at larger particle sizes.

To our knowledge, no studies have investigated the combined effects of substrate dispersion method and particle size on *in vitro* digestibility and gas production kinetics. We hypothesized that incubating samples in filter bags reduces *in vitro* digestibility, as well as the extent and rate of gas production and composition, with this effect being greater for processed feeds with larger particle sizes. Our objective was to evaluate whether the method of substrate dispersion and particle size affects *in vitro* digestibility, gas production kinetics and composition, and fermentation characteristics in different feed types.

2 Materials and methods

All animal care and handling procedures were approved by the Animal Care and Use Committee of the University of Kentucky (protocol 2023-4296).

2.1 Substrates, processing, and chemical composition

Alfalfa hay, tall fescue hay, ground corn, soybean meal, and a total mixed ration (TMR; Table 1) were used as substrates. Substrates were chosen because of their common use in ruminant diets and contrasting chemical compositions. All substrates were ground using a knife mill fitted with a 2-mm screen sieve (Model 3 Wiley Mill, Thomas Scientific, Swedesboro, NJ, USA). Subsequently, half of each substrate was ground through the same mill using a 1-mm screen sieve.

Chemical composition of the basal diet and substrates are presented in Table 1. Dry matter concentration was analyzed by oven-drying for 24 h at 105°C. Total nitrogen (N) concentration was analyzed by combustion using a CN628 Carbon/Nitrogen Determinator (Leco Corporation, St. Joseph, MI; AOAC, 1995; method 990.03). Crude protein concentration was calculated by

TABLE 1 Chemical composition of basal diet and feed samples.

Item, g/kg DM[a]	Alfalfa hay	TMR[b]	Tall fescue hay	Ground corn	Soybean meal
DM, g/kg as fed	921	367	916	887	897
CP	169	154	97.0	127	518
aNDF	453	496	638	125	122
EE	17.9	33.1	24.0	41.2	16.8
Starch	6.00	118	13.0	652	16.0

[a]DM, dry matter; CP, crude protein; aNDF, neutral detergent fiber assayed with a heat-stable α-amylase and expressed inclusive of residual ash, EE, ether extract.
[b]Total mixed ration contained 72.5% corn silage, 10.0% cracked corn, 7.50% dried corn distillers' grains with solubles, 7.41% soybean meal, 1.1% finely ground corn, 0.79% calcium carbonate, 0.54% trace mineral premix, 0.11% tallow, 0.02% Vitamin A, D, & E premix, 0.01% Rumensin 90.

multiplying N concentration × 6.25. Crude fat concentration was analyzed by high-temperature solvent extraction with diethyl ether using an ANKOM X15 Extractor (ANOKM Technology Method 2, Macedon, NY, USA). Total starch concentration was quantified using an enzymatic-colorimetric method (AOAC, 2023; method 2014.10), with the colorimetric step substituted by amperometric detection using a YIS-2950D-1 Biochemistry Analyzer (YSI Life Sciences, Yellow Springs, OH). Neutral detergent fiber (aNDF) concentration was quantified using an ANKOM200 Fiber Analyzer (ANOKM Technology, Macedon, NY, USA) with heat-stable α-amylase and omitting sodium sulfite.

2.2 Treatments, experimental design, and *in vitro* incubation

Treatments were structured in a 2 × 2 × 5 factorial arrangement, as follows: two substrate dispersion methods inside the bottles (loose substrates and into filter bags; 25 μm pore size, ~44 cm^2 surface area; Cat. #F57; Ankom Technology, Macedon, NY, USA), two particle sizes (1 mm and 2 mm), and five feeds (alfalfa hay, tall fescue hay, ground corn, soybean meal, and TMR). The experiment was replicated over four daily incubations, with two bottles per treatment in each incubation.

Approximately 500 mg of each feed was weighed directly into 250-mL incubation bottles (Cat. #7056; Ankom Technology, Macedon, NY, USA) or weighed into acetone-cleaned, labeled, filter bags. Filter bags were heat-sealed and secured inside the bottles using silicone-coated, steel-lined cable ties to ensure they remained fully submerged throughout the incubation period. On the morning of incubation, 2 mL of deionized water was added to each bottle to wet the substrate and promote uniform dispersion of the inoculum throughout the substrate.

Two ruminally-cannulated Angus × Holstein crossbred steers (body weight: 522 ± 11 kg) were used as inoculum donors. Steers were fed daily at 0700 h with a corn-silage-based diet formulated to exceed requirements for ruminally degradable protein, metabolizable protein, vitamins, and minerals (Table 1; NASEM, 2016). Approximately 4 h after feeding, ruminal contents (both liquid and solid digesta) were collected from the solid-liquid interface of the rumen mat of each steer's rumen mat and pooled. The pooled ruminal contents were immediately placed into insulated bottles (YETI Rambler Gallon Jug, Yeti Holdings, Inc., Austin, TX) and transported to the laboratory.

Upon arrival, ruminal contents were blended under a CO2 headspace for 30 seconds and then strained through four layers of cheesecloth. A mixture of buffer solution, macro- and micro-mineral solutions, and reducing solution was prepared as described by Goering and Van Soest (1970). The combined solution was maintained at 39°C on a hot plate equipped with a temperature probe, with continuous agitation using a stir bar and constant infusion with CO2. After 30 minutes, rumen fluid was added into the buffered solution and mixed thoroughly at a buffer: rumen fluid ratio of 4.5:1. Subsequently, 100 mL of the prepared medium was dispensed into each incubation bottle using a FlexiPump Pro dispensing pump (Interscience International, St. Nom, France).

Bottles were placed in a 39°C water bath to equilibrate. The valves on the bottle caps were simultaneously opened to release any accumulated pressure. Then, valves were connected to an automated pressure transducer system (Memograph M RSG45 Data Manager; Endress+Hauser, Greenwood, IN) described previously by Seeforth and Trotta (2025), with cumulative gas pressure readings recorded every 5 minutes over a 72-h incubation period.

2.3 Sampling and measurements

At the end of the 72-h incubation period, bottles were placed in an ice bath to halt fermentation. After 15 minutes, a 10-mL gas sample was collected from each bottle using a syringe. The gas sample was collected into an empty vacuum evacuated test tube (Vacutainer®, Becton, Dickinson and Company, East Rutherford, NJ, USA) using a line connected to the vent valve on the bottle cap. Gas samples were analyzed for methane concentration by gas chromatography with flame ionization detection (HP 7890A, Agilent Technologies, Santa Clara, CA, USA) as described by Trotta et al. (2023). Following gas sampling, bottles were opened, and the pH of the incubation medium was immediately measured using a pH meter (HM-21P, DKK TOA Electronics Ltd., Tokyo, Japan). A 1-mL aliquot of the fluid was then transferred into 1.5 mL centrifuge tubes containing 0.1 mL of 85 mM 2-ethyl butyrate (internal standard) and 0.1 mL of 50% metaphosphoric acid for

volatile fatty acids (VFA) and ammonia analysis. Samples were frozen at -20°C for subsequent analysis.

The VFA were analyzed by gas chromatography with flame ionization detection (8890 GC System; Agilent Technologies Inc., Santa Clara, CA, USA) as described by Trotta et al. (2023). Ammonia concentration was quantified using the glutamate dehydrogenase method (Kun and Kearney, 1974), adapted for use in a multimode plate reader (BioTek Synergy HTX; Agilent Technologies Inc., Santa Clara, CA, USA) as described by Trotta et al. (2024).

For loose incubation, the contents of each bottle were carefully transferred to acetone-rinsed, labeled, and pre-weighed F57 filter bags. Samples were filtered using a vacuum pump and then heat-sealed. All filter bags, including the ones initially used for substrate incubations, were washed thoroughly with tap water until the rinse water ran clear, and sequentially oven-dried at 55°C and 105°C for 24 h. After drying, filter bags were then placed in a desiccator and weighed to estimate the apparent undigested DM residue. Subsequently, filter bags were processed in neutral detergent solution with heat-stable α-amylase (17,400 liquefon units/g; Spezyme Fred; Genencor International, Inc., Palo Alto, CA) using an ANKOM200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY, USA). Filter bags were washed three times with hot distilled water, submerged in acetone for 3 min, and sequentially oven-dried at 55°C and 105°C for 24 h, placed in a desiccator and weighed to estimate the true undigested DM residue.

2.4 Calculations

The apparent *in vitro* DM digestibility (apparent IVDMD, g/kg), true *in vitro* DM digestibility (true IVDMD, g/kg), and *in vitro* NDF digestibility (IVNDFD, g/kg) were calculated using the following equations (Equations 1–3):

$$\text{Apparent IVDMD} = \frac{\text{DMi} - \text{DMr}}{\text{DMi}} \times 1000 \quad (1)$$

$$\text{True IVDMD} = \frac{\text{DMi} - \text{NDFr}}{\text{DMi}} \times 1000 \quad (2)$$

$$\text{IVNDFD} = \frac{\text{NDFi} - \text{NDFr}}{\text{NDFi}} \times 1000 \quad (3)$$

where *DMi* is the mass of DM incubated; *DMr* is the mass of the apparently undigested DM residue; *NDFi* is the mass of NDF incubated; *NDFr* is the mass of the undigested NDF residue.

Assuming ideal gas behavior, constant temperature, and a closed system, total gas production (GP, in mL) was calculated from the vessel pressure corrected to current atmospheric pressure into standard atmospheric pressure (101.33 kPa) using the following equation (Equation 4):

$$\text{GP} = \frac{\Delta P \times V_{\text{headspace}}}{P_{\text{STP}}} \times 1000 \quad (4)$$

where ΔP is the net pressure change in kPa (corrected for atmospheric pressure), $V_{\text{headspace}}$ is the headspace volume (L), and P_{STP} is the standardized pressure (kPa). The volume of the headspace was estimated for each bottle by subtracting the volume of the contents (inoculum, substrate, filter bag, and clip) from the total volume of the bottle. The volume of the contents was estimated from the difference between the bottle weight at filling and the empty bottle. The total volume of each bottle was determined by subtracting the weight of the empty bottle from that of the water-filled bottle. Methane production was obtained by multiplying the total gas production by the methane concentration in the headspace gas.

The cumulative gas production curve was adjusted individually for each bottle using a first-order exponential model using the PROC NLIN procedure in SAS (version 9.4; SAS Institute Inc., Cary, NC, USA), as follows (Equation 5):

$$G(t) = G \times \left[1 - e^{(-k \times \text{time})}\right] \quad (5)$$

where G(t) is the cumulative gas production (mL) at time t, G is the asymptotic gas production (mL), and k is the fractional rate of gas production (h^{-1}).

2.5 Statistical analysis

All analyses were performed using GLIMMIX procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Data were analyzed according to a completely randomized design including the fixed effects of substrate dispersion method, particle size, feed, and their interactions. Observations from two bottles in each treatment were averaged for each day of incubation, and the averaged bottle was considered the experimental unit (n = 4). When a significant two-way interaction between method or particle size and feed was detected, simple effects of method or particle size were evaluated within each feed level using an F-test. Least squares means and their standard errors were computed for each method and feed combination. Significance was declared at $P < 0.05$ and tendency was considered at $0.05 < P < 0.10$.

3 Results

3.1 *In vitro* digestibility and gas production characteristics

There was no interaction ($P \geq 0.70$) between substrate dispersion method and particle size on *in vitro* digestibility (Table 2). Incubating samples using filter bags decreased ($P \leq 0.001$) all *in vitro* digestibility characteristics compared with loose incubation. However, we observed an interaction ($P \leq 0.003$) between dispersion method and feed type for all *in vitro* digestibility characteristics. Overall, filter bags decreased ($P \leq$

TABLE 2 Effects of substrate dispersion method (M), particle size (PS), and feed (F) on *in vitro* digestibility and gas production and composition.

Item[a]	1-mm		2-mm		SEM[b]	*P*-value					
	Loose	Filter bag	Loose	Filter bag		M	PS	M × PS	M × F	PS × F	M × PS × F
Digestibility, g/kg DM											
Apparent IVDMD	774	750	767	738	6.64	<0.001	0.16	0.74	0.003	0.15	0.98
True IVDMD	842	819	835	813	4.10	<0.001	0.097	0.92	<0.001	0.012	0.93
IVNDFD	641	601	645	607	11.8	<0.001	0.69	0.93	<0.001	0.17	0.94
Gas production, mL/g DM											
Maximum Gas Production	173	148	177	148	2.94	<0.001	0.58	0.53	<0.001	0.89	0.80
Gas Production$_{72h}$	166	145	166	145	2.80	<0.001	0.93	0.98	0.028	0.82	0.97
Rate, h^{-1}	0.064	0.069	0.062	0.072	0.004	0.098	0.91	0.57	0.002	0.96	0.98
Methane, %	5.23	4.09	5.21	3.92	0.071	<0.001	0.17	0.30	<0.001	0.94	0.95
Methane	8.70	5.89	8.67	5.68	0.173	<0.001	0.47	0.59	<0.001	0.79	0.96
Gas production, mL/g digested DM											
Maximum Gas Production	208	180	214	182	3.76	<0.001	0.27	0.48	<0.001	0.95	0.85
Gas Production$_{72h}$	199	177	201	179	3.97	<0.001	0.61	0.98	0.31	0.99	0.99
Methane	10.4	7.17	10.4	6.96	0.232	<0.001	0.69	0.60	<0.001	0.97	0.97

1-mm, substrates processed through 1-mm sieve; 2-mm, substrates processed through 2-mm sieve; Loose, substrates incubated loose in bottles; Filter bag, substrates incubated using filter bags.
[a]IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fiber digestibility; Gas Production$_{72h}$, gas production after 72 h of incubation; Maximum Gas Production, asymptotic gas production from the adjusted model.
[b]SEM, standard error of the mean.

0.022) true IVDMD in forages but had no effect (P ≥ 0.32) on concentrate feeds (Figure 1B). Similarly, filter bag incubation reduced (P ≤ 0.013) both apparent IVDMD and IVNDFD in TMR and tall fescue hay (Figures 1A, C). In contrast, filter bags did not influence (P > 0.11) apparent IVDMD but decreased (P< 0.013) IVNDFD in ground corn. For soybean meal, using filter bags did not affect (P > 0.15) apparent IVDMD but increased IVNDF (P< 0.002). In addition, filter bag incubation tended to decrease (P ≥ 0.087) both apparent IVDMD and IVNDFD in alfalfa hay.

True IVDMD tended to be greater (P = 0.097) for substrates ground to 1 mm compared with substrates ground to 2 mm (Table 2). Likewise, we observed an interaction (P< 0.012) between particle size and feed type on true IVDMD. Tall fescue substrates ground to 2 mm had decreased (P< 0.001) true IVDMD compared with those ground to 1 mm (Figure 2). No effects (P ≥ 0.35) of particle size were detected for the other feeds. Particle size did not influence (P ≥ 0.16) apparent IVDMD or IVDNDF (Table 2).

No effect of particle size (P ≥ 0.17) or interaction effect between method and particle size (P ≥ 0.30) was found on gas production characteristics (Table 2). Filter bag incubation decreased (P ≤ 0.001) total gas and methane production, whether expressed as mL/g incubated DM or mL/g digested DM. Similarly, methane concentration in headspace gas decreased (P< 0.001) when substrates were incubated in filter bags compared with loose substrates. Nevertheless, the effect of method dispersion on gas production characteristics depended (P ≤ 0.028) on feed type. Using filter bags decreased (P ≤ 0.013) total gas production at 72 h across all feeds, except for soybean meal, which was unaffected (P > 0.22; Figure 1D). Likewise, filter bag incubation decreased (P ≤ 0.001) asymptotic gas production in TMR and tall fescue, tended to decrease it in alfalfa hay (P = 0.059), but had no effect in ground corn or soybean meal (P ≥ 0.15; Figure 3A). When expressed as mL/g digested substrate, filter bags decreased (P ≤ 0.001) asymptotic gas production in TMR and tall fescue but did not influence (P ≥ 0.15) asymptotic gas production in alfalfa hay, ground corn, and soybean meal (Figure 4A). Using filter bags decreased (P ≤ 0.015) both methane production and the concentration of methane in the headspace across all feeds, even though the magnitude was greater for TMR, tall fescue hay, and ground corn (Figures 3C, D, 4B).

Incubating substrates using filter bags tended to increase (P = 0.098) gas production rate (Table 2). We observed an interaction (P< 0.002) between substrate dispersion method and feed type on gas production rate. Filter bag incubation increased (P< 0.003) gas production rate in tall fescue hay and tended to increase gas production rate in TMR (P = 0.056) and alfalfa hay (P = 0.097; Figure 3B). Conversely, filter bag incubation decreased (P< 0.034) gas production rate in ground corn and had no effect (P > 0.45) on soybean meal.

3.2 Fermentation characteristics

There were no interactions (P ≥ 0.74) between method and particle size for pH or ammonia concentration (Table 3). Media pH

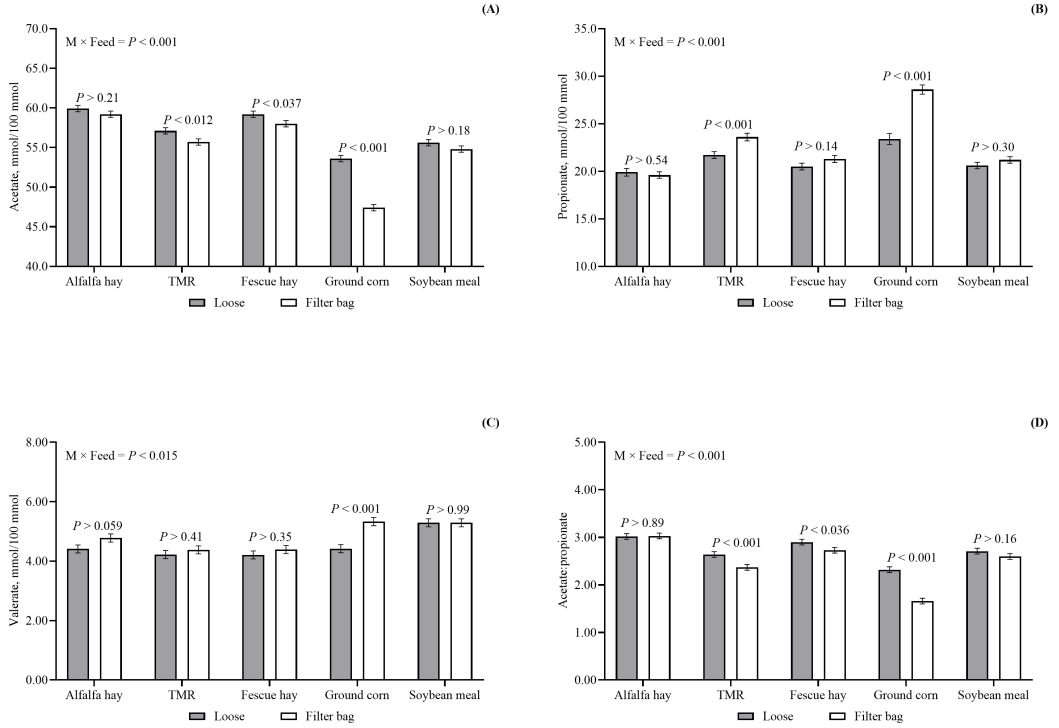

FIGURE 1
Effects of substrate method dispersion on apparent *in vitro* dry matter digestibility [apparent IVDMD, **(A)**], true *in vitro* dry matter digestibility [true IVDMD, **(B)**], *in vitro* neutral detergent fiber digestibility [IVNDFD, **(C)**], and gas production at 72 h [GP72h, **(D)**], according to the type of feed. [Loose, substrates incubated loose in bottles; Filter bag, substrates incubated using filter bags].

was greater (P< 0.022) for substrates incubated using filter bags, whereas it was decreased (P< 0.036) with 1 mm particle size. On average, final pH ranged from 6.93 to 6.97, showing that this effect was of low relevance or magnitude. Substrates ground to 1 mm tended to have a greater (P = 0.064) ammonia concentration than those ground to 2 mm. Nevertheless, the effect of particle size on ammonia concentration tended (P = 0.073) to depend on feed type. Specifically, processing samples to 2 mm decreased (P< 0.036) and tended to decrease (P = 0.094) ammonia concentrations in tall fescue hay and alfalfa hay, respectively. In contrast, particle size did not affect (P ≥ 0.11) ammonia concentration for the remaining feeds. Furthermore, filter bag incubation tended to decrease (P = 0.068) ammonia concentration compared with loose incubation.

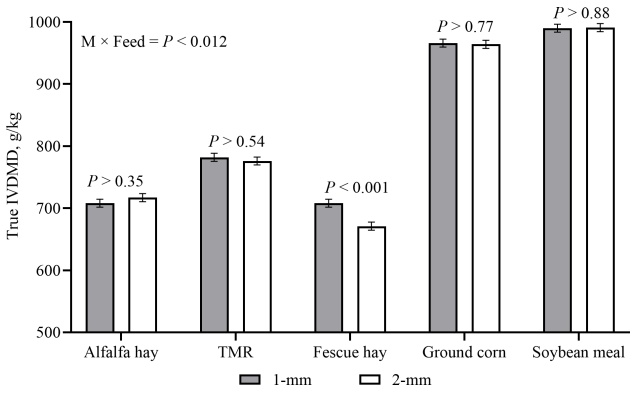

FIGURE 2
Effects of particle size on true *in vitro* dry matter digestibility (true IVDMD) according to the type of feed. [1-mm, substrates processed through 1-mm sieve; 2-mm, substrates processed through 2-mm sieve].

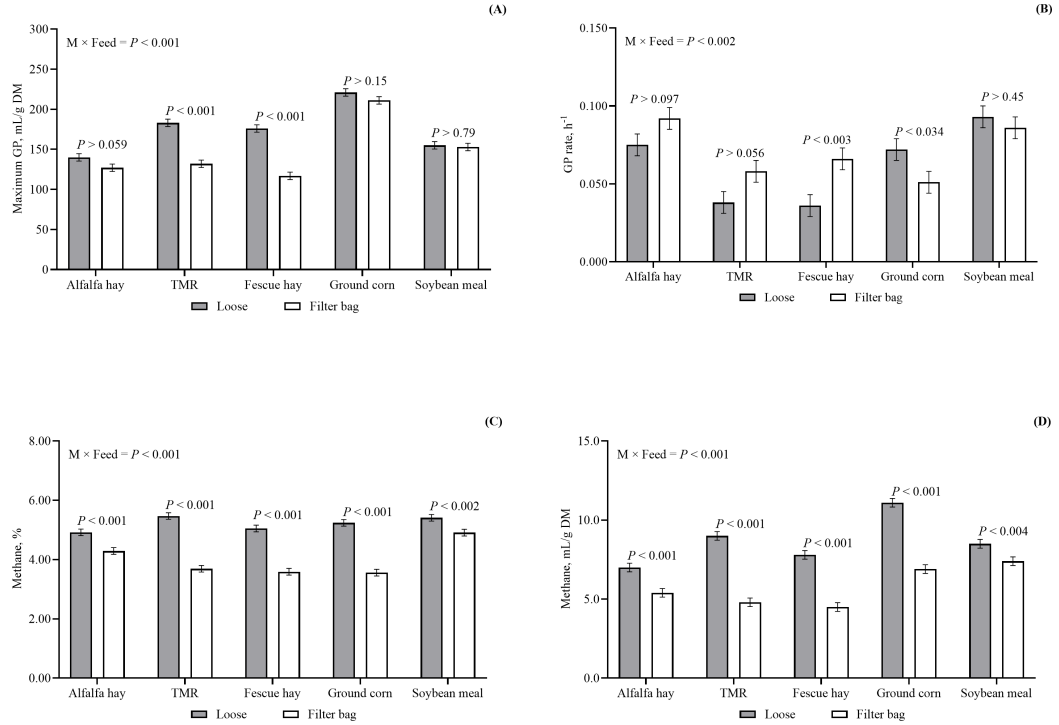

FIGURE 3
Effects of substrate method dispersion on maximum gas production [Maximum GP, **(A)**], gas production rate [GP rate, **(B)**], methane concentration **(C)**, and methane production **(D)** according to the type of feed. [Loose, substrates incubated loose in bottles; Filter bag, substrates incubated using filter bags].

There was no interaction (P > 0.73) between method and particle size on total VFA concentration (Table 3). Substrates incubated in filter bags had a lesser molar proportion of acetate and a greater molar proportion of propionate than those incubated in bottles (P ≤ 0.001). As a result, filter bag incubation decreased (P< 0.001) acetate-to-propionate ratio. However, the effects of substrate dispersion method on acetate and propionate proportions, and acetate-to-propionate ratio varied (P ≤ 0.001) by feed type. The use of filter bags decreased both (P ≤ 0.036) acetate and the acetate-to-propionate ratio in tall fescue hay, TMR, and ground corn, but had no effect (P ≥ 0.16) on alfalfa hay and soybean meal (Figures 5A, C). Likewise, the molar proportion of propionate was increased (P ≤ 0.001) in TMR and ground corn incubated in filter bags but remained unchanged (P ≥ 0.14) in alfalfa hay, tall fescue hay, and soybean meal (Figure 5B).

Filter bag incubation tended to increase (P = 0.099) butyrate molar proportion. On average, filter bag incubation increased (P< 0.001) molar proportion of valerate and tended to decrease (P = 0.088) the molar proportion of isobutyrate compared with loose incubation (Table 3). However, the effect of method on isobutyrate molar proportion tended to depend (P = 0.083) on feed type. The isobutyrate molar proportion was less (P< 0.004) in ground corn samples incubated in filter bags, while it was not affected (P ≥ 0.18) for the other feeds. Similarly, the effect of method on valerate molar proportion depended on particle size (P = 0.071) and feed type (P< 0.015). Filter bag incubation increased (P< 0.001) the molar proportion of valerate in samples ground to 2 mm, but had no effect (P > 0.17) on samples ground to 1 mm. Furthermore, filter bags tended to increase (P = 0.059) and increased (P<0.001) valerate molar proportions in alfalfa hay and ground corn, respectively, while filter bags did not influence valerate in the other feeds (Figure 5D). No effects (P ≥ 0.12) of particle size or interaction with feed type (P ≥ 0.81) were observed on VFA molar proportions.

4 Discussion

In this study, we aimed to investigate whether the substrate dispersion method and substrate particle size affected *in vitro* digestibility, gas production kinetics and composition, and fermentation characteristics across different substrate types. Contrary to our hypothesis, the overall effects of substrate dispersion methods were independent of substrate particle size. In fact, the effect of particle size was limited on *in vitro* digestibility and gas production. The effects of substrate particle size on *in vitro* digestibility and gas production are inconsistent across studies (Wilman and Adesogan, 2000; Lowman et al., 2002; Bossen et al., 2008; Damiran et al., 2008; Tagawa et al., 2017). Such variability may be attributed to differences in incubation time and substrate type. The lack of a particle size effect observed in the present study may be explained by the long incubation period (72 h). Under long-term incubation, the smaller surface area of substrates ground

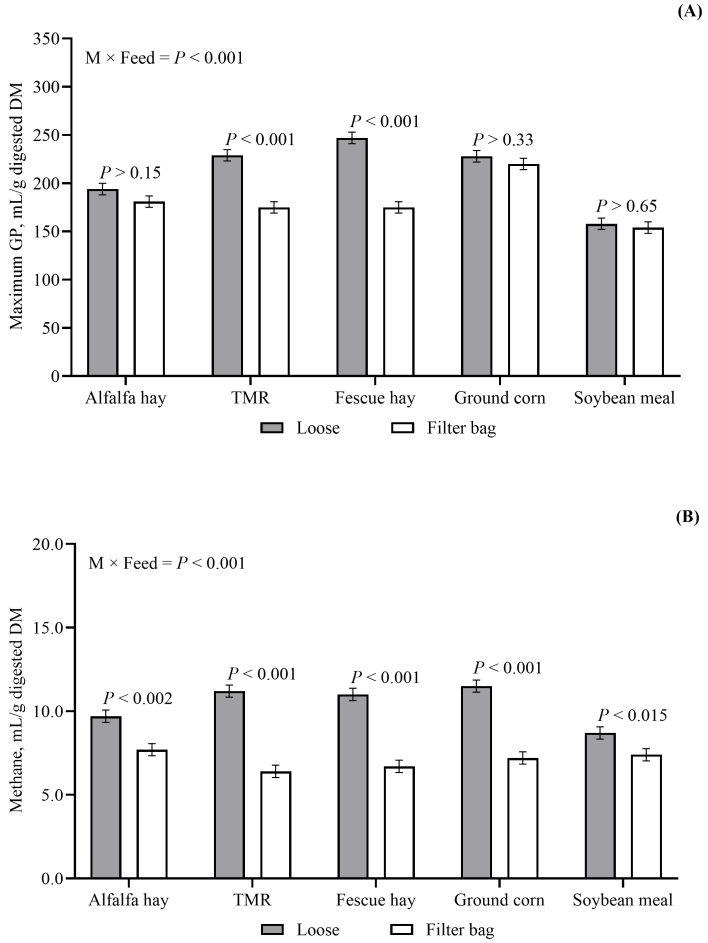

FIGURE 4
Effects of substrate method dispersion on maximum gas production [Maximum GP, **(A)**] and methane production **(B)** according to the type of feed. [Loose, substrates incubated loose in bottles; Filter bag, substrates incubated using filter bags].

to 2 mm may have been compensated by the extended time available for microbial digestion. Indeed, previous studies (Tagawa et al., 2017) have reported that particle size affects DM digestibility at early incubation times but not after 24 h of incubation. Similarly, increasing particle size in long-term incubation (48 h to 96 h) did not affect true DM or NDF digestibility (Wilman and Adesogan, 2000; Bossen et al., 2008). Moreover, some authors (Lowman et al., 2002) have reported that increasing particle size decreases total gas production in forages but not in concentrate feeds. In our study, we observed a trend toward decreased true IVDMD in tall fescue hay ground to 2 mm, although gas production was unaffected. Additionally, tall fescue hay processed to 2 mm showed lower ammonia concentrations in the fermentation medium compared with substrates ground to 1 mm, which may indicate reduced substrate degradability. Although these differences were small, the results suggest that grinding substrates to 2 mm may reduce *in vitro* digestibility in slowly digestible feeds.

Overall, the effects of substrate dispersion method on digestibility and total gas varied by feed type. For forages, filter bag incubation decreased apparent IVDMD, true IVDMD, and IVNDFD by 6.43%, 5.09%, and 11.8%, respectively. Among concentrate feeds, ground corn incubated in filter bags showed reductions of 2.55%, 0.81%, and 11.4% in apparent IVDMD, true IVDMD, and IVNDFD, respectively, whereas soybean meal showed increases of 2.22%, 0.93%, and 9.35%, respectively. Total gas production decreased by 17.1% in forages and 6.75% in concentrates when incubated in filter bags. Therefore, changing the method of substrate dispersion impacted digestibility and gas production to a greater extent in forages, while the effects on concentrate feeds, although present, were relatively minor. Similar results have been reported by other authors (Krizsan et al., 2013; Schlau et al., 2021; García et al., 2024) when comparing substrates incubated in filter bags versus dispersed freely in the medium. In addition, the greater digestibility of soybean meal incubated in filter bags was unexpected, particularly because it was not accompanied by greater gas production. In fact, gas production was decreased for soybean meal incubated in filter bags.

Several factors may explain the differences in *in vitro* digestibility and total gas production between substrate dispersion

TABLE 3 Effects of substrate dispersion method (M), particle size (PS), and feed (F) on *in vitro* fermentation characteristics.

Item[a]	1-mm		2-mm		SEM[b]	*P*-value					
	Loose	Filter bag	Loose	Filter bag		M	PS	M × PS	M × F	PS × F	M × PS × F
Media pH	6.93	6.95	6.95	6.97	0.008	0.022	0.036	0.74	0.50	0.84	0.90
Ammonia, m*M*	35.0	33.4	33.4	32.9	0.56	0.068	0.064	0.30	0.42	0.073	0.65
Total *VFA*, m*M*	51.1	50.8	49.9	48.7	1.47	0.62	0.27	0.73	0.93	0.99	0.92
VFA, mmol/100 mmol											
Acetate	57.1	55.4	57.1	54.7	0.25	<0.001	0.17	0.20	<0.001	0.89	0.97
Propionate	21.2	22.9	21.2	22.8	0.25	<0.001	0.78	0.93	<0.001	0.93	0.96
Butyrate	9.77	9.94	9.89	10.2	0.131	0.099	0.19	0.72	0.10	0.82	0.76
Isobutyrate	2.60	2.50	2.56	2.54	0.032	0.088	0.91	0.23	0.083	0.93	0.93
Valerate	4.52	4.69	4.50	4.98	0.086	<0.001	0.12	0.071	0.015	0.95	0.96
Isovalerate	4.79	4.62	4.80	4.83	0.085	0.41	0.21	0.22	0.64	0.81	0.98
Acetate: propionate	2.71	2.49	2.72	2.46	0.035	<0.001	0.82	0.65	<0.001	0.94	0.97

1-mm, substrates processed through 1-mm sieve; 2-mm, substrates processed through 2-mm sieve; Loose, substrates incubated loose in bottles; Filter bag, substrates incubated using filter bags.
[a]VFA, volatile fatty acids.
[b]SEM, standard error of the mean.

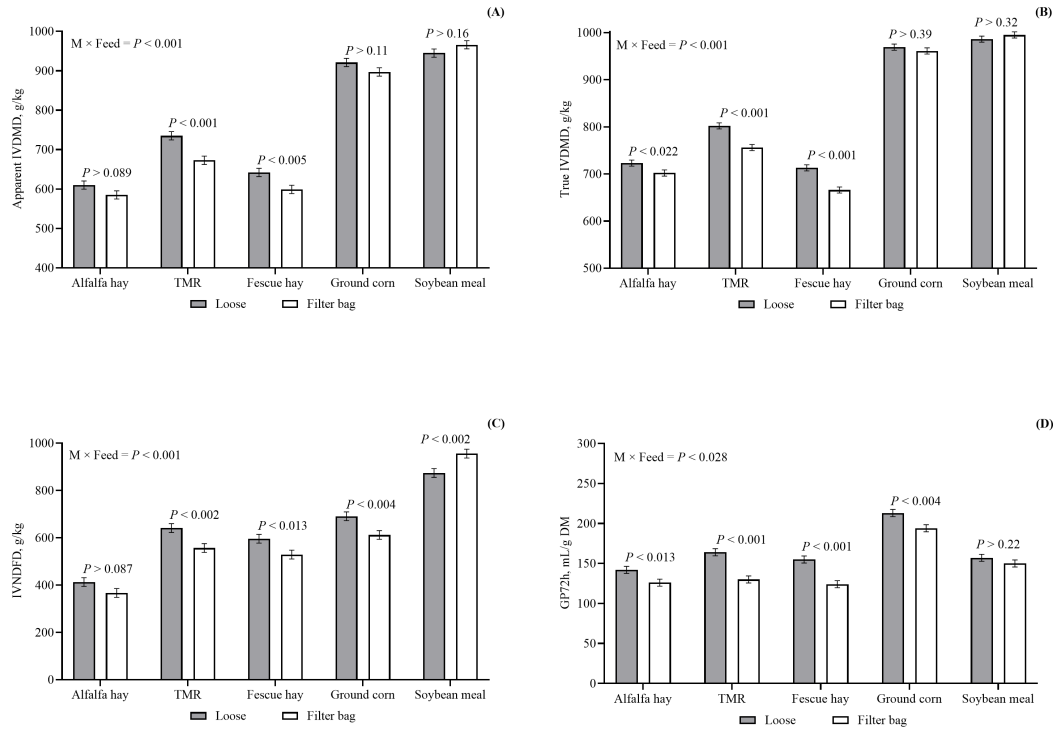

FIGURE 5
Effects of substrate method dispersion on molar proportions of acetate **(A)**, propionate **(B)**, valerate **(C)**, and acetate-to-propionate ratio **(D)** according to the type of feed [Loose, substrates incubated loose in bottles; Filter bag, substrates incubated using filter bags].

methods (substrates in filter bags vs. loose substrates). Feed particles can aggregate in filter bags as they become hydrated. This may impair the flow of incubation medium and the associated microbes through the sample, thereby limiting microbial access to the substrate. A similar pattern has been suggested when filter bags are used in pressurized systems for estimating fiber content in feedstuffs, and has been proposed as one of the reasons for the greater fiber values obtained with filter bags compared with conventional loose refluxing systems (Barbosa et al., 2015).

Filter bags used in the current study had an average pore size of 25 μm (#F57, Ankom Technology). Based on size, bacteria should be able to readily pass through the pores in the bag. Hence, no compromise in microbial degradation would be expected due to bacterial exclusion. However, the filter bags used in this study are composed of non-woven fabric, which results in an irregular pore structure (Valente et al., 2011a). Previous *in situ* incubation studies (Meyer and Mackie, 1986) reported a decrease in bacterial counts inside filter bags as pore size decreased, although this effect was more strongly associated with the proportion of pore surface area than with pore size itself. Furthermore, this pore size is smaller than the average width of most rumen ciliate protozoa genera (Williams and Coleman, 1992). Early studies (Lindberg et al., 1984) using *in situ* incubation reported a linear increase of total protozoa inside the bags as pore size increased from 10 μm to 36 μm. Likewise, increasing bag pore size from 40 μm to 100 μm enhanced protozoal counts as well as dry matter and fiber digestibility of substrates incubated in a semi-continuous culture system (Carro et al., 1995). Protozoa are known to play a key role in the ruminal degradation of organic matter (Williams and Coleman, 1992). Several studies have shown that partial or total defaunation decreases fiber digestibility (Newbold et al., 2015; Li et al., 2018; Dai and Faciola, 2019). Hence, it is possible that the 25 μm pore size and the irregular pore structure of the filter bags used may have restricted the entry of some protozoa into the bags, which could have contributed to the decrease in dry matter and fiber digestibility in the current study. This may also help explain why the effects of filter bags were more pronounced for forages than for concentrates, given the key role of protozoa in fiber degradation.

The use of filter bags in *in situ* or *in vitro* incubation assays assumes that the environment inside the bag is identical or at least similar to that outside the bag. However, a body of evidence suggests that this assumption may not be entirely valid (Meyer and Mackie, 1986; Lindberg et al., 1984; Marinucci et al., 1992; Valente et al., 2011b). Some authors (Úden et al., 1974; Marinucci et al., 1992) have reported limited fluid exchange between the filter bag and the surrounding medium. This limitation has been attributed to the small pore size of the bags and, more importantly, to the lack of pressure against filter bags or physical agitation especially in *in vitro* systems (Marinucci et al., 1992). Such factors may be critical for dislodging particles or facilitating fluid movement through the filter material (Marinucci et al., 1992). As a consequence, gas accumulation and a decline in pH within the bags have been observed, impairing microbial degradation, particularly by fiber-degrading microorganisms (Úden et al., 1974; Nocek et al., 1979; Marinucci et al., 1992). Unfortunately, we did not measure pH and VFA concentrations inside the bags, and the buffered media pH exhibited minimal variation. Nonetheless, we observed that using filter bags shifted the fermentation profile toward increased propionate compared with loose incubation, particularly for the TMR and corn, and to a lesser extent for tall fescue hay. Similarly, Sampaio et al. (2024) reported changes in the VFA profile towards propionate when substrates were incubated in filter bags.

Two main reasons may explain this shift in the fermentation profile toward propionate. First, even though we cannot conclude from this study, it is possible that the microenvironment inside the bags (e.g., VFA and gas accumulation) might have favored the activity of bacteria that degrade non-fibrous carbohydrates (e.g., amylolytic bacteria) while impairing fibrolytic bacteria, thereby altering the fermentation pattern toward propionate. Secondly, the increase in propionate proportion could be a direct effect of a potential decrease in protozoal entry into the bags. Indeed, it has been observed that decreasing ruminal protozoa shifts the fermentation pattern from acetate toward propionate both *in vivo* (Li et al., 2018; Dai and Faciola, 2019) and *in vitro* (Spanghero et al., 2022). This shift has been attributed to impaired fiber digestibility in the absence of protozoa (Newbold et al., 2015; Dai and Faciola, 2019). Additionally, protozoa compete with amylolytic bacteria for dietary starch, which is mostly fermented into acetate by protozoa (Williams and Coleman, 1992). Hence, a potential decrease in protozoa within the bags may have favored starch utilization by amylolytic bacteria, resulting in increased propionate proportion. This likely accounts for the more pronounced increase in propionate observed for TMR and corn incubated in filter bags, given their high starch contents.

In the current study, one of the most notable effects of using filter bags was the decrease in methane production. Methane production decreased by 34.4% for forages and 26.2% for concentrate feeds when incubated in filter bags. Similar findings have been reported in previous studies (Ramin et al., 2013; Sampaio et al., 2024) comparing substrates incubated in filter bags versus directly in bottles. For instance, Sampaio et al. (2024) observed that methane production decreased by 47.2% in a high-forage diet and 68.8% in a high-concentrate diet with filter bag incubation. Likewise, Ramin et al. (2013) reported an average decrease of 8% in methane production across feeds, although the magnitude of the filter bag's effect varied considerably depending on the type of feed. At first glance, the decrease in methane production can be attributed, at least partially, to the decreased amount of digested organic matter in samples incubated using filter bags. Indeed, there is a well-established positive linear relationship between the amount of digested organic matter and methane production in the rumen (Dai et al., 2022). To account for differences in digestibility, we expressed methane production relative to the amount of substrate digested. Even after this correction, substrates incubated in filter bags had decreased methane production compared with substrates incubated in bottles, particularly for TMR, tall fescue, and corn. In addition, methane concentration in the headspace gas was reduced by 24.8% for forages and 20.6% for concentrate feeds under filter bag incubation. These results appear to suggest that the use of filter bags altered ruminal fermentation and the fermentation end-products, as previously discussed.

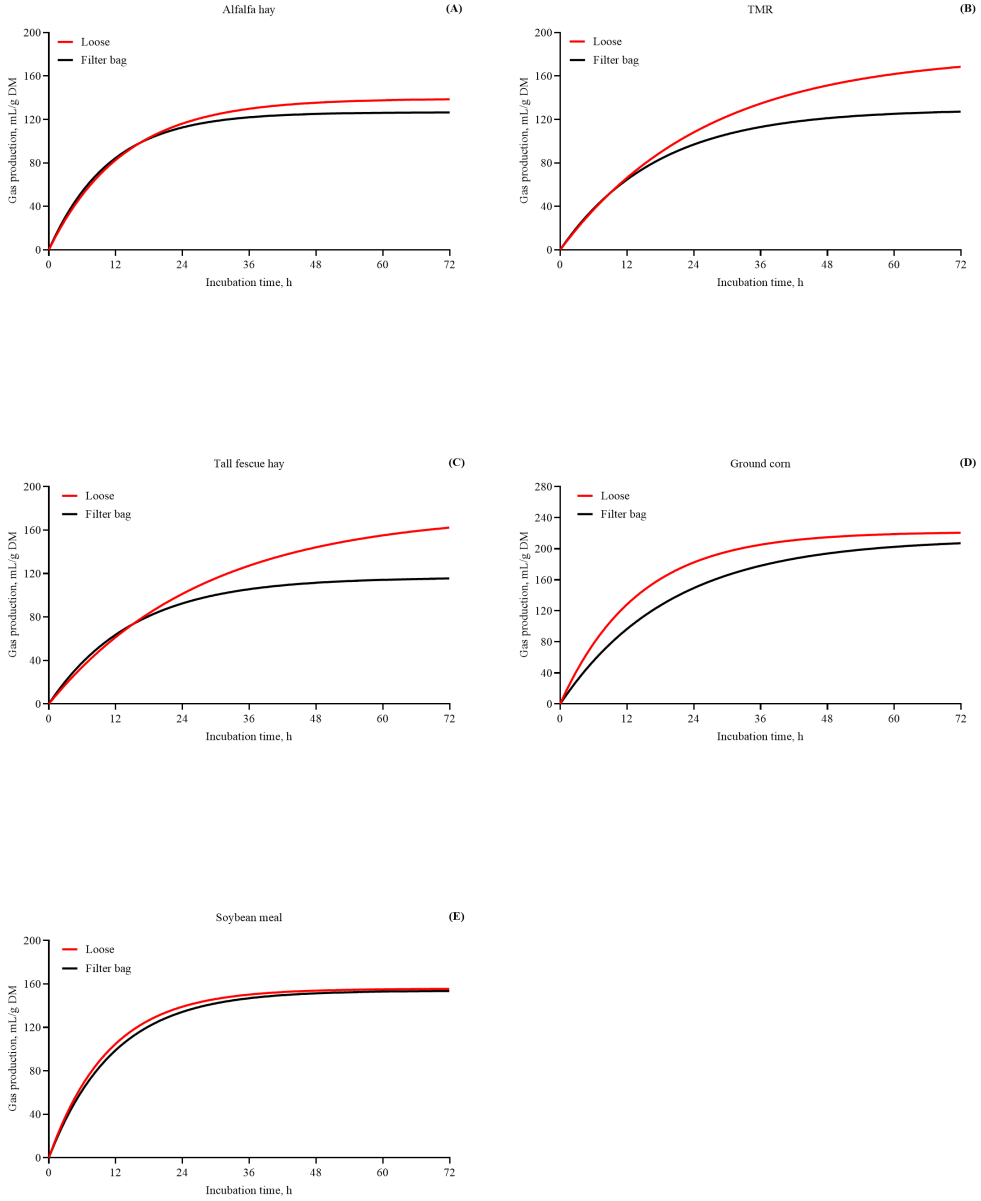

FIGURE 6
Cumulative gas production over a 72-h incubation period according to the substrate method dispersion and feed type [**(A)** Alfalfa hay, **(B)** TMR, **(C)** Tall fescue hay, **(D)** Ground corn, **(E)** Soybean meal; Loose, substrates incubated loose in bottles; Filter bag, substrates incubated using filter bags].

There could be multiple explanations for greater propionate proportions observed with filter bag incubations. First, the shift in the fermentation profile from acetate to propionate can result in decreased metabolic hydrogen (H2) availability for methanogens (Janssen, 2010), which could partially account for the decreases in methane production observed in samples incubated in filter bags. Second, the potential restriction of ciliate protozoa influx into the filter bags may have further contributed to the decrease in methane production. Rumen protozoa play a key role in methanogenesis, primarily due to their capacity to generate H_2 in hydrogenosomes and to harbor both epi- and endosymbiotic methanogens, protecting them from oxygen toxicity (Fenchel and Finlay, 2006). Indeed, one of the most consistent effects of decreasing protozoa in the rumen is a concomitant decrease in methane production (Newbold et al., 2015; Li et al., 2018; Dai and Faciola, 2019; Dai et al., 2022; Spanghero et al., 2022). This effect has been attributed, at least in part, to decreased fiber degradation in the absence of rumen protozoa (Dai and Faciola, 2019). However, even when accounting for known methane-related variables (e.g., feed intake, fiber digestibility), Dai et al. (2022) reported that a decrease in total protozoa counts, especially isotrichids, was associated with lower methane output. Together, these findings appear to suggest that the

use of filter bags alters the fermentation pattern, ultimately impairing methane production.

Also, we observed that samples incubated in filter bags tended to exhibit greater gas production rates compared with those incubated directly in bottles. At first glance, this finding appears to contradict our previous argument that filter bags affect microbial degradation processes. However, a closer examination of the gas production rates and the estimated gas production curves suggests a different interpretation. In general, filter bags increased the gas production rate in forages but either decreased or did not influence the gas production rate in concentrate feeds. A noteworthy observation relates to the asymptotes of the fitted gas production curves (Figure 6). Gas production from forages incubated in filter bags tended to plateau much earlier, whereas gas production from the same feeds incubated in bottles continued to increase and had not reached a plateau even after a 72 h-incubation. Asymptotic gas production and gas production rate were negatively correlated (data not shown). Thus, the greater gas production rates observed for forages in filter bags likely reflect a modeling artifact resulting from the lower asymptotic gas production. In fact, the rapid stabilization in gas production, coupled with the overall lower gas production, suggests an environment inside the filter bags that is not conducive to sustained microbial degradation. This interpretation is further supported by the gas production patterns observed for concentrate feeds. As gas production reached a plateau at similar time points and showed a minimal difference in asymptotic gas production between incubation methods, the gas production rate was either lower (e.g., for corn) or unaffected (e.g., for soybean meal) in samples incubated in filter bags.

5 Conclusions

Incubating samples within filter bags alters *in vitro* digestibility, gas production, and fermentation characteristics regardless of particle size. These effects are more pronounced in forage-based feeds than concentrate feeds. Future research should focus on elucidating the compositional and functional differences in microbial communities residing inside the filter bags compared with those in the surrounding incubation medium and the impact of restricting protozoa access on feedstuff characterization.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by University of Kentucky IACUC Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. EM: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. RT: Conceptualization, Project administration, Supervision, Writing – review & editing. ED: Writing – review & editing. DH: Conceptualization, Methodology, Project administration, Writing – review & editing.

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RUNNING HEAD: MITIGATION OF FESCUE TOXICOSIS WITH SOYBEANS

Soybean meal mitigates vasoconstriction and serotonin suppression during fescue toxicosis in beef cattle

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Lay Summary

Fescue toxicosis, caused by the consumption of ergot alkaloids from toxic endophyte-infected tall fescue (TE) decreases the growth of over 9 million calves in the Southeastern U.S. annually. Our objectives were to evaluate the effects of soybean hulls, whole soybean, and soybean meal supplementation on the mitigation of ergot alkaloid-induced vasoconstriction, heat stress susceptibility, and systemic immune activation in beef cattle during fescue toxicosis. Feed intake, meal duration, or meal size were not affected by treatments. Steers receiving TE increased the number of meals compared with steers fed non-toxic endophyte-infected tall fescue seed (NTE) and TE + soybean meal. Soybean meal supplementation mitigated ergot alkaloid-induced peripheral vasoconstriction, and promoted vasodilation, as evidenced by a 10.4% increase in caudal artery cross-sectional area relative to baseline, and decreased systolic blood pressure compared with TE steers. Measures of heat stress (respiration rate, and rectal temperature) were not influenced by soybean feed supplementation. Soybean meal supplementation increased serum serotonin by up to 34% compared with other TE-containing treatments. Among soybean-based feeds evaluated, soybean meal was the most effective because of the mitigation of ergot alkaloid-induced vasoconstriction, promotion of vasodilation, and restoration of serum serotonin, and normal feeding behavior in beef cattle challenged with fescue toxicosis.

Teaser Text

Soybean meal supplementation mitigated ergot alkaloid-induced vasoconstriction, promoted peripheral vasodilation, and restored serum serotonin and normal feeding behavior in beef cattle challenged with fescue toxicosis.

List of Abbreviations

5-HIAA, 5-hydroxyindoleacetic acid; 5-HTP, 5-hydroxytryptophan; AICC, akaike's information criterion with correction; ATP, adenosine triphosphate; AUC, area under the curve; BW, body weight; Cr-EDTA, chromium-ethylenediaminetetraacetic acid; DM, dry matter; HTR2A, 5-hydroxytryptamine receptor 2A; HTR4, 5-hydroxytryptamine receptor 4; MAO, monoamine oxidase; NEFA, non-esterified fatty acid; NTE, non-toxic endophyte-infected tall fescue; SBH, soybean hulls; SBM, soybean meal; TE, toxic endophyte-infected tall fescue; WSB, whole soybean.

ABSTRACT

The objectives were to evaluate the effects of soybean hulls, whole soybeans, or soybean meal supplementation on the mitigation of vasoconstrictive, heat stress, and inflammatory responses in beef cattle during fescue toxicosis. Ten ruminally-cannulated Angus ´ Holstein steers were used in a 5 ´ 10 Latin rectangle design. Five treatments were evaluated: 1) non-toxic endophyte-infected tall fescue seed (NTE), 2) toxic endophyte-infected tall fescue seed (TE), 3) TE + soybean hulls (SBH), 4) TE + whole soybean (WSB), or 5) TE + soybean meal (SBM). Treatment periods lasted 7 d, followed by a 14-d washout. Feed intake and feeding behavior were recorded daily. Caudal artery hemodynamics were assessed before feeding (0 h), 4 h, and 8 h after the morning feeding on days -1 (baseline) and 7. Physiological stress measures (skin and rectal temperatures, respiration rate, blood pressure, heart rate) were collected at 0 h, 4 h, and 8 h on days 1 and 7. Blood samples were collected at 0 h and 4-h on days 1 and 7. Treatments did not influence ($P \geq 0.35$) total dry matter (DM) intake, basal diet DM intake, meal duration, or meal size. Steers receiving TE or TE + WSB consumed a greater ($P = 0.02$) number of meals compared with NTE and TE + SBH steers. Relative caudal artery cross-sectional area decreased ($P = 0.02$) by 8.62% for steers receiving TE and increased ($P = 0.02$) by 10.4% for steers receiving TE + SBM. Systolic blood pressure of the caudal artery tended to increase ($P = 0.06$) in steers fed TE compared with steers receiving NTE or TE + SBM. Respiration rate, rectal temperature, and neck skin temperature were not influenced by soybean feed supplementation ($P \geq 0.24$). Rump skin temperature decreased ($P = 0.02$) in steers receiving TE compared with NTE and TE + SBM. Soybean meal supplementation increased ($P = 0.01$) serum serotonin concentration by up to 34% compared with other TE-containing treatments. Dietary supplementation of soybean-based feeds has the potential to alleviate symptoms of fescue toxicosis in cattle. Among soybean-based feeds evaluated, soybean meal was the most effective because of the mitigation of ergot alkaloid-induced vasoconstriction,

promotion of vasodilation, and restoration of serum serotonin and normal feeding behavior in beef cattle challenged with fescue toxicosis.

Keywords: blood flow, ergot alkaloid, fescue toxicosis, ruminant, serotonin, soybean

INTRODUCTION

Most tall fescue (*Lolium arundinaceum*) is infected with the endophyte, *Epichlöe coenophialia*, that enhances strand persistence, forage productivity, and drought tolerance (Dinkins et al., 2017). However, consumption of toxic ergot alkaloids produced by the endophyte can lead to fescue toxicosis in ruminant livestock species, resulting in negative impacts on animal growth, reproduction, and lactation (Strickland et al., 2011; Klotz, 2015). Symptoms of fescue toxicosis include decreases in feed intake, body weight gain, milk production, reproductive performance and increases in respiration rate, body temperature, and vasoconstriction (Strickland et al., 2011). Mechanisms describing how ergot alkaloid ingestion relates to the development of clinical symptoms of fescue toxicosis are not completely understood. Ergot alkaloids share structural similarity with monoamine neurotransmitters such as serotonin, dopamine, epinephrine, and norepinephrine (Berde, 1980), enabling interactions with their respective receptors to alter numerous physiological and metabolic functions (Klotz, 2015). It has been estimated that fescue toxicosis decreases the weaning weight of over 9 million beef calves in the Southeastern United States every year (Kallenbach, 2015), translating to a $2 billion annual loss in economic revenue for the beef industry (Kallenbach, 2015). There are few solutions that are both economically feasible and effective in preventing the onset or promoting amelioration of fescue toxicosis.

Some studies have reported positive effects when supplementing soy-based feeds to ruminants grazing toxic tall fescue or exposed to ergot alkaloids. Soybean hull supplementation increased average daily gain, increased serum prolactin, and improved hair coat scores of steers grazing toxic endophyte-infected tall fescue (Aiken et al., 2008; Carter et al., 2010). Soybean meal supplementation partially attenuated ergot alkaloid-induced vasoconstriction of the carotid artery in goats (Aiken et al., 2016; Harlow et al., 2022). However, interpretation of these responses is complicated by potential confounding effects from decreased dry matter intake

associated with ergot alkaloid ingestion, and increased dry matter intake with supplemental feeding. Differences in nutrient intake, dilution of ergot alkaloid intake, and uncontrolled environmental conditions have made it difficult to delineate factors associated with the onset and amelioration of fescue toxicosis in grazing ruminants (Koontz et al., 2012; Nicol and Klotz, 2016).

To our knowledge, no studies have directly compared the effects of soybean hull, whole soybean, or soybean meal supplementation on physiological responses to a fescue toxicosis challenge in beef cattle. Therefore, the objectives of this experiment were to evaluate the effects of soybean hulls, whole soybeans, and soybean meal supplementation on the mitigation of fescue toxicosis in beef cattle. The hypothesis of this experiment is soybean hull, whole soybean, and soybean meal supplementation will mitigate clinical symptoms of fescue toxicosis, including peripheral vasoconstriction, serotonin suppression, increased susceptibility to heat stress, and increased immune system activation.

MATERIALS AND METHODS

All animal care and handling procedures were approved by the University of Kentucky Animal Care and Use Committee (2023-4296).

Animals, diet, and housing

Ten Angus ´ Holstein steers [body weight (**BW)** = 221 ± 5.3 kg] were ruminally cannulated (Harmon and Richards, 1997) for use in a 5 ´ 10 Latin Rectangle design experiment. Steers were housed indoors in individual pens (3 m × 3 m) in the Intensive Research Building of the University of Kentucky C. Oran Little Research Center in Versailles, KY. To simulate summer environmental conditions, the room was maintained with a 16:8 h light:dark cycle, and ambient temperature was increased to 32°C during the light phase and decreased to 21°C during the dark phase (Koontz et al., 2012; Valente et al., 2020). Steers were initially fed a corn silage-

based diet devoid of soy-based feed ingredients (Table 1) once daily to supply 1.5 times the net energy required for maintenance (NASEM, 2016).

Experimental design and treatments

Rows and columns of the Latin Rectangle were randomized, and steers were randomly assigned to treatment sequences. During each experimental period, two steers received one of five treatments: 1) non-toxic novel endophyte-infected tall fescue seed (**NTE**; Estancia Forage Tall Fescue Grass Seed; 0 mg ergovaline + ergovalinine/kg; negative control), 2) toxic endophyte-infected tall fescue seed (**TE**; Kentucky 31 Tall Fescue Grass Seed; 9.34 mg ergovaline + ergovalinine/kg; positive control), 3) TE supplemented with soybean hulls (**TE + SBH**), 4) TE supplemented with raw whole soybeans (**TE + WSB**), and 5) TE supplemented with solvent-extracted soybean meal (**TE + SBM**). All seeds and supplements were dried equalize dry matter (**DM**) concentration and then ground through a 3-mm screen using a hammer mill to minimize variation in particle size. Ground seeds and supplemental treatments were administered through the rumen cannula immediately before feeding (Koontz et al., 2012; Ahn et al., 2020; Valente et al., 2020). The TE dose provided 15 mg ergovaline + ergovalinine·kg BW^{-1}·d^{-1}, which was previously shown to effectively induce fescue toxicosis and reduce serum serotonin concentration (Ahn et al., 2020; Valente et al., 2020). The amount of TE and NTE seeds administered were balanced to ensure equivalent daily seed intake. Soy-based feeds were supplemented at 2.75 g/kg of BW per day on a DM basis to provide approximately 680 g/d for a 250-kg steer. To equalize DM intake among treatments, steers receiving NTE and TE alone were supplemented with an equivalent amount of ground corn. The selected supplementation rate reflects common practices among beef producers feeding soy-based feeds to growing cattle (Lehmkuhler and VanValin, 2021), allowed supplementation rates to be scaled with each experimental period, maintained dietary ether extract below 6% of DM, and was comparable to the 2.61 g/kg of BW dose used by Harlow et al. (2022), who

reported increased carotid artery luminal diameter in goats ruminally-dosed with TE seed and supplemented with soybean meal. Treatment periods were 7 days in duration and were separated by 14-d washout periods. During washout periods, steers were housed outdoors in group pens for the first 10 days and then returned indoors during the final 4 days to allow acclimation to housing conditions. Baseline measurements were collected on the final day of each washout period (day −1). During washout periods, steers were fed the basal diet at 1.5 times the net energy required for maintenance.

Dry matter intake, feeding behavior, and nutrient analysis

Dry matter intake was measured and adjusted daily. All steers were pair-fed to the treatment with the lowest daily DM intake based on the DM intake measured during the previous day. The basal diet and orts were collected daily and analyzed for DM concentration by forced-air oven drying at 105 ºC for 24 h. Distribution of DMI throughout the day was monitored at 1-min intervals using an electronic scale equipped with a data logger attached to each animal's feed bunk (Campbell Scientific, Logan, Utah, USA). Feeding behavior variables, including meal frequency, meal size, and meal duration, were calculated for each animal on each experimental day (Egert-McLean et al., 2019).

Samples of the basal diet (Table 1) were collected for nutrient analysis including DM, total starch, crude protein, crude fat, neutral detergent fiber, acid detergent fiber, and mineral concentrations. Diet samples were partially dried at 55°C for 48 h in a forced-air oven and subsequently ground to pass a 1-mm screen using a Wiley mill. Dry matter concentration was determined by oven-drying for 3 h at 105°C (NFTA 2.1.4.). Total starch concentration was determined according to AOAC method 2014.10 by first gelatinizing samples in water, followed by two-step enzymatic hydrolysis with α-amylase and glucoamylase in acetate buffer (AOAC, 2023). Free glucose concentration was measured using the glucose oxidase electrode of a YSI Series 2950 D-1 Biochemistry Analyzer (YSI Inc., Yellow Springs, OH) and then

multiplied by 0.9 to convert to anhydroglucose equivalents, as found in starch (McCleary et al., 1994). Nitrogen concentration was determined by combustion (AOAC, 1999) (method 990.03) using a CN628 Carbon/Nitrogen Determinator (Leco Corporation, St. Joseph, MI), and crude protein concentration was calculated by multiplying N concentration × 6.25. Crude fat concentration was determined by ether extraction using an ANKOM X15 Extractor (ANKOM Technology Method 2, Macedon, NY). Neutral detergent fiber and acid detergent fiber concentrations were determined sequentially using the filter bag technique (ANKOM Technology Methods 14 and 15, respectively) with an automated fiber analyzer (ANKOM DELTA; ANKOM Technology, Macedon, NY). For mineral analysis, samples were digested in 50-mL MARSXPress vessels (CEM Corporation, Matthews, NC) using a MARS 6 Microwave Digestion System (CEM Corporation, Matthews, NC), and calcium and phosphorus concentrations were determined using inductively coupled plasma-optical emission spectroscopy (iCAP PRO XP ICP-OES; Thermo Fisher Scientific Inc., Beverly, MA). Total aglycone and individual isoflavone concentrations (Table 2) were quantified using liquid chromatography-mass spectrometry (Davis et al., 2023). Tall fescue seed samples were analyzed for ergovaline + ergovalinine concentrations using high-performance liquid chromatography with fluorescence detection (Yates and Powell, 1988; Klotz et al., 2018).

Caudal artery hemodynamics

Caudal artery cross-sectional area and hemodynamics at the 4th coccygeal vertebrae were assessed using color Doppler ultrasonography (Terason usmart 3300 NexGen ultrasound; Terason, Burlington, MA, USA). Measurements were collected at 0-h (before feeding), 4-h and 8-h after the morning feeding on days –1 (baseline, last day of the washout period prior to treatment administration) and 7 of each experimental period. For assessment of caudal artery cross-sectional area, the uSmart 15L4 linear array transducer (12 MHz) was positioned at the 4th coccygeal vertebrae in cross-sectional orientation and arterial pulsations were recorded as

video clips (5 cm depth, 4.5 MHz frequency, 3.0 Hz pulse repetitive frequency, and 40.0 gain). The caudal artery diameter was traced during five separate peak systolic phases and averaged. Cross-sectional area was calculated as πr^2, where r is the vessel radius (Aiken et al., 2007; Aiken et al., 2009; Davis et al., 2023).

Following the measurement of arterial cross-sectional area, the transducer was repositioned at the same anatomical location in longitudinal orientation and three consecutive cardiac cycle waveforms were recorded (2-cm depth, 24˚ correction angle, 0.5 mm sample volume). Peak systolic velocity, end diastolic velocity, and heart rate were calculated using built-in software functions of the ultrasound instrument. Mean velocity was calculated manually by tracing each cardiac cycle waveform using the ultrasound software. Pulsatility index was calculated as the difference between peak systolic velocity and end diastolic velocity divided by mean velocity. Resistance index was calculated as the difference between peak systolic velocity and end diastolic velocity divided by peak systolic velocity. Blood flow was calculated as mean velocity × cross-sectional area × 60 s (Lemley et al., 2012). Stroke volume was calculated as blood flow (mL/min) / heart rate (beats/min). Caudal artery cross-sectional area, blood flow, and stroke volume were expressed as absolute measurements and measurements relative to baseline. Measurements relative to baseline were calculated as:

$$\text{Change from baseline (\%)} = \frac{Response_{treatment\ period} - Baseline_{washout\ period}}{Baseline_{washout\ period}} \times 100$$

For measurements expressed relative to baseline, positive percent changes indicated vasodilatory activity and negative percent changes indicated vasoconstrictive activity.

Physiological stress measures

Skin temperature (neck and rump), rectal temperature, respiration rate, heart rate, and blood pressure were assessed as indicators of heat stress and fescue toxicosis (Al-Qaisi et al., 2019). Measurements were recorded at 0 h (before feeding), 4 h, and 8 h after the morning feeding on days 1 and 7 of each experimental period. To facilitate accurate measurements, hair

in the neck and rump areas were clipped on day −1 of each period and, skin temperatures were measured using a digital infrared thermometer (Fluke Corporation, Everett, WA, USA). Respiration rate was determined by counting flank movements for 15 s and multiplying by four to obtain breaths per minute. Blood pressure and heart rate were measured using an adjustable (16 cm to 24 cm) blood pressure cuff that was wrapped around the tail head and connected to a digital monitor (Lifesource A&D Engineering, Inc., San Jose, CA, USA) as described previously (Eisemann et al., 2014).

Blood collection and analysis

Blood (40 mL) was collected from the jugular vein with a syringe on day 1 at 0 h (before feeding and treatment administration) and on day 7 at 4 h after feeding. Blood was dispensed from syringes into two 10-mL tubes containing K_2EDTA and two 10-mL tubes with clot activator. Steers had continuous access to feed and water during blood sampling. Vacutainer tubes for plasma were centrifuged immediately after collection, whereas vacutainer tubes for serum were allowed to clot at room temperature for 45 min to 60 min before centrifugation. Whole blood was centrifuged (2,000 × *g*; 20 min; 4°C) and the supernatants were aliquoted into 2-mL tubes, and stored at −80°C until analysis.

Plasma glucose concentration was analyzed using the hexokinase/glucose-6-phosphate dehydrogenase method (Farrance, 1987) with Infinity Hexokinase Reagent (ThermoFisher Scientific Inc., Middletown, VA, USA). Plasma non-esterified fatty acid (**NEFA**) concentration was analyzed using the acyl CoA synthetase-acyl CoA oxidase-peroxidase method (Okabe et al., 1980) with the LabAssay NEFA FFA kit (FUJIFILM Wako Pure Chemical Corporation, Richmond, VA, USA). Plasma β-hydroxybutyrate concentration was analyzed using the β-hydroxybutyrate dehydrogenase/tetrazolium salt method (McMurray et al., 1984) with the Fisherbrand β-Hydroxybutyrate Liquicolor Reagent Kit (Stanbio Laboratory L.P., Boerne, TX, USA). Plasma urea concentration was quantified using the *o*-phthaldialdehyde/primaquine

diphosphate method described by Jung et al. (1975) and modified by Zawada et al. (2009). All colorimetric assays were adapted for use with a multi-mode plate reader (BioTek Synergy HTX; Agilent Technologies Inc., Santa Clara, CA, USA). Detailed protocols are provided in Supplementary Materials and Methods.

Acute phase proteins (serum amyloid A, haptoglobin) have been used as positive biomarkers of immune system activation and inflammation in cattle (Kvidera et al., 2017; Horst et al., 2020; Pate et al., 2021). Cholesterol, total bilirubin, and albumin have been used as negative biomarkers of immune system activation and inflammation in cattle (Rodriguez-Jimenez et al., 2025). Serum amyloid A (TP807; Tridelta Phase range SAA, Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland) and haptoglobin (HAPT-11; Cow Haptoglobin ELISA Kit; Life Diagnostics, West Chester, PA, USA) concentrations were analyzed using ELISA kits according to the manufacturer's instructions. Plasma albumin concentration was quantified using the bromocresol green method (Doumas et al., 1971). Plasma cholesterol concentration was analyzed using the cholesterol esterase-cholesterol oxidase-peroxidase method (Allain et al., 1974; Roeschlau et al., 1974) with Infinity Cholesterol Liquid Stable Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). Serum total bilirubin concentration was analyzed using the Jendrassik-Grof method (Shull et al., 1980). All procedures were adapted for use with a multi-mode plate reader (BioTek Synergy HTX; Agilent Technologies Inc., Santa Clara, CA, USA). Full procedure descriptions are available in Supplementary Materials and Methods.

The concentrations of tryptophan, kynurenine, 5-hydroxytryptophan (**5-HTP**), serotonin, and 5-hydroxyindolacetic acid (**5-HIAA**) in serum were quantified by high-performance liquid chromatography (**HPLC**) with modified methods from Sa et al. (2012). Briefly, 500 μL of serum was deproteinized by mixing with an equal volume of 5% (v/v) perchloric acid containing 50 mM kynurenic acid for use as an internal standard. Samples were

chilled at 4ºC for 5 min, vortexed, and centrifuged (20,000 × g; 10 min; 4 °C). The acidified supernatants (800 µL) were transferred to 2 mL new tubes and buffered with 410 µL of 0.6 M KOH. The samples were chilled at 4ºC for 5 min and centrifuged (20,000 × g; 10 min; 4 °C). A 10-µL aliquot of the resulting supernatant was then injected into the HPLC system (1260 Infinity II HPLC System; Agilent Technologies Inc., Santa Clara, CA, USA) using an autosampler. Chromatographic separation was performed using an ACE C18-PFP column (4.6 × 150 mm, 3 µm; Advanced Chromatography Technologies, Aberdeen, Scotland) maintained at 39 °C. The mobile phase consisted of 50 mM KH_2PO_4 and methanol (85:15 v/v; pH = 4.3) at a flow rate of 1.0 mL/min. Separated tryptophan metabolites were detected by fluorescence (tryptophan, 5-HTP, serotonin, 5-HIAA) or UV detection (kynurenine). The fluorescence detector was operated with an excitation wavelength of 278 nm and an emission wavelength of 338 nm, and the UV detector was set at 365 nm. The concentrations of tryptophan metabolites were quantified based on peak areas relative to a mixed analytical standard solution [50 µM L-tryptophan (T-0254; Sigma-Aldrich), 5 µM L-kynurenine (K9625; Sigma-Aldrich), 5 µM 5-hydroxy-L-tryptophan (H0531, TCI AMERICA), 5 µM serotonin hydrochloride (S0370, TCI AMERICA), and 5 µM 5-hydroxyindole-3-acetic acid (H8876; Sigma-Aldrich)] containing 15 µM kynurenic acid in KH_2PO_4 (pH = 4.3). Peak integration was performed electronically using OpenLAB ChemStation software (Agilent Technologies Inc., Santa Clara, CA, USA).

Total-tract gastrointestinal permeability

Total-tract gastrointestinal permeability was assessed using Cr-EDTA as a paracellular leak permeability marker (Wood et al., 2015; Horst et al., 2020; Bertens et al., 2024). On day 4, temporary intravenous catheters (14 Ga × 13 cm, Mila International, Inc., Florence, KY, USA) were inserted into the jugular vein. Before feeding and treatment administration on day 5, Cr-EDTA solution (180 mM) (Binnerts et al., 1968) was pulse-dosed through the ruminal cannula at a dose of 0.369 mmol/kg BW (Bertens et al., 2024). Blood (20 mL) was collected

through the jugular vein catheter at 2, 8, 20, 40, and 48 h after Cr-EDTA infusion (Bertens et al., 2024) and added to two 10-mL Vacutainer tubes containing sodium heparin. After collection of individual blood samples, 10 mL of heparinized saline was infused through the jugular catheter to flush residual blood and maintain patency.

Vacutainer tubes were centrifuged (2,500 × *g*; 15 min; 4 °C), plasma was aliquoted into 2-mL tubes, and stored at −80 °C. Plasma Cr concentrations were determined using atomic absorption spectroscopy (AAnalyst 200; PerkinElmer Inc., Shelton, CT, USA). Absorbance was measured at 357.87 nm. Plasma Cr area under the curve was calculated using the trapezoidal method in GraphPad Prism 9.4 (GraphPad Software, San Diego, CA, USA).

Statistical analyses

All analyses were performed using the GLIMMIX procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). For variables containing repeated measures, the fit of covariance structures were evaluated and selected based on the smallest Akaike's information criterion with correction (AICC). Feed intake and feeding behavior variables were analyzed as repeated measures using the random statement. The model included fixed effects of treatment, day, and the day × treatment interaction with random effects of animal and period. Caudal artery hemodynamics and plasma Cr concentrations were analyzed using the repeated measures statement, including the fixed effects of treatment, time, and the time × treatment interaction with random effects of animal and period. Plasma and serum metabolites, physiological stress indicators, and plasma Cr AUC were analyzed considering the fixed effect of treatment and the random effects of animal and period. Outliers were identified and removed if studentized residuals exceeded ± 2.5 (single measures) or ± 3.0 (repeated measures). Degrees of freedom were estimated using the Kenward–Roger method. Least squares means were compared using Fisher's least significant difference approach, protected by a significant *F*-test. Statistical significance was declared at $P < 0.05$, and tendencies were declared when $0.05 < P < 0.10$.

RESULTS

Dry matter intake and feeding behavior

There were no interactions ($P \geq 0.16$) between treatments and days for feed intake and feeding behavior variables (Table 3). Treatments did not influence ($P \geq 0.35$) total DM intake, basal diet DM intake, meal duration, or meal size. Steers receiving TE or TE + WSB consumed a greater ($P = 0.02$) number of meals compared with NTE and TE + SBH steers.

Caudal artery hemodynamics

There was a tendency for heart rate ($P = 0.06$) and blood flow relative to baseline ($P = 0.08$) among treatment and time (data not shown). The time × treatment interactions were not significant ($P \geq 0.39$) for other hemodynamic variables (Table 4).

Caudal artery cross-sectional area tended to decrease ($P = 0.06$) for steers ruminally-dosed with TE (Table 4). When expressed relative to baseline, caudal artery cross-sectional area decreased ($P = 0.02$) by 15.1 percentage units for steers receiving TE compared with NTE. The relative caudal artery cross-sectional area increased ($P = 0.02$) by 19 and 11 percentage units for steers ruminally-dosed with TE + SBM or TE + SBH compared with TE, respectively. Relative caudal artery cross-sectional area did not differ ($P \geq 0.15$) for TE + WSB compared with NTE or TE controls. Peak systolic velocity, end diastolic velocity, mean velocity, and resistance index were not influenced ($P \geq 0.59$) by soybean feed supplementation. Steers receiving TE and TE + SBH had greater ($P = 0.01$) pulsatility indexes compared with steers receiving TE + WSB. When expressed relative to baseline, caudal artery blood flow tended to increase ($P = 0.08$) for TE + SBH and TE + SBM compared with TE. Absolute blood flow and stroke volume were not influenced ($P \geq 0.12$) by soybean feed supplementation.

Physiological stress indicators

Treatments did not influence ($P \geq 0.38$) rectal temperature or neck temperature (Table 5). Conversely, rump skin temperature decreased ($P = 0.02$) in steers receiving TE compared

with NTE and TE + SBM. Skin temperatures were intermediate in steers fed TE + SBH and TE + WSB, not differing from the other treatments ($P > 0.05$). Respiration rate was not affected by treatments ($P = 0.24$). Treatments tended to affect systolic pressure ($P = 0.06$) but did not influence diastolic pressure ($P = 0.67$). Systolic pressure tended to increase ($P = 0.06$) in TE steers compared with NTE steers. Systolic pressure tended to decrease ($P = 0.06$) in TE + SBM steers compared with TE steers. Furthermore, steers receiving NTE, TE + SBM, and TE + WSB showed greater ($P < 0.01$) heart rate compared with those fed TE and TE + SBH.

Serotonin metabolites

Serum tryptophan concentration was not affected by treatments ($P = 0.12$; Table 6). Serum kynurenine concentration was affected by treatment ($P = 0.03$) as concentrations were greater for steers receiving TE + SBM and TE + SBH compared with NTE and TE controls. Serum 5-HTP concentration tended to be greater ($P = 0.10$) for all TE treatments. Serum serotonin concentration was greater ($P = 0.01$) in NTE and TE + SBM. Serum 5-HIAA concentration tended to be greater ($P = 0.09$) in steers fed all TE treatments.

Metabolic and inflammatory biomarkers

Plasma glucose concentration was greater ($P = 0.02$) in all TE treatments compared with those receiving NTE (Table 7). Plasma NEFA concentration was not affected by treatments ($P = 0.25$). Conversely, plasma β-hydroxybutyrate concentration was greater ($P = 0.02$) in steers fed NTE compared with those receiving TE. Plasma urea concentration was greater ($P < 0.01$) in steers receiving NTE compared with those receiving TE. Among soybean-fed steers, plasma urea concentration was greatest in TE + SBM, intermediate in TE + WSB, and least in TE + SBH ($P < 0.01$).

Serum amyloid A concentration tended to differ among treatments ($P = 0.08$), as concentrations were generally greater in the soy-containing treatments. Serum haptoglobin and plasma albumin concentrations were not affected by treatments ($P > 0.30$). Serum total bilirubin

concentration was increased ($P = 0.03$) for the TE treatments with the exception of TE + SBM which was lower and similar to NTE. There were no differences between NTE, TE, and TE + SBM on plasma cholesterol concentration ($P > 0.05$). However, plasma cholesterol concentration was greater ($P = 0.01$) in steers receiving TE + SBH and intermediate for steers fed TE + WSB.

Total-tract gastrointestinal permeability

There was no effect of treatments ($P = 0.72$) or interaction between treatments and time ($P = 0.77$) on plasma Cr concentrations (Figure 1A). Additionally, the plasma Cr AUC was not affected by treatments ($P = 0.16$; Figure 1B).

DISCUSSION

The objectives of the current study were to evaluate the effects of SBH, WSB, and SBM supplementation on ergot alkaloid-induced vasoconstriction, susceptibility to heat stress, and systemic immune activation in beef cattle during fescue toxicosis. Previous studies have reported benefits of soybean feeds supplementation in steers grazing toxic endophyte-infected tall fescue, including increased average daily gain (Aiken et al., 2008), restored serum prolactin, and improved hair coat scores (Carter et al., 2010). More recently, SBM supplementation partially alleviated ergot alkaloid-induced vasoconstriction in goats (Harlow et al., 2022). However, a clear mechanism explaining the magnitude of responses observed across studies remains difficult to delineate, partly due to potential confounding effects caused by decreased feed intake in animals exposed to ergot alkaloids, and the effects of supplements on forage and ergot alkaloid intake. To our knowledge, no studies have directly compared use of soybean feeds to mitigate symptoms of fescue toxicosis in beef cattle with controlled environmental heat loads and equalized feed intakes.

Mitigation of ergot alkaloid-induced vasoconstriction

One of the predominant negative symptoms of fescue toxicosis is increased vasoconstriction, leading to decreased peripheral blood flow in several ruminant livestock species (Aiken et al., 2007, 2009; Harlow et al., 2022; Davis et al., 2023). Persistent vasoconstriction during fescue toxicosis is thought to be attributed to increased interactions of ergot alkaloids (e.g., ergovaline) with serotonergic receptors in blood vessels (Klotz et al., 2013; Trotta et al., 2018). In the present study, TE ingestion decreased the cross-sectional area of the caudal artery by 8.62% and tended to decrease peripheral blood flow by 7.91% relative to baseline. Supporting these observations, systolic blood pressure of the caudal artery was 8.8% greater in TE steers compared with NTE steers. These results confirm that ergot alkaloid-induced vasoconstriction occurred in TE steers.

Previous research demonstrated that TE + SBM supplementation to goats increased carotid arterial cross-sectional area by 30.7% compared with goats receiving TE alone (Harlow et al., 2022). However, goats receiving TE had decreased hay intake compared with goats receiving TE + SBM, which prevented assessing the direct impact of SBM on alleviating ergot alkaloid-induced vasoconstriction (Harlow et al., 2022). In the current study, SBM supplementation to steers increased the relative cross-sectional area of the caudal artery by 20.8% compared with TE under pair-fed conditions. Likewise, SBM increased caudal arterial blood flow by 12.9% compared with NTE and by 22.6% relative to TE. These observations demonstrate that SBM supplementation mitigated ergot alkaloid-induced vasoconstriction and promoted peripheral vasodilation to increase blood flow in steers, independent of changes in feed intake.

Previous research has demonstrated that SBH supplementation increased average daily gain of steers grazing TE pastures (Aiken et al., 2008; Carter et al., 2010), increased digestible organic matter intake, and total-tract organic matter digestibility (Fieser and Vanzant, 2004), and increased duodenal microbial N flow, and nitrogen retention (Fieser and Vanzant, 2004;

Richards et al., 2006). In the current study, supplementing TE + also mitigated ergot alkaloid-induced vasoconstriction (+13.4% relative to TE) and increased caudal artery blood flow (+12.1% relative TE), although to a lesser extent than SBM supplementation. It is possible that beneficial effects of SBH supplementation could result from positive associative effects on forage intake, digestibility, and nitrogen utilization, and/or increased estrogenic activity mediated by isoflavones (Fieser and Vanzant, 2004; Richards et al., 2006; Shappell et al., 2015).

Legumes, including red clover, white clover, and soybean-based feeds, contain greater concentrations of flavonoids compared with corn-based feeds (Bhagwat and Haytowitz, 2015; Harlow et al., 2022). Studies have reported partial or complete reversal of ergot alkaloid-induced vasoconstriction in ruminants supplemented with legumes containing isoflavones, including SBM (Aiken et al., 2016; Harlow et al., 2022; Davis et al., 2023). However, the mode of action of these responses was unclear because of confounding effects on DM and nutrient intake and potential phytoestrogenic properties of isoflavones in the vasculature (Wu et al., 2010; Aiken et al., 2016). In the current study, the total aglycone dose was 0.443 mg/kg of BW in TE + SBH, 3.04 mg/kg of BW in TE + WSB, and 4.42 mg/kg of BW in TE + SBM. These doses were similar to those reported by Harlow et al. (2022) (3.39 mg/kg of BW; SBM) and greater than those reported by Davis et al. (2023) (0.47 mg/kg of BW; red clover). In both studies, supplementation with legumes (e.g., SBM and red clover) mitigated ergot alkaloid-induced vasoconstriction. It could be possible that partial attenuation of ergot alkaloid-induced vasoconstriction observed in steers receiving TE + SBM was mediated by the antimicrobial and/or vasodilatory properties of isoflavones. However, the mechanisms of vascular relaxation with isoflavones have not been well-studied in ruminants. One study did not observe vascular responses to isoflavones using isolated bovine mesenteric blood vessels (Jia et al., 2015).

Serotonin metabolism

Increased ruminal tryptophan availability with SBM supplementation could have occurred because of increased dietary supply of tryptophan from SBM and/or inhibition of ruminal amino acid-utilizing bacteria by isoflavones in SBM (Flythe et al., 2013; Harlow et al., 2017). Estimated total tryptophan intake was 3.28 g/d in both NTE and TE, 3.70 g/d in TE + SBH, 7.84 g/d in TE + WSB, and 9.30 g/d in TE + SBM in the current study. Estimates of tryptophan degradation by ruminal microbes are approximately 35% to 50% (Yokoyama and Carlson, 1974; Mohammed et al., 2003; Ma et al., 2012); however, ruminal tryptophan degradation is strongly inhibited by gram-negative bactericidal compounds such as monensin (Hammod and Carlson, 1980). More recently, Jardon et al. (2025) reported that duodenal tryptophan flow from a high-concentrate diet containing SBM was greater than dietary tryptophan intake in steers. Additionally, 89% to 94% of the net duodenal tryptophan flow from SBM-containing diets was not from microbial origin, suggesting that tryptophan was mostly ruminally undegradable. Therefore, it could be speculated that SBM may have increased postruminal tryptophan flow, and subsequent serotonin synthesis in enterochromaffin cells of the small intestine in the current study (Valente et al., 2020) resulting in the observed serum serotonin. The low degradability of tryptophan in the rumen, presence of monensin in the basal diet, increased supply of tryptophan from WSB and SBM, and increased concentrations of isoflavones with SBH, WSB, and SBM support this hypothesis. However, serum tryptophan concentrations were only numerically greater with soybean-containing treatments compared with NTE and TE.

The effects of SBM supplementation on the mitigation of ergot alkaloid-induced vasoconstriction could result from several factors including direct effects of serotonin on vascular relaxation, direct effects of isoflavones on vascular relaxation, or indirect effects of isoflavones on ruminal tryptophan metabolism. In prior experiments, ergot alkaloid ingestion decreased feed intake and serum or plasma serotonin concentration in cattle (Valente et al.,

2020, 2024a). Moreover, increasing exposure to ergot alkaloids increases vasoconstriction of isolated bovine peripheral blood vessels via activation of serotonergic receptors involved in contraction, 5-hydroxytryptamine receptor 2A (**HTR2A**) (Klotz et al., 2013; Trotta et al., 2018). Increasing serotonin concentration after ergot alkaloid exposure results in dose-dependent vasorelaxation of isolated bovine blood vessels (Trotta et al., 2023), which is mediated via the activation of serotonergic receptors involved in relaxation, 5-hydroxytryptamine receptor 4 (**HTR4**) (Trotta et al., 2024). Soybean meal supplementation increased serum serotonin concentration compared with TE by 1.42 μM. Previous research demonstrated that a 1 μM increase in extracellular serotonin concentration could elicit a maximal relaxation of isolated bovine peripheral blood vessels via HTR4 activation (Trotta et al., 2024). Overall, these data suggest that the increased vasodilation occurring with SBM supplementation during fescue toxicosis is partially mediated by increased serotonin.

One possible explanation for the increase in both serotonin and kynurenine with SBM supplementation would be a greater availability of tryptophan to the animal, despite no apparent increase in serum tryptophan concentration. Tryptophan is the sole precursor for serotonin synthesis, which occurs predominantly in the distal gastrointestinal tract (approximately 90%) and to a lesser extent in the central nervous system (about 10%) (Boadle-Biber et al., 1993). The rate-limiting step of serotonin synthesis is the hydroxylation of tryptophan to 5-hydroxytryptophan by tryptophan hydroxylase 1. This intermediate is then decarboxylated by aromatic L-amino acid decarboxylase to form serotonin (Roth et al., 2021). Importantly, serotonin synthesis competes with the kynurenine pathway. In healthy adult mammals, more than 95% of dietary tryptophan is catabolized primarily in the liver through the kynurenine pathway (Yao et al., 2011). Taken together, the increases in serum serotonin and kynurenine concentrations suggest that intestinal serotonin and hepatic kynurenine pathways were stimulated by SBM supplementation, which resulted in increased utilization of tryptophan.

Serum concentrations of 5-HTP and 5-HIAA tended to increase in steers receiving TE compared with NTE. In contrast, previous studies did not observe changes in serum 5-HTP or 5-HIAA concentrations in cattle challenged with TE (Valente et al., 2020, 2024a). 5-hydroxyindoleacetic acid is the primary product of serotonin catabolism, formed by the sequential actions of monoamine oxidase and aldehyde dehydrogenase, which metabolizes serotonin to 5-hydroxyindole acetaldehyde and then to 5-HIAA for urinary excretion (O'Mahony et al., 2015). Increased serum 5-HIAA concentration with TE suggests that ergot alkaloids may have increased serotonin catabolism in the current study. This may be partially responsible for the decreased serotonin observed in fescue toxicosis (Valente et al., 2020). The degradation of serotonin serves as a mechanism to inhibit serotonergic signaling and is regulated by monoamine oxidase activity (Scotton et al., 2019). Considering that exposure to ergot alkaloids causes vasoconstriction in bovine peripheral blood vessels via activation of HTR2A (Klotz et al., 2013; Trotta et al., 2018), we speculate that the increase in serotonin catabolism, evidenced by elevated 5-HIAA, may potentially represent an attempt to attenuate serotonergic activity resulting from persistent stimulation with increased ergot alkaloid interactions (Shi et al., 2008; Valente et al., 2021).

Feed intake behavior and susceptibility to heat stress

It is well-documented that heat stress and ergot alkaloid ingestion result in a reduction in feed intake (Klotz, 2015; Ahn et al., 2020; Valente et al., 2024a, 2024b). To avoid confounding the intake depression induced by heat stress and ergot alkaloids, all steers were exposed to identical environmental conditions and were pair-fed to the lowest DM intake observed from the previous day. Consequently, there were no differences in either basal diet DM intake or total diet DM intake among treatments. This ensures that the differences reported here are attributable to the nutritive value and/or bioactive compounds of soybean feeds, rather than indirect effects resulting from changes in voluntary feed intake.

Despite the absence of differences in feed intake, feeding behavior was influenced by ergot alkaloids and the inclusion of soy-based feeds. Steers receiving TE had a greater number of meals per day that tended to be shorter in duration and smaller in size compared with steers receiving NTE. Previous studies have reported an increase in number of meals (Ahn et al., 2020) and decreased grazing time (Schmidt and Osborn, 1993; Seman et al., 1997) in cattle exposed to ergot alkaloids. According to the theory of minimal discomfort in production animals (Forbes, 2007), the greater meal frequency observed in TE steers may represent an adaptive response to minimize discomfort associated with ergot alkaloid exposure. Notably, supplementation of SBH restored feeding behavior to a pattern comparable to NTE, whereas SBM supplementation produced an intermediate response. The mechanisms underlying the altered feeding behavior in steers receiving TE, as well as its restoration with soybean supplementation, require further investigation.

One of the key issues associated with ergot-induced vasoconstriction is the reduced ability of animals to thermoregulate, which can exacerbate the negative effects of heat stress (Hemken et al., 1981; Al-Haidary et al., 2001; Ferguson et al., 2021). Several studies have reported decreased skin temperature at peripheral sites in cattle exposed to ergot alkaloids under heat stress conditions (Al-Haidary et al., 1995; Browning et al. 1998; Poudel et al., 2025). In the current study, steers receiving TE exhibited decreased rump skin temperature compared with NTE, whereas TE + SBM supplementation increased rump skin temperature compared with TE steers. The decrease in rump skin temperature observed in TE steers could indicate impaired heat dissipation and may potentially be related to reduced peripheral blood flow. These findings may relate to previous research demonstrating that ergot alkaloid ingestion results in decreased whole-body heat production under fasting (Koontz et al., 2013) and low energy intake conditions (Koontz et al., 2014). Increased rump temperature with TE + SBM suggests that SBM supplementation during fescue toxicosis increased heat dissipation.

Markers of nutrient metabolism and inflammation

In the present study, steers receiving TE had increased plasma glucose concentrations compared with NTE steers. The effects of ergot alkaloids on plasma glucose concentration in cattle have been variable. Previous studies have reported either unchanged serum glucose (Oliver et al., 2000), increased plasma glucose (Browning, 2003; Eisemann et al., 2014; King et al., 2024), or decreased serum glucose (Jackson et al., 2015) in cattle challenged with ergot alkaloids. Such discrepancies may be attributed to differences in DM intake between NTE and TE treatments, the dose of ergot alkaloids, and the duration of exposure. Increased circulating glucose during fescue toxicosis might be attributed to reduced insulin sensitivity and possibly impaired glucose uptake by peripheral tissues (Ferguson et al., 2023; King et al., 2024). Ergot alkaloids from TE increase the hepatic expression of phosphoenolpyruvate carboxykinase, a key regulatory enzyme of gluconeogenesis (Brown et al., 2009). Therefore, increased plasma glucose concentration with TE in the current study might have resulted from greater gluconeogenesis, reduced insulin sensitivity, or a combination of factors. Greater plasma glucose concentration with TE may reflect increased mobilization of energy for use by peripheral tissues.

Additionally, we observed decreased plasma urea concentration in TE steers compared with NTE. Similarly, King et al. (2024) reported decreased plasma NEFA concentration in steers receiving increasing concentrations of ergovaline. Although a direct effect of ergot alkaloids on plasma urea and NEFA concentrations cannot be concluded from the present study, we speculate that the increased number of meals consumed throughout the day by TE steers may have resulted in a more distributed nutrient intake, thereby reducing peaks in urea and NEFA compared with NTE steers, which consumed their feed more rapidly. Among the steers supplemented with soybeans, plasma urea concentration was least in TE + SBH, intermediate

in TE + WSB, and greatest in TE + SBM. This reflects a direct effect of increased protein intake, due to the greater crude protein concentration of the soybean supplements.

Previous research in cattle has demonstrated that increased heat stress and nutrient restriction increase gastrointestinal permeability (Koch et al., 2019; Fontoura et al., 2022; Bertens et al., 2024). Because of the increased environmental temperature and decreased feed intake with fescue toxicosis in field conditions, it is reasonable to speculate that ergot alkaloids may exacerbate alterations in gut barrier function and intestinal permeability because of increased vasoconstriction of ruminal and mesenteric vasculature in cattle (Trotta et al., 2018; Trotta et al., 2023). Conversely, no differences were observed between TE and NTE groups regarding markers of inflammatory responses and immune system activation. It is likely that the fescue toxicosis challenge of the current study was not successful at eliciting an immune response. This is evidenced by: 1) a lack of change in positive inflammation markers, 2) a lack of change in negative inflammation markers, and 3) a lack of change in total-tract gastrointestinal permeability to Cr-EDTA. Reasons for the lack of response could be too short of a challenge period (7 d) to elicit an immune response or a lack of measurements at baseline to observe changes with the fescue toxicosis challenge. Despite the combined stress of ergot alkaloids, heat stress, and feed restriction, the effects of soybean feeds on decreasing systemic immune activation could not be adequately evaluated in the current study.

CONCLUSIONS

Dietary supplementation of soybean-based feeds has the potential to mitigate fescue toxicosis in cattle. Among the tested feeds (SBH, WSB, SBM), SBM was the most effective. Soybean meal supplementation mitigated ergot alkaloid-induced vasoconstriction and promoted vasodilation in steers challenged with ergot alkaloids from toxic endophyte-infected tall fescue seed. This was evidenced by increased caudal artery cross-sectional area, increased blood flow, decreased systolic blood pressure, and increased heart rate with SBM

supplementation during fescue toxicosis. Increased serum serotonin concentration may be associated with positive responses observed with SBM supplementation. More research is needed to investigate if the mitigation of ergot alkaloid-induced vasoconstriction with SBM is mediated by increased dietary supply of essential amino acids, isoflavones, or a combination of both compounds. This will aid in efforts to develop dietary strategies to mitigate fescue toxicosis in beef cattle and utilize widely available feedstuffs for functional inclusion in beef cattle diets.

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The authors declare no real or perceived conflict of interest.

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Author Contributions

Luiz C. O. de Sousa (Data Curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing), Brittany E. Davis (Conceptualization, Methodology, Resources, Writing – review & editing), David L. Harmon (Methodology, Resources, Supervision, Writing – review & editing), Ronald J. Trotta (Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing).

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Table 1. Composition of basal diet fed to steers.

Item	
Ingredient composition	
Corn silage, %	72.5
Dried corn distillers' grains with solubles, %	17.5
Finely ground corn, %	7.18
Limestone, %	1.76
Trace mineral premix, %[1]	0.46
Urea, %	0.33
Tallow, %	0.23
Vitamin A, D, & E premix, %[2]	0.02
Rumensin 90, %[3]	0.01
Tylan 40, %[4]	0.01
Chemical composition	
Dry matter, %	40.4
Total starch, % of DM	40.1
Neutral detergent fiber, % of DM	27.7
Acid detergent fiber, % of DM	16.2
Crude protein, % of DM	10.3
Crude fat, % of DM	4.17
Ca, % of DM	0.780
P, % of DM	0.290
Net energy for maintenance, Mcal/kg[5]	1.72
Net energy for gain, Mcal/kg[5]	1.10

[1]Contained: 56.34% Cl, 36.53% Na, 1.2% S, 0.06% Ca, 9.29 g Fe/kg, 5.52 g Zn/kg, 4.79 g Mn/kg, 1.84 g Cu/kg, 120 mg I/kg, 68.9 mg Co/kg, and 18.5 mg Se/kg on a DM basis.
[2]Composed of vitamin A acetate (1,814 kIU/kg), D-activated animal sterol (source of vitamin D_3; 363 kIU/kg), vitamin E supplement (227 IU/kg), roughage products, calcium carbonate, and mineral oil.
[3]Contained 199 mg monensin per gram of premix.
[4]Contained 18.1 g tylosin phosphate per kilogram of premix.
[5]Calculated according to NASEM (2016).

Table 2. Chemical composition, individual and total aglycone concentration of the basal diet, corn, and soybean feeds.

Item	Feed				
	Basal diet	Corn	Soybean hulls	Whole soybean	Soybean meal
Crude protein, % DM	10.3	9.30	12.1	33.1	50.0
NEg, Mcal/kg	1.10	1.53	0.70	1.81	1.42
Isoflavones, µg/g DM					
Biochanin A	<10	<10	<10	<10	<10
Formononetin	<10	<10	<10	<10	<10
Genistein	<10	<10	66.5	601	860
Daidzein	<10	<10	91.0	478	705
Total aglycones	<10	<10	157.5	1079	1565

Table 3. Effects of soybean hulls, whole soybeans, or soybean meal on feed intake and feeding behavior in steers receiving endophyte-infected tall fescue seed.

	Treatment[1]						*P*-value[3]		
Item	NTE	TE	TE + SBH	TE + WSB	TE + SBM	SEM[2]	Trt	Day	Day × Trt
Total DM intake, kg	4.47	4.34	4.33	4.32	4.38	0.322	0.84	<0.01	0.96
Basal diet DM intake, kg	3.37	3.26	3.24	3.23	3.31	0.269	0.80	<0.01	0.99
Supplement DM intake, kg	0.690	0.686	0.698	0.670	0.674	0.042	0.35	-	-
Fescue seed DM intake, kg	0.395	0.399	0.399	0.399	0.399	0.013	-	-	-
Number of meals	6.13[b]	7.24[a]	5.95[b]	7.28[a]	6.62[ab]	0.670	0.02	<0.01	0.16
Meal duration, min	24.9	20.8	23.8	20.2	23.4	3.16	0.47	<0.01	0.18
Meal size, kg as-fed	1.28	1.03	1.36	1.02	1.16	0.183	0.45	<0.01	0.35

[1]NTE, non-toxic endophyte; TE, toxic endophyte; TE + SBH, toxic endophyte + soybean hulls; TE + WSB, toxic endophyte + whole soybean; TE + SBM, toxic endophyte + soybean meal.
[2]SEM, standard error of the mean (n = 10 steers per treatment).
[3]Trt, main effect of treatment; Day, main effect of experimental day; Day × Trt: interaction of treatment with experimental day.
[a,b]Means within a row with different superscripts differ at $P < 0.05$.

Table 4. Effects of soybean hulls, whole soybeans, or soybean meal on caudal artery hemodynamics in steers receiving endophyte-infected tall fescue seed.

	Treatment[1]						*P*-value[3]		
Item	NTE	TE	TE + SBH	TE + WSB	TE + SBM	SEM[2]	Trt	Time	Time × Trt
Cross-sectional area, mm^2	19.1^{xy}	17.0^{z}	18.6^{xy}	18.1^{yz}	19.6^{x}	0.73	0.06	<0.01	0.92
Cross-sectional area, % change from baseline	6.51^{ab}	-8.62^{c}	2.42^{ab}	-0.378^{bc}	10.4^{a}	4.67	0.02	<0.01	0.70
Peak systolic velocity, cm/s	22.3	22.9	23.8	22.9	22.9	1.08	0.84	0.02	0.43
End diastolic velocity, cm/s	2.68	2.81	2.75	2.84	2.81	0.085	0.59	<0.01	0.44
Mean velocity, cm/s	10.2	10.2	10.8	10.6	10.6	0.47	0.77	<0.01	0.39
Resistance index	0.874	0.873	0.876	0.869	0.877	0.007	0.84	0.08	0.78
Pulsatility index	1.93^{ab}	1.96^{a}	1.97^{a}	1.85^{c}	1.90^{bc}	0.026	0.01	<0.01	0.66
Heart rate	70.2^{b}	71.8^{b}	70.6^{b}	77.9^{a}	73.8^{ab}	3.06	0.02	<0.01	0.06
Blood flow, mL/min	121	106	121	113	123	8.05	0.23	<0.01	0.47
Blood flow, % change from baseline	1.73^{xyz}	-7.91^{z}	4.38^{xy}	-1.38^{yz}	12.9^{x}	6.69	0.08	<0.01	0.08
Stroke volume, mL/beat	1.71	1.51	1.72	1.51	1.66	0.161	0.12	<0.01	0.38
Stroke volume, % change from baseline	0.145	0.025	0.135	0.036	0.178	0.101	0.18	0.015	0.32

[1]NTE, non-toxic endophyte; TE, toxic endophyte; TE + SBH, toxic endophyte + soybean hulls; TE + WSB, toxic endophyte + whole soybean; TE + SBM, toxic endophyte + soybean meal.

[2]SEM, standard error of the mean (n = 10 steers per treatment).

[3]Trt, main effect of treatment; Time, main effect of time; Time × Trt: interaction of time with treatment.

[abc]Means within a row with different superscripts differ at $P < 0.05$.

[xyz]Means within a row with different superscripts differ at $P < 0.10$.

Table 5. Effects of soybean hulls, whole soybeans, or soybean meal on physiological stress indicators in steers receiving endophyte-infected tall fescue seed.

	Treatment[1]						
Item	NTE	TE	TE + SBH	TE + WSB	TE + SBM	SEM[2]	*P*-value
Rectal temperature, ºC	39.4	39.2	39.3	39.3	39.2	0.149	0.38
Neck temperature, ºC	37.2	36.9	37.0	37.0	37.1	0.226	0.64
Rump temperature, ºC	37.0^{a}	36.6^{b}	36.9^{ab}	36.8^{ab}	37.1^{a}	0.217	0.02
Respiration rate, breaths/min	72.4	78.8	74.4	85.1	84.2	7.89	0.24
Systolic pressure, mm Hg	95.6^{yz}	104^{x}	102^{xy}	98.6^{xyz}	93.0^{z}	4.42	0.06
Diastolic pressure, mm Hg	46.6	48.5	46.2	46.1	45.8	2.87	0.67
Heart rate, beats/min	87.2^{a}	82.4^{b}	80.0^{b}	90.6^{a}	91.3^{a}	3.95	<0.01

[1]NTE, non-toxic endophyte; TE, toxic endophyte; TE + SBH, toxic endophyte + soybean hulls; TE + WSB, toxic endophyte + whole soybean; TE + SBM, toxic endophyte + soybean meal.
[2]SEM, standard error of the mean (n = 10 steers per treatment).
[ab]Means within a row with different superscripts differ at $P < 0.05$.
[xyz]Means within a row with different superscripts differ at $P < 0.10$.

Table 6. Effects of soybean hulls, whole soybeans, or soybean meal on serum concentrations of tryptophan metabolites in steers receiving endophyte-infected tall fescue seed.

	Treatment[1]						
Item	NTE	TE	TE + SBH	TE + WSB	TE + SBM	SEM[2]	*P*-value
Tryptophan, μM	19.4	21.0	21.8	23.3	24.5	2.51	0.28
Kynurenine, μM	5.81^{c}	5.81^{c}	6.78^{ab}	6.32^{bc}	7.24^{a}	0.54	0.03
5-hydroxytryptophan, μM	0.133^{y}	0.163^{x}	0.159^{x}	0.155^{x}	0.155^{x}	0.014	0.10
Serotonin, μM	10.2^{ab}	9.78^{b}	8.33^{c}	9.84^{b}	11.2^{a}	1.03	0.01
5-hydroxyindoleacetic acid, μM	0.367^{y}	0.409^{x}	0.393^{x}	0.398^{x}	0.390^{xy}	0.029	0.09

[1]NTE, non-toxic endophyte; TE, toxic endophyte; TE + SBH, toxic endophyte + soybean hulls; TE + WSB, toxic endophyte + whole soybean; TE + SBM, toxic endophyte + soybean meal.

[2]SEM, standard error of the mean (n = 10 steers per treatment).

[abc]Means within a row with different superscripts differ at $P < 0.05$.

[xy]Means within a row with different superscripts differ at $P < 0.10$.

Table 7. Effects of soybean hulls, whole soybeans, or soybean meal on metabolic and inflammatory biomarkers in steers receiving endophyte-infected tall fescue seed.

	Treatment[1]						
Item	NTE	TE	TE + SBH	TE + WSB	TE + SBM	SEM[2]	*P*-value
Metabolic markers							
Glucose, mM	3.73^{b}	4.13^{a}	4.09^{a}	4.23^{a}	4.29^{a}	0.201	0.02
Non-esterified fatty acids, mM	99.8	101	111	125	96.4	16.5	0.25
β-hydroxybutyrate, mM	0.881^{a}	0.627^{b}	0.706^{b}	0.704^{b}	0.712^{b}	0.058	0.02
Urea, mM	1.40^{d}	1.04^{e}	1.70^{c}	3.00^{b}	3.29^{a}	0.21	<0.01
Positive inflammatory markers							
Amyloid A, µg/mL	69.2^{yz}	57.3^{z}	83.4^{xy}	82.4^{xyz}	98.2^{x}	32.2	0.08
Haptoglobin, µg/mL	104	194	155	74.1	87.2	93.0	0.31
Negative inflammatory markers							
Albumin, g/dL	2.85	2.80	2.83	2.82	2.87	0.075	0.88
Bilirubin, mg/dL	0.725^{bc}	0.797^{ab}	0.859^{a}	0.776^{ab}	0.675^{c}	0.059	0.03
Cholesterol, mg/dL	68.9^{bc}	68.3^{c}	85.3^{a}	75.7^{ab}	70.7^{bc}	4.77	0.01

[1]NTE, non-toxic endophyte; TE, toxic endophyte; TE + SBH, toxic endophyte + soybean hulls; TE + WSB, toxic endophyte + whole soybean; TE + SBM, toxic endophyte + soybean meal.

[2]SEM, standard error of the mean (n = 10 steers per treatment).

[abc]Means within a row with different superscripts differ at $P < 0.05$.

[xyz]Means within a row with different superscripts differ at $P < 0.10$

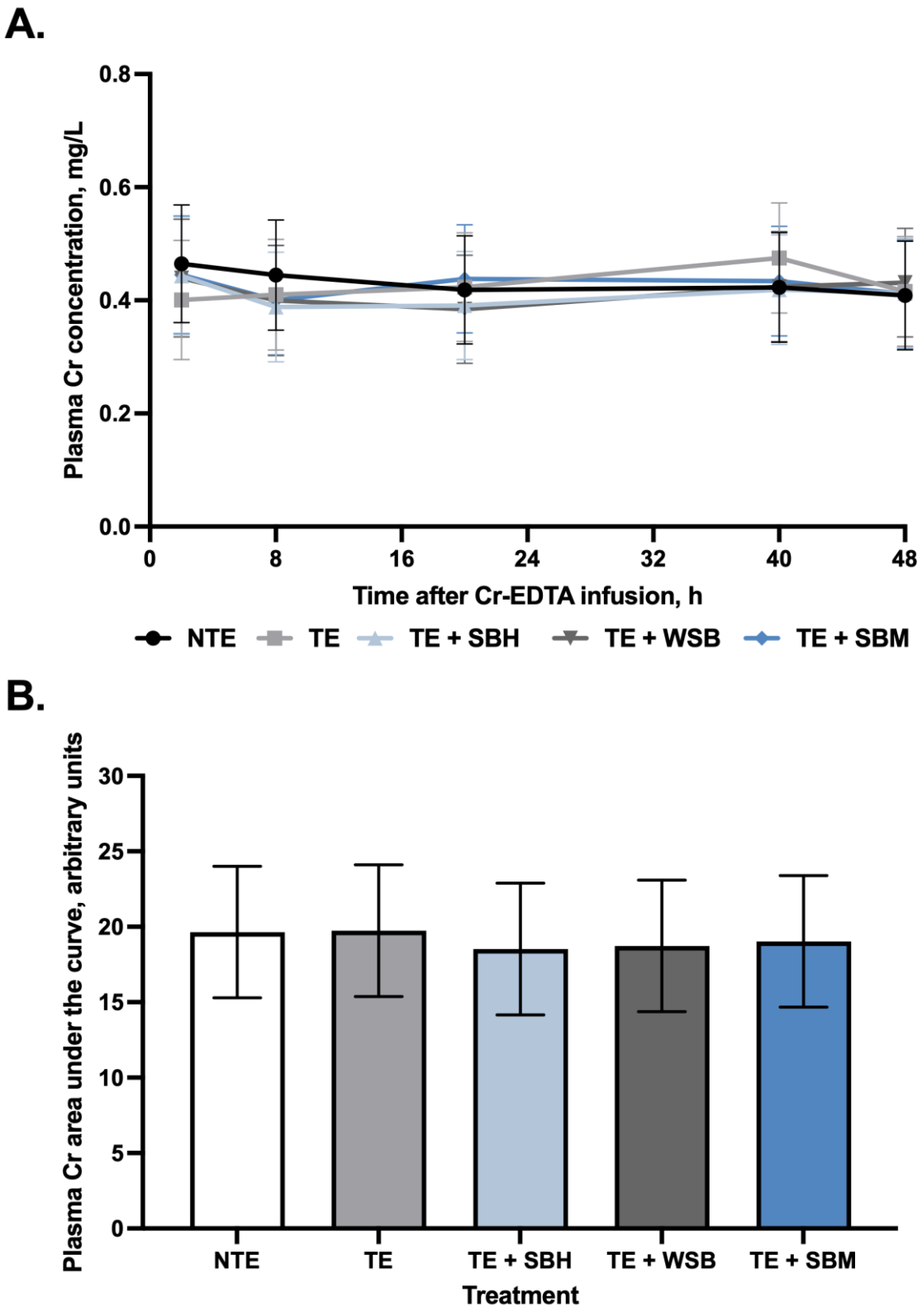
A.
Plasma Cr concentration, mg/L
0.0
0.2
0.4
0.6
0.8
0
8
16
24
32
40
48
Time after Cr-EDTA infusion, h
NTE
TE
TE + SBH
TE + WSB
TE + SBM
B.
Plasma Cr area under the curve, arbitrary units
0
5
10
15
20
25
30
NTE
TE
TE + SBH
TE + WSB
TE + SBM
Treatment

Figure 1.

General Conclusions

Based on the findings obtained in this thesis, we conclude:

1) Guanidinoacetic acid supplementation decreases skeletal muscle mobilization and enhances the placental vascularization of beef cows during late gestation. However, providing dietary GAA to beef cows at late gestation does not affect the performance of their offspring. Importantly, GAA supplementation may induce a deficiency of methyl groups, potentially attenuating the benefits of improved placental blood flow;

2) Incubating samples within filter bags compromises *in vitro* digestibility, gas production, and fermentation characteristics regardless of particle size. These effects are more pronounced in forage feeds than concentrate feeds. Future research should focus on elucidating the compositional and functional differences in microbial communities residing inside the filter bags compared with those in the surrounding incubation medium and the impact of restricting protozoa access on feedstuff evaluation; and

3) Soybean meal supplementation mitigates ergot alkaloid-induced vasoconstriction and promotes vasodilation in steers challenged with ergot alkaloids from toxic endophyte-infected tall fescue seed. Further research is warranted to determine whether this mitigation is driven by the increased dietary supply of essential amino acids, isoflavones, or their combined effects. Elucidating these mechanisms will support the development of nutritional strategies to alleviate fescue toxicosis and optimize the functional use of widely available feedstuffs in beef cattle diets.