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ABUNDANCE OF *Succinivibrionaceae* BACTERIA IN THE FECES OF CALVES FED TANNINS, AMINO ACIDS, AND THEIR COMBINATION

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Abstract

The purpose of this study was to determine how common *Succinivibrionaceae* bacteria are in calf feces. Four female calves, with an average age of 6.48 ± 0.51 months, participated in a Latin square trial featuring four dietary treatments: control (C), tannin (T), amino acid (AA), and a tannin-amino acid mixture (TM). The duration of each treatment was 14 days, whereby faecal samples were harvested on day 14. The 14-day treatment trial included the collection of faecal samples on day 14 for the extraction of DNA to conduct Next-Generation Sequencing (NGS) analysis. Results showed that the *Succinivibrionaceae* population grew extensively when treated with T, AA, and TM compared to C, with AA being the most effective. Analysis of the detected bacteria revealed five genera and five species. The bacterial genus *Ruminobacter* dominated in the C (53%), AA (62%), and TM (58%) treatment groups, while *Succinivibrio* dominated the T treatment group (78%). The species *Ruminobacter amylophilus* dominated treatments C, AA, and TM, but the species *Succinivibrio dextrinsolvens* dominated treatment T. The results suggest that the addition of tannins, amino acids, or their combination to the gut environment may help reduce methane emissions by altering microbiome composition.

Keywords: amino acids, calve, methane, *Succinivibrionaceae*, tannin

KELIMPAHAN BAKTERI Succinivibrionaceae PADA FESES PEDET YANG DIBERI PAKAN TANIN, ASAM AMINO, DAN CAMPURANNYA

Abstrak

Penelitian ini bertujuan untuk menentukan kelimpahan bakteri Succinivibrionaceae pada feses pedet. Objek penelitian menggunakan empat ekor anak sapi betina berusia 6,48 ± 0,51 bulan. Rancangan penelitian menggunakan rancangan bujur sangkar latin dengan empat perlakuan diet: kontrol (C), tanin (T), asam amino (AA), dan campuran tanin-asam amino (TM). Setiap pedet mendapat perlakuan selama 14 hari per periode dan sampel feses dikumpulkan pada hari ke-14 setiap periode. Ekstraksi DNA pada sampel feses dan dilakukan analisis metagenomik dengan metode Next-Generation Sequencing (NGS). Hasil sekuensing menunjukkan bahwa perlakuan T, AA, dan TM meningkatkan kelimpahan bakteri Succinivibrionaceae dibandingkan dengan perlakuan C, dengan kelimpahan tertinggi pada perlakuan AA. Terdapat lima genus dan lima spesies bakteri Succinivibrionaceae yang berhasil diidentifikasi. Genus Ruminobacter mendominasi pada perlakuan C (53%), AA (62%), dan TM (58%), sementara genus Succinivibrio mendominasi pada perlakuan T (78%). Pada tingkat spesies, Ruminobacter amylophilus mendominasi pada perlakuan C (53%), AA (62%), dan TM (58%), sedangkan Succinivibrio dextrinsolvens mendominasi pada perlakuan T (78%). Hasil penelitian menunjukkan penambahan tanin, asam amino, atau kombinasi keduanya dapat mengurangi produksi metana dengan memodulasi komposisi mikrobiota usus.

Kata Kunci: asam amino, metan, pedet, Succinivibrionaceae, tanin

INTRODUCTION

Practices that are good for the environment must support sustainable livestock farming. Livestock farming is a significant cause of the greenhouse effect because ruminant animals release methane gas. Animal agriculture contributes significantly to greenhouse gas emissions, accounting for 14.5% of global emissions, which is comparable to the transportation sector

(Kristiansen *et al.,* 2020). Methane production significantly impacts the environment and causes an animal to lose 2% to 12% of the gross energy consumed, which is converted to enteric CH4 during ruminal digestion, contributing approximately 6% of global anthropogenic greenhouse gas emissions (Beauchemin *et al.,* 2020). To effectively combat methane production, it is essential to implement targeted management strategies that incorporate innovative feed additives and supplements. By doing so, we can significantly mitigate environmental impacts and promote sustainable practices in livestock management.

Tannin is a commonly used feed additive in animal nutrition. Tannins are secondary polyphenolic metabolites that plants make on their own. They exhibit numerous bioactive effects, including antibacterial, antioxidant, anthelmintic, anti-inflammatory, immunomodulatory, and anti-diabetic properties (Esmaeili *et al*., 2021). Adding tannins to calf feeds as feed additives has been demonstrated to increase growth rates and reduce the incidence of diarrhea (Liepa *et al*., 2018). Tannins also directly prevent methane production, inhibit protein degradation, and indirectly decrease the amount of hydrogen (H_2) needed for methanogenesis (Hassan *et al*., 2020). Some research, however, has discovered that tannins at high levels can reduce the number of fibrolytic bacteria in the rumen, which would make fiber less digestible (Battelli *et al*., 2024).

Methionine and lysine are the two most important amino acids for the growth and development of weaned calves. However, these amino acids break down very easily in the rumen, which renders absorption less efficient (Schwab & Broderick, 2017). Tannins are capable of binding to proteins, and when combined with amino acids, they can prevent these nutrients from breaking down in the rumen. Nevertheless, less research has been conducted on this interaction. Amino acid supplements have been shown to encourage the development of fibrolytic bacteria (Teklebrhan & Tan, 2022).

The gut microbiota comprises a diverse array of microorganisms, including bacteria, viruses, fungi, and archaea. This microbial ecosystem plays a crucial role in digestion, metabolism, immune function, and overall health. Research suggests that the gut microbiome may interact with immune functioning, health, and growth in calves (Amin & Seifert, 2021). The microbiome may also assist in methane generation in ruminants (Kamke *et al*., 2016). Several earlier studies have proposed that bacteria from the family *Succinivibrionaceae* may be indicators of low methane-emitting animals (Wallace *et al*., 2015). The increased abundance of such bacteria was associated with better digestion efficiency and less diarrhea in ruminants. However, little is known about the abundance of these bacteria in ruminant feces in response to dietary interventions with feed additives and feed supplements. Determining the effect of these feed additives on gut microbial communities, particularly in relation to the level of *Succinivibrionaceae*, may aid in formulating nutritional strategies to enhance animal performance and minimize environmental impact. Hence, this study aimed to investigate the prevalence of *Succinivibrionaceae* in the feces of calves under diets with feed additives or feed supplements, such as tannins, amino acids, and their combinations.

MATERIALS AND METHODS

The Animal Care and Use Committee of the Ethical Clearance, BRIN issued Number 004/KE.02/SK/01/2024 to approve all experimental procedures. The research took place between January and June 2024 at two sites, including PT Sumber Citarasa Alam dairy farm in Ciawi, Bogor Regency, and the Genomics and Environment Laboratory of KST Soekarno, located in Cibinong, Bogor Regency.

Experimental Animals and Diets

Four Friesian Holstein post-weaned heifer calves (109.00±18.17 kg of live weight, aged 6 to 7 months) were used for the experiment conducted at PT Sumber Citarasa Alam, Ciawi, Bogor, Indonesia. The dairy company PT Sumber Citarasa Alam also provided the total mixed ration (TMR) that the calves received as their daily feed. Every calf received 20 kilograms of forage and 2 kilograms of concentrate through the TMR mixture. The TMR provided contained 5.90 kg dry matter (DM). Tannin, amino acids, and a mixture of tannin and amino acids were added on top and then mixed into the TMR. The tannin used was a commercial extract from the

Chestnut tree (Hydrolyzable tannin, Saviolife, Italy), while the amino acids were DL-methionine and L-lysine (PT Cheil Jedang, Indonesia). The study tested four different diets: (1) a control group eating TMR without any extras; (2) TMR with tannin at 1% of dry matter; (3) TMR with methionine (0.3% of dry matter) and lysine (0.7% of dry matter); and (4) TMR with a mix of tannin (1%), methionine (0.3%), and lysine (0.7%). The formulas for the diets were based on the dry matter requirements listed in the 2001 NRC Nutrient Requirements of Dairy Cattle. The dosage of tannin and amino acids used is based on references from previous studies (Mazinani *et al*., 2020; Liu *et al*., 2013)

Maintenance and Sample Collection

We first recorded the weight of the calves at the beginning of each testing period. All treatments ran for two weeks. For the analysis of gut microbiota, we sampled fecal matter using rectal swabs, a non-invasive and easy method for microbiota collection. The swabs were subsequently transferred into tubes containing 10% glycerol and stored at -80°C until processing was completed. Fecal samples were collected on the final day of each treatment period, i.e., on day 14.

DNA Extraction and Metagenomic Sequencing Using Next-Generation Sequencing (NGS)

DNA was extracted from calf fecal samples using the Total DNA Mini Kit (Blood/Cultured Cell) from Geneaid, following the manufacturer's protocol. The concentration and purity of the extracted DNA were measured with a NanoPhotometer from IMPLEN, Germany. The DNA samples were then pooled by their treatment groups and subjected to next-generation sequencing (NGS) targeting the full-length 16S rRNA gene using Oxford Nanopore Technologies (ONT). This was followed by bioinformatics analysis. The primers used for amplification were 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Bahram *et al.,* 2018), which targeted an approximately 1,500 bp region.

DNA concentration was measured with both NanoDrop spectrophotometers and a Qubit fluorometer. We prepared libraries using kits from Oxford Nanopore Technology. We performed nanopore sequencing using MinKNOW software version 24.02.16. Basecalling was performed using Dorado version 7.3.11 with the high-accuracy model (Wick *et al*., 2019). We visualized the quality of FASTQ files using NanoPlot and filtered the quality with NanoFilt (de Coster *et al*., 2018; Nygaard *et al*., 2020). We classified filtered reads using the Centrifuge classifier (Kim *et al*., 2016). We built a bacteria index using the NCBI 16S RefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/).

RESULTS AND DISCUSSION

Abundance of *Succinivibrionaceae* Identified in Calves' Feces

Succinivibrionaceae is a family of bacteria in the phylum *Proteobacteria*. Some members, like *Succinivibrio dextrinosolvens*, are known to indicate low methane production in ruminants (Wallace *et al*., 2015; Hailemariam *et al.,* 2020). These bacteria are key producers of succinate in the rumen. Succinate is a precursor to propionic acid, which competes with the methanogenesis pathway for the use of hydrogen (H_2). As a result, a higher population of *Succinivibrionaceae* is associated with lower methane production, as hydrogen is redirected toward forming propionic acid instead of methane (Kamke *et al*., 2016; Ramayo-Caldas *et al*., 2020).

A metagenomic analysis using next-generation sequencing (NGS) on calf fecal samples showed that *Succinivibrionaceae* increased in response to diets with feed additives. The relative abundance of *Succinivibrionaceae* in C, T, AA, and TM was 0.20%, 0.31%, 0.57%, and 0.39%, respectively, from all bacteria that had been identified. from all bacteria that had been identified.

Figure 1 presents the abundance of *Succinivibrionaceae* bacteria. The AA treatment had the highest abundance (434 Operational Taxonomic Units [OTUs]), followed by the TM treatment (307 OTUs) and the T treatment (249 OTUs). The control (C) treatment showed the lowest abundance (143 OTUs).

The rise in *Succinivibrionaceae* observed in the T, AA, and TM treatments compared to the control suggests that supplementing feed can alter the rumen microbial profile. This shift can lead to more efficient fermentation and decrease methane gas production. The AA treatment showed the highest levels of *Succinivibrionaceae*. The provision of these amino acids will stimulate methionine and lysine-resistant rumen microorganisms, especially *Succinivibrionaceae*, which break down complex carbohydrates into succinate. It is essential to note that these amino acids may enhance the activity of fermentative bacteria and regulate nitrogen balance within the rumen (Teklebrhan & Tan, 2022). This increase may significantly lower methane emissions.

Tannin supplementation showed more complex interactions. At the applied dose, tannins did not seem to inhibit *Succinivibrionaceae* and may have even created a more supportive environment for them compared to the control group. This aligns with the selective antimicrobial properties of tannins, which can inhibit methanogenic archaea (Fagundes *et al.*, 2020). Hassan *et al.* (2020) found that tannins, at specific doses, can reduce methanogenesis by shifting the rumen microbial population toward succinate-producing bacteria.

Results from the TM group suggest that the effect of amino acids in increasing *Succinivibrionaceae* may be slightly reduced by the presence of tannins. However, their abundance remained higher than in the control and tannin treatments. This suggests a potential interaction between tannins and amino acids in influencing the microbiota. While tannins may slightly inhibit certain metabolic processes or nutrient absorption in bacteria, the positive effects of amino acids appear to be more significant.

Relative Abundance of *Succinivibrionaceae* in Calf Feces at the Genus and Species Levels

In calf feces, five main genera were found in the family *Succinivibrionaceae*: *Ruminobacter, Succinivibrio, Succinimonas, Succinatimonas*, and *Anaerobiospirillum*. *Ruminobacter* was the most prevalent in the C (53%), AA (62%), and TM (58%) treatments. In contrast, *Succinivibrio* dominated the T (78%) treatment within the *Succinivibrionaceae* group (Figure 2). *Succinivibrio* and *Ruminobacter* are known as amylolytic bacteria, which can ferment carbohydrates, mainly starch, into succinate, their primary product. Succinate is a crucial precursor for propionate production in the rumen, providing an energy source for calves. Converting succinate into propionate also lowers methane production (McCabe *et al.*, 2015).

High levels of *Succinivibrio* in the T treatment may be associated with reduced methane production and the prevention of diarrhea in calves. *Succinivibrio* can compete with harmful bacteria in the gut, such as *E. coli* and *Salmonella*, making it harder for them to establish infections and cause issues like diarrhea. In this study, the abundance of *E. coli* was lower in the T and TM treatments (1% and 1% of *Enterobacteriaceae*, or 10% and 9% of *Escherichia*, respectively) compared to the C treatment (6% of *Enterobacteriaceae*, or 50% of *Escherichia*). Higher succinate production, supported by increased levels of *Succinivibrio*, is associated with increased propionate production and thus less hydrogen available for methane formation. According to Lee *et al.* (2024), Succinivibrio showed a negative relationship to methane yield for both sheep and dairy cattle. This finding was in agreement with prior studies that tannins inhibit methanogenic archaea but stimulate certain fermentative bacteria, such as *Succinivibrio* (Pérez-Ruchel *et al.*, 2023).

High numbers of *Ruminobacter* in the AA and TM treatments suggest possible improvement in carbohydrate fermentation and propionate production as a result of increasing methionine and lysine in the diets (Wang *et al.*, 2024). Providing amino acids in the diet meets the nutritional requirement of *Ruminobacter*, thereby favoring its growth compared to *Succinivibrio* in that environment. This suggests that introducing amino acids into the starch-digesting bacterial community can alter the growth preferences of the community. The TM treatment exhibited a more balanced mix of microbes, with a higher proportion of *Ruminobacter* and *Succinivibrio*, highlighting the potential benefits of combining tannins and amino acids in maintaining diversity among fermentative microbes. The lack of a dominant genus might suggest a stable microbiota, which could support the digestive health of calves.

At the species level, five bacteria were identified in the family *Succinivibrionaceae*: *Ruminobacter amylophilus, Succinivibrio dextrinosolvens, Succinimonas amylolytica, Succinatimonas hippei*, and *Anaerobiospirillum thomasii* (Figure 3). *R.*

amylophilus was the main species in the C, AA, and TM treatment groups, with relative abundances of 53%, 62%, and 58%, respectively. Conversely, a significant shift occurred in the T treatment group, where *S. dextrinosolvens* was dominant, making up 78% of the *Succinivibrionaceae* population.

The study's results suggest that tannin supplementation significantly boosts the growth of *S. dextrinosolvens*. This rise in *S. dextrinosolvens* coincides with a drop in *R. amylophilus*, indicating possible competitive interactions between these two species. *S. dextrinosolvens* is key in redirecting the hydrogen metabolic pathway to the propionate pathway, which reduces the hydrogen available for methane-producing microbes and lowers methane gas output (Kamke *et al.,* 2016). According to a study by Haelamariam *et al*. (2020), one of the primary studies focused on *S. dextrinosolvens* in the rumen to investigate its ability to reduce methane emissions by increasing propionate levels. Based on the current study, this argument is further supported by the role played by tannins in increasing the level of essential bacteria, such as *S. Dextrinosolvens,* which help reduce methane levels.

Some of the effects of amino acid supplementation include a unique pattern of modification of the microbes, increasing the prevalence of *R. amylophilus* and *S. amylolytica* while decreasing the levels of *S. dextrinosolvens*, another notable feature of amino acid's microbial effects. The effects of amino acids appear to focus on enhancing microbial proliferation and nutrient utilization, thereby having indirect effects on methane through improved rumen fermentation efficiency and increased microbial protein synthesis. Supplementation with an amino acid, such as lysine or methionine, enhances microbial growth and boosts fermentation of carbohydrates (Mazinani *et al*., 2020). Although producing succinate, *R. amylophilus* is an important starch-fermenting bacteriumits; however, the effect on methane reduction is relatively insignificant compared to that of *Succinivibrio*.

The resultant microbial profiling from the combination of tannins and amino acids is complex. Amino acids seem to lessen the strongly stimulating effect of tannins on *S. dextrinosolvens*, while also enhancing *S. amylolytica* to the highest levels observed. This combined effect creates a balanced and intricate relationship, protecting dietary proteins from excessive breakdown by tannins while ensuring that sufficient amino acids are available for healthy microbial growth and function.

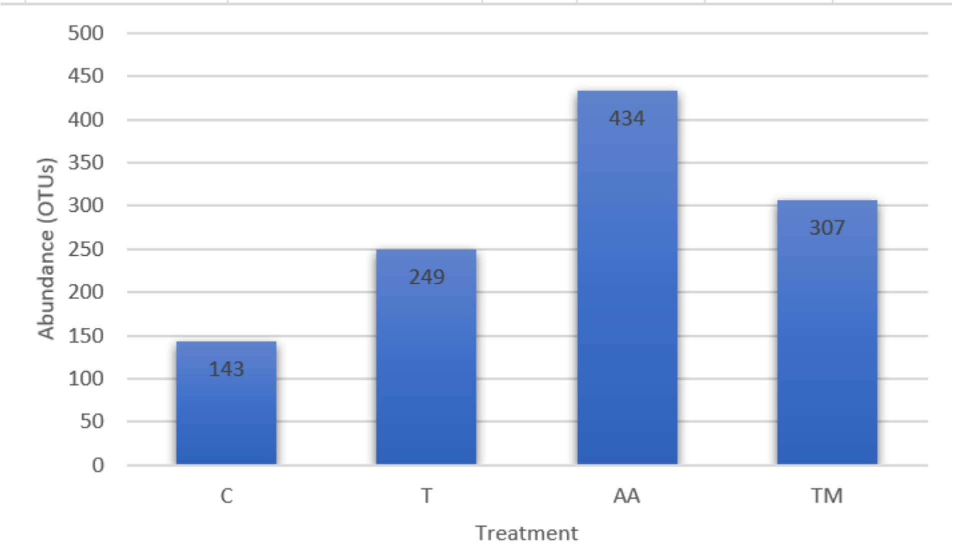
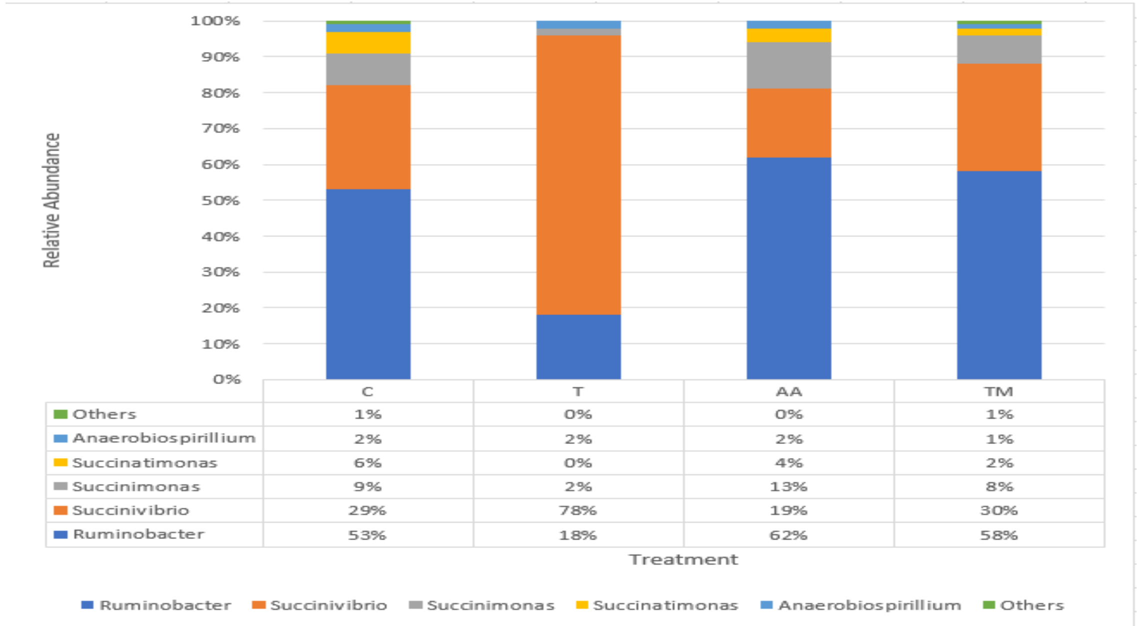


Figure 1. Abundance of *Succinivibrionaceae* Identified in Feces of Calves Fed Control (C), Tannin (T), Amino Acids (AA), and mixture of tannin and amino acids (TM).



	C	T	AA	TM
■ Others	1%	0%	0%	1%
■ Anaerobiospirillium	2%	2%	2%	1%
■ Succinatimonas	6%	0%	4%	2%
■ Succinimonas	9%	2%	13%	8%
■ Succinivibrio	29%	78%	19%	30%
■ Ruminobacter	53%	18%	62%	58%

Figure 2. Relative Genus-Level Abundance of *Succinivibrionaceae* in Feces of Calves Fed Control (C), Tannin (T), Amino Acids (AA), and mixture of tannin and amino acids (TM).

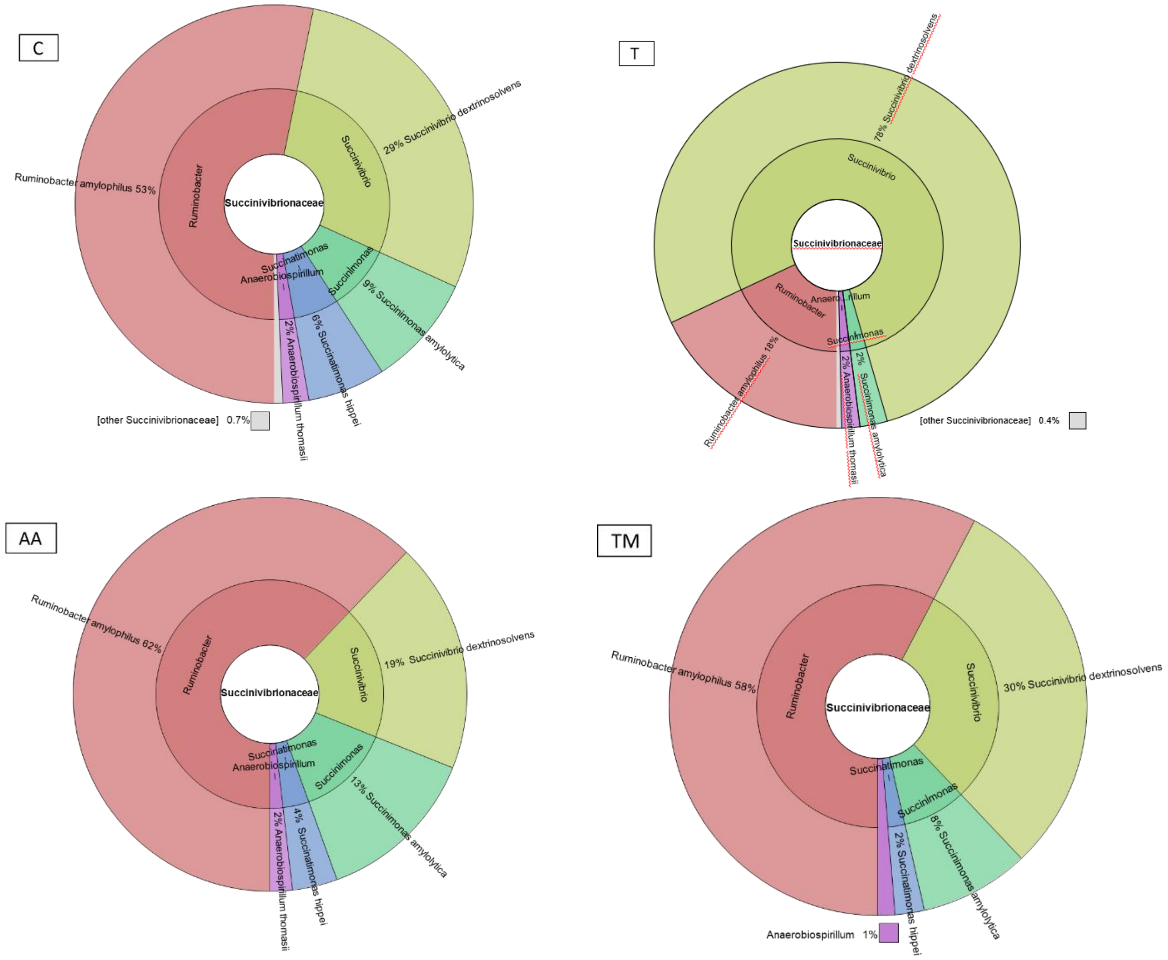


Figure 3. Species-Level Abundance of *Succinivibrionaceae* in Feces of Calves Fed Control (C), Tannin (T), Amino Acids (AA), and a mixture of tannin and amino acids (TM).

CONCLUSIONS

The addition of tannin, amino acids (lysine and methionine), and their mixture to calf feed increases the abundance of *Succinivibrionaceae* bacteria, which is associated with an indirect reduction in methane production by calves. *Succinivibrio* and *Ruminobacter* were the main genera in all treatments, with different relative abundances. At the species level, *S. dextrinosolvens* and *R. amylophilus* were prevalent in all treatments, with varying proportions. *Succinivibrionaceae* bacteria, identified by their metabolic activity, can significantly reduce methane production in calves.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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