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"Luiz de Queiroz" College of Agriculture

Performance and metabolism of ruminants fed with *Bacillus licheniformis* and *Bacillus subtilis*

Bruno Augusto Valverde Arthur

Thesis presented to obtain the degree of Doctor
in Science. Area: Animal Science and Pastures

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“Laziness reduces both health and wealth”

Robert T. Kiyosaki

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RESUMO

Desempenho e metabolismo de ruminantes alimentados com *Bacillus licheniformis* e *Bacillus subtilis*

O uso de antibióticos como aditivo alimentar para animais é gradualmente banido devido ao risco de acúmulo de resíduos em produtos de origem animal e ao surgimento de cepas bacterianas resistentes a antibióticos. Como alternativa a atender os anseios por maior sustentabilidade na produção de alimentos, surgem os probióticos. Este produto tem como ação modificar o ambiente ruminal através do estímulo ao crescimento de microrganismos, estabilização do pH, mudanças no padrão de fermentação da microbiota e aumento da digestibilidade e utilização de nutrientes, principalmente os microrganismos avaliados no trabalho atual que possuem como característica maior utilização de nutrientes, alterando a fermentação ruminal para uma maior síntese de propionato. O objetivo deste trabalho foi avaliar o efeito de probiótico a base de *Bacillus licheniformis* ($1.6x10^9$ UFC/g) e *Bacillus subitilis* ($1.6x10^9$ UFC/g) sobre o desempenho animal, comportamento ingestivo e digestibilidade aparente no trato total em ruminantes de grande porte. Para o estudo de desempenho animal, foram utilizadas sessenta vacas holandesas, esses animais foram blocados pela produção de leite (21,1 ± 4,5 kg/d), dias em lactação (139,93 ± 72,5 dias) e peso vivo (536,2 ± 58,6 kg) e se eram primíparas ou multíparas. As vacas receberam uma mesma dieta à base de silagem, variando apenas o tratamento (carbonato de cálcio CON ou probiótico PROB) que foi adicionado à dieta de cada vaca uma vez ao dia pela manhã (3 g/d), juntamente com uma solução de açúcar para aderir o produto e aumentar a palatabilidade para consumo imediato do probiótico. Foram utilizados para o estudo de digestibilidade dos nutrientes 18 animais nelore machos, fistulados no rúmen, 16 deles em blocos de quadro e randomizados nas baias para consumo de dieta. Assim, foram alocados 8 animais por tratamento (com ou sem probiótico). Os outros dois animais foram usados para determinar o iFDN de amostras do experimento de desempenho animal. Os touros receberam a mesma dieta e foram alimentados da mesma maneira que os animais do experimento anterior. Os dados foram analisados usando o procedimento PROC MIXED da SAS (SAS Inst., Inc., Cary, NC) para um projeto de bloco aleatório completo. O modelo estatístico incluiu o efeito fixo do tratamento e o efeito aleatório do bloqueio. As diferenças ocorreram na redução do consumo de matéria seca dos animais PROB, sem alteração na produção de leite, aumentando assim a eficiência alimentar. A composição do leite também foi modificada, com aumento de gordura (%) para os animais PROB e tendência de redução de proteína (%) para os mesmos, resultando em maior produção de proteína (kg/d), ainda em características do leite, os animais PROB apresentaram menor MUN (mg/dL) em comparação CON. A digestibilidade total da matéria seca foi maior para PROB, assim como digestibilidade de FDN e de amido, sendo apenas a digestibilidade de proteína bruta menor para o PROB em comparação ao CON. A glicose plasmática foi maior para PROB em todos os horários coletados após a primeira refeição (0, 1, 2, 3, 6h) com exceção do horário 12h onde o tratamento CON apresentou maior glicose plasmática. BUN foi menor em todos os horários coletados após a primeira refeição para o tratamento PROB (0, 1, 2, 3, 6 e 12h). Já nos dados coletados no trabalho de digestibilidade dos nutrientes com os bois Nelore os valores de amônia ruminal (mg/dL) foi menor para PROB no horário 0h após a primeira refeição e maior para PROB no horário 24h após primeira refeição e apresentou tendência em ser menor nos demais horários para PROB após primeira refeição (12, 48 e 72h). O pH ruminal foi maior para PROB em todos os horários de coleta após a primeira refeição. Como conclusão, o probiótico foi eficaz em aumentar eficiência alimentar dos animais em lactação.

Palavras-chave: Saúde animal; Digestibilidade de fibra; Aproveitamento do amido; Contagem de Células Somáticas; Alternativa aos antibióticos.

ABSTRACT

Performance and metabolism of ruminants fed with *Bacillus licheniformis* and *Bacillus subtilis*

The use of antibiotics as a feed additive for animals is gradually banned due to the risk of residue accumulation in animal products and the emergence of antibiotic-resistant bacterial strains. As an alternative to meeting the desire for greater sustainability in food production, probiotics emerge. This product's action is to modify the rumen environment by stimulating the growth of microorganisms, stabilizing pH, changes in the microbiota fermentation pattern and increasing digestibility and use of nutrients, especially the microorganisms evaluated in the current work that have the characteristic of greater use. of nutrients, altering rumen fermentation towards greater propionate synthesis. The objective of this work was to evaluate the effect of probiotics based on Bacillus licheniformis (1.6x109 CFU/g) and Bacillus subitilis (1.6x109 CFU/g) on animal performance, ingestive behavior and apparent digestibility in the total tract in large ruminants. . For the animal performance study, sixty Holstein cows were used, these animals were blocked by milk production (21.1 ± 4.5 kg/d), days in lactation (139.93 ± 72.5 days) and live weight (536.2 ± 58.6 kg) and whether they were primiparous or multiparous. The cows received the same silage-based diet, varying only the treatment (CON calcium carbonate or PROB probiotic) that was added to each cow's diet once a day in the morning (3 g/d), together with a solution of sugar to adhere the product and increase palatability for immediate consumption of the probiotic. Eighteen male Nelore animals were used to study nutrient digestibility, fistulated in the rumen, 16 of them in frame blocks and randomized in stalls for diet consumption. Thus, 8 animals were allocated per treatment (with or without probiotic). The other two animals were used to determine the iNDF of samples from the animal performance experiment. The bulls received the same diet and were fed in the same way as the animals in the previous experiment. Data were analyzed using the PROC MIXED procedure from SAS (SAS Inst., Inc., Cary, NC) for a randomized complete block design. The statistical model included the fixed effect of treatment and the random effect of blocking. The differences occurred in the reduction in dry matter consumption of PROB animals, without changing milk production, thus increasing feed efficiency. The composition of the milk was also modified, with an increase in fat (%) for PROB animals and a tendency towards a reduction in protein (%) for them, resulting in greater protein production (kg/d), still in terms of milk characteristics, PROB animals had lower MUN (mg/dL) compared to CON. Total dry matter digestibility was higher for PROB, as well as NDF and starch digestibility, with only crude protein digestibility being lower for PROB compared to CON. Plasma glucose was higher for PROB at all times collected after the first meal (0, 1, 2, 3, 6h) with the exception of 12h where the CON treatment showed higher plasma glucose. BUN was lower at all times collected after the first meal for PROB treatment (0, 1, 2, 3, 6 and 12h). In the data collected in the nutrient digestibility work with Nelore cattle, rumen ammonia values (mg/dL) were lower for PROB at 0h after the first meal and higher for PROB at 24h after the first meal and showed a tendency to be lower at other times for PROB after the first meal (12, 48 and 72h). Rumen pH was higher for PROB at all collection times after the first meal. In conclusion, the probiotic was effective in increasing the feed efficiency of lactating animals.

Keywords: Animal health; Fiber digestibility; Use of starch; Somatic cell count; Alternative to antibiotics.

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INTRODUCTION

Dairy products in particular have emerged as a key source of revenue for farmers and have contributed to the expansion of developing countries. Livestock farming is a dynamic industry that is essential to meeting the growing demand for animal sourced products (Thornton, 2010; Tona, 2021). One of the largest industrial sectors, dairy production produced almost 930 million tons of milk globally in 2020 (FAO 2022). The pressing need for cutting-edge methods to improve cow health and productivity and make dairy production sustainable in a fast-changing environment is brought on by the increasing strain of the world population, limited arable land, and climate change. (Britt et al., 2018). Despite rising demand, low animal productivity remains a problem for the dairy industry in low- and middle-income nations.

A large disease burden in the livestock chain can also result in public health problems including the emergence of antibiotic resistance. As a result, any unfavorable reduction in milk supply induced by illness or starvation in lactating animals can entail significant economic losses (Sharma et al., 2018a). Since the use of antibiotics as growth promoters has been strictly regulated in many countries to prevent the evolution and spread of antibiotic resistance through the food system. A popular feed addition called monensin has been linked to benefits such increasing the effectiveness of rumen fermentation and energy metabolism. However, due to the possibility of residue buildup in animal products and the advent of bacterial strains resistant to antibiotics, the use of antibiotics as an additive in livestock feed is being gradually outlawed.

The use of natural and affordable probiotics-based supplements as alternatives to antibiotics to promote animal growth and health has increased in recent years in the livestock industry (Sharma et al., 2018a). Probiotics are living, non-pathogenic bacteria that are frequently also somewhat naturally present in the digestive system which, when supplied in sufficient quantities, promote the host's health (FAO/WHO, 2001).

Probiotics have been shown in several studies to alter gut balance, increase calf performance (Meyer et al., 2001; Timmerman et al., 2005), and reduce calf scours (Wehnes et al., 2009), the bacteria changed how calves ferment their rumens. Probiotics can function in a variety of ways. These include limiting the growth of pathogenic microbes on mucosal surfaces through a mechanism of competitive exclusion (competition for receptors), competing for nutrients, promoting mucosal and systemic host immunity, promoting the

growth of other commensal bacteria, and producing antimicrobial substances (La Ragione et al., 2001; McNaught and MacFie, 2001; Hong et al., 2005; Leser et al., 2009).

B. subtilis has also demonstrated probiotic characteristics since it has been demonstrated to inhibit pathogens like *Salmonella typhimurium*, Clostridium species, Campylobacter species, Streptococcus species, *Escherichia coli*, and *Staphylococcus aureus* (Teo and Tan, 2005; Guo et al., 2006; Teo and Tan, 2006), among others (Teo and Tan, 2005; Teo and Tan, 2006), it is believed to be antimicrobial.

The performance of non-ruminants (Fritts et al., 2000; Hooge, 2008; Zhang et al., 2012, 2013; Lee et al., 2014) and calves (Sun et al., 2010 and 2011) has been enhanced by the addition of *Bacillus subtilis*. *Bacillus subtilis* has been shown to improve immune function (Sun et al., 2010), increase anaerobiosis in the digestive tract, which favors the native proliferation of Lactobacilli able to produce lactic acid and inhibit pathogenic bacterial growth (Maruta et al., 1996; Sanders et al., 2003), and early lactation dairy cows without any negative effects (Peng et al., 2011).

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Early lactation dairy cows with *B. subtilis* natto supplementation showed improved lactation performance, probably due to changes in the rumen fermentation pattern (Peng et al., 2009). According to Qiao et al., *B. subtilis* had no discernible impact on the characteristics of rumen fermentation, duodenal microbial N flow, and ruminal apparent nutrient digestibility while *B. licheniformis* increased the ruminal apparent nutrient digestibility of neutral detergent fiber, acid detergent fiber, and organic matter.

The addition of several Bacillus species to dairy cow diets has been linked to improved fiber digestibility (Qiao et al., 2009), higher milk and milk ingredient yields (fat, protein, and lactose; Sun et al., 2013), and lower enteric methane emissions (Wang et al., 2016). Recent research by Pech-Cervantes et al. (2019) showed that *B. subtilis* has a higher capacity to manufacture and release expansin-like proteins than *Trichoderma reesei* (Liu et al., 2015). Additionally, when *B. subtilis* was added to a medium rather than cellulase alone, cellulase's hydrolytic activity enhanced (Pech-Cervantes et al., 2019).

The effects of *B. subtilis* feeding on ruminants have been the subject of few investigations, and the results are inconsistent. *B. licheniformis* and *B. subtilis* together boosted milk production in ewes, and *B. subtilis* natto altered rumen fermentation patterns in calves, according to (Kritas et al., 2006; Sun et al., 2011). According to Qiao et al. (2010), *B. subtilis* had no impact on dairy cows' milk production or rumen fermentation. Another study, now more recent, also obtained divergent results from the literature, without changes in milk production and dry matter consumption, with the investigation finding an increase in fat digestibility, promoting an increase in milk fat and consequently an increase in corrected milk for fat (Oyebade et al., 2023). The level of direct-fed microbial inclusion in the food, diet composition, feed intake, and feeding frequency, as well as animal characteristics like age, physiological stage, health, and stress state, are further variances between researches.

Therefore, the present study aims to help enrich the database regarding the use of *B. subitilis* and *B. licheniformis* on the performance of dairy cows and digestibility of nutrients in the rumen.

LITERATURE REVIEW

According to Yoon and Stern (1995), direct-fed microbials has been used to refer to live microbial cultures, culture extracts, enzyme preparations, or other combinations of those items. Numerous bacteria and fungi have been classified as probiotics over the years (*Lactobacillus* spp., *Saccharomyces cerevisiae*, *Bifidobacterium* spp., and *Bacillus* spp., are highlighted (Holzapfel et al., 2001; Luize et al., 2022. (Choct, 2009; Punyia et al., 2015; Markowiak and Ślizewska, 2018; Zommiti and Ferchichi, 2021). Probiotic supplementation helps maintain gut microbiota homeostasis, which enhances feed conversion effectiveness and, ultimately, increases milk and meat output. (Jinturkar et al., 2009; Maake et al., 2021; Mani et al., 2021). Additionally, probiotics have been shown to lower levels of stress-related indicators like cortisol (Zhang et al., 2016) and competitive exclusion of pathogenic - microorganisms (Fujiwara et al., 2009).

Probiotics have been shown in several studies to alter gut balance, increase calf performance (Meyer et al., 2001; Timmerman et al., 2005), and reduce calf scours (Wehnes et al., 2009). Probiotics can function in a variety of ways. These include limiting the growth of pathogenic microbes on mucosal surfaces through a mechanism of competitive exclusion (competition for receptors), competing for nutrients, promoting mucosal and systemic host immunity, promoting the growth of other commensal bacteria, and producing antimicrobial substances (La Ragione et al., 2001; McNaught and MacFie, 2001; Hong et al., 2005; Leser et al., 2009). The focus on the use of probiotics is due to the fact that it seeks ways to prevent diseases with the aim of reducing the use of antibiotics. These include altered rumen fermentation patterns, altered rumen microbial populations, enhanced meal digestibility, increased intestinal nutrient flow, and immune system regulation (Yoon and Stern, 1995; Krehbiel et al., 2003). The objectives of rumen microbial studies are enhancements in feed utilization, animal production and health, and animal food safety. By promoting favorable fermentation, reducing ruminal diseases, and eliminating pathogens, these objectives can be accomplished. In order to increase animal performance, feed efficiency, and disease prevention, a number of feed additives have been utilized. (Seo et al., 2010).

A number of authors have examined the impact of direct-fed microbial supplements on cow performance or rumen fermentation (Martin and Nisbet, 1992; Jouany, 1994; Newbold, 1995; Nocek and Kautz, 2006). Although direct-fed microbial supplements increased milk output, component yield, feed efficiency, and animal health, the animals' reactions to DFM varied. Additionally, it is challenging to evaluate the outcomes of direct-fed microbial studies

done on dairy calves because so many different organisms, strains of organisms, and combinations of several organisms have been added. The level of direct-fed microbial inclusion in the food, diet composition, feed intake, and feeding frequency, as well as animal characteristics like age, physiological stage, health, and stress state, are further variances between research (Wagner et al., 1990).

Although some bacterial direct-fed microbials (**DFM**) have recently been discovered to have positive effects in the rumen, others have potential positive effects on the post-ruminal gastrointestinal tract. The intestinal system may benefit from the presence of lactic acid generating bacteria (**LAB**). Nevertheless, some researchers have hypothesized that LAB may also have beneficial effects in the rumen. LAB like lactobacilli and enterococci may prevent ruminal acidosis in dairy cows by promoting the growth of ruminal microorganisms that are adapted to the presence of lactic acid in the rumen (Yoon and Stern, 1995) and by stimulating lactic acid-utilizing bacteria (**LUB**).

LUB have also been recommended as DFM and have been successfully utilized to lower lactate concentrations and keep ruminal pH constant. When fed a highly fermentable diet, *Megasphaera elsdenii* may utilize lactate and prevent sharp pH reductions brought on by lactate buildup in the rumen (Kung and Hession, 1995), and the supplementation of *M. elsdenii* was suggested as a way of preventing acute acidosis in transition animals.

Lactate is fermented to propionate by protonibacteria. Increases in propionate production in the rumen lead to increases in hepatic glucose production since propionate is the primary precursor for gluconeogenesis in early lactation dairy cows (Reynolds et al., 2003), increasing the substrates available for the synthesis of lactose, increasing energy efficiency, and decreasing ketosis (Weiss et al., 2008). Additionally, a rise in propionate may lower the hydrogen available in the rumen for the generation of methane. According to Stein et al. (2006), specific propionibacteria species have been shown to alter rumen fermentation and increase the molar fraction of ruminal propionate.

Rose (1987) proposed that yeasts in the rumen eliminate oxygen. In order to maintain metabolic activity, yeast cells in the rumen need oxygen that is present on the surfaces of recently consumed grain. With the addition of yeast, Jouany et al. (1999) saw a considerable drop in redox potential in the rumen of up to -20 mV. This modification improves the growing environment for strictly anaerobic cellulolytic bacteria, encourages their attachment to forage particles, and accelerates the initial rate of cellulolysis (Roger et al., 1990). Additionally, *S. cerevisiae* was able to outcompete other starch-utilizing bacteria for starch fermentation, which prevented lactate buildup in the rumen (Chaucheyras et al., 1995; Lynch

and Martin, 2002). According to Chaucheyras et al. (1995), S. cerevisiae can provide growth factors like organic acids or vitamins, which can encourage ruminal populations of LUB and cellulolytic bacteria.

Modulate immune cells and stimulate immune function, modulate microbial balance in the gastrointestinal tract (**GIT**), attach to the intestinal mucosa and prevent potential pathogen establishment, maintain lower pH in the GIT thereby inhibiting growth of pathogens, produce antibacterial compounds such as bacteriocin and hydrogen peroxide, and prevent illness brought on by intestinal pathogens or stress.

E. coli enterotoxin-producing strains cause diarrhea by adhering to intestinal epithelial cells and mucus (Jones and Rutter, 1972). According to Lee et al. (2003), *L. rhamonsus* might hydrophobically interface with epithelial cells and prevent other pathogens from adhering to the enterocytic receptor. Pathogens are displaced by steric hindrance, and they finally separate from the enterocytic receptor. Additionally, according to Forestier et al. (2001), L. *rhamonsus* reduces the adherence of enteropathogenic and enterotoxigenic *Klebsiella pneumonia* and *E. coli*. In additional studies, mice's digestive tracts were able to cling to LAB, defending the mice against *Salmonella Dublin* DSPV 595T (Frizzo et al., 2010). The primary metabolic end products of LAB are lactate and acetate. These acids have crucial roles in reducing intracellular pH by entering microbial cells and interfering with vital cell functions (Holzapfel et al., 1995). Due to their properties of probiotics and competitive exclusion, hydrogen peroxide and a number of bacteriocins generated by LAB are also significant substances. The oxidation of sulfhydryl groups in metabolic enzymes like glucose transport enzymes, hexokinase, and glycerol aldehyde-3-phosphate dehydrogenase by hydrogen peroxide can block glycolysis (Dicks and Botes, 2010). These enzymes include glycerol aldehyde-3-phosphate dehydrogenase and hexokinase. According to Holzapfel et al. (1995), LAB may create hydrogen peroxide that efficiently inhibits *Pseudomonas spp*. and S. *aureus*. Reuterin is produced by L. *reuteri* when it is grown anaerobically with glucose and glycerol (Dicks and Botes, 2010), and it prevents substrates from binding to the ribonucleotide reductase subunit, preventing target bacteria from making DNA (Dobrogosz et al., 1989).

Another method of action recognized by DFM is the modification of host immunological function. Dendritic cells, natural killer cells, macrophages, neutrophils, and T and B lymphocytes are only a few of the immune cells found in the GIT, and they collect in Peyer's patches, lamina propria, and intraepithelial areas (Krebiel et al., 2003). DFM are directly taken up by intestinal epithelial cells through transcytosis after being delivered to the

GIT. They are then engulfed by dendritic cells, macrophages, or antigen-presenting cells, which ultimately triggers an immunological response (Dicks and Botes, 2010). Different LAB strains trigger the production of immune-stimulating cytokines by macrophages.

Young calves may be at danger of intestinal proliferation of harmful organisms since they must digest a large amount of diet nutrients in their intestines. In unfamiliar situations like transportation, weaning, immunization, and dehorning, newborn calves are frequently anxious (Krehbiel et al., 2003). Calves and cows are quickly separated in intensive farming systems before the gut microbiota of the calves has fully colonized. Diarrhea and weight loss may be more likely in this circumstance. In stressful intestinal settings, the introduction of large doses of advantageous bacteria may facilitate colonization and hasten the restoration of normal GIT function in scouring calves (Kung Jr, 2001). Many studies indicated that LAB could regulate diarrhea incidence as well as improve weight gain and feed efficiency when used as a DFM source.

In certain studies, young calves were also vaccinated with LAB to enhance growth performance (Frizzo et al., 2010). To create an intestinal imbalance, young calves were given milk replacer and a significant amount of spray-dried whey powder. According to Frizzo et al. (2010), under these circumstances, calves administered probiotics exhibited greater daily gains, total feed intake, starter diet consumption, and lower fecal consistency indices, indicating that the incidence of diarrhea was decreased. *Propionibacterium jensenii*, a bacterial strain discovered in Australia, was the subject of Adams et al.'s (2008) investigation of the impact of growth performance. While dairy propionibacteria are infrequently utilized, LAB is a major component of most bacterial DFM for newborn calves. Propionibacteria can raise the concentration of propionate and butyrate in the rumen, promoting rumen growth. These calves showed increased weight growth during the preweaning and postweaning periods, and fecal recovery of *P. jensenii* from the treatment groups at the end of week 2 indicated effective gastrointestinal transit of the bacterium.

Dairy cows experience stress during transition periods, which are defined as the three weeks before and three weeks following calving (Grummer, 1995). This stress is brought on by calving, switching to diets high in rapidly fermented carbohydrate sources, and lactation. A subacute acidosis in dairy cows may result from sudden changes that take place during this time (Oetzel et al., 2007; Chiquette et al., 2008). Preventing ruminal acidosis in beef cattle who are finishing is also crucial. This condition is brought on by highly fermentable diets. The growth performance, milk and meat production, and feed efficiency of dairy and beef

cattle fed DFM were all enhanced in numerous tests (Ghorbani et al., 2002; Nocek et al., 2002; Krehbiel et al., 2003; Stein et al., 2006).

During the postpartum period, DFM enhanced dry matter intake, milk output, and milk protein content. For cows receiving DFM during the postpartum period, blood sugar and insulin levels were greater whereas non-esterified fatty acids (**NEFA**) levels were decreased (Nocek et al., 2003). In a different study, cows fed with E. *faecium* with yeast had more ruminally accessible dry matter (**DM**), ingested more DM during the pre- and postpartum periods, and produced more milk per cow per day (Nocek and Kautz, 2006). Between cows supplemented with DFM and controls, the 3.5% fat-corrected milk showed no changes. Yield and percentage of protein in milk, as well as milk fat yield, were same. DFM-consuming cows had lower betahydroxybutyrate levels both before and on the first postpartum day, as well as greater blood glucose levels postpartum. According to Oetzel et al. (2007), the combination of E. *faecium* and S. *cerevisiae* boosted milk fat percentages when used as DFM for cows in their first lactation and milk protein percentages when used as DFM for cows in their second and subsequent lactations. Additionally, compared to cows getting a placebo, second-lactation cows receiving DFM received fewer antimicrobial treatments prior to 85 days in milk (**DIM**).

Since they can adapt to unfavorable conditions by producing spores, several species of spore-forming bacteria from the genus Bacillus (such as *B. licheniformis* and *B. subtilis*) have been identified as non-pathogenic additives and are now frequently used in animal feeds (de Boer et al., 1994; Hong et al., 2005). As a dietary probiotic addition, *B. licheniformis* improved production performance in developing pigs, broiler chickens, and laying hens (Lei et al., 2013, Liu et al., 2012, Davis et al., 2008, Jorgensen et al., 2016, and Pan et al., 2017).

More than 2,700 different species of *Bacillus* spp. have currently been identified (www.lpsn.dsmz.de). Bacilli are Gram-positive, spore-forming, aerobic, and facultatively anaerobic bacteria. The potential advantages of *Bacillus* spp. on the health and performance of monogastric animals, including direct and indirect pathogen suppression, immunostimulatory effects, and nutritional digestibility and utilization, have recently been examined by Luise et al. (2022). In fact, past research found that numerous *Bacillus* spp. can produce a diverse range of fibrolytic, amylolytic, lipolytic, and proteolytic enzymes that might improve nutritional digestibility and performance in animals (Ghani et al., 2013; Elshaghabee et al., 2017; Su et al., 2020).

For use in human, poultry, and swine probiotic bacteria, *Bacillus* species have proven successful (Cutting, 2011; Luise et al., 2022). And has been employed as probiotics in the

past (Luise et al., 2022) and has a variety of uses and is stable, allowing it to be incorporated into various ruminant supplements. Only a small number of studies in ruminants examined the effects of these probiotics on performance, rumen fermentation profile, and health in calves, beef steers, and lactating dairy cows (Sun et al., 2013; Deng et al., 2021; Lucey et al., 2021.

For their part in preventing infectious diseases and boosting animal productivity, *Bacillus licheniformis* and *Bacillus subtilis* have drawn attention (Holzapfel et al., 2001 (ARTIGO 5); Alexopoulos et al., 2004; Chen et al., 2009). *Bacillus subtilis* is a transitory digestive tract bacterium that is not harmful to animals and has the ability to produce spores that can withstand both heat and cold. The microbe is allegedly capable of boosting immunity and diet digestibility as an animal feed probiotic.

The natural tendency of the consumer market is toward the production of human food from animals supplemented with live microorganisms as an alternative to chemical feed additives. Probiotics may improve immune system and gut physiology when added to a diet, according to Reid (2008). *Bacillus subtilis* and other spore-forming bacteria have been utilized as probiotic supplements for both people and animals (Cutting, 2011). To function as a probiotic in animal feed, the spores must germinate at the intestinal lumen. After the supplementation is stopped, the amount of *Bacillus subtilis* bacteria in the digestive tract decreases (Sanders et al., 2003). This justifies daily administration of *Bacillus subtilis* dietary supplements. When compared to probiotic supplementation in the form of vegetative cells, probiotics based on bacterial spores have the potential to be more resistant to the low gastric pH and can survive passage through the stomach (Hoa et al., 2000).

According to studies on *B. subtilis* effects on ruminants, the bacteria changed how calves ferment their rumens and enhanced ewes milk production (Qiao et al., 2009). *Bacillus* sp. is not typically seen in the gastrointestinal tract, unlike lactic acid bacteria. In an endosymbiotic association with their host, *Bacillus* sp. is eventually expelled in the feces after briefly being able to survive and grow in the GIT (Hong et al., 2005). Furthermore, these bacteria's spore forms can endure in the gastrointestinal system (Casula and Cutting, 2002).

B. subtilis has also demonstrated probiotic characteristics since it has been demonstrated to inhibit pathogens like *Salmonella typhimurium*, Clostridium species, Campylobacter species, Streptococcus species, *Escherichia coli*, and *Staphylococcus aureus* (Teo and Tan, 2005; Guo et al., 2006; Teo and Tan, 2006), among others (Teo and Tan, 2005; Teo and Tan, 2006), it is believed to be antimicrobial.

According to available data from in vitro research, some anaerobic bacteria, including Lactobacillus sp. and Bifidobacterium sp., can grow more readily when exposed to B. subtilis

natto (Hosoi et al., 2000). The performance of non-ruminants (Fritts et al., 2000; Hooge, 2008; Zhang et al., 2012, 2013; Lee et al., 2014) and calves (Sun et al., 2010 and 2011) has been enhanced by the addition of *Bacillus subtilis*. *Bacillus subtilis* has been shown to improve immune function (Sun et al., 2010), increase anaerobiosis in the digestive tract, which favors the native proliferation of Lactobacilli able to produce lactic acid and inhibit pathogenic bacterial growth (Maruta et al., 1996; Sanders et al., 2003), and early lactation dairy cows without any negative effects (Peng et al., 2011). Early lactation dairy cows with *B. subtilis* natto supplementation showed improved lactation performance, probably due to changes in the rumen fermentation pattern (Peng et al., 2009). According to Qiao et al., 2009 *B. subtilis* had no discernible impact on the characteristics of rumen fermentation, duodenal microbial N flow, and ruminal apparent nutrient digestibility while *B. licheniformis* increased the ruminal apparent nutrient digestibility of neutral detergent fiber, acid detergent fiber, and organic matter. A diverse microbiota made up of numerous different species of microorganisms inhabits the gastrointestinal system of healthy animals (Frizzo et al., 2008). To encourage effective digestion and maximal nutrition absorption, the microbial balance in the digestive tract's microbiota is crucial. According to Walter et al. (2003), it can make the host more capable of eliminating pathogen germs and so avoid various diseases.

The effects of *B. subtilis* feeding on ruminants have been the subject of few investigations, and the results are inconsistent. *B. licheniformis* and *B. subtilis* together boosted milk production in ewes, and *B. subtilis* natto altered rumen fermentation patterns in calves, according to (Kritas et al., 2006; Sun et al., 2011). According to Qiao et al. (2010), *B. subtilis* had no impact on dairy cows' milk production or rumen fermentation. Additionally, according to Sun et al. (2010), *B. subtilis* natto raised serum IgG and IFN-c levels in the DFM-fed calves.

Forages make up a significant portion of the diets of both beef and dairy cattle (Beauchemin et al., 2003; Alvarez et al., 2009), but the amount of fiber and the type (warm- or cool-season) of these feedstuffs limit rumen digestibility and, as a result, herd productivity (Bohnert et al., 2011, Adesogan et al., 2014; Romero et al. Treatment of forage with fibrolytic enzymes has been suggested as an alternative to increase forage digestibility (Dean et al., 2005), but results have been inconsistent (Beauchemin et al., 2003), primarily because the substrate type limits the accessibility and, consequently, the effectiveness of fibrolytic enzymes in hydrolyzing cellulose into glucose (Zhang et al., 2015). Expansion-like proteins, which can be expressed by a number of bacteria and fungi, can loosen, expand, or disturb

plant cell wall constituents like cellulose and hemicellulose to alleviate this latter problem (Liu et al., 2015). Recent research by Pech-Cervantes et al. (2019) showed that *B. subtilis* has a higher capacity to manufacture and release expansin-like proteins than *Trichoderma reesei* (Liu et al., 2015). Additionally, when *B. subtilis* was added to a medium rather than cellulase alone, cellulase's hydrolytic activity enhanced (Pech-Cervantes et al., 2019).

In order to encourage a larger NDF digestibility of forage sources frequently fed to cattle, Ferraretto et al. (2015) reported that hybrid selection for corn silage, for instance, might be utilized as an alternative. When it comes to the performance of dairy and beef cattle, starch digestibility is crucial (Ferraretto et al., 2013; Vander Pol et al., 2008; Owens et al., 2016).

To reduce the amount of fecal starch in beef and dairy cattle, it is essential to maximize starch digestion in the rumen and, as a result, to increase total starch digestibility (Ferraretto et al., 2013; Owens et al., 2016). Grain processing (Owens et al., 1997, 2016; Marques et al., 2016) and the use of enzymes, such as amylases (Mora et al., 2002), are two technologies that can support these improvements. According to Ferraretto et al. (2011), feeding an exogenous amylase to nursing Holstein cows had only marginally positive impacts on milk output, composition, and production efficiency.

To increase the productivity and health of dairy cows, DFM are frequently provided (McAllister et al., 2011); The nutritional value and health advantages of food products with an animal origin have drawn increasing attention in recent years (Henchion et al., 2017). Bovine milk contains a significant amount of fat, which ranges in content from 3 to 6% depending on the breed, nutrition, lactation stage, and season (Linn, 1988; Palmquist et al., 1993). Thousands of lipid species with various health benefits can be found in the very complex fats found in cow's milk (Sichien et al., 2009).

According to (Oyebade et al., 2022, unpublished), adding a combination of *Lactobacillus animalis*, *Propionibacterium freudenreichii*, *Bacillus subtilis,* and *Bacillus licheniformis* to one's diet improved the amount of milk fat and fat corrected milk (**FCM**) that could be produced from an ether extract. It is believed that altered milk fatty acid composition results from increased milk fat yield brought on by enhanced dietary fat digestion and uptake by the mammary gland (Palmquist and Jenkins, 1980). 7,143 different lipid species were found and identified in total (https://doi.org/10.13140/ RG.2.2.17897.57444). All milk samples contained triglycerides (**TG**), which was followed by diglycerides (**DG**), sterol lipids, and fatty acid (**FA**) as the most prevalent lipid species.

Long-chain fatty acids (**LCFA**) are primarily obtained from feed and extensively biohydrogenated by rumen microbes in ruminants, resulting in a high percentage of saturated fats leaving the rumen (Bionaz et al., 2020). The extent to which ruminal microbes are involved in lipid metabolism, such as deesterification and biohydrogenation, has a major impact on the LCFA profiles of animal products, such as milk and meat (Demeyer & Doreau, 1999). Microbial variables that affect the rate of lipolysis and biohydrogenation are principally responsible for unprotected unsaturated fatty acid (**UFA**) escape from this process (Jenkins, 1993). With health-promoting benefits like anticarcinogenic and antiatherosclerotic effects, polyunsaturated FA in milk and other dairy products are recognized to improve the health status of consumers (Jensen, 2002; Micha and Mozaffarian, 2010). In their comprehensive study, Mozaffarian et al. (2010) demonstrated that a switch from saturated fatty acid (**SFA**) to a greater polyunsaturated fatty acid (**PUFA**) intake would considerably lower risks of coronary heart disease. Additionally, it has been suggested that PUFA consumption is linked to a decreased risk of type 2 diabetes (Salmerón et al., 2001). Ceramides and sphingosines, which are the breakdown products of sphingolipids, are known to decrease intestinal inflammation and have a favorable impact on cell regulation (Vesper et al., 1999).

Since gastrointestinal illnesses in calves at this period affect their future growth and productivity, it is crucial to reduce their prevalence (Rosmini et al., 2004). Probiotics were initially developed for use in production animals based on their prospective advantages (Fuller, 1999), and they were later employed as an alternative to antibiotics with the goals of preserving the microbiota's balance, balancing digestive function, and enhancing the health of the animal. Vanbelle et al. (1990) claimed that after effective oral dosing, they are able to establish themselves in the gastrointestinal tract and maintain or increase the natural microbiota, assisting in the prevention of colonization by pathogenic microorganisms, and ensuring better utilization of nutrients. What strikes out in some of these studies is that the expected effects of probiotics are more noticeable when the animals are under stress, which often occurs within the first two weeks of life (Timmerman et al., 2005; Cruyagen et al., 1995; Ridell et al., 2010).

Bacteria are the principal source of branched-chain FA (**BCFA**). As a result, the main source of these nutrients in the diet of North Americans is food products from ruminants (Ran-Ressler et al., 2014). Ruminants can receive BCFA through the digestion of rumen bacteria, which can then be exported into milk or transported to tissues. Numerous studies

have demonstrated that the diet of dairy cows affects the BCFA composition of milk (Villeneuve et al., 2013; Baumann et al., 2016; Leduc et al., 2017), despite the fact that these FA are still only minor components of milk fat, accounting for just around 2% of all dairy FA (Ran-Ressler et al., 2011b).

Bacillus subtilis and *Bacillus licheniformis* are also given to commercial herds as probiotics. These bacteria have very high BCFA concentrations in their cell walls (Kaneda, 1977). The addition of several Bacillus species to dairy cow diets has been linked to improved fiber digestibility (Qiao et al., 2009), higher milk and milk ingredient yields (fat, protein, and lactose; Sun et al., 2013), and lower enteric methane emissions (Wang et al., 2016). Additionally, dairy ewes' milk output and milk fat and protein concentrations increased when they were given a direct-fed microbial supplement including live *Bacillus subtilis* and *Bacillus licheniformis* (Kritas et al., 2006).

Because methane (CH4) has a greenhouse gas potential that is 28 times greater than that of carbon dioxide, its impact on global warming is receiving more attention (IPCC, 2014). Over 90% of the CH4 produced by ruminants comes from microbial fermentation in the rumen, making ruminants the principal CH4 producers in the livestock industry globally (McAllister et al., 2015). According to Jeyanathan et al. (2014), ruminal CH4 emissions also account for 5% to 9% of the dietary gross energy loss in ruminants. Therefore, it is crucial for the ecology and the economy to reduce CH4 emissions from ruminants.

However, other mechanisms such as immunomodulation, antimicrobial production, and competitive exclusion may also be at play (Krehbiel et al., 2003; Hong et al., 2005; Mongkolthanaruk, 2012; FAO, 2016). One potential mechanism for this improvement is that the extracellular enzymes secreted by vegetative cells in Bacillus species enhance nutrient digestion in the animal's digestive tract (Leser et al., 2008). *B. licheniformis*-containing probiotics were given to breastfeeding sheep to improve milk production, milk fat, and milk protein content (Kritas et al., 2006). In sheep, certain bacterial inoculants reduced the amount of methane that was produced in a test tube (Nollet et al., 1998; Ellis et al., 2016), while others had no effect on in vivo methane emissions (Mwenya et al., 2004).

Dairy cattle produce their highest amount of milk about three months after giving birth. Most health issues, including most cases of mastitis, manifest themselves during this time period (Andersen et al., 2011). The overall milk output will not be as high as it could be before mastitis if it occurs during this time (Rajala-Schultz et al., 1999). Mastitis that

develops prior to the peak of lactation does, in fact, have a significant impact on subsequent milk production and is linked to substantially greater rates of culling (Seegers et al., 2003). Dairy cows who have mastitis, a highly frequent inflammatory illness of the mammary gland, produce less milk and milk of lower quality. Probiotics may be used as an alternative to antibiotics to prevent mastitis, and doing so may reduce the likelihood that bacteria may grow that are resistant to medicines. Additionally, according to Green et al. (2002), the risk of infection during the ensuing breastfeeding phase rises when intramammary infection occurs during the dry period. According to Sordillo (2005), possible causes for the onset of mastitis during this time include immunosuppression's reduction of resistance to bacterial exposure, the increased supply of milk nutrients for bacterial growth in the perinatal period, the stress of the impending parturition, and the negative energy balance brought on by parturition. In addition, infection during the dry period and subsequent lactation can alter the environment of the mammary gland, and a previous infection can weaken innate defenses. (Green et al., 2002).

Because mastitis reduces milk production and lowers milk quality, it has a significant negative economic impact on the dairy sector (Andersen et al., 2011; Rajala-Schultz et al., 1999). Mastitis' repeated recurrence is a frustrating feature. Clinical mastitis recurrence rates are about 50%, which leads to higher culling rates and shorter life spans for the animals in the herd (Bar et al., 2008; Wente et al., 2020). According to Olivera et al. (2013) and Ruegg (2017), mastitis can be brought on by a variety of pathogenic bacteria, including *Streptococcus agalactiae*, *Staphylococcus aureus*, *Streptococcus spp*., coliforms *Escherichia coli*, *Klebsiella spp.*, and *Mycoplasma spp*. Because they frequently infect the mammary gland soon after drying off and before parturition, when immunosuppression of cows raises the frequency of mastitis compared to the incidence during lactation, these bacteria are particularly crucial (Sordillo, 2005).

To stop the development of mastitis, certain vaccinations have received approval in a number of nations (Tashakkori et al., 2020). Despite various bacteria being known to cause mastitis, one drawback of the vaccines is that the targets, such as *S. aureus* and *E. coli*, rely on their makeup. Intramammary antibiotic infusion is the most widely used treatment for cow mastitis. Antibiotics have been widely utilized in veterinary medicine to combat bacterial infections, even though they are not always necessary in situations of clinical mastitis. However, overusing antibiotics increases the chance of developing germs that are resistant to them, reducing their ability to treat disease in the future. Therefore, it is necessary to

investigate other methods of mastitis treatment. Antibiotics may be substituted with probiotic microorganisms. For the purpose of enhancing the microbial habitat in the gut, the word "probiotic" is used to refer to advantageous microorganisms for both humans and animals. Probiotics include a number of well-known bacteria, including Lactobacillus and Bifidobacterium (Suez et al., 2019). To confirm that the animals given the probiotics can be protected from illness, such as mastitis in dairy cattle.

Bacillus subtilis produces spores with a high level of tolerance to abrasive environments. Digestive enzymes, such as pepsin in the stomach, help *B. subtilis* spores germinate after injection. This causes an increase in oxygen consumption and a subsequent rise in anaerobic conditions in the gut. Numerous advantageous bacteria subsequently flourish, as a result. Since 1986, the *B. subtilis* has been widely utilized as a probiotic feed supplement for cattle (Jeong & Kim, 2014).

MATERIALS AND METHODS

This study was conducted at the Free Stall barn "Professor Vidal Pedroso de Faria" of the Department of Animal Science at Luiz de Queiroz College of Agriculture – University of São Paulo, in Piracicaba, SP, Brazil, from October 14, 2021 to January 2022. All procedures using animals was follow the guidelines recommended by the Animal Care and Use Committee of the ESALQ/USP. Sixty Holstein cows were used to study animal performance, these animals were blocked by milk yield (21.1 ± 4.5 kg/d), days in milk (139.93 ± 72.5 days) and live weight (536.2 ± 58.6 kg) and whether it was primiparous or multiparous. The cows received the same diet (Table 1.), varying only the treatment calcium carbonate (n = 30; **CONT**) and *B. subitilis* and *B. liqueniformis*, (3,2 x 10^9 CFU/g; n = 30 **PROB**) that was top dress to the diet of each cow once per day in the morning (3 g/d), together with a sugar solution in order to adhere the product and increase palatability for immediate consumption of the probiotic. The experiment was conducted for 105 consecutive days. Over these 105 days, 3 weeks of collections were spaced, from the 29th to the 35th, 64th to 70th and 99th to 105th day, which the collections that will be described below were made. The only measurements taken daily were animal consumption and milk production. The 35th, 70th and 105th ended what we call cycles within that calendar day period.

The experimental diets were provided twice daily, at roughly 6:00 and 16:00 hours. A feed monitoring system (Intergado Ltda., Contagem, Minas Gerais, Brazil) that was validated by Chizzotti et al. (2015) was used to measure each cow's intake during the study. Daily ingredient samples were taken during sampling week (the final week of each cycle), and for each experimental cycle, composite samples were created. Similar to that, daily ort samples were taken and then combined for each cow and each cycle. Composite samples were ground to pass through a 1-mm screen in a Willey mill (A. H. Thomas Scientific, Philadelphia, PA) after being dried in a forced-air oven at 55oC for 72 hours. As stated by AOAC International (2012), the DM, OM (method 942.05), and ether extract (method 2003.05) were measured.

The Dumas technique was used to calculate the concentration of CP following measurement with a N analyzer (Leco FP-2000; Leco Corp., St. Joseph, MI, USA) (Wiles et al., 1998). Heat-stable -amylase and sodium sulfite were used to analyze the NDF using a TE-149 fiber analyzer (TECNAL Equipamentos para Laboratórios, Piracicaba, Brazil) (method 2002.04, AOAC, 2012). The analysis of starch followed Hall et al. (2015). By incubating ruminants in situ for 288 hours, indigestible NDF (**iNDF**) was measured (Huhtanen et al., 1994). Using the Penn State Particle Separator (**PSPS**), which was described by Kononnoff et

al. (2003a), the particle size distribution of total mix ration (**TMR**) was measured on undried and unground samples.

At 0600 and 1600 hours, cows were milked twice daily, and milk production was monitored regularly. On days 29, 30, 64, 65, 99 and 100, milk samples were collected throughout the course of four successive milkings. Mid-infrared analysis (Bentley 2000. Bentley Instruments Inc., Chaska, MN, USA) was used in Clínica do Leite (Piracicaba, Brazil) to measure the composition of milk and the milk urea nitrogen (**MUN**) level. Each experimental cycle, three skilled evaluators measured the body condition score (BCS; scale of 1 to 5; Wildman et al., 1982), and the average was used to describe the experimental units.

Fecal grab samples were taken every 8 hours from day 29 to 31, 64 to 66 and 99 to 101 and then composed by cycle. Using iNDF as an internal marker, total-tract apparent digestibility of dry matter (**DM**), neutral detergent fiber (**NDF**), starch, and crude protein (**CP**) was calculated (Huhtanen et al., 1994).

On day 22, the chewing habit was assessed by monitoring each cow's buccal activity continuously for 24 hours at intervals of 10 minutes. The buccal activities that were observed were drinking water, eating, thinking, and laziness. The sum of eating and ruminating time was the chewing time, expressed in minutes per day. The dry matter intake (**DMI**) of the day of chewing evaluation was used to compute the amount of chewing, eating, and ruminating per unit of DMI.

On day 32, 67 and 102 blood samples from the coccygeal arteries were taken to test for serum plasma urea-N (**PUN**), glucose. PUN samples were collected before and after the first daily feeding at 1, 2, 3, 6 and 12 hours. The blood was drawn into vacutainer tubes containing EDTA and promptly chilled. The plasma was then spun at 1,500 x g for 15 minutes and frozen at -20°C. A laboratory kit (Urea 500. Doles Reagentes para Laboratórios Ltda, Goiânia, Brazil) was used to analyze the PUN content. Before and after the first daily feeding 1, 2, 3, 6 and 12 hours, plasma glucose samples were taken in tubes containing sodium fluoride and EDTA. A laboratory kit (Glicose Enzimática Lquida, Doles Reagentes para Laboratórios Ltda, Goiânia, Brazil) was used to analyze them.

For the study of nutrient digestibility, 18 fistulated Nelore bulls were allocated in individual pens and received the same diet as the cows. The objective of this continuation is the ruminal evaluation (NH3-N and pH) of large ruminants supplemented with PROB. The conduction of this extension of work lasted 35 days (28 of rumen adaptation and 7 of

collections). In the same housing there were 2 fistulated Nellore bulls also individualized whose objective was the incubation for 288 hours to obtain the iNDF. Samples were collected 12 ± 0.35 h after the morning feeding. Ruminal pH was measured immediately (DIGIMED DM20, São Paulo, Brazil). A ruminal sample was instantaneously frozen in liquid nitrogen to stop fermentation and was kept frozen at -20^{o}C. The NH_3-N concentration was determined with a colorimetric method described by Chaney and Marbach (1962) and adapted for a microplate reader (BioRad, Hercules, CA, USA) with a 550 nm absorbance filter.

Table 1. Ingredient composition of experimental diets

Item	CONT	PROB
Diet ingredient, % of DM		
Corn silage	56.1	56.1
Soybean meal	12.4	12.4
Corn kernel, finely ground	20.1	20.1
Citrus pulp	9	9
Mineral and vitamin mix[1]	1.75	1.75
Urea	0.67	0.67
Probiotic	0	0.02
Calcium carbonate	0.02	0

[1]20.00 % Ca; 15.6 % P; 3.5 % S; 3.0 % Mg; 150 mg/kg Co; 2,000 mg/kg Cu; 200 mg/kg I; 5,000 mg/kg Mn; 11,900 mg/kg Zn; 82 mg/kg Se; 100,000 UI/kg Vitamin A; 220,000 UI/kg Vitamin D3; 6,200 UI/kg Vitamin E.

Table 2. Nutrient composition and particle size of experimental diets

Item	CONT	PROB
Dry matter, % of as fed	50.63	50.63
Nutrien composition of diet , % of DM		
Crude protein	12.93	12.93
NDF	43.17	43.17
Ether extract	3.04	3.04
Ash	6.84	6.84
Starch	20.6	20.6
PSPS[1], % of fed retained		
19-mm	4.13	4.13
8-mm	49.94	49.94
4-mm	20.3	20.3
Bottom pan	25.64	25.64

[1]Particle size distribution was measured using the Penn State particle size separator as described by Kononnoff et al. (2003).

STATISTICAL ANALYSIS

SAS (SAS Institute Inc., Cary, NC)'s MIXED technique was used to analyze the data. The cow within blocks (1 to 30) was a random effect, whereas the effects of treatment (CONT and PROB) were fixed effects. To measure the treatment impact, the treatment component served as the mistake. The first order autoregressive, compound symmetry, unstructured, and variance compounds were the options used to establish the covariance structure. Statistical significance and trends were considered at $P \leq 0.05$ and $P > 0.06$ to $P \leq 0.10$, respectively. The same was done for the nutrient digestibility study with Nellore cattle.

RESULTS

Cows fed with the PROB treatment had lower dry matter intake (-1.1 kg/d) and maintained milk production compared to the CONT treatment, causing feed efficiency to be higher for the PROB treatment (Table 3).

Milk composition showed difference between treatments. PROB showed an increase in the percentage of milk fat (+ 18.5%) compared to CONT, since the percentage of protein in milk tended to be lower for PROB (- 4.9%), but a statistically significant reduction in protein production in kg /d (-0.06 kg/d). In addition to these effects, there was also a trend towards reduced lactose production for the PROB treatment (-0.05 kg/d). Still regarding milk composition, MUN showed a reduction for the PROB treatment compared to the CONT (- 0.5 mg/dL) (Table 3).

Table 3. Effect of probiotic on the performance of dairy cows.

Item	***CONT***	***PROB***	***SEM***	***Trt***
Dry matter intake, kg/d	15.4	14.3	0.37	0.03
Milk yield, kg/d	20.0	19.9	0.66	0.77
Feed Efficiency, kg milk/kg DMI	1.31	1.45	0.050	0.04
FCM, kg/d	20.2	20.1	0.42	0.82
Fat-corrected feed efficiency, kg milk/kg DMI	1.40	1.47	0.059	0.39
ECM, kg/d	20.3	19.7	0.43	0.36
Energy-corrected feed efficiency, kg milk/kg DMI	1.39	1.46	0.058	0.45
Milk content, %				
Fat	3.35	3.97	0.068	< 0.0001
Protein	3.23	3.08	0.071	0.08
Lactose	4.13	4.11	0.03	0.56
Milk yield, kg/d				
Fat	0.70	0.73	0.016	0.36
Protein	0.65	0.59	0.018	0.01
Lactose	0.85	0.80	0.019	0.10
SCC, x 1000	173.0	140.6	33.58	0.49
MUN, mg/dL	13.0	12.5	0.11	< 0.01

As already shown, the DMI was lower for the PROB treatment, and this also resulted in lower consumption for nutritional partitions, such as NDF (- 0.74 kg/d), starch (- 0.39 kg/d) and CP (- 0.31 kg/d). d) (Table 4).

Table 4. Intake of dry matter, NDF, starch and CP of dairy cows

Item	***CONT***	***PROB***	***SEM***	***Trt***
Dry matter intake, kg/d	15.4	14.3	0.37	0.03
NDF intake, kg/d	6.85	6.11	0.15	<0.0001
Starch intake, kg/d	3.22	2.83	0.07	<0.0001
CP intake, kg/d	2.00	1.69	0.05	<0.0001

The results of the analyzes of the animals' feces showed lower levels of NDF, CP and higher starch for the PROB treatment in relation to the CONT. Furthermore, the estimated fecal output was lower for the PROB treatment (Table 5). Following this line of reasoning, the digestibility results indicated lower dry matter digestibility for the PROB treatment. However, there was greater digestibility of the starch and NDF fractions, on the other hand, the CP digestibility was lower for the PROB treatment, as well as the iNDF was also lower (Table 6). The evolution of starch digestibility can be seen in Figure 1 over the incubation time in Nellore cattle, where in the first 12 hours after incubation the PROB treatment showed 9.64% more starch digestibility compared to the CONT treatment, since with 24 hours of incubation, the PROB treatment had 5.54% less starch digestibility compared to the CONT treatment, the same was repeated at 48 hours (- 2.43%). However, at the last incubation time, at 72 hours, the PROB treatment showed higher digestibility again (+ 1.76%) compared to the CONT treatment.

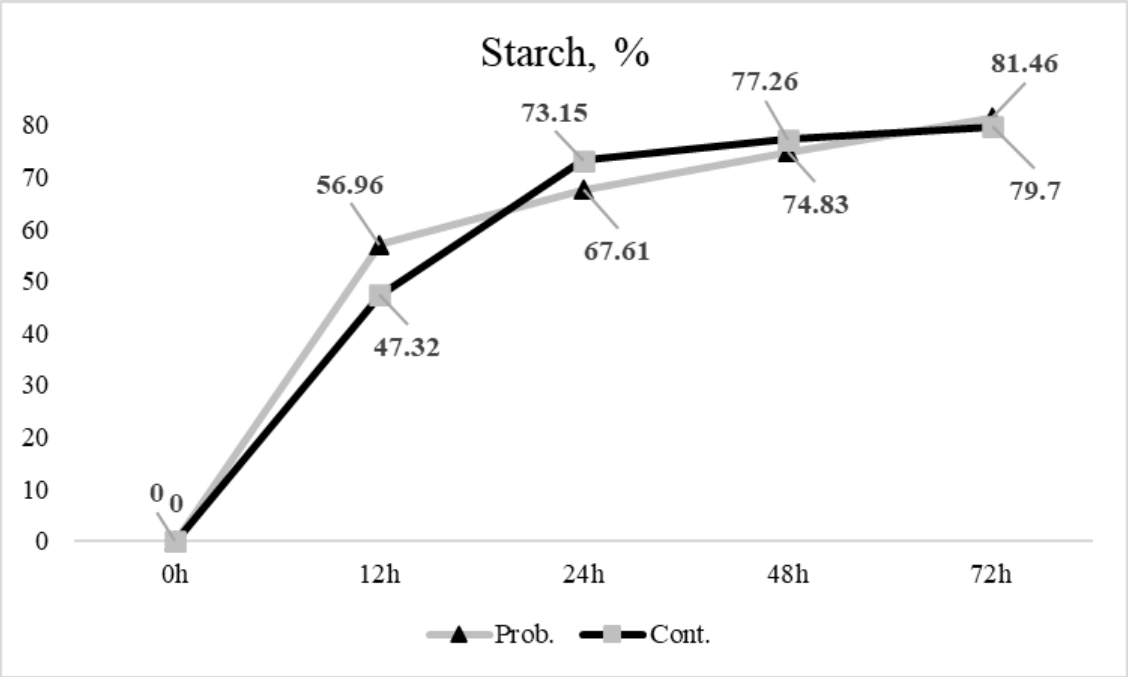
Table 5. Estimation of starch, NDF, CP in the feces and fecal production of dairy cows

Item	***CONT***	***PROB***	***SEM***	***Trt***
Fecal starch, %	1.94	1.95	0.07	<0.0001
Fecal NDF, %	52.56	50.93	0.004	<0.0001
Fecal CP, %	15.54	15.37	0.003	<0.0001
Fecal production estimate, kg/d	6.72	5.96	0.18	<0.0001

Table 6. Dry matter, NDF, starch and CP digestibility and iNDF of diet

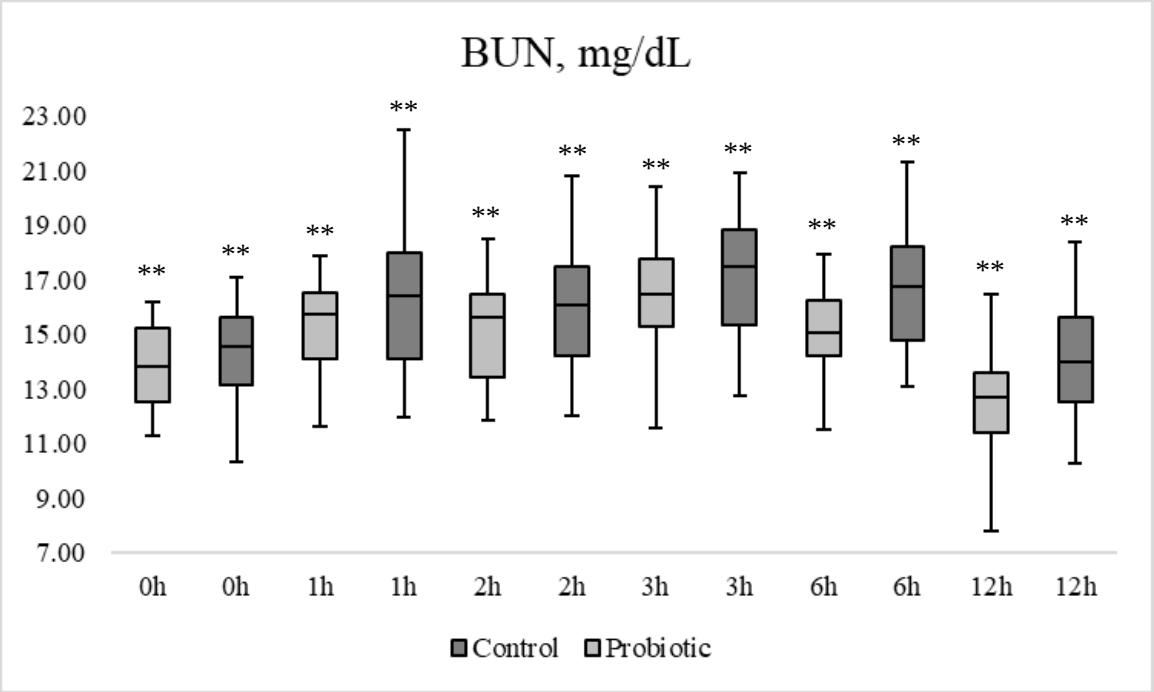
Item	*CONT*	*PROB*	*SEM*	*Trt*
Dry matter digestibility, %	56.20	56.19	0.54	<0.0001
NDF digestibility, %	46.87	49.00	1.02	<0.0001
Starch digestibility, %	95.55	95.66	0.19	<0.0001
CP digestibility, %	45.41	43.11	1.75	<0.0001
iNDF, %	45.62	46.83	0.99	<0.0001

Figure 1. Starch digestibility over time of incubation in Nellore bulls.



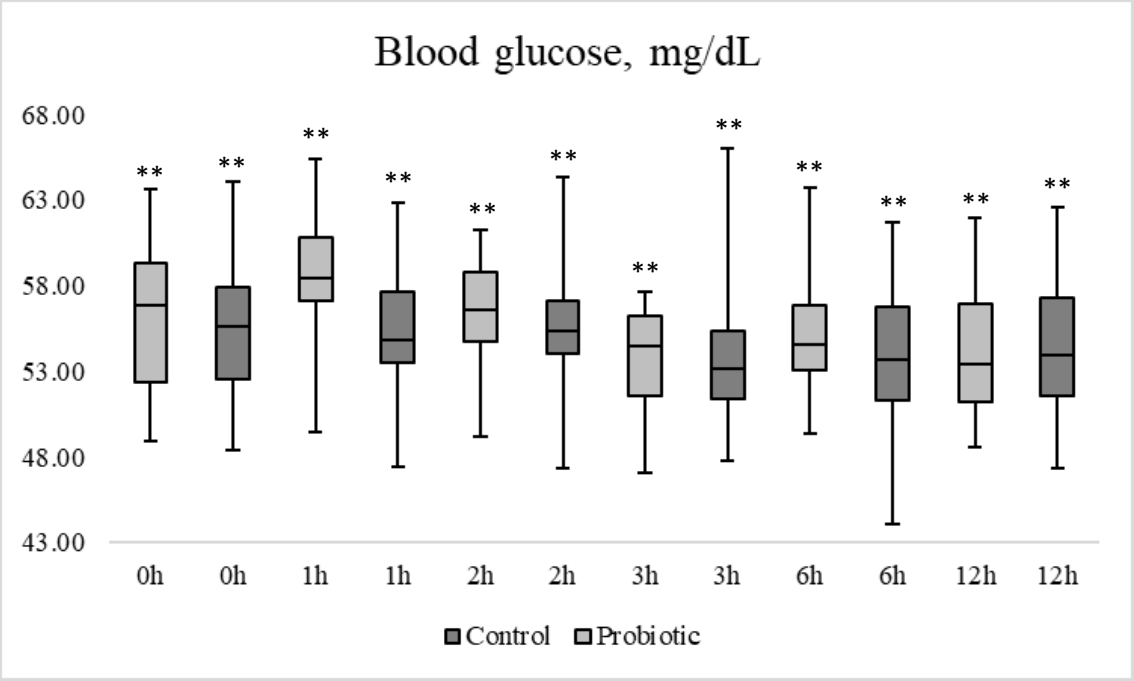
***Improvement of starch digestibility in the rumen of Nellore steers fed with CONT and PROB treatment**

Figure 2. Blood urea nitrogen in dairy cows fed with CONT or PROB



**** $P \leq 0.05$**

Figure 3. Blood glucose in dairy cows fed with CONT or PROB

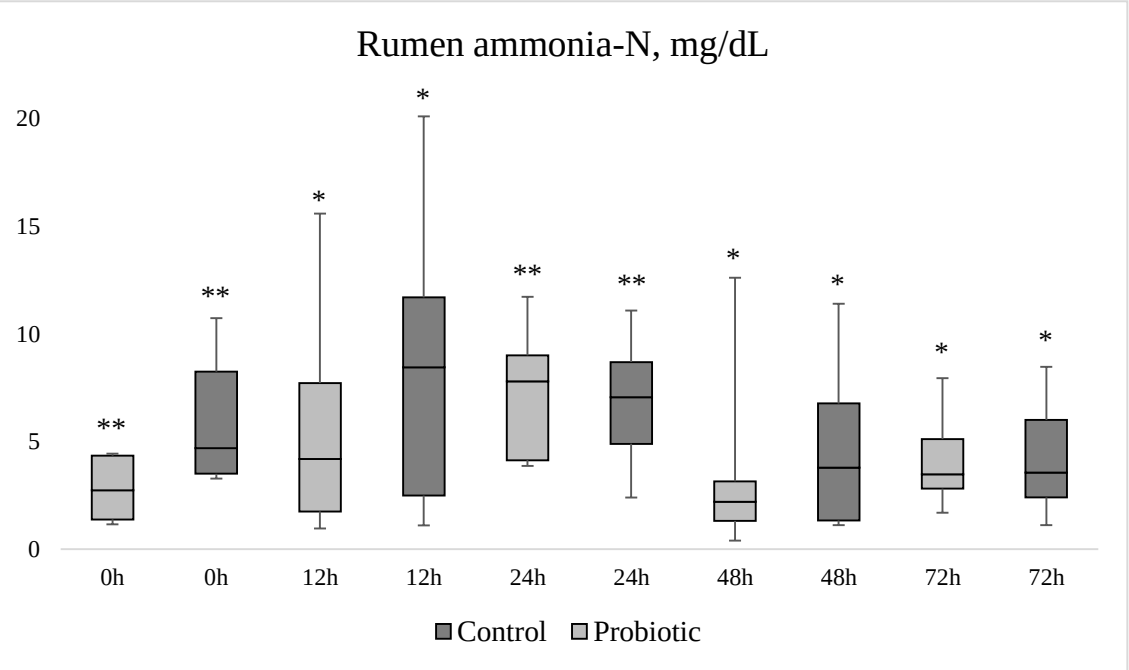


**** $P \leq 0.05$**

For the blood samples collected from lactating cows, at all collection times, the PROB treatment had lower BUN compared to the CONT treatment (Figure 2). Regarding blood glucose, at the collection times (0, 1, 2, 3 and 6h), there was a higher concentration of glucose in the blood, only at 12h was the glucose lower in the PROB treatment when compared to the CONT treatment (Figure 3).

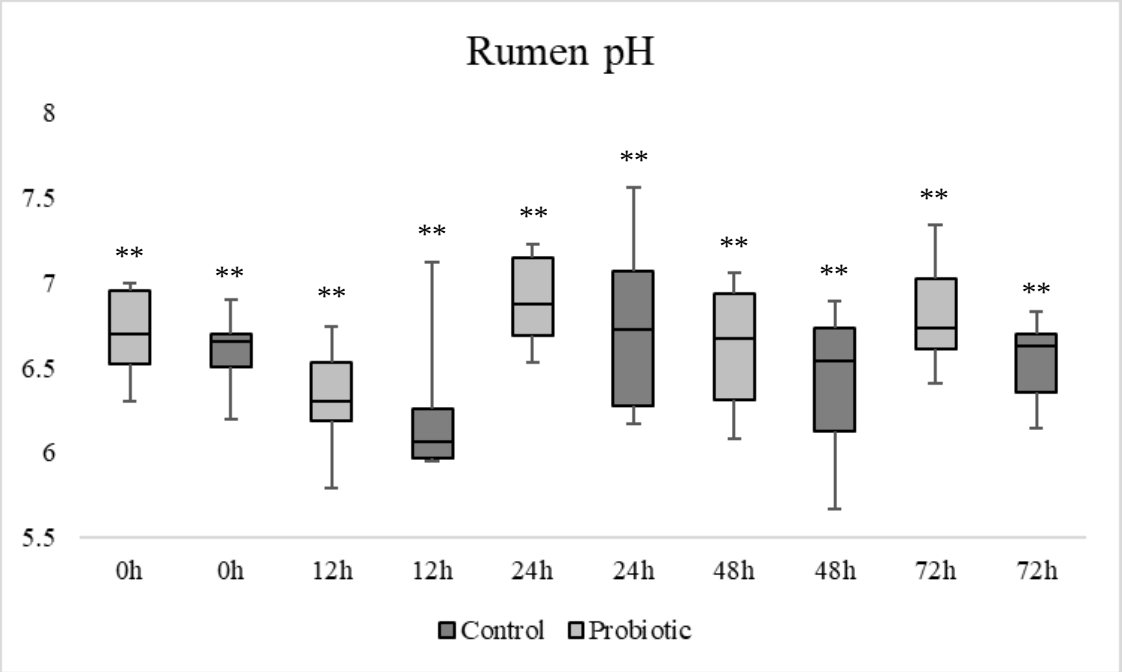
NH3-N values in the rumen of fistulated Nellore cattle were lower for the PROB treatment when compared to the CONT treatment in practically all collection times, with exceptions to the times (24 and 72 hours, referenced by the time of incubation of the samples), where the value was higher for the PROB treatment (Figure 4).
The rumen pH was higher for the PROB treatment compared to the CONT treatment at all times of sample collection (Figure 5).

Figure 4. Rumen ammonia-N in Nellore bulls fed with CONT and PROB



***0.10 ≤ P > 0.06**
**** P ≤ 0.05**

Figure 5. Rumen pH in Nellore bulls fed with CONT or PROB



** $P \leq 0.05$

DISCUSSION

The DMI in the present study showed a reduction for the animals that were treated with PROB, (Qiao et al., 2009) did not find alteration in the consumption when animals were fed with Bacillus, which suggests that there is no increase in palatability by the addition of Bacillus, By contrast, Nocek et al. (2000, 2003) as well as Soder and Holden (1999) reported that direct fed microbial increased dry matter intake in early lactation. Milk production was not altered with the addition of the probiotic. However, feed efficiency does, as demonstrated in another studies Wang et al. (2001) and Schingoethe et al. (2004).

Rumen pH, NH3-N and VFA concentrations are the important indicators that reflect rumen function and stability of the intraruminal milieu. Treatment with probiotics resulted in an increase in ruminal pH at all collection times and a reduction in NH3-N at all collection times, except for the time 24 hours after the first collection. Studies (ARTICLE 2) explained that consumption of Bacillus subtillis contained the acetate concentration and increased the concentration of propionate and valerate, satisfied the ratio of propionate acetate (Erasmus et al., 1992; Harrison et al., 1998; Hernández et al., 2009; Sun et al., 2012).

Rumen total VFA and acetate were increased by dietary *B. licheniformis* supplementation in dairy cows (Qiao et al., 2010), which may be associated with the unique specialization of *B. licheniformis* in starch hydrolysis and the use of propionate as a carbon source. It has been postulated that propionate is the major glucogenic precursor, which may increase blood glucose availability via gluconeogenesis (Stein et al., 2006) and subsequently increase milk lactose production improves energetic efficiency, and lessens ketosis (Sauer et al., 1989; Weiss et al., 2008). Available evidence has also demonstrated that higher levels of ruminal propionate increase milk yield (Oba and Allen, 2003), as well as lactose and protein contents in milk (Rigout et al., 2003; Stein et al., 2006). (ARTIGO 7). This is because propionate is the primary precursor for gluconeogenesis in early lactation dairy cows (Reynolds et al., 2003). Additionally, a rise in propionate may lower the hydrogen available in the rumen for the generation of methane. According to Stein et al. (2006), specific propionibacteria species have been shown to alter rumen fermentation and increase the molar fraction of ruminal propionate. In lactating ruminant animals, the mammary gland uses 60–85% of the total glucose present, and lactose production is responsible for 50–85% of this consumption (Knowlton et al., 1998). Since lactose is the primary osmoregulatory in the mammary gland intake of water (Lemosquet et al., 2004), the availability of glucose to the

mammary gland has a significant impact on milk supply. The greater lactose in milk and higher milk output were associated with higher propionate levels in DFM treatments.

The results of the current study may indicate the same effect, as the PROB treatment maintained the same milk production as the control treatment and reduced protein and lactose levels, in contrast, there was an increase in the fat content of the animals' milk in the probiotic treatment. According to Peng et al., 2012(ARTIGO10); Sun et al., 2013, dairy cows treated with a *B. subtilis* probiotic showed increased rumen total VFA and a higher molar percentage of propionate but a lower molar proportion of acetate. In contrast, the probiotic *B. amyloliquefaciens* reduced ruminal total VFA concentrations in sheep. These conflicting findings are a result of variations in microbial species and strains, dosage, animal species, physiological conditions, feeding schedules, and other variables between research. Unfortunately, the rumen microbial output and fermentation profile were not examined in this experiment to see if they may account for the variation in milk and protein discharges.

Because methanogenesis competes with propionate production for available hydrogen, higher propionate production in the rumen may have resulted in decreased methane levels and improved feed efficiency (Moss et al., 2000). The enhanced use of propionate for the synthesis of milk components, i.e., decreased heat increment, could result in additional efficiency advantages (West, 1999).

A drop in the acetate to propionate ratio in the rumen is the result of greater ruminal propionate creation being stoichiometrically connected with decreased CH4 production because methanogenesis competes with it for available H2 (Krehbiel et al., 2003; Seo et al., 2010). In the study conducted by researchers Seo et al., 2010, this did not happen, since CH4 synthesis was reduced and ruminal VFA were not altered, the authors attribute this effect to a possible stress response of ruminal bacteria after administration of B. licheniformis How ruminal methanogenesis is a complicated biochemical process influenced by many variables, including ruminal production of formate as a substrate for methanogenesis and ruminal abundance of H2-producing and H2-consuming bacteria, proteobacteria, fungi, and ciliated protozoa, other underlying mechanisms may also be involved (Tapio et al., 2017). Asshan and Qiao's (2007) finding that Bacillus cultures had no impact on methane generation in vitro. All these results lead to greater efficiency and energy use in animal metabolism, which justifies, in the present study, the maintenance of milk production in the PROB treatment compared to the PROB treatment, even with lower dry matter intake.

The literature related to bacillus in cattle feed suggests increases in digestibility after the use of these products. According to Qiao et al. (2010), supplementing with *B. licheniformis* increases the number of cellulolytic bacteria in the rumen, improving the digestibility of NDF and ADF. Amylases and proteases secreted by *B. licheniformis* may also help with ruminal and intestinal nutrient digestion. Because *B. licheniformis* stimulates bacteria that break down cellulose, higher VFA and acetate concentrations with *B. licheniformis* supplementation were likely happening. This is because more fiber was broken down in the rumen. Ghorbani et al. (2002) reported the identical information. The same can be observed in the present study, in which there was an increase in NDF digestibility (+2.13%).

Direct fed microbial culture supplementation has been shown by several researches (Nocek et al., 2002; Oellermann and Arambel, 1990; Wiedmtiel et al., 1987) to increase the total tract digestibility of NDF and ADF. According to Krehbiel et al. (2003), introducing direct-fed microbial culture to meals high in concentrate improved the proportion and number of cellulose-digesting bacteria in the rumen. The increased ruminal digestibility of NDF and ADF is due to this increase in cellulose-digesting bacteria. It has been shown by Mould and rskov (1993) and Wanapat et al. (2000) that cellulose digestion is constrained when ruminal pH falls below 6.0.

B. licheniformis supplementation appears to boost fiber-digesting microorganisms, even though it did not significantly increase the amount of milk fat (Qiao et al., 2009). Although the phenomena has been described previously (Piva et al., 1993), the present analysis is still unable to fully explain this little discrepancy. According to Harri and Pekka (1996), butyric acid also seems to have a particular impact on milk fat content. Increased butyrate supply increased fat content, despite a constant ratio of acetate to propionate of approximately 3.5 and the ratio of (acetate + butyrate) to propionate >4.0 (Huhtanen et al., 1993), which is considered to be a threshold for milk fat content (Sutton, 1980).

Treatment of pasture with fibrolytic enzymes is an alternative to boost fodder digestibility (Dean et al. Expansion-like proteins, which can be expressed by a variety of bacteria and fungi, can loosen, expand, or disturb plant cell wall constituents like cellulose and hemicellulose in order to alleviate this last issue (Liu et al., 2015). Recent research by Pech-Cervantes et al. (2019) showed that *B. subtilis* has a higher capacity to manufacture and release expansin-like proteins than *Trichoderma reesei* (Liu et al., 2015). Additionally, compared to cellulase alone, *B. subtilis* incubated in a medium boosted the hydrolytic activity

of cellulase (Pech-Cervantes et al., 2019). Different bacilli strains create various enzymes, such as cellulases in *B. licheniformis* and expansin-like proteins in *B. subtilis* (Pech-Cervantes et al., 2019; da Silva et al., 2021; Luise et al., 2022). Both here and in a subsequent study by Pech-Cervantes et al. (2019), it was found that these enzymes can have cumulative effects when combined.

Indeed, in substrates with a higher concentration of hemicellulose, Bunterngsook et al. (2014) observed greater synergism between expansin-like proteins and fibrolytic enzymes. This is likely because the hydrogen bonds between hemicellulose and cellulose are broken, increasing the accessibility of cellulases to cell wall polysaccharides (Saloheimo et al., 2002). According to Ferraretto et al. (2015), choosing hybrids for milho silage, for example, could be used as an alternative to encourage greater digestibility of forage sources frequently provided to cattle. Recently, Pech-Cervantes et al. (2019) reported that inoculating *B. subtilis* with a fibrolytic enzyme increased the NDF digestibility (+8,5%) in a total milk ration (TMR), leading to increased milk production (Ferraretto et al., 2013).

Therefore, the elevated relative long-chain polyunsaturated fatty acids concentrations seen in this study may have been caused by the altered milk lipoma from B. *subitilis* and B. *licheniformis* supplementation. This impact could be felt at the rumen level, for example by changing lipid metabolism to raise the amount of unsaturated fatty acids in the duodenum that is accessible for incorporating into milk fat. As an alternative, the effect could be a more intricate post-ruminal biological effect in the host animal itself, such as direct-fed microbials effects that could affect the de novo fatty acids system in the mammary gland, post-absorptive lipid metabolism, and the makeup of lipids that reach the gland. As a result, these microbes can produce unsatured fatty acids species with a variety of lengths and branching patterns (Fulco, 1969; Diaz et al., 2002; Altabe et al., 2003). Previous research has demonstrated that specific strains of *B. subtilis and B. licheniformis* possess an acyl lipid desaturase, an iron-dependent integral membrane protein capable of selectively introducing cis double bonds into long-chain fatty acids. Therefore, it is conceivable that dietary B. *subitilis* + B. *licheniformis* supplementation may have increased the desaturation of ruminal lipid content, which may have resulted in an increase in the flow of long-chain polyunsaturated into the duodenum and subsequent direct absorption of these fatty acids in the small intestine. Reduced biohydrogenation of dietary polyunsaturated fatty acids may have been caused by changes in the population of ruminal microbes responsible for biohydrogenation, which may also have been caused by the effects of other microbes or ruminal conditions like ruminal pH. This may have explained the reduced biohydrogenation of dietary polyunsaturated fatty acids A.

According to Pattnaik et al. (2001), some Butyrivibrio strains, which play a significant role in the biohydrogenation of unsaturated fatty acids in the rumen, are susceptible to a bacteriocin from the lichen *B. licheniformis*.

Due to the fact that free fatty acids are more easily absorbed in the lower gastrointestinal tract than esterified fats and saturated fatty acid, as well as the fact that unsaturated fatty acids are more easily absorbed than saturated fatty acid (Elliott et al., 1999; Daley et al., 2018). The degree of rumen lipolysis and biohydrogenation may be reduced by rumen pH 6.0, which is predicted to increase the duodenal outflow of long-chain polyunsaturated fatty acid (Doreau et al., 1997; Dewanckele et al., 2017). Similar to this, studies feeding B. *subtilis* and B. *licheniformis* to multiparous cows showed that the microbial additive had no impact on rumen pH but increased concentrations of branched-chain fatty acids in milk, which are frequently produced by bacilli and used as part of their cell wall (Lamontagne et al., 2019). Since many ruminal microorganisms, particularly fibrolytic bacteria, are toxic to high amounts of unsaturated fatty acid (Jenkins, 1993), high ruminal concentrations of unsaturated fatty acid are predicted to limit rumen microbial fermentation and fiber digestibility.

According to Oliveria et al. (2016), certain Bacillus strains are capable of producing fibrolytic enzymes including cellulase and xylanase as well as digesting enzymes. The ability of *B. subtilis* and *B. licheniformis* to work together has been shown in numerous studies to boost the digestibility of a range of fiber sources, including grasses and legumes (Qiao et al., 2010; Oliveira et al., 2016). Oyebade et al., 2023 De novo fatty acid, which is frequently used to indicate rumen fermentation conditions, was present in greater relative amounts in the milk of dairy cows fed B. *subitilis* and B. *licheniformis* (Woolpert et al., 2016). This is because acetate and butyrate, which are predominantly obtained through ruminal fermentation of fibrous feed, are used to manufacture de novo fatty acids in milk primarily in the mammary gland (Palmquist et al., 1993).

In the study conducted by researchers Pen et al., 2012, supplementation with fermentation products revealed that the release of NEFA from cow adipose tissue in direct-fed microbial was less than that of control cows. The greater propionate levels in the rumen may be responsible for the reduced NEFA in the direct-fed microbial treatments. According to certain studies (Oba and Allen, 2003; DeFrain et al., 2005; Liu et al., 2010), the plasma NEFA concentration dropped linearly with increasing propionate infusion. By reducing the activity of fatty acyl-CoA dehydrogenase (Shaw and Engel, 1985; Emery et al., 1992) or by reducing

the transport of fatty acids to mitochondria (Jesse et al., 1986), propionate can impede the oxidation of fatty acids, resulting in a decrease in lipolysis in adipose tissue. In the present study the NEFA levels of the evaluated cows were not measured. However, the literature that references the subject brings us this discussion that allows us to rely on a concept of greater metabolic efficiency when feeding with bacillus is used.

In the referenced study (Peng et al., 2012), the supplementation of *B. subtilis* natto fermentation product had no effect on the protein yield or milk fat %. With the increase in the supplementation of fermentation product, fat yield increased linearly. Bell (1995) hypothesized that a sizable portion of milk fat production in the early lactation may be accounted for by mammary uptake of NEFA. The higher milk output and increased NEFA uptake by the mammary of the cows fed direct-fed microbial may be the root causes of the higher fat yield. Similarly, with increased *B. subtilis* natto fermentation product supplementation, milk protein tended to linearly decline (Peng et al., 2012). The author attributed this effect to the dilution due to the increase in milk production. However, the same did not happen in the present study, which takes us in another direction, which can be explained by the lower rates of ruminal BUN and NH3-N, added to the lower CP digestibility that the PROB treatment presented compared to the CONT treatment, reflecting in lower protein intake in the mammary gland.

Isovalerate and isobutyrate are employed by some bacteria as primers for the synthesis of odd-chain and even-chain iso branched-chain fatty acid, respectively, according to Vlaeminck et al. (2006). However, the higher concentration of branched-chain fatty acid in milk fat cannot be directly attributed to the rise in rumen isoacid concentration. The deamination of branched amino acids may be encouraged by *Bacillus subtilis* and *Bacillus licheniformis* to improve the availability of the isoacids required for the synthesis of their longer odd- and even-iso fatty acid constituents. However, French et al. (2012) shown that milk branched-chain fatty acid concentrations are only slightly impacted by ruminal infusion of isoacids, demonstrating that the availability of primer is not the limiting factor for branched-chain fatty acid synthesis in the rumen. Nevertheless, a higher ruminal branched-chain fatty acid metabolism can be connected indirectly to an increase in isoacids in the rumen. For instance, it has been shown that isoacids are crucial metabolites for some cellulolytic bacteria, supporting the use of ammonia and boosting cellulose digestion (Andries et al., 1987). Additionally, it has been demonstrated that ruminal branched-chain fatty acid synthesis is linked to cellulolytic bacteria (Fievez et al., 2012). Reinforcing again the results

obtained in the increase of digestibility of the fibrous obtainment of the evaluations evaluated in the present study.

Ruminants, which can deliver 35–65% metabolizable protein, benefit from the fraction and ratio of amino acids in bacteria (Storm and rskov, 1983). Because rumen microorganisms produce a considerable amount of their cell protein from ammonia N, microbial protein made in the rumen provides the majority of absorbable amino acids in the small intestine (Hristov et al., 2004).

Study results (Qiao et al., 2009), following morning feeding, B. *licheniformis* reduced the ammonia nitrogen content at 0.5, 1, 3, and 6 hours. This showed that additional ammonia was likely used to create microbial nitrogen, which may have been a direct effect of increased microbial activity. According to Khan et al. (2006), Bach et al. (2005), and Alexander et al. (1996), the ruminal ammonia reduction has typically been atributed to increased microbial protein synthesis and enhanced ammonia assimilation.

It is known that ruminal microbial population is of great importance for rumen fermentation and milk production. Increased numbers of certain groups of bacteria, such as amylolytic and proteolytic bacteria number, and reduced protozoal number in B. *subtilis* natto treated period might accelerate ruminal recycling of bacterial N (Ghorbani et al., 2002) and thus resulting in higher ruminal NH3 concentrations. It is known that protease and amylase are the metabolites of the *B. subtilis* natto. However, in the present study, the concentration of ruminal NH3 fell in the treatment with probiotics, going against what the studies say about increased proteolysis and higher ruminal NH3. In another study (Jia et al., 2018) probiotics supplementation had no effect on rumen pH but decreased the concentration of ammoniacal nitrogen and increased the content of microbial proteins The result indicated that probiotics could improve the efficiency of rumen fermentation, which is in accordance with the research of *B. subtilis natto* applied in dairy cows (Sun et al., 2016). The change of ruminal NH3-N and microbial crude protein level that reflects the nitrogen utilization of the rumen microbials might attribute to the lower consumption and same milk production resulting in higher feed efficiency. (ARTIGO 6) The increased surface area of the rumen papillae (Sun et al., 2011), increased total ruminal bacterial populations with decreased protozoan populations (Sun et al., 2013), and increased ruminal N assimilation by rumen bacteria for microbial protein synthesis (Qiao et al., 2010) are all likely related to the lowered ruminal NH3-N by Bacillus probiotic supplementation. However, supplementing dairy cows with a *B. subtilis* probiotic led to an increase in ruminal NH3-N, which was then linked to improved dietary protein degradation

by higher populations of proteolytic bacteria in the rumen (Qiao et al., 2010; Sun et al., 2013). In the current study, ruminal NH3-N levels were lower for the PROB treatment. The same can be found in the literature that references this work, thus attributing this reduction of ruminal NH3-N to a greater assimilation of N by the bacteria that colonize the rumen.

The use of N increased with the use of probiotics. Although, the CP intake data are already smaller for the probiotic, the fecal CP data are also, however, what allows us to consider that the use of CP was better for the probiotic treatment is the CP digestibility that was higher with the use of probiotics in the diet. Wethers supplemented with *B. licheniformis* had improved nitrogen utilization efficiency (N retention/N intake); this improvement was largely attributable to improved N digestion as fecal N outputs were significantly reduced while urinary N excretions were unaffected in *B. licheniformis*-supplemented animals. Le et al. (2017) found that although the apparent N digestibility in these animals remained unaffected, the utilization efficiency of N was increased by 84% in ewes fed with *B. amyloliquefaciens* due to a 24% decrease in the urine N excretion rate (urinary N/N intake). The results of these authors (Le et al., 2017) and ours show that increased N uptake by rumen microorganisms and/or decreased deamination of amino acids (protein turnover) in the animal's body are likely connected with improved N utilization in probiotic-supplemented ruminants. In fact, dietary *B. licheniformis* supplementation increased the non-ammonia N flow, the duodenal microbial protein flow, and the ratio of bacteria N to N intake in the rumen of dairy cows (Qiao et al., 2010).

Souza et al., 2017, combining the milk protein response with a decrease in MUN shows that the *Bacillus subtilis* spores may have altered systemically or in the rumen protein metabolism. The same happened in the present study, reaffirming the effect of *Bacillus subtilis* on protein metabolism in ruminants. Cows fed *Bacillus subtilis* showed an increase in ruminal ammonia 1 and 6 hours after feeding, according to Qiao et al. (2010). *Bacillus subtilis* natto supplementation, according to Sun et al. (2013), increased ruminal ammonia concentration. In nursing cows, a *Bacillus subtilis* supplement had no influence on blood urea-N levels, although Peng et al. (2012) did notice a tendency for an increase in ruminal ammonia concentration. The literature that is currently accessible implies that supplementing with *Bacillus subtilis* may enhance ruminal ammonia accumulation, which is the opposite of the trend in which MUN and BUN were decreased in the current study.

The addition of *B. licheniformis* enhanced milk protein. Due to ruminal ammonia N high digestibility in the small intestine, a higher microbial protein synthesis in the rumen may

lead to a more efficient transfer of ruminal ammonia N into body and milk protein (Hvelplund and Hesselholt, 1987; Lapierre and Lobley, 2001; Blouin et al., 2002; Sarwar et al., 2004).

According to several investigations, increased levels of microbial crude protein in the intestine contribute favorably to better milk yields (Buttery and Foulds, 1985). As a result, the amount of microbial nitrogen entering the duodenum rose and more milk protein was produced. In the current study, this effect was not the same as that reported by these authors, since milk protein was lower for the PROB treatment. The addition of *B. subtilis* raised the ammonia nitrogen content 6h after morning feeding. This suggests that *B. subtilis* can probably induce microorganisms that produce proteases. In the present study, only the 24h and 72h hours showed a higher NH3-N content, which cannot be explained since the times are only a reference to the incubation period, but the animals were fed daily at the same time. Therefore, the collection times (0, 24, 48 and 72h) were performed at the time when the animals would receive food.

Stage of lactation is a plausible factor in the response of dairy cows to nutritional manipulations (Oba and Allen, 1999). Cows in this experiment were in a more advanced stage of lactation, this could probably be the reason why there was no effect on milk production. Souza et al., 2017, the authors found greater fat deposition in cows with more advanced IMD, which may have been a sign of greater tissue deposition as lactation progressed. The DIM reference of this cited experiment was 84 days, while the DIM of our study was close to 140 days.

Experimental evidence of the effectiveness of the product as a supplement for the majority of the lactating herd, and not just for a certain set of cows in early lactation, would be the identification of a positive reaction in lactation performance in cows in late lactation. The reduction in plasma concentration of non-esterified fatty acids after supplementation of a B. subtilis fermentation product to cows in early lactation (29 DIM) suggests that lipid metabolism was responsive to the probiotic, although the mechanism has not been clarified (Peng et al., 2012). However, lactating cows in late lactation produced more milk and protein after being fed B. subtilis natto for 7 weeks (Liang-ce et al., 2012), demonstrating that cows in early and late lactation can benefit of probiotic supplementation. The same was not observed in our study, since milk production was not altered and protein production in milk was reduced in the PROB treatment compared to the CONT. On the other hand, this advanced

DIM can justify some results obtained in the present study that differ from what is mentioned in the literature corresponding to the theme.

The impact of probiotics on animal output is greatest during a time of stress, according to Riddell et al. (2010). Therefore, it's probable that Bacillus probiotics benefit animals' immune systems, which in turn indirectly aids in maintaining high output when faced with pathogen or climatic problems. This may account for the variations in performance seen by Kritas et al. (2006) when ewes and their young were housed in an "old-fashioned traditional barn" with daily access to pasture and no technology boosting the animals' welfare. The controlled environment in which the animals were housed throughout the current experiment may have assisted in preventing metabolic stress, which could have restricted the impact of the probiotic on animal performance.

The environment is closely related to the health of the animals, and especially with the mastitis rates in the herd. The literature on the subject also supports the reduction in cases of mastitis in cows fed with probiotics. In the current study, no batch was exposed to situations that could condition the appearance of mastitis. However, even so, there was only a numerical and non-statistical variation that demonstrated a reduction in SCC, the main indicator of mastitis in dairy herds, for the group that received the probiotic treatment. Urakawa et al., 2022, compared to the previous lactation and the control cows, feeding dramatically reduced the incidence of mastitis. Additionally, the SCC in milk was controlled at a low level by the *B. subtilis*.

Animal immune systems may be affected by the supplementation of bacterial spores (Cutting, 2011; Sun et al., 2010). B. *subtilis* natto supplementation was found by Sun et al. (2013) to reduce SCC, but the precise mechanism of action was unknown, and our findings did not corroborate this finding. In neither of the tests, a treatment impact on SCC was found.

Metabolic disorders can affect dairy cows at different stages of development. However, the most critical occurs in the transition period from the end of pregnancy to postpartum, this period will determine the rest of lactation. The redirection of resources toward the mammary gland in support of milk production disturbs the homeostatic balance in postpartum dairy cows. A high degree of body fat mobilization occurs as a result of the high metabolic priority for milk production and the restricted feed intake around parturition. To free up glucose for milk production and to ensure that nutrients derived from tissues and the diet are distributed toward the mammary gland, many peripheral organs switch their

metabolic fuel source from carbohydrate to fat utilization under these circumstances (Bauman & Bruce Currie, 1980; Kuhla et al., 2016). It is well-known that the plasma metabolite concentrations in dairy cows change considerably before and after parturition, including plasma glucose, NEFA, BUN, and T-chol (Guretzky et al., 2006; Laeger et al., 2013), among others (Chandra et al., 2013; Dyck et al., 2011; Laeger et al., 2013; Salin et al., 2018). Dairy cows incur viral or metabolic disease during the transition from late gestation to early lactation because of these significant metabolic alterations (LeBlanc, 2010).

The related increased formation of reactive oxygen species (ROS) results in increased oxidative stress due to the metabolic demands associated with late pregnancy, parturition, and rising milk production in lactation (Chandra et al., 2013). Due to inadequate feed intake during pregnancy, freshly calved dairy cows with high genetic potential and high milk yield typically have reduced milk yields compared to their potential and are more susceptible to production illnesses. Feeding *B. subtilis* natto, a strain similar to *B. subtilis*, has reportedly been shown to boost milk yield and improve feed efficiency as a result of an increase in propionate in the lumen (Peng et al., 2012). As a result, treating dairy cows with *B. subtilis* may also improve feed intake effectiveness and restore the availability of nutrients around parturition. We speculate that the *B. subtilis* may have lessened the animals' stress under these conditions. In the present study, the transition period of the cows was not evaluated, since they entered the design already in lactation. However, the benefits of using probiotics extend to other metabolic disorders throughout lactation.

CONCLUSIONS

The use of probiotics based on B. licheniformis and B. subtilis was effective in making changes in milk composition, increasing fat, an ingredient in milk that is very desired by the production chain. There was no increase in dry matter intake, in fact the opposite was observed, that is, a reduction in dry matter intake. However, there was no reduction in milk production, which shows that the PROB treatment promoted greater feed efficiency for the animals that were fed with it.

As for ruminal health, PROB was effective in maintaining high ruminal pH, since at all times of ruminal fluid collection, the pH of the PROB treatment was higher than the CONT treatment.

The blend of the two bacillus was effective in increasing the digestibility, mainly of the NDF and starch fractions, a very favorable point for its recommendation since they are fractions considered as pillars in the formulations of diets for dairy cattle.

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