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Characterization of organic milk production systems in brazil and the effect of a feed additive on intake, milk yield, and methane emission of holstein or holstein×gyr crossbred dairy cows.

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Magister Scientiae

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AMANDA BARBOSA SANT'ANA

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Co-advisers: Polyana Pizzi Rotta
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ABSTRACT

SANT'ANA, Amanda Barbosa, M.Sc., Universidade Federal de Viçosa, February, 2024. **Characterization of organic milk production systems in brazil and the effect of a feed additive on intake, milk yield, and methane emission of holstein or holstein×gyr crossbred dairy cows.**. Adviser: Alex Lopes da Silva. Co-advisers: Polyana Pizzi Rotta and Fernanda Samarini Machado.

This dissertation was composed by two different studies, where the aim of the first study was to characterize organic milk production systems in Brazil and the aim of the second study was to evaluate the effect of a dietary supplement based on condensed tannins on the intake, milk yield, and methane (CH4) emission of Holstein or Holstein×Gyr crossbred lactating dairy cows. In the first study, a descriptive analysis was carried out in which the variables were geographical location, herd size, animal production, feed used, health and reproduction management, organic inputs used, feed production management, and transportation of products. The characteristics of the systems were evaluated according to the level of production, with farmers divided into 3 groups, with the upper extract comprising farms with an average production of over 16 L/cow per day (HIG), the medium extract with a production between 10.5 and 16 L/cow per day (MED) and the lower extract with an average production of less than 10.5 L/cow per day (LOW). The variables were subjected to binomial logistic regression and comparisons were made by odds ratio. The average area of the properties was 107 ha (minimum 3 ha and maximum 1,450 ha), the area for organic milk production was 44 ha (minimum 1 ha and maximum 550 ha). The average daily milk production was 645 L/day (minimum of 12 L/day and maximum of 5,000 L/day), with an average production of 13 L/cow per day (minimum of 4 L/cow per day and maximum of 25 L/cow per day). The herds have an average of 58 cows (minimum 2 cows and maximum 310 cows) and 40 lactating cows (minimum 2 and maximum 255 cows). The average annual production was 7,517 L/ha per year (minimum 21 L/ha per year and maximum 29,877 L/ha per year). The average number of family workers was 2 (minimum 2 and maximum 7), the average number of external workers was 3 (minimum 2 and maximum 16). The HIG and MED farms were found to be 90% less likely to produce cheese. In addition, HIG and MED farms were 10.7 and 6.6 times more likely to have Holstein×Jersey crosses in their herd, respectively. The MED farms were 80% less likely to have Urochloa spp pastures, while HIG farms were 93.2% less likely to have Urochloa spp pastures and 92% less likely to use chopped grass to supplement the herd. However, the odds of having Megathyrsus maximus pastures was 4.66

times greater for HIG. In addition, HIG farms were 4.5 times more likely to use any type of management software. The analysis of the distribution of certified organic dairy farms revealed a significant concentration in the Southeast region of Brazil. The distribution of certified organic dairy farms shows a concentration in the southeastern region of Brazil, where production is mainly focused on milk, while other regions have more diversified organic production. The HIG farms are more likely to use specialized cattle breeds, herd supplementation, pastures formed by higher-yielding forage species with greater nutritional value, and management software. These results emphasize the need for public policies that promote the adoption of technological and sustainable practices to increase the efficiency and productivity of the organic dairy sector. Concerning the second study, sixteen dairy cows (8 Holstein and 8 Holstein×Gyr) were allocated to two treatments: a control group (CON; no supplementation) and a supplemented group (SUP) that received 2.7 g/kg DM of the additive (Muucare Nature®). The trial lasted 84 days, including a 24-day adaptation period. The animals were housed in tie stalls and kept under identical conditions. Rumen fluid was collected via an esophagus on days 22 and 52, and the microbial composition was later analyzed by 16S rRNA sequencing. The VFA concentrations and rumen ammonia nitrogen were also quantified. Methane emission was measured using the sulfur hexafluoride tracer technique. The additive increased the apparent digestibility of DM and OM by about 6% without affecting feed intake or milk yield. Methane emission per ECM (g/kg) was reduced by almost 30%, and methane emission per milk yield (g/kg) showed a similar trend. The SUP cows showed higher propionate production and a lower acetate-to-propionate ratio, indicating a shift in fermentation. The microbial diversity in the rumen was altered, with a reduced alpha diversity and a different community composition, including an increased abundance of Prevotella ruminicola. The total amount of methanogens was unchanged, although one species, Methanobrevibacter smithii, tended to be less abundant. The additive reduced methane emission and improved nutrient digestibility, rumen fermentation, and nitrogen efficiency. These results indicate that the additive based on Acacia tannins and Saccharomyces cerevisiae yeast is a sustainable tool to reduce methane emissions in dairy production systems without compromising milk production.

Keywords: bovines ; organic milk ; survey

RESUMO

SANT'ANA, Amanda Barbosa, M.Sc., Universidade Federal de Viçosa, fevereiro de 2024. **Caracterização dos sistemas de produção de leite orgânico no Brasil e efeito de um aditivo alimentar sobre o consumo, produção de leite e emissão de metano de vacas leiteiras Holandesas ou cruzamento Holandês×Gir.**. Orientador: Alex Lopes da Silva. Coorientadores: Polyana Pizzi Rotta e Fernanda Samarini Machado.

Esta dissertação foi composta por dois diferentes estudos, onde o objetivo do primeiro estudo foi caracterizar os sistemas de produção de leite orgânico no Brasil, enquanto o objetivo do segundo estudo foi avaliar o efeito de um suplemento alimentar à base de taninos condensados sobre o consumo, desempenho e emissões de metano (CH4). No primeiro estudo, foi realizada uma análise descritiva em que as variáveis foram localização geográfica, tamanho do rebanho, produção animal, ração utilizada, manejo sanitário e reprodutivo, insumos orgânicos utilizados, manejo alimentar e transporte dos produtos. As características dos sistemas foram avaliadas de acordo com o nível de produção, com os produtores divididos em 3 grupos, sendo o nível superior composto por fazendas com produção média acima de 16 L/vaca por dia (ALTO), o nível médio com produção entre 10,5 e 16 L/vaca por dia (MÉDIO) e o nível inferior com produção média menor que 10,5 L/vaca por dia (BAIXO). As variáveis foram submetidas à regressão logística binomial e as comparações foram feitas por razão de chances. A área média das propriedades era de 107 ha (mínimo 3 ha e máximo 1.450 ha), a área para produção de leite orgânico era de 44 ha (mínimo 1 ha e máximo 550 ha). A produção média diária de leite era de 645 L/dia (mínimo 12 L/dia e máximo 5.000 L/dia), com uma produção média de 13 L/vaca por dia (mínimo 4 L/vaca por dia e máximo 25 L/vaca por dia). Os rebanhos têm uma média de 58 vacas (mínimo 2 vacas e máximo 310 vacas) e 40 vacas em lactação (mínimo 2 e máximo 255 vacas). A produção média anual era de 7.517 L/ha por ano (mínimo 21 L/ha por ano e máximo 29.877 L/ha por ano). O número médio de trabalhadores familiares foi de 2 (mínimo 2 e máximo 7), e o número médio de trabalhadores externos foi de 3 (mínimo 2 e máximo 16). Verificou-se que as fazendas HIG e MED tinham 90% menos probabilidade de produzir queijo. Além disso, as fazendas HIG e MED tinham 10,7 e 6,6 vezes mais probabilidade de ter cruzamentos de raça Holandesa × Jersey em seu rebanho, respectivamente. As fazendas MED tiveram 80% menos probabilidade de ter pastagens de Urochloa spp, enquanto as fazendas HIG tiveram 93,2% menos probabilidade de ter pastagens de Urochloa spp e 92% menos probabilidade de usar capim

picado para suplementar o rebanho. No entanto, a probabilidade de ter pastagens de Megathyrsus maximus foi 4,66 vezes maior para HIG. Além disso, as fazendas HIG tiveram 4,5 vezes mais probabilidade de usar qualquer tipo de software de gestão. A análise da distribuição de fazendas leiteiras orgânicas certificadas revelou uma concentração significativa na região Sudeste do Brasil. A distribuição de fazendas leiteiras orgânicas certificadas mostra uma concentração na região Sudeste do Brasil, onde a produção é focada principalmente em leite, enquanto outras regiões têm produção orgânica mais diversificada. As fazendas HIG são mais propensas a usar raças bovinas especializadas, suplementação de rebanho, pastagens formadas por espécies forrageiras de maior produtividade e maior valor nutricional e software de gestão. Esses resultados reforçam a necessidade de políticas públicas que promovam a adoção de práticas tecnológicas e sustentáveis para aumentar a eficiência e a produtividade do setor de laticínios orgânicos. No segundo estudo, dezesseis vacas leiteiras (8 Holandesas e 8 Holandesas × Gir) foram alocadas em dois tratamentos: um grupo controle (CON; sem suplementação) e um grupo suplementado (SUP) que recebeu 2,7 g/kg MS do aditivo (Muucare Nature®). O ensaio teve duração de 84 dias, incluindo um período de adaptação de 24 dias. Os animais foram alojados em baias individuais e mantidos em condições idênticas. O fluido ruminal foi coletado via esôfago nos dias 22 e 52, e a composição microbiana foi posteriormente analisada por sequenciamento de 16S rRNA. As concentrações de AGV e nitrogênio amoniacal ruminal também foram quantificadas. A emissão de metano foi medida usando a técnica do traçador hexafluoreto de enxofre. O aditivo aumentou a digestibilidade aparente da MS e MO em cerca de 6% sem afetar o consumo de ração ou a produção de leite. A emissão de metano por ECM (g/kg) foi reduzida em quase 30%, e a emissão de metano por produção de leite (g/kg) mostrou uma tendência semelhante. As vacas SUP apresentaram maior produção de propionato e menor relação acetato-propionato, indicando uma mudança na fermentação. A diversidade microbiana no rúmen foi alterada, com redução da diversidade alfa e composição diferente da comunidade, incluindo aumento da abundância de Prevotella ruminicola. A quantidade total de metanógenos permaneceu inalterada, embora uma espécie, Methanobrevibacter smithii, tenha tendido a ser menos abundante. O aditivo reduziu a emissão de metano e melhorou a digestibilidade dos nutrientes, a fermentação ruminal e a eficiência do nitrogênio. Esses resultados indicam que o aditivo à base de taninos de acácia e levedura Saccharomyces cerevisiae é uma ferramenta sustentável para reduzir as emissões de metano na produção leiteira sem comprometer a produção de leite.

Palavras-chave: bovinos; leite orgânico; pesquisa

SUMMARY

1. GENERAL INTRODUCTION

In the contemporary scenario, the growing concern about climate change, a widely discussed topic in the Paris Agreement, reflects a significant shift in consumer habits and priorities. As a result, there is an increased awareness and preference for niche products (Vieira Junior et al., 2019), driving a rising demand for healthier and environmentally friendly foods, with a special emphasis on organic products.

As a result, new products are being introduced to the market, combining the principles of sustainability and social responsibility (Henz, 2022). This trend towards naturalness has, to some extent, led to a rejection of products from conventional livestock farming, while simultaneously driving the production of organic milk, seen as a tastier and healthier option, although scientific evidence to support this claim is still lacking (Brazil Dairy Trends, 2017). In the same context, consumers are each time more worried about the impact of livestock on climate change. The methane eliminated by cattle is the result of ruminal fermentation, which plays a very important role in ruminant production, as it allows these animals to transform feeds of low nutritional value for humans into foods with high bioavailability, such as meat and milk. Most feeds used in ruminant production are not eligible for human consumption (Mottet et al., 2017). Thus, modifying ruminal fermentation becomes a tool with great potential for reducing the production of this gas (Vendramini, 2014).

In the current market, ruminal additives are being employed to reduce methane emissions produced by animals, but they are still perceived as an additional cost in the production process. In organic systems, it is essential to ensure that all additives used comply with the principles of organic agriculture, which include the prohibition of harmful synthetic and chemical substances, according to Instruction Normative No. 46/2011 of the Ministry of Agriculture, Livestock, and Supply (MAPA). Therefore, it is crucial to select additives derived from natural sources and approved for use in organic systems. Tannins can be found in natural sources such as fruit peels and plants, and they have been studied for their ability to improve animal digestive health and reduce greenhouse gas emissions (Berça et al., 2023). Similarly, yeasts are natural microorganisms widely used in animal production, including milk production, promoting a healthy rumen microbiota and contributing to the digestive health and well-being of cows (Amin and Mao, 2020; Sanchez et al., 2021). When combined, these additives offer a holistic approach to maximizing milk production and minimizing environmental impact. Therefore, it is possible to use tannins and yeast in both conventional and organic milk production systems, as long as they are selected and applied appropriately, following the principles and regulations of organic production.

With the growing demand for sustainably sourced dairy products, the use of additives containing tannins and yeast is becoming an increasingly relevant practice in the agricultural industry. In addition to improving productive efficiency, these additives also offer an opportunity for producers to diversify their revenue sources through the commercialization of carbon credits and premium dairy products. Additives containing condensed tannins and *Saccharomyces cerevisiae* yeast represent one such innovation, providing a promising solution to address the challenges of modern dairy production and build a more sustainable future for the agricultural industry as a whole.

Thus, it was hypothesized that: 1) the herd's production level influences the productivity and commercialization aspects of organic milk production systems; 2) lactating cows that received feed supplement based on condensed tannins present greater milk yield and lower methane emission compared to the control group; 3) the response to supplementation based on condensed tannins and *Saccharomyces cerevisiae* yeast would be different for Holstein and Holstein×Gyr crossbred cows. Forward, the objective of this study was to characterize organic milk production systems in Brazil, as well as to evaluate the effects of using an additive based on condensed tannins and *Saccharomyces cerevisiae* yeast on intake, milk yield, and methane emissions of Holstein or Holstein×Gyr lactating cows.

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2. Chapter 1

Characterization of organic milk production systems in Brazil

ABSTRACT

The aim was to characterize the organic dairy systems in Brazil. It was hypothesized that the production level of the herd influences the productivity and marketing aspects of organic milk production systems. A descriptive analysis was carried out in which the variables were geographical location, herd size, animal production, feed used, health and reproduction management, organic inputs used, feed production management, and transportation of products. The characteristics of the systems were evaluated according to the level of production, with farmers divided into 3 groups, with the upper extract comprising farms with an average production of over 16 L/cow per day (**HIG**), the medium extract with a production between 10.5 and 16 L/cow per day (**MED**) and the lower extract with an average production of less than 10.5 L/cow per day (**LOW**). The variables were subjected to binomial logistic regression and comparisons were made by odds ratio. The average area of the properties was 107 ha (minimum 3 ha and maximum 1,450 ha), the area for organic milk production was 44 ha (minimum 1 ha and maximum 550 ha). The average daily milk production was 645 L/day (minimum of 12 L/day and maximum of 5,000 L/day), with an average production of 13 L/cow per day (minimum of 4 L/cow per day and maximum of 25 L/cow per day). The herds have an average of 58 cows (minimum 2 cows and maximum 310 cows) and 40 lactating cows (minimum 2 and maximum 255 cows). The average annual production was 7,517 L/ha per year (minimum 21 L/ha per year and maximum 29,877 L/ha per year). The average number of family workers was 2 (minimum 2 and maximum 7), the average number of external workers was 3 (minimum 2 and maximum 16). The HIG and MED farms were found to be 90% less likely to produce cheese. In addition, HIG and MED farms were 10.7 and 6.6 times more likely to have Holstein×Jersey crosses in their herd, respectively. The MED farms were 80% less likely to have *Urochloa* spp pastures, while HIG farms were 93.2% less likely to have *Urochloa* spp pastures and 92% less likely to use chopped grass to supplement the herd. However, the odds of having *Megathyrsus maximus* pastures was 4.66 times greater for HIG. In addition, HIG farms were 4.5 times more likely to use any type of management software. The analysis of the distribution of certified organic dairy farms revealed a significant concentration in the Southeast region of Brazil. The distribution of certified organic dairy farms shows a concentration in the southeastern region of Brazil, where production is mainly focused on milk, while other regions have more diversified organic production. The HIG farms are more likely to use specialized cattle breeds, herd supplementation, pastures formed by higher-yielding forage species with greater nutritional value, and management software. These results emphasize the need for public policies that promote the adoption of technological and sustainable practices to increase the efficiency and productivity of the organic dairy sector.

Keywords: Bovines. Organic milk. Survey

Nonstandard abbreviations:

CNPO = National Register of Organic Producers

CNPJ = Corporate Taxpayer Identification Number

CPF = Social Security Number

GMO - Genetically modified organisms

HIG = higher production level

IBGE = Brazilian Institute of Geography and Statistics

LOW = lower production level

MID = medium production level

OCS = Social Control Organizations

PGS = Participatory Guarantee System

INTRODUCTION

In recent decades, the profile of consumers has changed, with a strong tendency to favor products of differentiated quality and a greater concern about the type of production, mainly due to issues related to environmental impact, health risks and ethical considerations during the production process (Lima et al., 2020)

Organic products fit this profile, as food is produced while respecting the natural cycle of resource conservation (Piao et al., 2021). The global organic cultivation area increased by 26.6% between 2021 and 2022 (96.6 million ha), with a global market of €134.8 billion. With 996.44 ha and 24,205 organic producers and a market of €778 million, Brazil ranks 12th in terms of organically farmed area (Willer et al., 2024). According to a study by KPMG (2018), certified organic milk accounts for around 0.9% of all milk produced worldwide, with the market volume mainly concentrated in countries such as the USA, Germany and France (Kyrylov et al., 2018).

Although there are segments of the dairy industry in developed countries that have the potential to comply with organic production principles, milk production in developing countries generally takes place on a small scale and the activity is considered quite fragmented, with an undefined level of consistency (KPMG, 2018). In Brazil, there is a lack of accurate and reliable data on production, which makes it difficult to structure the chain and develop public policies and marketing strategies (Machado et al., 2021). This scenario makes the growth of organic dairy farming in Brazil rather slow compared to other countries. There are two official sources of information in Brazil: Brazilian Institute of Geography and Statistics (**IBGE**) data, collected as part of the agricultural census, and data from the National Registry of Organic Producers (**CNPO**), provided by the Brazilian Ministry of Agriculture and Livestock. These data were systematized by Embrapa and are available via the “Organic Milk Observatory” (https://leiteorganico.cnpgl.embrapa.br/).

According to the 2017 Agricultural Census (IBGE, 2019), Brazil had 64,690 farms practicing organic agriculture, accounting for approximately 1.4% of all certified organic farms globally (Willer et al., 2024). Out of these, 36,689 were exclusively dedicated to organic farming, 17,612 to organic livestock production, and 10,389 combined both activities. This number, however, reflects all establishments self-declared as organic and does not necessarily imply certification. In contrast, data from the National Register of Organic Producers (CNPO) reported only 15,856 certified organic production units in 2017, corresponding to 0.3% of all farms in the country (Vilela et al., 2019). The CNPO figures refer strictly to certified producers, which may explain the difference. According to data from BRASIL (2021), the number of producers registered with the CNPO increased from 5,900 in 2012 to 17,700 in 2019 (Holmström et al., 2020), reaching 25,919 in 2024 (BRASIL, 2025).

Despite this increase, a major obstacle to the development of the organic sector in Brazil is the lack of accurate data on indicators for the production, commercialization and consumption of organic products (Lima et al., 2020). There are no official records of production indicators such as the number of animals in the herd, the number of lactating cows, the amount of milk produced, etc. In addition, the lack of coordination between milk producers and organic input suppliers makes it difficult to develop commercial strategies for farmers and the processing industry (Machado et al., 2021).

The aim of this study was therefore to characterize the organic milk production systems in Brazil. It was hypothesized that the production level of the herd influences milk production efficiency, as well as marketing and the use of technology in organic systems.

MATERIAL AND METHODS

Based on data from the CNPO of the Brazilian Ministry of Agriculture and Livestock, a search was carried out for registered producers whose activities include the production of dairy cows in the period between August 2019 and August 2021.

The CNPO is mandatory and its database is maintained and updated by the conformity assessment organisms or state agricultural authorities, with production units labeled with the following information:

1) Certification system (public or private certifier, participatory guarantee system (**PGS**), or social control organization (**OCS**)) and name of the unit;
2) Federation Unit and city;
3) Farmer's data (name, Social Security Number (CPF)/Corporate Taxpayer Identification Number (CNPJ), contact);
4) Scope (animal and/or plant primary production, processing of products of animal and/or plant origin, extractivism, processing of inputs, etc.);
5) Description of the production activity.

The CNPO data was systematized in order to identify organic milk producers and to analyze the type of certification and activity (exclusively organic milk production or production of milk and other organic products on the same farm). In December 2019, 145 organic milk producers were registered with the CNPO, representing a total of 103 production units (farms) in Brazil, since some properties had more than one rural producer registered.

Survey Application

The research was previously submitted to the Human Research Ethics Committee of the Universidade Federal de Viçosa (Minas Gerais, Brazil) for evaluation and approved under protocol no. 5.664.620.

Based on the contacts provided by the CNPO, telephone interviews were conducted with 53 farmers in 2019 and information was obtained from 35 farmers via an online survey in 2020 and 31 farmers in 2021, with five farms in the conversion phase at the time of the survey. As some properties have more than one farmer registered on them, the farms were considered to be places of "primary production" of milk, i.e. large or small farms, excluding enterprises dedicated solely to milk processing not counted. The workforce was considered familiar when all farming activities were carried out exclusively by members of the farmer's family, without the involvement of any hired external workers.

Over the three years in which the study was conducted, 119 responses were received, covering 90 farms out of 103. Of these, six were excluded because they are farms that produce buffalo *(Bubalus bubalis*) milk rather than cow's (*Bos taurus taurus* and *Bos taurus indicus)* milk, giving a total of 84 farms, which represents 81.5% of the total organic dairy farms. The survey collected various information to characterize the organic dairy farm: geographical location, herd size, daily milk production, feed used, health and reproduction management, inputs used, management of feed production for the herd, and product transport (Supplemental File 1).

Statistical Analyses

For the statistical analysis, the consistency of the data was checked. This included duplicate responses and checking for biological sense between related variables reported by farmers, and inconsistent data were excluded from the results. A descriptive analysis of the data set was then carried out. The variables were: geographical location, herd size, animal production, feed used, health and reproduction management, organic inputs used, production management of feed for the herd, and transportation of products.

To evaluate the characteristics of the systems according to the production level (L/cow per day), the farms were grouped into terciles, using the 33.33rd and 66.66th percentiles as cut-off points. The top tercile (**HIG**) included 28 farms with an average production of over 16 L/cow per day, the medium tercile (**MED**) included 29 farms with a production between 10.5 and 16 L/cow per day, while the bottom tercile (**LOW**) included 27 farms with an average production of less than 10.5 L/cow per day (Figure 1). For farms allowing calves to be present during milking, calves were prevented from suckling on milk recording days, ensuring that recorded milk yield reflected actual production. Calves remained in contact only to stimulate milk let-down, so terciles were based solely on recorded milk yield, maintaining data consistency and preventing classification bias.

Subsequently, the variables of interest were subjected to binomial logistic regression, followed by the estimation of the odds ratio for the production levels using the logit link function available in the glm function of R (R Core Team, 2025), with a significance threshold of $P < 0.05$. The contrasts tested were HIG *vs.* LOW (base group), MED *vs.* LOW (base group), and HIG *vs.* MED (base group).

RESULTS AND DISCUSSION

Organic milk production units in Brazil

A total of 84 organic dairy farms were identified in this study, spread across 11 states and the Federal District (Figure 2). The present study also analyzed the number of certified organic dairy farms by region in Brazil (Figure 3A). We found that the majority of certified farms are located in the Southeast region, which accounts for approximately 75% of the total. In contrast, the South region represents around 19%, while the remaining 6% are distributed across the Northeast and Midwest regions.

As observed, the Southeast region of Brazil stands out with a high number of certified organic dairy farms, which is due to the investments made by private companies (Nestlé and Danone) in the production of organic milk powder between 2018 and 2020 (Machado et al., 2021). During this period, both companies launched organic products on the market, encouraging the entry of new producers and the modernization of certified farms (Lima et al., 2020). Danone collected organic milk from eight suppliers in Minas Gerais and São Paulo (both located in the southeast). Nestlé had around forty producers in its organic milk project, with a daily collection of around 35 thousand liters of milk in São Paulo (Machado et al., 2021).

The distribution of organic dairy farms in the Brazilian states reflects the diversity of production practices observed. São Paulo is the state with the highest number of certified farms (53.6%), followed by Minas Gerais, Paraná, Rio de Janeiro and Santa Catarina. Interestingly, this distribution reflects the production patterns observed on these farms. While 53% of all certified farms in Brazil focus exclusively on the production of milk or milk and dairy products, the remaining 47% have a more diversified production approach that includes various other organic products such as fruits, vegetables, grains, and others. The geographical distribution of certified farms therefore not only illustrates the regional differences but also reflects the broader spectrum of production diversity within the organic dairy sector in Brazil.

This diversified approach of production predominates in most regions of Brazil (Figure 3B). In contrast, farms producing only organic milk and milk and other organic products predominate in the Southeast (Figure 3B). In the state of São Paulo, 36 farms are dedicated exclusively to the production of organic milk and 9 farms have a diversified production approach. We also found that 6 farms in Minas Gerais and 4 farms in Rio de Janeiro produce only organic milk. The greater number of farms dedicated exclusively to the production of milk and dairy products in the Southeast can be attributed in part to the significant investment in the organic system by large dairy companies mentioned earlier.

Although there has been growth in organic milk production over the years, reflecting a global expansion trend, the pace of this growth in Brazil has been rather moderate. The entry of large companies into the dairy sector and their investments in the collection of organic milk in Brazil have thus contributed to the growth and structuring of the organic milk production chain. This impact is particularly noticeable in the Southeast region, where the sector grew between 2018 and 2021 (Silvano, 2018; Machado et al., 2021).

Regarding the use of organic milk production, the survey revealed that 74.7% of producers market their milk as organic, either by selling it directly at outdoor fairs or by selling it to industries or cooperatives. In contrast, 25.3% stated that they sell it as conventional due to a lack of alternatives. Of the producers who sold organic milk as conventional milk, 38% discontinued organic milk production. These results point to a number of challenges faced by organic producers, including issues related to market demand, access to organic markets, logistical constraints and production costs. The lack of demand or access to organic markets in certain regions may result in producers opting to sell their milk as conventional milk to secure a stable source of income.

The analysis of the production of dairy products and other organic products using logistic regression revealed a statistically significant difference in cheese production (P=0.01). The MED and HIG farms were 89% less likely to produce cheese than LOW farms, with an odds ratio confidence interval of 0.03 to 0.31 (Figure 4A and 4B). This scenario can be attributed to the fact that most MED and HIG farms operate at a scale that enables the transportation of a large proportion of fresh milk to the milk processing plants (Figure 4C).

Alternatively, no statistically significant differences were found in the production of butter, yogurt and other organic milk derivatives (P>0.05), indicating that the economic viability of producing these products was similar across different production levels. It is worth noting that organic milk derivatives fetch a higher price compared to conventional dairy products (KPMG, 2018), which provides an additional incentive to produce these higher value-added products.

No statistically significant differences were found in the production of organic products such as vegetables, cereals and others (P>0.05). Therefore, regardless of the production level on the farm, the probability of producing one of these products remains the same (Figure 4D, 4E, and 4F).

Certification systems for organic milk production in Brazil

Brazilian legislation establishes three types of organic certification: audit certification, participatory certification, and social control within direct sales (BRASIL, 2025). Audit certification is conducted by accredited public or private bodies that issue certificates after verifying compliance with organic standards through independent audits. Participatory certification involves a collective system of evaluation among producers, technicians, and consumers, typically used by small-scale farmers and recognized by the government. Social control within direct sales applies to family farmers who sell directly to consumers and are monitored through organized groups registered with government oversight, without the need for formal certification.

In Brazil, the most important organic certification bodies recognized by audits include the Biodynamic Institute Certifications, Ecocert Brasil and TECPAR. This study found that the majority of organic farms in Brazil obtain certification through audits conducted by the certification companies, covering 71.4% of registered farms (Table 2). In the south-east of the country, this certification method is particularly widespread in the states of São Paulo and Minas Gerais.

Certification can also take place through a PGS, in which the members themselves (e.g. producers, processors and traders) guarantee the organic quality of their products and which is based on trust, social networks, and knowledge sharing (IFOAM, 2018). Some of the most important organic certification bodies recognized as PGS in Brazil are the Ecovida Association of Participatory Certification, the Association of Organic Farmers of the State of Rio de Janeiro, the Brota Cerrado of Serra da Canastra Association of Participatory Certification and the Natural Agriculture Association of Campinas.

The PGS-certified dairy farmers account for 26.2% of the total number of certifications in Brazil and are very present in the southern region and the state of Rio de Janeiro (Figure 2), as these are pioneer locations for non-governmental organizations such as the Association of Organic Farmers of the State of Rio de Janeiro and the Ecovida Association for Participatory Certification in the south of the country (Vilela et al., 2019). In addition, both southern Brazil and the state of Rio de Janeiro have a diverse supply of agricultural products, which can encourage demand for organic certification. PGS certification can be an attractive option for farmers who produce a variety of organic crops and are looking for a more accessible and participatory certification system.

The OCS is a way to involve producers who only need to consider selling their products directly to consumers. According to Candiotto (2018), one of the requirements of these programs is that 30% of the food purchased must come from family farms, with producers of certified organic food receiving an additional 30% over and above the amount paid for conventional food, making them important mechanisms for strengthening and expanding organic agriculture. With only 2.4% of all certifications, the OCS (e.g. Association of Agroecological Farmers of Bom Jardim) is only present in the Northeast region, in the state of PE, with 2 certified organic farms (Table 2). The Northeast region of Brazil, known for its strong tradition of family farming, is more affected by OCS due to several factors. The OCS emphasizes the involvement of the local community in the certification process, which is consistent with areas where there is strong community organization and a growing interest in organic production. In addition, in some regions of the Northeast, there is broader access to resources and support for the development of local organic initiatives, which further favors the presence of the OCS.

Characterization of the production systems

The farms in the present study had an average size of 107 ha, ranging from 3 to 1,450 ha. The average area devoted to organic milk production was 44 ha, ranging from 1 to 550 ha. The herd consisted of 58 cows, ranging from 2 to 310 animals depending on the farm. An average of 40 lactating cows were identified, ranging from 2 to 255 animals with an average daily milk production of 13 L/day, ranging from 4 to 25 L/day. The average daily total milk production of the farms was 645 L/day, with farms producing between 12 and 5,000 L/day. Annual productivity averaged 7,517 L/ha/year, ranging from 21 to 29,877 L/ha/year. Regarding the family labour force, the number of family workers averaged 2 per farm and varied between 2 and 7, while the number of external workers averaged 3 and varied between 2 and 16 employees per farm. In terms of milking management, 65% of producers use mechanical milking without the calf, while 35% use mechanical milking with the calf present.

Brazilian legislation on organic farming, represented by BRASIL (2021), recommends the preference of breeds adapted to the specific climate and management of each region. The most common breeds in organic milk production include Holstein×Gyr, Jersey and Holstein×Jersey. However, some producers keep two or more breeds on their farms.

Based on the results of the logistic regression (Figure 5), it was found that the probability of a producer breeding Holstein×Gyr, Jersey or other breeds was similar at all production levels (*P*>0.05). However, a difference was observed for the Holstein×Jersey crosses between HIG vs LOW (Figure 5A) and MED vs LOW (Figure 5B; *P*<0.05). Farmers at HIG were 10.7 times more likely to have Holstein×Jersey crossbreds in their herd than farmers at the LOW (*P*<0.01). The MED farmers were 6.57 times more likely to have Holstein×Jersey crossbreds in their herd than LOW (*P*=0.02). This result suggests that HIG farmers have a particular preference to crossbred animals, possibly due to their production characteristics, especially milk yield and quality, as well as their adaptability to different environmental conditions and management systems. These aspects are in line with the requirements of more intensive and efficient production systems.

Furthermore, this pattern could reflect a deliberate strategy of breed selection by producers aimed at maximizing productivity and efficiency on their farms. In addition to increasing the volume of milk produced, the pursuit of higher milk solids content may make the dairy industry in Brazil more competitive. The trend indicates that the quality payment system will become more prevalent (Sorio, 2018). However, payment based on solids content is still in its infancy, as most premiums are paid based on milk volume rather than composition (Sorio, 2018).

Finally, regarding the use of reproductive technology, 47.5% of respondents stated that they use artificial insemination in their herd, 31.1% use natural mating and 21.3% use both methods. According to the legislation, artificial insemination is allowed, but the semen used must preferably come from organically reared animals. However, in vitro fertilization, embryo transfer and estrogen synchronization techniques are not allowed (BRASIL, 2021).

Feed use and management

According to BRASIL (2021), which establishes the technical regulations for organic production systems, provides for maximum use of the grazing system, with the proportion of fresh, dried or ensiled feed being at least 60% of the dry matter of the feed, although this proportion may be reduced to 50% for lactating animals, for a maximum period of 3 months from the start of lactation. As far as production systems are concerned, the farms in this study predominantly practiced pasture-based milk production with rotational grazing and semi-fencing. The predominant forage species mentioned by the participating producers were *Urochloa* spp, *Megathyrsus* spp and *Cynodon* spp.

A difference was observed in farms with pastures consisting of forage plants of the *Urochloa* spp and *Megathyrsus* spp (*P*<0.05; Figure 6A and 6B). The HIG farms were 93.2% less likely to have *Urochloa* spp in their pastures than LOW farms (*P*<0.01). Similarly, MED farms were 80% less likely to have *Urochloa* spp in their pastures than LOW farms (*P*=0.04). As for the pastures formed by *Megathyrsus* spp, the probability that they contained *Megathyrsus* spp was 4.66 times higher in HIG farms than in LOW farms (*P*=0.03). However, the probability that the pasture consisted of forage plants of the genus *Cynodon* spp, native willows or other forage plants was similar at all production levels (*P*>0.05). No differences were observed between HIG and MED farms (Figure 6C; *P*>0.05).

Most Brazilian pastures consist of forage species from the genera *Urochloa* and *Megathyrsus* (Vilela et al., 2019). *Megathyrsus* pastures are widely used in MED to HIG production systems, as they are forage crops that have high productivity and consequently higher soil fertility requirements (Lima et al., 2020), resulting in higher implementation and maintenance costs. On the other hand, *Urochloa* spp pastures adapt well to LOW systems with, as they are more resistant to acidic and low fertility soils, generally with lower productivity compared to *Megathyrsus* (Lima et al., 2020). Thus, the use of *Urochloa* spp can reduce production costs in terms of implementation or maintenance.

Based on the results of the logistic regression analysis, there was a statistically significant difference in the form of roughage supplementation used on the farms (*P*<0.05; Figure 6D and 6E). No statistically significant difference was observed for sugar cane supplementation or the provision of other types of supplementations (*P*>0.05). However, in terms of grass supplementation, HIG farms were found to be 92% less likely to use grass as a form of herd supplementation compared to farms to LOW farms (*P*=0.02). This suggests that the type of supplementation may vary depending on the production level of the farm, with higher-producing farms tending to favor other forms of supplementation over grass. Similarly to the observed for the pastures, no differences were observed between HIG and MED farms for the roughage used (Figure 6F; *P*>0.05)

In general, silage (corn, sorghum and grass) was the predominant feed in all farms, regardless of production level, with more than half of the respondents opting for this type of supplement. Silva et al. (2023) conducted a study in 2019 and reported that in Rio de Janeiro, most producers use rotational grazing and supplementation with some type of roughage. The most used are chopped elephant grass (86%), chopped sugarcane (57%), hay (43%) and silage from grass, corn or sorghum (43%).

Feed planning in production units in Santa Catarina and Paraná is a major challenge (Pacheco, 2013). Of the 30 participants in the survey, 40% did not carry out feed planning. Of those who did plan their herd's diet, 50% invested in annual summer grazing and 70% in annual winter grazing. For the supply of concentrates, 52.4% opted for commercial feed, while 47.6% prepared the concentrates on their own farm. In addition, half of the organic grain used was produced on site and supplemented with external products, while 40% of farmers purchased inputs from suppliers. Only 10% were able to produce all the feed on their own farm.

Herd monitoring and management

Farmers were asked if they monitor the farm and 95% said that they use some kind of tool to monitor information: Notebook, spreadsheet, or management software (Figure 7). Based on the logistic regression analysis, no statistically significant difference was found in relation to the use of a notebook or spreadsheet for monitoring control, i.e. the probability of using a notebook or spreadsheet for monitoring control was the same at all production levels ($P>0.05$). However, a difference was observed in the use of monitoring and management software ($P=0.033$), with HIG farms being 4.5 times more likely to use some type of monitoring and management software than LOW farms. Additionally, no differences were observed between HIG and MED farms in the use of monitoring and management software (Figure 7C; $P>0.05$).

Only 39.3% of the farmers who responded to the survey use specialized technical assistance, and of those, 18% use some type of management software. This suggests that as production levels increase, farmers are more likely to use more advanced technologies such as management software to improve the control and monitoring of farming activities and make the management of operations more efficient and accurate. In this context, one of the great challenges for Brazilian agriculture is to spread the use of technologies, such as software, in rural farms, especially in small farms. In addition to being accessible, management software can be free of charge and simplify the collection and processing of production data, enabling the planning of medium and long-term actions (Melo et al., 2021).

We also examined the share of organic dairy farming in farmers' family incomes (Figure 8). The overwhelming number of farmers for whom organic dairy production is an important part of their income indicates a strong economic dependence on this activity. We found that for about 45% of farmers who responded to the survey, organic milk production accounts for more than 75% of their income source, while for 21% of farmers it accounts for between 50% and 75% and for 9.8% of farmers it accounts for between 25% and 50%. However, the fact that there are states with a variety of organic production where milk accounts for less than 25% of family income underlines the need to consider strategies to strengthen the economic potential of organic milk production in the country.

The main challenges for organic milk production in Brazil

The present study examined the main challenges faced by farmers in organic dairy farming. In this sense, one of the main problems is the difficulty of marketing organic milk due to several factors, such as limited market access and competition with conventional products. In addition, the lack of organic inputs such as non-genetically modified organisms (**GMO**) and organic corn and soybean, as well as high market prices, are an obstacle. Another critical problem is the lack of knowledge about ecological systems and the lack of specialized technical advice, which often leads to inappropriate production practices. In addition, the certification process is perceived as costly and bureaucratic, which discourages many farmers from continuing their activities.

As far as health challenges are concerned, many farms are struggling with considerable difficulties. The control of endo- and ectoparasites in the herd and on pasture and the treatment of mastitis are proving to be outstanding problems. The limited availability of suitable treatment options for animals kept in organic systems exacerbates these challenges. Wallenbeck et al. (2019) have shown a high prevalence of mastitis and metabolic diseases in organic systems in Europe.

In this context, some farmers on the farms studied used antibiotics, while others took measures such as drying and separating contaminated udder and homeopathic treatments. In some countries, the use of antibiotics or anthelmintics is permitted, but the withdrawal period is quite long. In Brazil, antibiotics or other unauthorized drugs may be used at most twice a year, if necessary, with a withdrawal period of at least 96 hours or twice the withdrawal period indicated on the label. If it is necessary to exceed the maximum permitted number, the animal must be removed from the organic system (BRASIL, 2021).

Although some studies show good health indicators for organic farms due to the low incidence of disease (Rutherford et al., 2008; Levison et al., 2016), others indicate a high prevalence of disease (Krieger et al., 2017), reflecting the complexity and diversity of factors involved in animal health in organic systems. The use of antibiotics is controversial. Some farmers resort to them in case of illness, while others look for alternatives such as homeopathic and herbal treatments.

The main challenges of feed management in organic production systems include the scarcity and high cost of organic inputs and the difficulties associated with on-farm feed production (Escribano, 2018). Out of a group of 83 respondents, only 17 farmers stated that they grow any type of cereal or grain on their land. However, most farmers appear to rely on pasture as the main source of feed for their livestock, a decision that can impact livestock production depending on the genetics of the livestock (Honorato et al., 2014).

Although organic farmers can include up to 15% of conventional, non-GM concentrates in DMI according to BRASIL (2021). As Honorato et al. (2014) noted, organic farmers face challenges in sourcing these inputs, particularly due to the lack of testing for transgenes. This lack of testing is a significant obstacle, as regulations prohibit the use of transgenic products in any form (BRASIL, 2021). Testing for GMOs is crucial in the context of organic milk production to ensure that livestock feed, such as concentrates or supplements, meets organic standards and remains free of GMO. This is crucial for farmers as it allows them to maintain organic certification and offer consumers truly organic, GMO-free products.

When it comes to the herd, a number of challenges arise, focusing on the complexity of general management and in particular reproductive management. In addition, issues relating to genetics and the low productivity of organically reared animals are also a major concern. Of the 58 farmers surveyed, only 13 reported that they had no specific problems with their herd, highlighting the extent of these difficulties among farmers. According to Röös et al. (2018), the low productivity observed in organic systems can be partly attributed to the limited use of high-yielding breeds and the lower proportion of concentrates in the diet. These results underline the importance of effective management strategies and appropriate genetic selection to maximize herd productivity and welfare on organic dairy farms.

In terms of marketing, the majority of farmers pointed out the difficulty of selling their products, mainly due to the lack of processing plants for organic milk. Out of a total of 64 respondents, only 11 had no problems marketing their organic milk. When asked whether the milk produced was sold as organic milk, 21 of the 83 respondents stated that they marketed their milk as conventional milk to local dairies, large dairy companies or direct sales to consumers. Of these responses, 8 farmers in the state of São Paulo indicated that they had ceased their activity.

Between 2018 and 2020, as discussed previously, dairy companies such as Nestlé and Danone made significant investments in organic milk production in Brazil. These investments encouraged the entry of new farmers into the sector and the introduction of advanced technologies in already certified farms, especially in the Southeast. However, these companies have recently reduced and stopped collecting organic milk (Machado et al., 2021). These changes may therefore have influenced the decision of some farmers to abandon organic production.

In Brazil, the market for organic milk is still developing and the product remains more expensive than conventional milk due to several factors, including high certification costs, sustainable farming practices and the limited availability of organic inputs. In 2022, Brazil's gross domestic product per capita was around USD 8,917, while in the United States and European countries, where organic products are consumed on a larger scale, the gross domestic product per capita was over USD 48,000, according to the World Bank. In Brazil, organic milk may be perceived as a luxury item and develop into a niche market. Despite the growing interest in healthy and sustainable food, most Brazilians still prefer cheaper options. Although the consumption of organic food is gradually increasing in the country, there is still a large gap between demand and supply.

CONCLUSIONS

The analysis of the distribution of certified organic dairy farms revealed a concentration in the southeastern region of Brazil, where production is mainly focused on milk, while other regions have a more diversified organic production. The production level of the farms influenced decisions regarding cattle breeds, herd supplementation, pastures types, milk productivity and the adoption of management tools. Organic dairy farming is an important source of income for many farmers, with 45.1% of them deriving more than 75% of their total income from it. This data underlines the importance of technology in organic dairy production and its significant economic contribution to farmers. However, it is noteworthy that specialized technical support and the use of management software are still adopted by a minority of farmers, indicating the opportunity for greater integration of technology into farm management. These findings highlight the need for public policies that promote the adoption of technological and sustainable practices to increase the efficiency and productivity of the organic dairy sector.

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Supplemental File 1. The survey applied to organic milk dairy farmers.

- Farm name
- Farmer name
- State
- City
- Certified property? ☐ Yes ☐ No ☐ In conversion

- What is the certifier or Participatory Conformity Assessment Organization?
  - ☐ ASSOCIATION OF ORGANIC FARMERS OF THE STATE OF RIO DE JANEIRO
  - ☐ ECOCERT BRASIL
  - ☐ BIODYNAMIC INSTITUTE
  - ☐ ECOVIDA - Participatory Conformity Assessment Organization

- Which is the year of certification?
- Which is the farm total area (ha)?
- Which farm area is dedicated to milk production (ha)?
- Average milk farm production (L/day)
- Average production per animal (L/animal per day)
- Which is the total number of cows on the property? (dry and lactating cow)
- Which is the number of lactating cows?

- Which is the most representative breed of the herd:
  - ☐ Holstein
  - ☐ Holstein×Jersey crossbred
  - ☐ Jersey
  - ☐ Holstein×Gyr crossbred
  - ☐ Other:

- Type of reproduction program:
  - ☐ Artificial Insemination
  - ☐ Natural
  - ☐ Other:

- Gerencial tool used in zootechnical control
  - ☐ Notebook
  - ☐ Spreadsheet
  - ☐ Manager software
  - ☐ Does not zootechnical control
  - ☐ Other:

- Does the farm receive specialized consultancy or technical assistance?
  - ☐ Yes
  - ☐ No

- Do you use family labor? How many people?

- o Do you use external labor? How many people?
- o What is the production system: ☐ Confinament ☐ Grazing ☐ Both
- o Type of grazing: ☐ Continuous ☐ Rotacioned ☐ Other:
- o What is predominant the type of pasture?
- o What type of roughage is used for supplementation?
- o What is the type of milking and management used? ☐ Manual ☐ Mechanical ☐ With calf ☐ Without calf
- o Is the milk sold as organic? ☐ Yes ☐ No
- o What is milk's destiny? ☐ Own dairy ☐ Cooperative ☐ Nestlé ☐ Danone ☐ Other:
- o Which dairy products do you produce? ☐ Cheese ☐ Iogurt ☐ Cream cheese ☐ Butter ☐ Other: ☐ Do not produce dairy products
- o Is milk production the main source of income? ☐ Yes ☐ No
- o How much does milk represents in the producer's total income? ☐ 0% ☐ Until 25% ☐ 25 a 50% ☐ 50 a 75% ☐ >75%
- o Perform some other organic activity? ☐ Yes ☐ No If yes, what?
- o What is the main problem faced by organic dairy farming?
- o What are the challenges of organic dairy farming in feed production?
- o What are the challenges of organic dairy farming in a herd (genetics, reproduction, management)?
- o What are the challenges of organic dairy farming about marketing?

- What are the challenges of organic dairy farming about certification?

Table 1. Descriptive statistics for characterization of certified Brazilian organic dairy farms.

Predictor	Descriptive statistics					
	N[1]	Mean	Median	Minimum	Maximum	SD[2]
Area, ha						
Total	84	107	50	3	1,450	188.6
Organic dairy farming	82	44	25	1	550	70.6
Productivity						
Total milk, L/d	82	645	415	12	5,000	864.4
Milk yield, L/cow per day	82	13	14	4	25	5.1
Milk production, L/ha per year	800	7,517	6,387	21	29,877	6,437.3
Herd						
Total	84	58	45	2	310	55.9
Cows in lactation	84	40	28	2	255	43.2
Cows in lactation/total cows, %	84	66	70	15	100	19.9
Workforce						
Familiar	81	2	2	0	7	1.6
External	77	3	2	0	16	3.4

[1]N=number of properties, [2]SD=standard deviation.

Table 2. Number of organic dairy farms per Brazilian state, stratified by type of certification.

State	Type of certification		
	Certifier	PGS[1]	OCS[2]
São Paulo	44	1	
Minas Gerais	9	1	
Rio de Janeiro		7	
Espírito Santo	1		
Paraná	2	7	
Santa Catarina		4	
Rio Grande do Sul	1	2	
Pernambuco			2
Alagoas	1		
Distrito Federal	1		
Goiás	1		

OCS = Social Control Organizations, PGS = Participatory Guarantee System; Certifier = public or private organizations officially accredited by MAPA to carry out independent audits and issue organic certificates.

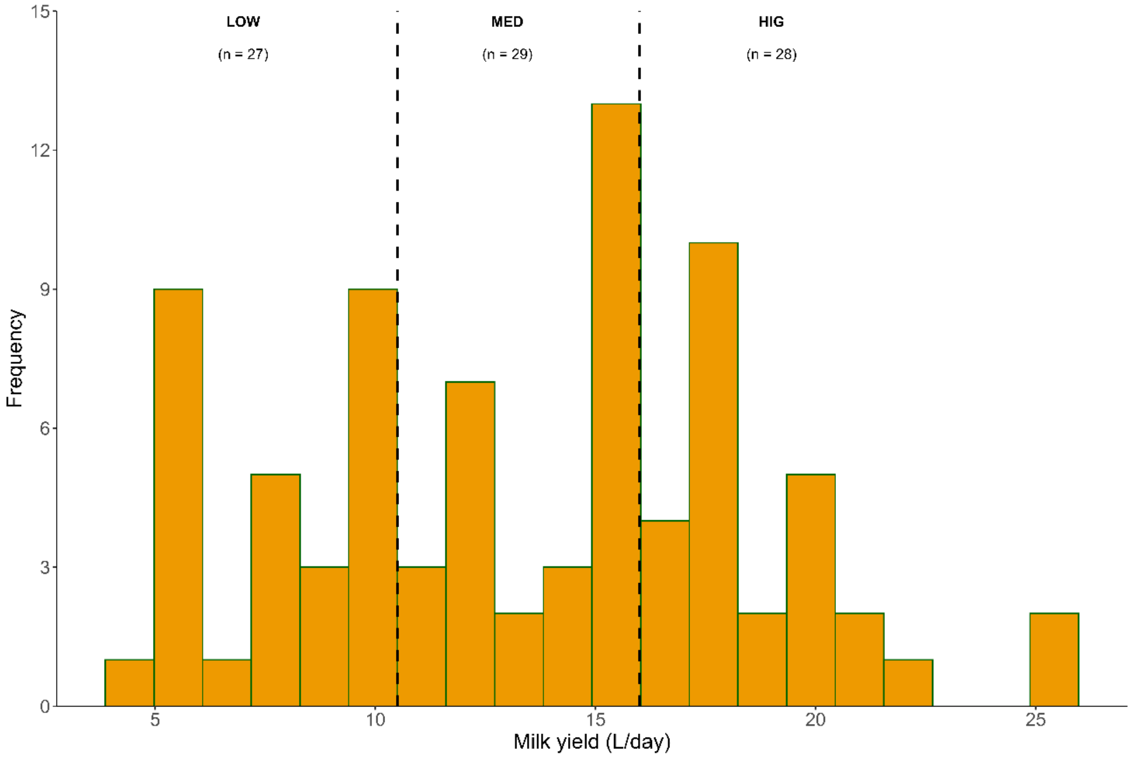

Figure 1. Frequency distribution of daily milk production categorized by production level.

LOW = low production level; MED = medium production level; HIG = high production level.

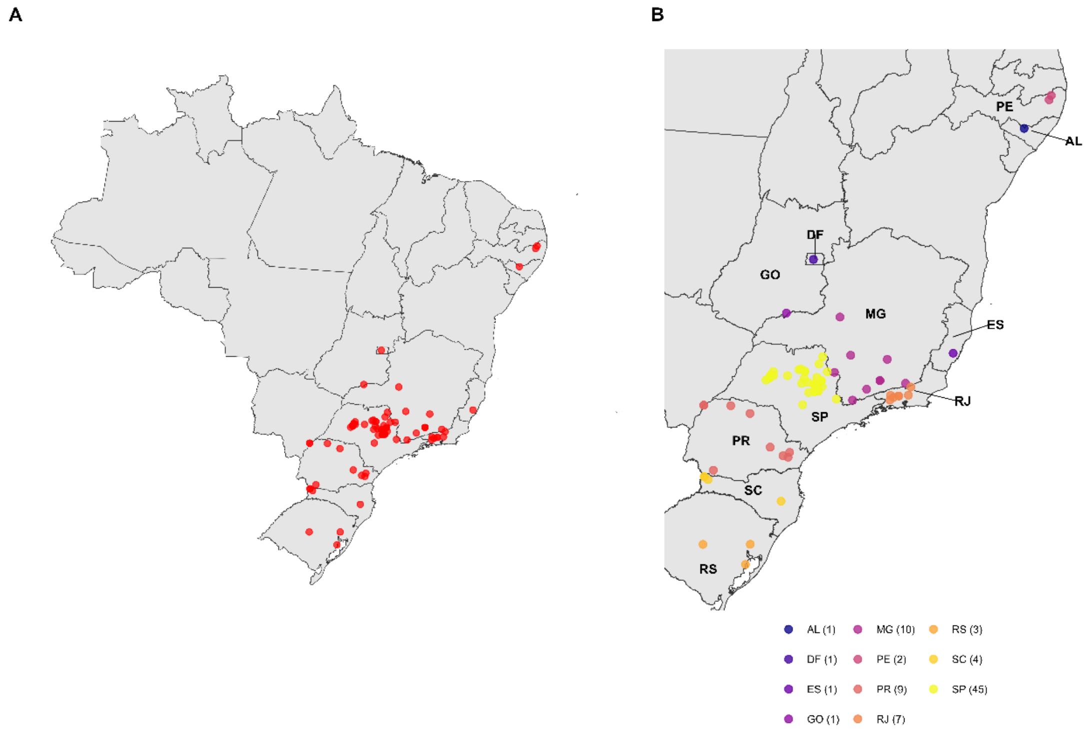

Figure 2. Distribution of organic dairy farms across the federal states in Brazil (A), with a zoomed-in view highlighting the main production regions (B).

SP = São Paulo, MG = Minas Gerais, PR = Paraná, RJ = Rio de Janeiro, SC = Santa Catarina, RS = Rio Grande do Sul, PE = Pernambuco, AL = Alagoas, ES = Espírito Santo, GO = Goiás.

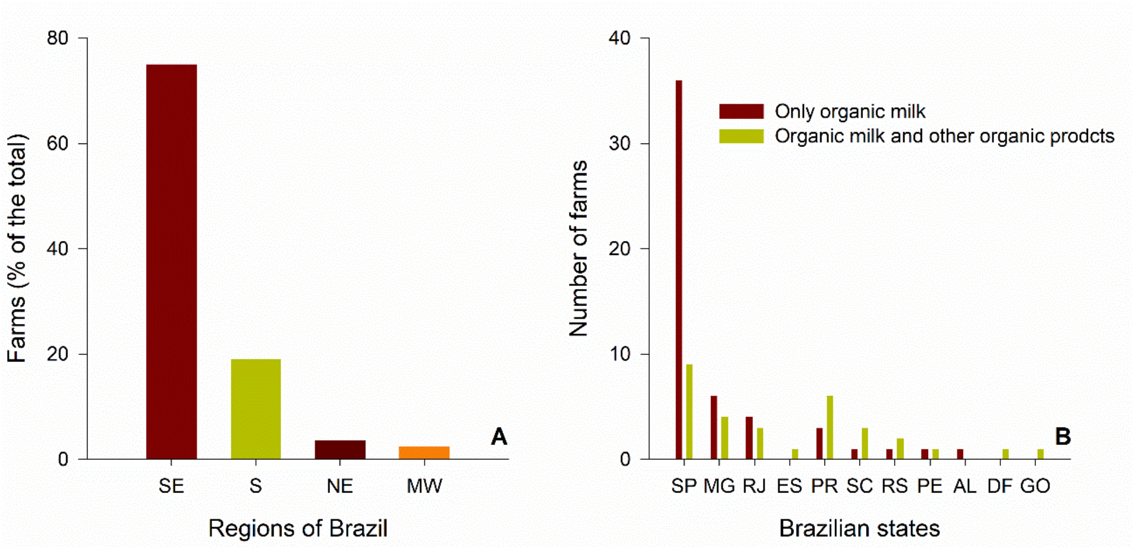

Figure 3. Distribution of organic milk production units by Brazilian region (A) and production of organic milk or milk and other organic products by Brazilian state (B).

SE = Southeast; S = South; NE = Northeast; MW = Midwest; SP = São Paulo, MG = Minas Gerais, RJ = Rio de Janeiro, ES = Espírito Santo, PR = Paraná, SC = Santa Catarina, RS = Rio Grande do Sul, PE = Pernambuco, AL = Alagoas, DF = Distrito Federal, GO = Goiás.

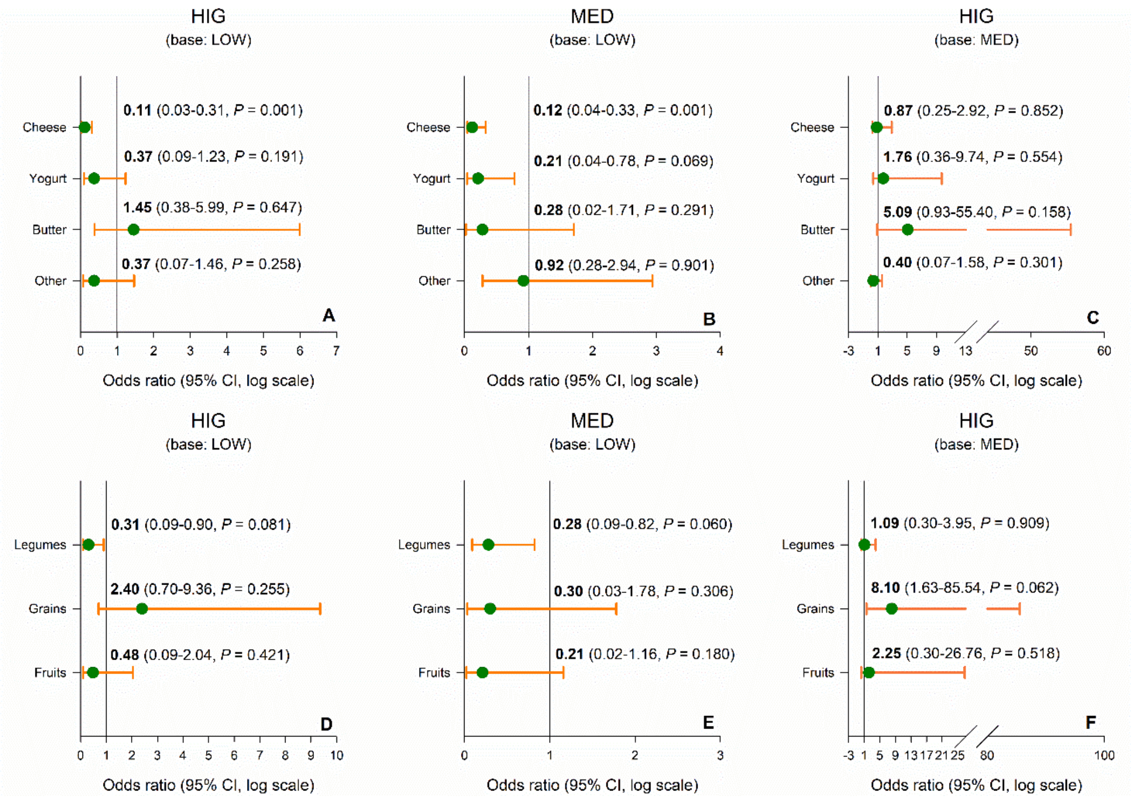

Figure 4. Effects and odds ratio from a logistic regression for the association between production level and organic dairy products (A, B, and C) and other organic activities (D, E, and F) of Brazilian organic dairy farms.

LOW = low production level; MED = medium production level; HIG = high production level.

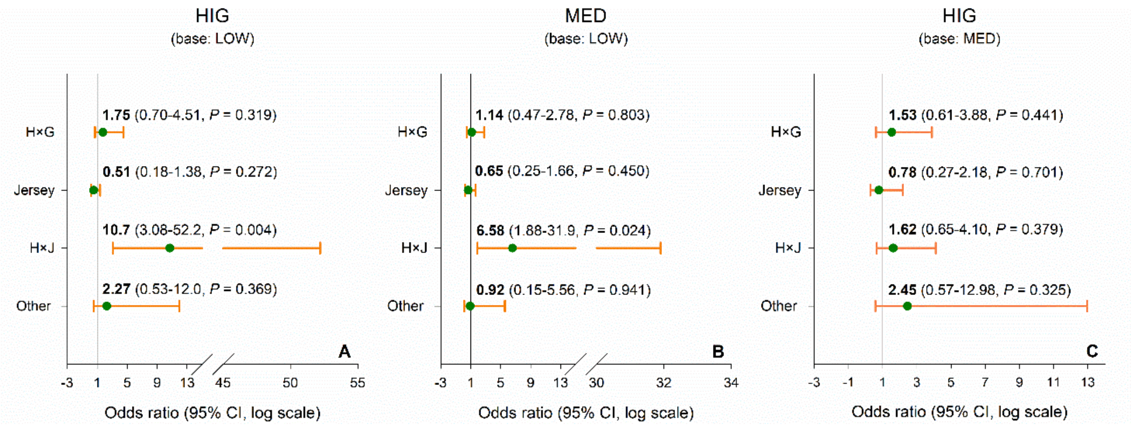

Figure 5. Effects and odds ratio from a logistic regression for the association between different production levels and dairy breeds of Brazilian organic dairy farms.

LOW = low production level; MED = medium production level; HIG = high production level; H×G = Holstein×Gyr crossbred; H×J = Holstein×Jersey crossbred.

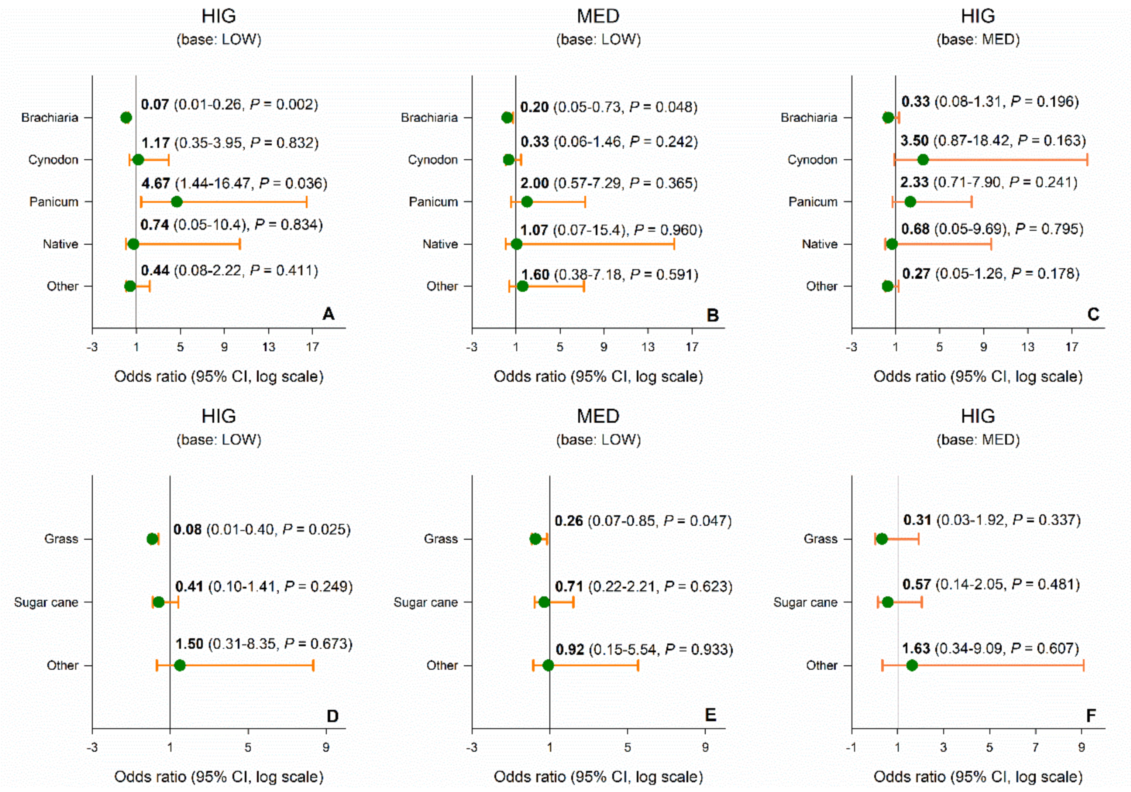

Figure 6. Effects and odds ratio from a logistic regression for the association between high and medium production levels when using grass (A, B, and C) or roughage supplementation (D, E, and F) in Brazilian organic dairy farms.

LOW = low production level; MED = medium production level; HIG = high production level.

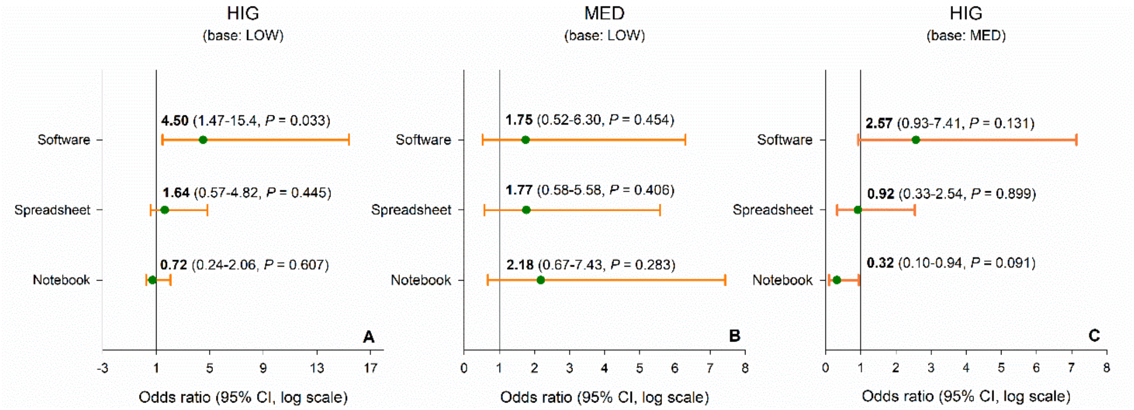

Figure 7. Effects and odds ratio from a logistic regression for the association between different production levels and herd management tools in Brazilian organic dairy farms.

LOW = low production level; MED = medium production level; HIG = high production level.

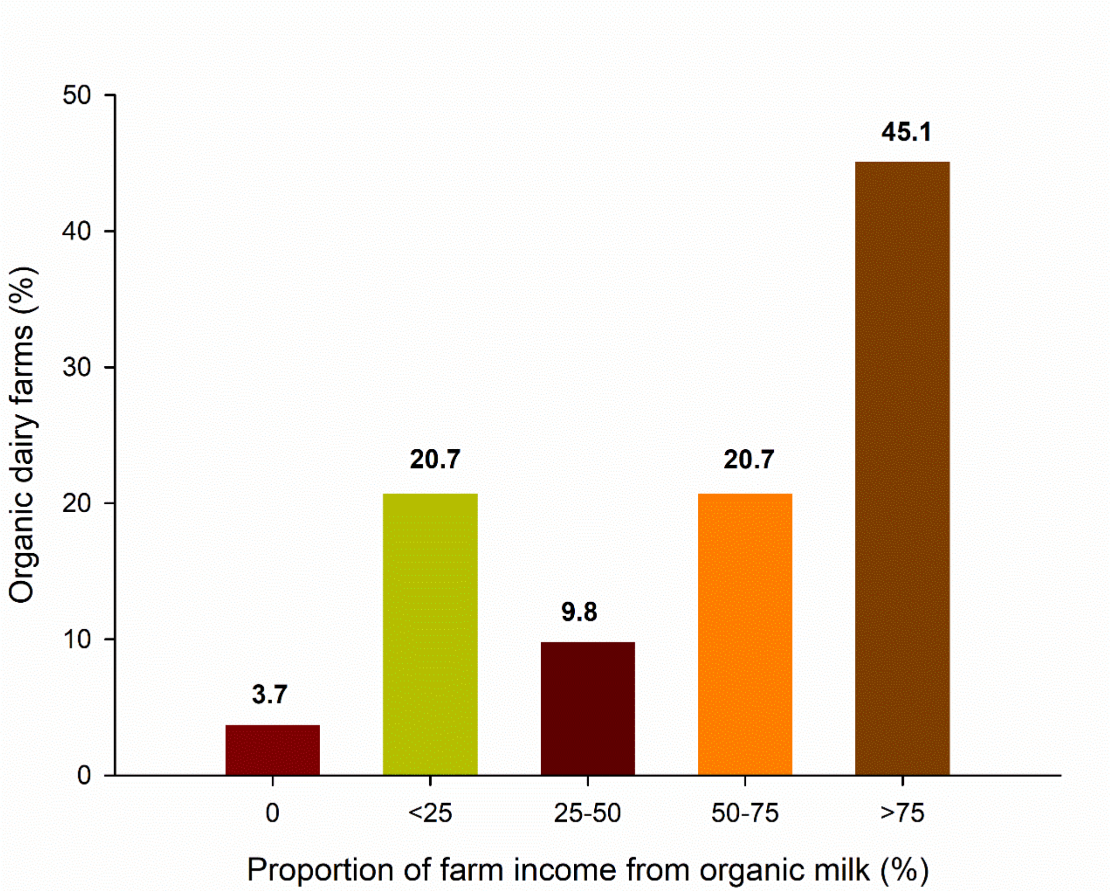

Figure 8. Percentage of organic dairy farms according to the proportion of farm income from organic milk production, by stratum.

3. Chapter 2

Dietary tannins and compounds from fermentation of *Saccharomyces cerevisiae* effects on intake, productive performance, enteric methane emission, and rumen microbial diversity of lactating dairy cows

ABSTRACT

The reduction of enteric methane emissions from ruminants is a central topic in research on sustainable livestock research. Feed additives targeting the rumen microbiome are among the strategies being explored to reduce methane emissions and improve nutrient efficiency. In this context, the aim of this study was to investigate the effects of a supplement composed by condensed tannins and *Saccharomyces cerevisiae* yeast compounds on intake, productive performance, nitrogen metabolism, methane emission, and rumen microbial biodiversity of lactating Holstein and Holstein×Gyr dairy cows. Sixteen dairy cows (8 Holstein and 8 Holstein×Gyr) were allocated to two treatments: a control group (**CON**; no supplementation) and a supplemented group (**SUP**) that received 2.7 g/kg DM of the additive (Muucare Nature®). The trial lasted 84 days, including a 24-day adaptation period. The animals were housed in tie stalls and kept under identical conditions. Rumen fluid was collected via an esophagus on days 22 and 52, and the microbial composition was later analyzed by 16S rRNA sequencing. The VFA concentrations and rumen ammonia nitrogen were also quantified. Methane emission was measured using the sulfur hexafluoride tracer technique. The additive increased the apparent digestibility of DM and OM by about 6% without affecting feed intake or milk yield. Methane emission per ECM (g/kg) was reduced by almost 30%, and methane emission per milk yield (g/kg) showed a similar trend. The SUP cows showed higher propionate production and a lower acetate-to-propionate ratio, indicating a shift in fermentation. The microbial diversity in the rumen was altered, with a reduced alpha diversity and a different community composition, including an increased abundance of *Prevotella ruminicola*. The total amount of methanogens was unchanged, although one species, *Methanobrevibacter smithii*, tended to be less abundant. The additive reduced methane emission and improved nutrient digestibility, rumen fermentation, and nitrogen efficiency. These results indicate that the additive based on Acacia tannins and *Saccharomyces cerevisiae* yeast is a sustainable tool to reduce methane emissions in dairy production systems without compromising milk production.

Keywords: Greenhouse gas reduction, Ruminal Modulationsupplement, Condensed Tannins from *Acacia mearnsii* Acacia.

Nonstandard abbreviations:

CON = control group; CO_2 = carbon dioxide;

SUP = supplemented group

H_2 = hydrogen

uNDF = undigestible NDF

ME/MY = methane emissions relative to milk yield

ME/ECM = methane emissions relative to energy corrected milk

ME/DMI = methane emissions relative to dry matter intake

ME/OMI = methane emissions relative to organic matter intake

ME/OMId = methane emissions relative to digestible organic matter intake

MY = milk yield; SF_6 = sulfur hexafluoride

INTRODUCTION

Methane is considered one of the main contributors to greenhouse gas emissions and it is estimated that ruminants are responsible for up to 68.2% of total emissions from agriculture (Berça et al., 2023) and 9.6% of total anthropogenic emissions in the world (US EPA, 2017). Strategies to reduce methane emissions on modern dairy farms aim to maintain high productivity through genetic approaches and feed management to alter the population and fermentative activity of the microbiota in the rumen. The products resulting from the breakdown of dietary components by the rumen microbiota led to the production of microbial protein, VFA, hydrogen (**H_2**) and carbon dioxide (**CO_2**) (Cunha et al., 2016). However, compounds such as H_2 and CO_2 are used as substrates by the methanogenic archaea present in the rumen and produce methane. To prevent the accumulation of H_2 in the rumen, which can create an unfavorable environment for fermentative processes, the synthesis of methane by methanogenic archaea is essential.

Therefore, the use of additives in the diet of dairy cows is one of the most important tools currently used. They can modulate rumen fermentation and improve the utilization of nutrients by microbiota, resulting in better animal performance. In this context, there is growing interest in society and science in the use of alternative additives that can improve animal performance, increase their efficiency while reduce methane emissions and making milk production more environmentally friendly, such as the use of polyphenols and yeasts (Hassan et al., 2020).

Polyphenols are natural compounds produced by plants that have been shown to be beneficial primarily due to their antioxidant and anti-inflammatory effects (Gessner et al., 2017). Tannins are plant polyphenol complexes found in legumes and other C3 plants (Berça et al., 2023). They can bind mainly to proteins and to a lesser extent to metal ions, amino acids, and polysaccharides (Goel and Makkar, 2012). Although tannins are considered an “anti-nutritional factor”, research has shown that some tannins have positive effects on milk production, milk quality, and animal welfare when used appropriately in cattle diets (Berça et al., 2023). In addition, the addition of tannins to feed can contribute to the reduction of methane emissions (Gessner et al., 2017; Cardoso-Gutierrez et al., 2021; Orzuna-Orzuna et al., 2021). Suggesting that the addition of tannins to feed can increase N fixation and energy utilization, which in turn reduces methane emissions and ammonia excretion (Wischer et al., 2014; Wang et al., 2021).

Supplementation with yeast and its products such as yeast cultures, autolyzed yeast, and yeast cell walls has also been shown to be beneficial by altering the proportion of VFA in the rumen, increasing propionate production relative to acetate and reducing ammonia levels in the rumen (Xiao et al., 2016; Amin and Mao, 2021), improved milk production and milk fat, increased nutrient digestibility by stimulating fiber digestion and stabilized rumen pH (Amin and Mao, 2021; Burdick Sanchez et al., 2021).

In addition, studies suggest that genetics have an influence on methane emissions (Villanueva et al., 2023; Matiello et al., 2024). Recently, a study showed that Holstein cows had a higher DMI and milk production (kg/BW0.75) compared to Holstein×Gyr crossbred cows and tended to produce less methane per kg of milk (Matiello et al., 2024).

To our knowledge, there is no study on products that represent a link between two natural technologies (tannins + yeast compounds) to reduce methane emissions, as some technologies currently available on the market are composed of synthetic and non-natural products. In this context, it was hypothesized that lactating cows receiving a dietary supplement based on tannins and yeast compounds from *Sacharomyces cerevisiae* would show higher milk production and lower methane emissions in comparison and that the response would be different in Holstein and Holstein×Gyr crossbred cows. The aim was to investigate the effect of a supplementary feed based on tannins and yeast compounds from fermentation of *Saccharomyces cerevisiae* on intake, productive performance, N metabolism, methane emissions, and microbial biodiversity in the rumen of lactating Holstein and Holstein×Gyr crossbred cows.

MATERIAL AND METHODS

All procedures involving the use of animals were previously reviewed by the Ethics Committee for the Use of Animals of the Department of Animal Science at the Universidade Federal de Viçosa and approved under protocol no. 057/2024. The experiment was conducted at the Teaching, Research, and Extension Dairy Farm of the Universidade Federal de Viçosa (UFV), Viçosa-Minas Gerais, Brazil.

Animals, Experimental Design, and Treatments

Sixteen lactating cows (8 Holsteins and 8 Holstein×Gyr crossbred) were assigned to a randomized block design, with groups formed based on DIM and milk yield (**MY**). Average ± SD of DIM and MY were 345 days and 19.6 kg/day for group 1, 175 days and 26.0 kg/day for group 2, and 88 days and 42.5 kg/day for group 3. The animals were then allocated to 2 treatments: a control treatment (**CON**; without additives) and a supplemented treatment (**SUP**) in which the diet contained an additive containing condensed tannins from *Acacia mearnsii* and yeast compounds from fermentation of *Saccharomyces cerevisiae*, administered at 2.7 g/kg DM (Table 5). The experimental period lasted 84 days, with the first 24 days serving to adapt the animals to the facilities. During the adaptation period, the animals were given experimental diets.

Diets were provided to each animal individually and consisted of corn silage, Tifton hay, whole cottonseed, and concentrate, formulated to meet nutritional requirements based on each group's initial milk production (Table 5). The diet was offered twice daily — at 0800 and 1430 hours — after the cows had returned from milking so that they could consume it ad libitum. The amount of feed was adjusted to give approximately 5 % orts (as-fed basis). Orts were removed and weighed daily before the feed was offered and individual intake was calculated as the difference between the feed offered and orts. Throughout the trial, cows had ad libitum access to water and individual water intake was recorded daily using a hydrometer system.

The animals were housed in a tie stall with sand bedding (2.0 × 1.22 m) and equipped with individual drinkers and feeders. Every morning, manure and wet bedding were removed and cleaned; dry sand was added. In the afternoon, in addition to cleaning, hydrated lime was applied to all beds to inhibit microbial growth. The cows were milked 3 times a day (0730, 1430, and 2030 hours) using a mechanical system equipped with an automatic extractor. After each milking, the cows were returned to the tie stall and given the experimental diet. All animals were weighed at the beginning and end of the experiment.

Milk Yield and Analysis

The MY was measured between the 15th and 17th and the 45th and 47th day and milk was collected from all animals. To collect the milk, collection cups were connected to the automatic milking system so that the milk samples could be taken from each animal individually and evenly throughout the entire milking process. When the milking set was disconnected, a volume of 40 mL of milk was collected in a sterile collection bottle. At the end of the 3 milkings of the day, a composite sample was prepared from the 3 collected samples for subsequent analysis of the milk composition in terms of fat, protein, lactose, and total solids using an automatic ultrasonic milk analyzer (Lactoscan S_LP (Milkotronic LTD, Nova Zagora, Bulgaria)) and the results obtained were averaged. In addition, a sample was collected in a vial with preservative (Bronopol). The vials with the samples were shaken twice at 15-minute intervals to completely dilute the preservative and stored in a polystyrene box with thermogel. The samples were then sent to a laboratory for analysis of SCC by flow cytometry and MUN by infrared.

The ECM was calculated according to NASEM (2021):

$$ECM = (0.252 \times M) + (12.30 \times F) + (7.77 \times P)$$

where: ECM = energy-corrected milk (kg/day), M = milk yield (kg/day), F = fat yield (kg/day), and P = protein yield (kg/day).

Intake, Digestibility, and Urine Collections

Between days 17–19 and 47–49, samples of orts, hay, cottonseed, and silage offered to the animals were collected. Composite samples were prepared at the end of each 3-day sampling period. In addition, samples of the concentrate offered were taken during the preparation of each batch.

On days 18 and 19, as well as 48 and 49, spot collections of feces and urine were performed. Fecal samples were collected manually directly from the rectal ampoule, with an interval of 4-hours, according to the method proposed by Morris et al. (2018). On the first day, collections were performed at 0600, 1000, 1400, 1800, and 2200 hours, and on the second day at 0200 hours, for a total of 6 collections.

Urine was collected in six equally spaced samplings over a 24-hour period, which were synchronized with the fecal collections, as described by Lee et al. (2019). A composite sample was prepared from the total urine volume, homogenized, and filtered through gauze. Two aliquots were then taken: a 50 mL undiluted sample for total N analysis, and a 10 mL sample that was immediately diluted with 40 mL of 0.036 N H_2SO_4 to prevent precipitation of uric acid. Both aliquots were stored at -15°C. The diluted sample was then analyzed for the concentrations of creatinine, urea, uric acid, and allantoin as described in the following section.

The estimation of microbial protein synthesis was based on uric acid and allantoin excretion in urine, using an average daily rate of 0.385 mmol/kg BW$^{0.75}$/day, according to the methodology of Chen and Gomes (1992). Daily urine volume was determined by creatinine excretion, using an average rate of 29.0 mg/kg BW/day, as reported by Valadares et al. (1999). Quantification of creatinine, uric acid, and urea was performed with a Mindray BS200E automated biochemistry analyzer using colorimetric kinetic, colorimetric enzymatic and timed kinetic methods, respectively. Allantoin analysis was performed using the colorimetric method outlined by Chen and Gomes (1992). Total N was quantified using the INCT-CA N-001/2 method as described in Detmann et al. (2025). In addition, the N balance was calculated as the difference between the N ingested and the N excreted via feces, urine, and secreted in milk.

Samples of feces, orts, and silage were partially dehydrated in a forced-air oven at 55°C according to the INCT–CA G-001/2 protocol. Subsequently, samples of feces, orts, silage, hay, cottonseed, and concentrates were then ground onto a 1 mm sieve using a knife mill. All samples were analyzed for DM (INCT–CA G-003/2), ashes (INCT–CA M-001/2), CP (INCT–CA N-001/2), ash-corrected NDF (INCT–CA F-002/2; INCT–CA M-002/2), and undigestible NDF (**uNDF**; INCT–CA F-009/2; Detmann et al., 2025). The uNDF was used as an internal marker to estimate fecal DM excretion as described by Morris et al. (2018).

Rumen Contents and Blood Collections

Rumen contents were collected on days 22 and 52 to evaluate rumen ammonia nitrogen (**RAN**) concentration, microbial diversity, and VFA profile. Sampling was performed 4 hours after the first feeding using an esophageal probe. The rumen contents were filtered through a triple layer of gauze. Two rumen fluid samples per animal were placed in Eppendorf tubes and stored at –15°C for subsequent analysis of RAN and VFA concentrations. An additional two samples were stored at –80°C in an ultra-low-temperature freezer for microbial diversity analysis.

The VFA were analyzed by high performance liquid chromatography, treating the samples as described by Siegfried et al. (1984) and separating them on a HPX 87H Biohad column, 300 × 7.8 mm, at 45°C using a chromatograph (Shimadzu model:

LC20AT) coupled to a RID-20A refractive index detector. The mobile phase used was sulfuric acid (H_2SO_4, 5 mmol/L) at a flow rate of 0.7 mL/min. The RAN was analyzed in a microdilution plate according to the methodology described by Chaney and Marbach (1962).

At the same time as the rumen content collection, blood samples were obtained via coccygeal vein puncture using vacuum tubes containing a separating gel and coagulation activator for subsequent analysis of serum glucose, total protein, BUN, and IGF-1 concentrations. The tubes were placed in a polystyrene box with ice and transported to the laboratory where they were centrifuged at 3,000 × g for 20 minutes. Two serum aliquots per animal were transferred to Eppendorf tubes and stored at –15°C until analysis. Serum urea nitrogen, glucose, and total protein were quantified by kinetic fixed-time methods, colorimetric enzymatic methods, and biuret methods, respectively, using commercial kits (Bioclin®) on an automated biochemistry analyzer (Mindray BS200E). The IGF-1 concentrations were determined by chemiluminescence using Siemens Immulite 2000 assay kits.

Microbial Biodiversity

Microbial diversity was assessed using a commercially available, matrix-specific DNA extraction kit, following the manufacturer's instructions. DNA quality and concentration were evaluated by spectrophotometry using a NanoDrop instrument (Thermo Scientific™). For bacterial analysis, a ~460-bp fragment of the V3–V4 hypervariable region of the 16S rRNA gene was amplified using primers 375F and 805R under the following PCR conditions: initial denaturation at 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min.

Amplicons were labeled with Illumina® Nextera dual-index barcodes under the following PCR conditions: 95°C for 3 min; 8 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; followed by a final extension at 72°C for 5 min. The resulting products were purified, pooled, and sequenced on an Illumina® NextSeq platform (Degnan and Ochman, 2012) using 300-base paired-end reads. Sequencing data were processed using the QIIME2 (Quantitative Insights Into Microbial Ecology) platform (Caporaso et al., 2011), following a workflow that included quality filtering and trimming of forward and reverse reads (R1 and R2 truncated at 170 bp) for bacterial community analysis.

The bioinformatics workflow included adapter removal, host read mapping, filtering of low-quality sequences, chimera removal, and taxonomic classification. Amplicon sequence variants were identified based on sequence homology and used to classify sequences into bacterial genera. Taxonomic assignment was performed using the Genome Taxonomy Database, version 207 (Parks et al., 2022), with in silico reads extracted from the same amplified V3–V4 regions of the 16S rRNA gene. Classification codes (e.g., NSJ-53 or sp001543345) correspond to reference genome identifiers used for operational taxonomic unit clustering. To ensure consistent coverage across all samples, bacterial community analysis was performed using 67,920 reads per sample, with data normalized to the lowest read count across samples. This normalization minimizes bias in comparisons between microbiome profiles with varying sequencing depths.

Methane Measures

The methane emissions were measured between days 23–30 and 53–60 using the sulfur hexafluoride (**SF_6**) tracer gas technique described by Johnson et al. (1994), with modifications proposed by Primavesi et al. (2004). Prior to the measurement period, a permeation capsule with a known SF_6 release rate was administered into the rumen via forced swallowing. The rate of CH_4 emission was calculated based on the concentrations of CH_4 and SF_6 measured in the collected samples and the known SF_6 release rate from the capsule.

Before sampling began, each animal was equipped with the SF_6 permeation capsule, a halter adapted to support the sampling apparatus, and a capillary tube system designed to collect exhaled air into a PVC canister (yoke). After each 24-hour collection period, the sampled yoke was replaced with a pre-evacuated yoke to initiate a new sampling cycle. The internal pressure of the sampled yoke was recorded and equalized. To collect ruminal air, a disposable syringe and fine needle were used to transfer gas into 30-mL penicillin bottles sealed with rubber stoppers and aluminum crimps, which had been previously evacuated using a manual vacuum pump. Five vials were prepared per yoke, sealed, packaged, and submitted for subsequent gas analysis.

The methane concentrations were determined using a gas chromatograph equipped with a flame ionization detector, while SF_6 concentrations were measured using a gas chromatograph fitted with an electron capture detector, as described by

Cunha et al. (2016) and Primavesi et al. (2004). Methane emissions were expressed as absolute values (g/day) and relative to MY (**ME/MY**; g/kg), ECM (**ME/ECM**; g/kg), DMI (**ME/DMI**; g/kg), OM intake (**ME/OMI**; g/kg), and digestible OM intake (**ME/OMId**; g/kg).

Statistical analyzes

Data were analyzed with the SAS GLIMMIX procedure (SAS Institute Inc., 2024) using the following model:

$$Y_{ijklm} = \mu + D_i + R_j + B_k + (D \times R)_{ij} + \delta_{ijkl} + P_m + (D \times P)_{im} + (R \times P)_{jm} + (D \times R \times P)_{ijm} + \epsilon_{ijklmn}$$

where: Y_{ijklmn} = observed variable; μ = overall average; D_i = fixed effect of treatment; R_j = fixed effect of breed; B_k = random effect of block; $D \times R_{ij}$ = fixed effect of interaction between treatment and breed; δ_{ijkl} = random error when the variance between animals within treatment is equal to the covariance between repeated measures within animals; P_m = fixed effect of collection time; $D \times P_{im}$ = fixed effect of interaction between treatment and collection time; $R \times P_{jm}$ = fixed effect of interaction between breed and collection time; $D \times R \times P_{ijm}$ = fixed effect of interaction between treatment, breed and collection time; and ε_{ijklmn} = random error.

The covariance structures evaluated included variance components, compound symmetry, heterogeneous compound symmetry, first-order autoregressive, heterogeneous first-order autoregressive, Toeplitz, and heterogeneous Toeplitz. The structure that yielded the lowest corrected Akaike Information Criterion (AICc) value was selected for the final model.

The SCC variable was fitted according to a lognormal distribution, while the Gaussian distribution was used for the other variables. Observations with an internal student residual of more than |2.5| were considered "outliers" and removed from the respective data set. When necessary, the Tukey test was used to separate the means, and in all cases, differences were explained when $P < 0.05$ and trend for $P < 0.10$.

Statistical comparisons of alpha diversity between groups were performed using the nonparametric Mann–Whitney U test (Mann and Whitney, 1947), with statistical significance declared when $P < 0.05$ and trend for $P < 0.10$. Beta diversity was assessed by permutational multivariate analysis of variance (perMANOVA) using the adonis2 function from the *vegan* package in R, with 10,000 permutations and a 95% confidence level. All analyses and visualizations were conducted in R. Alpha diversity metrics were calculated using the *phyloseq*, *vegan*, and *microbiome* packages. To identify taxa with differential relative abundance between groups, the Mann–Whitney U test was applied at all taxonomic ranks, using a significance of $P < 0.05$ and trend for $P < 0.10$.

RESULTS

Intake, Digestibility, and Productive Performance

No significant effects of treatment, breed or interaction were observed for water intake or intake of DM, OM, CP, and NDF expressed in kg/day, or for DM intake expressed in g/kg of BW ($P > 0.05$; Table 2). No treatment × breed interactions were detected for any parameter ($P > 0.05$).

Treatment affected DM and OM digestibilities ($P < 0.05$), with SUP cows having 6.3% greater DM digestibility (655 vs. 616 g/kg) and 6.7% greater OM digestibility (704 vs. 660 g/kg) than CON cows ($P = 0.02$ and $P < 0.001$, respectively; Table 2). A treatment × breed interaction was observed for DM digestibility ($P = 0.049$). Within CON, Holstein×Gyr crossbred cows had greater DM digestibility than Holstein cows (634 vs. 599 g/kg; $P = 0.034$). However, for SUP, no differences were observed between breeds (662 g/kg for Holstein and 645 g/kg for crossbreds).

Period effects were observed for intake of DM, OM, CP, and NDF (kg/day), relative DM intake (g/kg of BW), and water intake (all $P < 0.01$), as well as for DM, OM, and NDF digestibilities ($P < 0.05$), with greater values in period 2 than period 1 (Table S1).

No significant effects of treatment, breed, or treatment × breed interaction were observed for MY and milk composition ($P > 0.05$; Table 3). Although not statistically significant, Holstein cows produced numerically more milk than Holstein×Gyr crossbred cows (27.4 vs. 24.2 kg/day). Period effects were observed for MY ($P = 0.018$) and milk fat percentage ($P = 0.027$). Mean MY was greater in period 2 than period 1 (26.8 vs. 24.8 kg/day), whereas milk fat percentage was lower in period 2 than period 1 (3.08 vs. 3.44%; Table S1).

Methane Emission

Treatment affected ME/ECM (*P* = 0.023) and tended to affect ME/MY (*P* = 0.078). SUP cows had 29.6% lower ME/ECM than CON cows (6.33 vs. 9.08 g/kg), and 23.8% lower ME/MY than CON cows (5.78 vs. 7.59 g/kg; Table 4).

A treatment × breed interaction was observed for ME/DMI (*P* = 0.037). For CON no difference was observed between breeds. However, within SUP, Holstein × Gyr crossbred cows had lower ME/DMI than Holstein cows (6.33 vs. 10.1 g/kg of DM; Figure 1). Additionally, a trend for treatment × breed interaction was observed for both total methane emission and ME/OMId (Table 4), which followed similar patterns. No differences were observed between breeds within the CON treatment. However, within the SUP treatment, Holstein × Gyr crossbred cows exhibited lower total methane emissions compared with Holstein cows.

No effect of period was observed for any variable involved in the methane emission (Table S2).

Serum and Ruminal Parameters

When analyzing blood serum variables, no effects were observed between treatments, breeds, or interaction effects for GLU, BUN, TP, and IGF-1 (*P* > 0.05; Table 5). A period effect was observed for serum concentrations of IGF-1 (*P* = 0.02), indicating an increase in IGF-1 concentration at the end of the experiment with averages of 124 ng/mL in period 1 and 203 ng/mL in period 2 (Table S2). No effects of treatments, breeds, and interaction were observed for total VFA and concentrations of acetate and butyrate (*P* > 0.05). However, a trend for treatment was observed for propionate, where the SUP group presented a higher value compared with the CON group (Table 5).

Regarding the molar proportions of VFA, an effect of treatment was observed for propionate, as well as for the acetate-to-propionate ratio (P < 0.05). The SUP treatment had a higher molar proportion of propionate compared with the CON group (P = 0.03; Table 5). An effect of breed was observed for the molar proportion of propionate (P = 0.02), where Holstein × Gyr crossbred cows had higher propionate compared with Holstein lactating cows (17.5 vs. 19.8%, respectively).

An interaction effect between treatment and breed was observed for the molar proportion of butyrate (*P* = 0.03), wherein no difference was observed between breeds within the CON group. However, within the SUP group, Holstein cows had a higher percentage of butyrate compared with Holstein × Gyr crossbred cows (10.3 vs. 8.68 mmol/100 mmol, respectively).

No effect was observed for treatments, breeds, or interaction between treatment × breed for RAN (*P* > 0.05; Table 5). A period effect was observed for RAN (*P* < 0.01), indicating a general increase at the end of the experiment, with averages of 44.9 mg/dL in period 1 and 71.2 mg/dL in period 2 (Table S2).

Microbial Protein Synthesis and Nitrogen Metabolism

There was no significant effect for the treatments, breeds, or interaction between treatment × breed for urinary urea excretion, microbial protein synthesis, rumen degradable protein flow, and microbial efficiency (*P* > 0.05). A period effect was observed for all analyzed parameters (*P* < 0.10; Table 6), indicating a reduction over time in urinary urea excretion (*P* < 0.01; Table S3).

Regarding N balance, an effect of treatment was observed for fecal N excretion and milk N secretion (*P* < 0.10). The SUP group presented a reduction in fecal N excretion compared with the CON group (*P* = 0.02), with averages of 134 g/day for the SUP group and 161 g/day for the CON group. This result indicates that the additive was effective in reducing environmental impacts caused by nitrogen in the environment. Milk N secretion was higher for the SUP group compared with the CON group (*P* = 0.02), with averages of 119 g/day for the CON group and 148 g/day for the SUP group, indicating that the product was effective in converting nitrogen into milk protein. There was no effect between breeds or interaction between treatment × for any of the analyzed items (*P* > 0.05). There was a period effect for N intake and urinary N excretion (*P* < 0.05), indicating that overall, at the end of the experiment, there was an increase in N intake and urinary N excretion (Table S3).

Microbial Diversity

Overall, no interactions between treatment and breed were observed for alpha diversity, beta diversity, taxonomic composition, or differential abundance of taxa.

However, treatment effects were detected for both alpha and beta diversity analyses ($P < 0.05$; Figure 2). The SUP treatment exhibited lower alpha diversity and greater beta diversity compared with the CON group, indicating a more specialized microbial community structure. The Firmicutes to Bacteroidota ratio differed between treatments ($P = 0.036$), with lower values observed in the SUP group compared with the CON group.

At the species level, treatment affected the differential abundance of Prevotella ruminicola and Limivicinus sp002320035, with the SUP group exhibiting lower abundance of Limivicinus sp002320035 and higher abundance of Prevotella ruminicola compared with the CON group (Figure 3). The differential abundance of methanogenic archaea was not influenced by treatment at the species, genus, or family levels; however, a trend toward lower abundance of Methabrevibacter smithii was observed in SUP animals (Figure 4B; $P = 0.071$).

DISCUSSION

The present study was conducted to investigate the effect of a supplement tannins based on Acacia extract and compounds from fermentation of *Saccharomyces cerevisiae* (Muucare Nature®) on the intake, productive performance, and methane emissions of lactating Holstein and Holstein × Gyr crossbred cows.

The observed period effect on the intake of DM (kg/day or g/kg of BW), OM, CP, and NDF, and water intake indicates an overall increase in animal intake over time. In response, an increase in milk production and its nutrients (percentage of fat, protein, and lactose) was observed at the end of the experiment, but without treatment effect. It is well documented that moderate levels of tannins in the diet can improve nutrient utilization, by improving digestibility, without deleterious effect on intake and nutrient metabolism. (Fitri et al., 2022; Battelli et al., 2024).

The ability of yeast to stimulate the growth of fibrolytic bacteria is well documented. Pinloche et al. (2013) conducted a study with the inclusion of 0.5 g/day or 5 g/day of live *Saccharomyces cerevisiae* yeasts in the diet of Holstein cows at 40 DIM, fed a diet consisting of corn silage (61% of DM), concentrates (30% of DM), dehydrated alfalfa (9% of DM), and a mineral and vitamin mixture (1% of DM). The authors reported an increase in some fibrolytic bacterial groups (Fibrobacter and Ruminococcus) and lactate-utilizing bacteria (Megasphaera and Selenomonas), which stabilized the pH in the rumen by reducing the redox potential in the rumen and favoring fiber degradation. However, this pattern was not observed in our study, which emphasizes that these effects can be influenced by various factors such as diet, dose, etc.

Tannins can also alter digestive processes in the rumen by modulating the rumen microbiota and enabling cattle to convert carbohydrates into energy (Min et al., 2014; Carrasco et al., 2017). Carrasco et al. (2017) found that supplementation with 2% Chestnut tannin and Quebracho in cattle feed altered some fibrolytic, amylolytic, and ureolytic bacterial communities in the rumen and reduced methanogenic archaea. The study indicated an increase in cellulolytic and hemicellulolytic bacteria of the genus Ruminococcus and Firmicutes, while bacteria of the genera Prevotella and Fibrobacter decreased. The Firmicutes/Bacteroidota ratio was reduced in the SUP treatment, accompanied by an increased abundance of Prevotella spp. and especially Prevotella ruminicola. These results explain the increased digestibility of TM and OM by SUP treatment observed in this study, although this increase was not translated into higher MY.

Quirino et al. (2024) found that the microbial community of Holstein and Holstein × Gyr crossbred heifers fed the same diet in a pasture-based system showed differences in their composition, mainly related to the phylum of Bacteroidota and the abundance of members of the family Prevotellaceae and the genus Prevotella. In contrast, in our study we did not detect differences in microbial communities as a function of breed, suggesting that diet is the most important factor modulating microbial communities.

Similar to our results, meta-analyzes by Orzuna-Orzuna et al. (2021) and Berça (2023) as well as a study by Dai and Faciola (2019) report that tannins increase the concentration of butyrate and propionate in the rumen of cattle. The SUP treatment also led to a reduction in the acetate:propionate ratio. Interestingly, the effect of yeasts and tannins may differ depending on the breed. Holstein × Gyr crossbred cows exhibited higher propionate production in the rumen and showed better performance and efficiency by excreting less ME/DMI than Holstein cows when supplemented. The higher level of propionate observed suggests that supplementation with tannins and Saccharomyces cerevisiae yeasts altered the microbial population in the rumen, resulting in a change in the type and proportion of VFA produced. This finding was confirmed by an increased abundance of Prevotella spp. in the SUP treatment, particularly Prevotella ruminicola, which is associated with propionate production.

Bacteria of the genus Prevotella may be involved in propionate synthesis and play an important role in the rumen by utilizing polysaccharides and starch as substrates. In addition, studies have shown links between bacteria and rumen fermentation, feed efficiency, MY, milk fat, and methane emission (Betancur-Murillo et al., 2023; Shinkai et al., 2024). Shinkai et al. (2024) observed that ME/DMI and DMI were positively correlated with the proportion of VFA and negatively correlated with the expression of methanogenic genes in a study of thirty cows in the final phase of lactation fed TMR based on corn silage. In addition, the authors concluded that Prevotella can be associated with low methane production and increased propionate production in the rumen. The increase in propionate production has become an alternative pathway for the elimination of H_2 from the rumen, thereby reducing methane emissions (Orzuna-Orzuna et al., 2021).

The 29.6% ME/ECM reduction in methanogenesis and the trend towards a 23.9% ME/MY reduction observed in the SUP supplemented group can be explained by the negative correlation between the increase in propionate and the production of methane in the gut. In addition, we observed a tendency towards a lower abundance of Methanobrevibacter smithii in the SUP animals, although there was no difference for other Methanobrevibacter spp. in this study. This result confirms that there is a weak correlation between methanogenic archaea and methane emission in the rumen (Martínez-Álvaro et al., 2020; Betancur-Murillo et al., 2023). In our study, we did not observe SUP effect on acetate production, although a reduction in acetate could be expected due to the decrease in acetogenesis to eliminate metabolic H_2 (Berça et al., 2023). Thus, the results of this study support the proposed hypothesis that SUP is effective in reducing methane emissions in lactating dairy cows.

No differences in CP intake and digestibility were observed, but SUP treatment reduced fecal N excretion and increased N synthesis in milk. In agreement with our results, Zhang et al. (2019) found that supplementing lactating cows with 3% condensed tannins effectively reduced total, fecal, and urinary nitrogen excretion and increased its retention. In our study, despite the reduction in fecal N excretion, no increase in urinary N excretion was observed. Higher efficiency was likely a combination of the numerical increase in MY and milk protein content, although no reduction in rumen NH3-N concentration was observed in our study. Nitrogen that is not retained in tissues or excreted in milk is excreted in urine and feces, with urinary N being more susceptible to losses through leaching and volatilization compared to fecal N (Hristov et al., 2019). Battelli et al. (2024) observed a shift in N excretion from urine to feces; however, there was no improvement in N use efficiency.

CONCLUSIONS

The SUP treatment reduced ME/ECM by 29.6 % and improved DM digestibility (6.3 %) and OM digestibility (6.7 %) through favorable changes in the ruminal microbiome and fermentation profile, particularly through increased abundance of *Prevotella ruminicola* and increased propionate production. In addition, the SUP improved N efficiency by reducing fecal N excretion and increasing N excretion from milk. These results support that the additive based on Acacia tannins and *Saccharomyces cerevisiae* (Muucare Nature®) is a sustainable tool for methane emission reduction in dairy production systems without compromising milk production.

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Supplementary Table S1. Period effects on intake and apparent total-tract digestibilities of dry matter and nutrients, milk yield and composition of lactating cows.

	Period		
Item[1]	1	2	SEM
Intake, kg/day			
DM	19.4	20.7	0.62
DM, g/kg de BW	30.5	32.8	0.85
OM	17.5	18.6	0.56
CP	3.2	3.4	0.10
NDFa	5.7	5.9	0.16
Water, L/day	57.3	62.1	3.34
Digestibility, g/kg			
DM	620	650	5.8
OM	656	706	5.3
CP	714	711	9.7
NDFa	423	542	11.5
Milk production, kg/day			
Milk yield	24.8	26.7	2.29
ECM	22.8	22.3	1.98
Milk composition, %			
Fat	3.4	3.1	0.18
Protein	3.3	3.3	0.06
Lactose	4.9	5.0	0.06
Milk composition, kg/day			
Fat	0.85	0.87	0.103
Protein	0.83	0.88	0.095
Lactose	1.25	1.32	0.144
MUN, mg/dL	15.9	16.9	0.65
SCC, log SCC	4.6	4.4	0.41

[1] DM = dry matter, OM = organic matter, CP = crude protein, NDFap = neutral detergent fiber corrected for ash and protein, DEI = digestible energy intake. [1] ECM = Milk yield corrected for energy, MUN = Milk Urea Nitrogen; SCC = Somatic cell count.

Supplementary Table S2. Period effects on methane emission, serum parameters, production and profile of volatile fatty acids, and rumen ammonia nitrogen of lactating cows.

	Period		
Item[1]	1	2	SEM
Methane emission, g/day	158.8	190.6	28.8
ME/MY[1], g/L	6.4	7.0	0.64
ME/ECM[1], g/L	6.7	8.6	0.72
ME/DM, g/kg	8.3	9.3	1.46
ME/OM, g/kg	9.1	10.0	1.34
ME/OMd, g/kg	14.0	15.0	2.46
GLU, mg/dL	56.1	58.5	4.27
BUN, mg/dL	39.2	39.6	1.63
PT, g/dL	5.9	6.0	0.16
IGF-1, ng/mL	123.9	203.3	48.36
VFA, mmol/L			
Total	79,3	86,7	4,00
Acetate	55,5	62,3	3,25
Propionate	13,5	17,0	0,99
Butyrate	7,6	8,3	0,66
VFA, mmol/100mmol			
Acetate	72,2	70,9	1,08
Propionate	17,7	19,5	0,96
Butyrate	10,0	9,5	0,34
Acetate:propionate	4,1	3,9	0,27
RAN, mg/dL	44,9	71,2	6,55

[1] ME/MY = ratio between methane emission and milk yield, ME/ECM = ratio between methane emission and energy corrected milk, ME/DM = ratio between methane emission and dry matter intake, ME/OM = ratio between methane emission and organic matter intake, ME/OMd = ratio between methane emission and digestible organic matter intake, GLU = glucose, BUN = serum urea nitrogen, PT = total protein, IGF-1 = insulin-like growth factor type 1, VFA = amount of total volatile fatty acids, RAN = concentration of ammonia in the rumen.

Supplementary Table S3. Period effects on nitrogen balance and microbial protein synthesis of lactating cows.

Item[1]	Period		
	1	2	SEM
N metabolism			
Urinary urea excretion, g/d	84,8	76,7	4,89
Microbial protein synthesis, g/d	2751	2271	195,9
RUP flow, g/d	645	1142	120,6
Microbial efficiency[6], g/kg	240	183	16,6
N balance, g/d			
Intake	517	546	16,8
Fecal N	147	152	5,5
Urinary N	170	212	14,7
Milk N	133,6	132,7	13,7
Balance	63,4	44,5	16,3

[1] RUP=rumen undegradable protein, Microbial efficiency was calculated based on the relationship between microbial protein synthesis and digestible organic matter intake.

Table 1. Ingredients and chemical composition of experimental diets.

Items[1]	Treatments[2]	
	CON	SUP
Ingredients, g/kg DM		
Corn silage	564	564
Tifton hay	36	36
Cottonseed whole	37.6	37.6
Corn grain	177.4	176.1
Soybean meal	125.5	124.6
DDG	32.6	32.4
Urea	4.4	4.4
Mineral premix[3]	7.9	7.9
Limestone	3.6	3.5
Sulfur flower	0.3	0.3
Commercial buffer[4]	4.8	4.7
Sodium bicarbonate	5.9	5.9
Supplement[5]	-	2.7
Chemical composition, % DM		
DM, as fed	40.81	40.88
OM	86.98	86.01
CP	14.03	14.07
aNDF	34.87	34.44

[1] DDG = distillers dried grains with solubles, DM = dry matter, OM = organic matter, CP = crude protein, and aNDF = ash corrected neutral detergent fiber.

[2] CON=control, SUP= supplemented.

[3] Calcium: 40 g/kg, Cobalt: 280 mg/kg, Cupper: 11 g/kg, Sulfur: 200 mg/kg, Iodine: 600 mg/kg, Magnesium: 150 g/kg, Manganese:25 g/kg, Selenium: 250 mg/kg, Zinc: 40 g/kg, Vitamin A: 4000000 UI/kg, Vitamin D: 1000000 UI/kg, Vitamin E: 25000 UI/kg.

[4] Buffer composed of sodium bicarbonate, calcareous seaweed and magnesium oxide.

[5] Chemical composition of the supplement based on condensed tannins and *Saccharomyces cerevisiae* (SUP) - DM: 889 g/kg, OM: 496 g/kg; CP: 172 g/kg; aNDF: 180 g/kg.

Table 2. Intake and apparent total-tract digestibilities of dry matter and nutrients of Holstein or Holstein×Gyr lactating cows receiving a diet with or without a supplement based on condensed tannins and *Saccharomyces cerevisiae* yeasts.

Items[1]	Treatments[2]		SEM	Breed[3]		SEM	Probability[4]			
	CON	SUP		H	HG		T	B	T×B	P
Intake, kg/day										
DM	20.2	19.9	0.84	20.2	19.9	0.84	0.879	0.799	0.407	<0.001
DM, g/kg de BW	30.7	32.6	1.13	32.4	30.9	1.13	0.280	0.386	0.890	<0.001
OM	18.1	18.0	0.76	18.2	17.9	0.76	0.905	0.806	0.401	<0.001
CP	3.36	3.30	0.14	3.36	3.30	0.15	0.773	0.752	0.408	<0.001
NDFa	5.87	5.79	0.22	5.86	5.80	0.22	0.814	0.865	0.381	<0.001
Water intake, L/day	60.5	58.9	4.39	64.4	54.9	4.39	0.804	0.174	0.443	0.006
Digestibility, g/kg										
DM	616	655	8.0	630	641	7.24	0.020	0.293	0.049	<0.001
OM	660	704	7.0	678	685	6.30	0.004	0.412	0.104	<0.001
CP	693	732	13.9	713	713	11.2	0.081	0.971	0.177	0.738
NDFa	463	502	14.9	490	475	14.8	0.100	0.500	0.164	<0.001

[1] DM = dry matter, OM = organic matter, CP = crude protein, NDF = neutral detergent fiber.

[2] CON = control, SUP = supplemented.

[3] H = Holstein, HG = Holstein×Gyr.

[4] Probability of treatment effects: T = treatment effect, B = breed effect, T×B = interaction effect between treatment and breed, and P = period effect.

Table 3. Milk yield and composition of Holstein or Holstein×Gyr lactating cows receiving a diet with or without a supplement based on condensed tannins and *Saccharomyces cerevisiae* yeasts.

Items[1]	Treatments[2]		SEM	Breed[3]		SEM	Probability[4]			
	CON	SUP		H	HG		T	B	T×B	P
Milk yield, kg/day	25.3	26.3	3.01	27.4	24.2	2.96	0.812	0.429	0.752	0.018
ECM, kg/day	22.4	22.8	2.66	22.9	22.2	2.51	0.929	0.838	0.814	0.696
Milk composition, %										
Fat	3.29	3.23	0.18	3.15	3.37	0.18	0.740	0.167	0.980	0.027
Protein	3.28	3.35	0.07	3.30	3.33	0.07	0.398	0.635	0.485	0.706
Lactose	4.97	5.03	0.09	4.99	5.01	0.09	0.665	0.928	0.948	0.105
Milk composition, kg/day										
Fat	0.88	0.85	0.11	0.90	0.83	0.10	0.734	0.486	0.679	0.870
Protein	0.84	0.88	0.09	0.92	0.80	0.09	0.699	0.303	0.780	0.004
Lactose	1.26	1.32	0.14	1.38	1.20	0.14	0.699	0.306	0.778	0.005
MUN, mg/dL	16.9	15.9	0.78	16.5	16.3	0.74	0.358	0.819	0.350	0.076
SCC, log SCC	4.57	4.38	0.54	4.70	4.25	0.54	0.810	0.573	0.598	0.397

[1] ECM = milk yield corrected for energy, MUN = milk urea nitrogen; SCC = somatic cell count.

[2] CON = control, SUP = supplemented.

[3] H = Holstein, HG = Holstein×Gyr.

[4] Probability of treatment effects: T = treatment effect, B = breed effect, T×B = interaction effect between treatment and breed, and P = period effect.

Table 4. Methane emission of Holstein or Holstein×Gyr lactating cows receiving a diet with or without a supplement based on condensed tannins and *Saccharomyces cerevisiae* yeasts.

Items[1]	Treatments[2]		SEM	Breed[3]		SEM	Probability[4]			
	CON	SUP		H	HG		T	B	T×B	P
Methane emission, g/day	187	163	30.8	186	163	30.0	0.461	0.402	0.077	0.137
ME/MY, g/L	7.59	5.78	0.63	7.06	6.31	0.67	0.078	0.452	0.213	0.538
ME/ECM, g/L	9.00	6.33	0.70	8.57	6.76	0.75	0.023	0.110	0.814	0.111
ME/DM, g/kg	9.38	8.23	1.52	9.30	8.31	1.47	0.483	0.451	0.037	0.416
ME/OM, g/kg	10.4	8.79	1.57	9.94	9.23	1.67	0.443	0.797	0.108	0.473
ME/OMd, g/kg	15.9	13.1	2.57	15.2	13.8	2.48	0.322	0.543	0.069	0.624

[1] ME/MY = ratio between methane emission and milk yield, ME/ECM = ratio between methane emission and energy corrected milk, ME/DM = ratio between methane emission and dry matter intake, ME/OM = ratio between methane emission and organic matter intake, ME/OMd = ratio between methane emission and digestible organic matter intake.

[2] CON = control, SUP = supplemented.

[3] H = Holstein, HG = Holstein×Gyr.

[4] Probability of treatment effects: T = treatment effect, B = breed effect, T×B = interaction effect between treatment and breed, and P = period effect.

Table 5. Serum parameters, production and profile of volatile fatty acids, and rumen ammonia nitrogen of Holstein or Holstein×Gyr lactating cows receiving a diet with or without a supplement based on condensed tannins and *Saccharomyces cerevisiae* yeasts.

Items[1]	Treatments[2]		SEM	Breed[3]		SEM	Probability[4]			
	CON	SUP		H	HG		T	B	T×B	P
Serum parameters										
GLU, mg/dL	52.2	60.3	4.65	57.6	56.9	4.46	0.183	0.832	0.913	0.122
BUN, mg/dL	40.3	38.5	1.57	38.1	40.7	1.58	0.443	0.281	0.238	0.850
TP, g/dL	6.05	5.87	0.16	5.96	5.95	0.16	0.457	0.963	0.739	0.582
IGF-1, ng/mL	160	167	53.3	185	142	50.3	0.903	0.325	0.302	0.020
VFA, mmol/L										
Total	80.9	85.1	5.22	83.9	82.1	5.24	0.602	0.813	0.343	0.012
Acetate	58.1	59.7	3.05	60.2	57.6	3.17	0.736	0.587	0.111	0.170
Propionate	13.9	16.6	1.10	14.5	15.9	0.99	0.075	0.162	0.519	0.002
Butyrate	8.15	7.83	0.74	8.52	7.46	0.67	0.725	0.106	0.867	0.303
VFA, mmol/100mmol										
Acetate	72.8	70.4	1.20	72.4	70.8	1.08	0.134	0.166	0.103	0.259
Propionate	17.1	20.2	1.03	17.5	19.8	0.93	0.032	0.022	0.301	0.062
Butyrate	10.0	9.48	0.38	10.1	9.41	0.34	0.316	0.084	0.036	0.167
Acetate:propionate	4.46	3.56	0.30	4.23	3.79	0.27	0.022	0.092	0.361	0.260
RAN, mg/dL	55.5	60.6	7.52	51.1	65.1	7.76	0.658	0.240	0.657	0.008

[1] GLU = glucose, BUN = serum urea nitrogen, PT = total protein, IGF-1 = insulin-like growth factor type 1.

[2] CON = control, SUP = supplemented.

[3] H = Holstein, HG = Holstein×Gyr.

[4] Probability of treatment effects: T = treatment effect, B = breed effect, T×B = interaction effect between treatment and breed, and P = period effect.

Table 6. Nitrogen balance and microbial protein synthesis of Holstein or Holstein×Gyr lactating cows receiving a diet with or without a supplement based on condensed tannins and *Saccharomyces cerevisiae* yeasts.

Items[1]	Treatments[2]		SEM	Breed[3]		SEM	Probability[4]			
	CON	SUP		H	HG		T	B	T×B	P
N metabolism, g/day										
Urinary urea excretion	78.8	82.7	6.48	80.0	81.5	6.48	0.697	0.881	0.534	0.004
Microbial protein synthesis	2,601	2,421	220	2,638	2,385	220	0.478	0.384	0.488	0.009
RUP flow	849	939	140	746	1,042	126	0.688	0.217	0.325	0.043
Microbial efficiency[5], g/kg	222	203	17.0	216	208	17.2	0.303	0.693	0.378	0.002
N balance, g/day										
Intake	537	527	21.9	537	527	21.9	0.773	0.752	0.408	0.012
Fecal nitrogen	161	134	5.7	153	146	5.5	0.017	0.438	0.101	0.519
Urinary nitrogen	209	175	17.1	173	211	16.6	0.197	0.155	0.823	0.031
Milk nitrogen	119	148	12.5	139	128	12.6	0.016	0.316	0.598	0.952
Balance	53.0	31.0	23.2	55.9	28.1	22.6	0.520	0.415	0.295	0.232

[1] RUP = rumen undegradable protein.

[2] CON = control, SUP = supplemented.

[3] H = Holstein, HG = Holstein×Gyr.

[4] Probability of treatment effects: T = treatment effect, B = breed effect, T×B = interaction effect between treatment and breed, and P = period effect.

[5] Microbial efficiency was calculated based on the relationship between microbial protein synthesis and digestible organic matter intake.

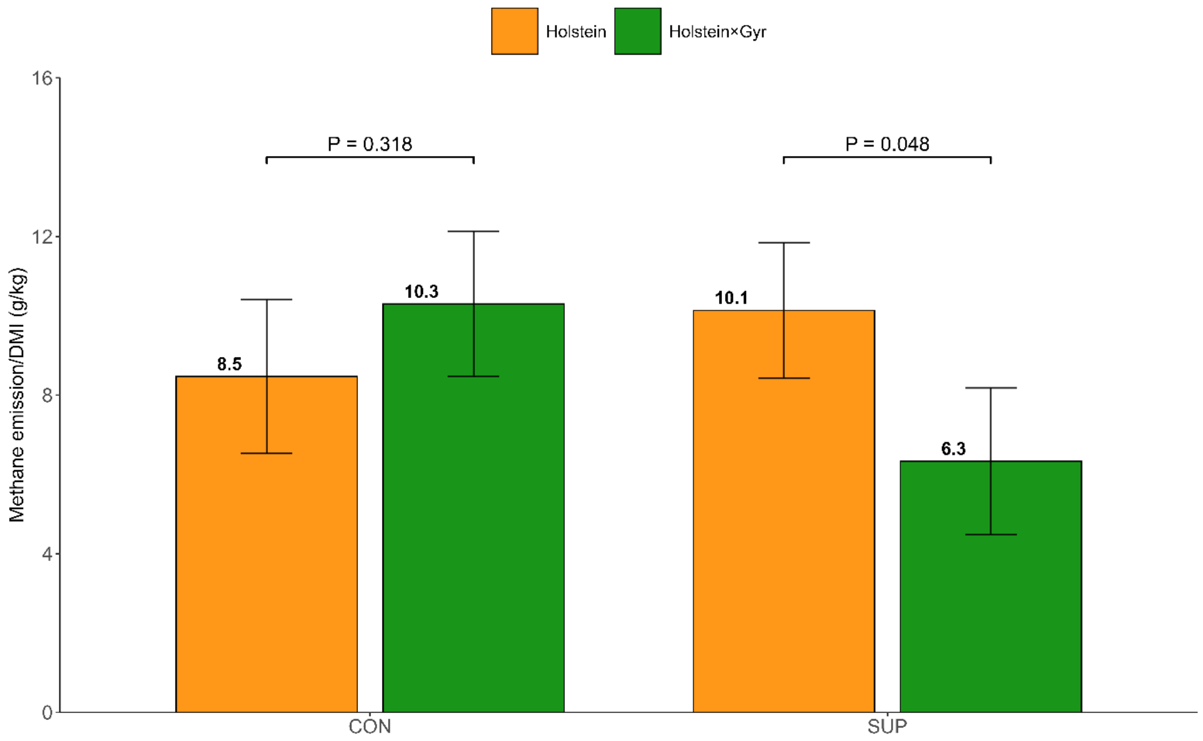

Figure 1. Methane emission per dry matter intake (g/Kg) showing interaction between treatment and breed. Values are least squares means ± SE. P-values show within-treatment breed comparisons.

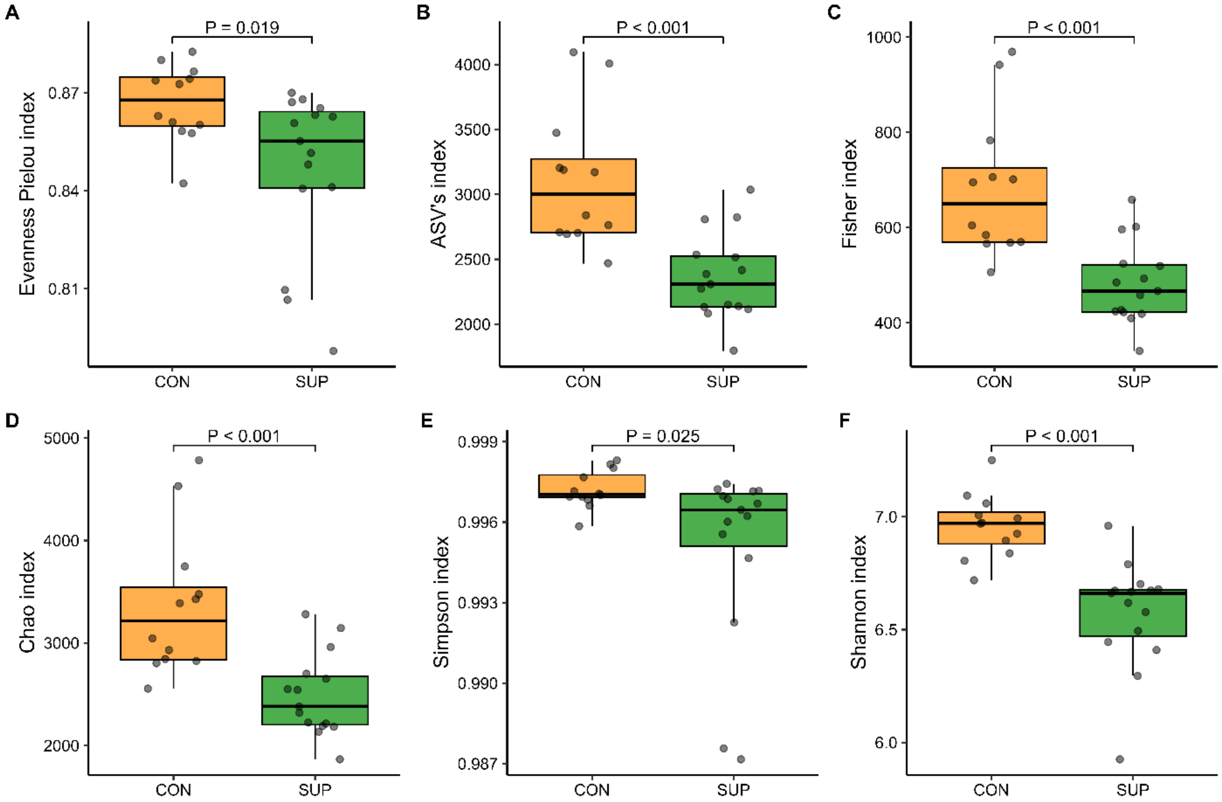

Figure 2. Alpha-diversity indices between control (CON) and supplemented (SUP) treatments: (A) Pielou's evenness, (B) observed ASVs, (C) Fisher index, (D) Chao1, (E) Simpson index, and (F) Shannon index. Data are presented as boxplots with individual d ata points (jitter).

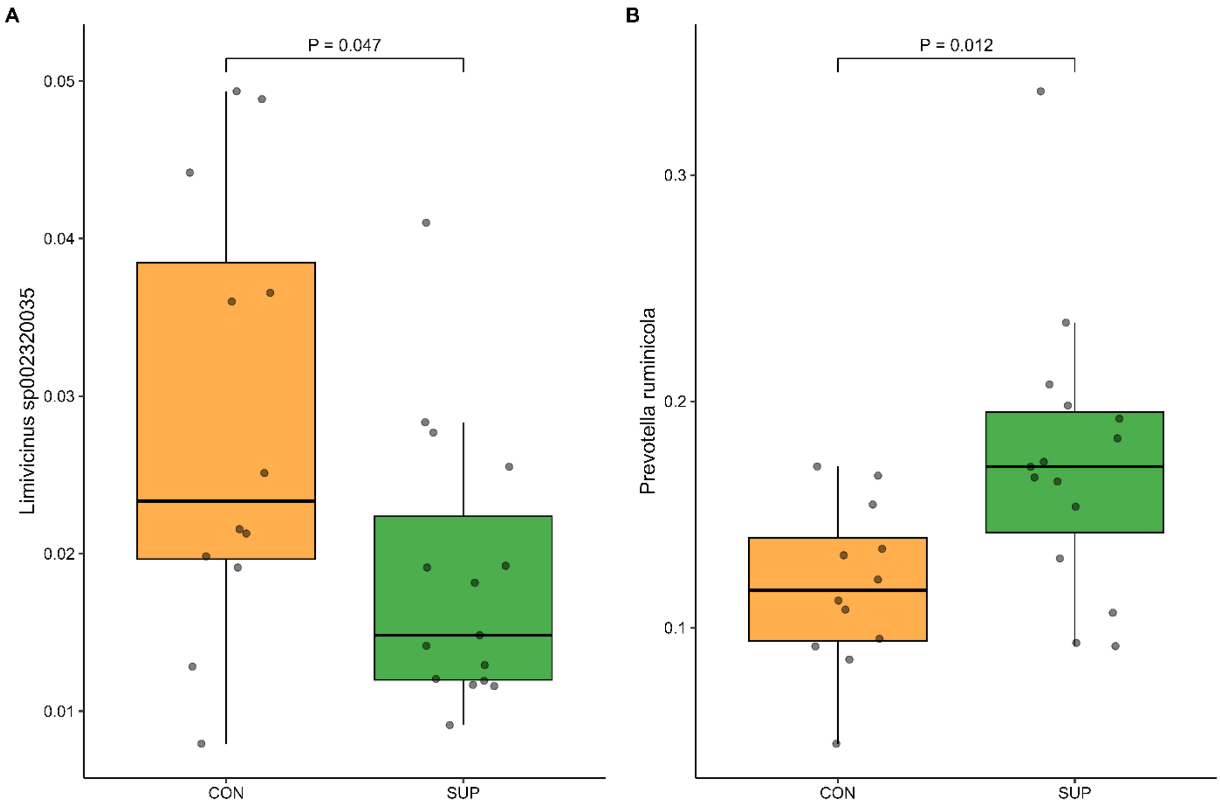

Figure 3. Differential abundance of taxa between control (CON) and supplemented (SUP) treatments for (A) Limivicinus species sp002320035 and (B) Prevotella ruminicola. Data are presented as boxplots with individual data points (jitter).

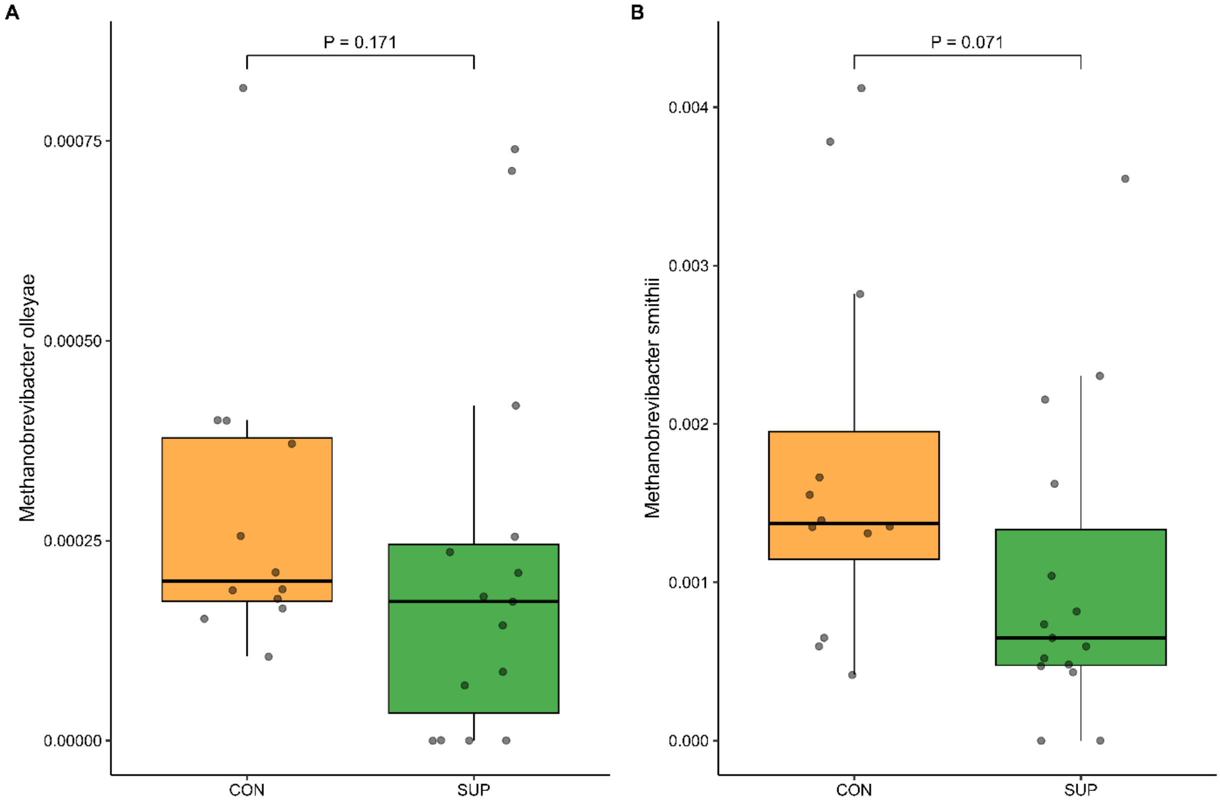

Figure 4. Differential abundance of taxa between control (CON) and supplemented (SUP) treatments for (A) Methanobrevibacter olleyae and (B) Methanobrevibacter smithii. Data are presented as boxplots with individual data points (jitter).