MARCELO DE BARROS ABREU

ASSOCIATION OF COW, FEEDING, AND ENVIRONMENTAL CONDITIONS WITH MILK PERFORMANCE IN ORGANIC DAIRIES, RUMEN-PROTECTED AMINO ACIDS SUPPLEMENTATION IN MID-LACTATION COWS, AND FEEDING PRACTICES MANAGEMENT IN BRAZILIAN DAIRIES

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

Adviser: Marcos Inácio Marcondes

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Marcos Inácio Marcondes
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ABSTRACT

ABREU, Marcelo de Barros, D.Sc., Universidade Federal de Viçosa, February, 2022. **Association of cow, feeding, and environmental conditions with milk performance in organic dairies, rumen-protected amino acids supplementation in mid-lactation cows, and feeding practices management in Brazilian dairies**. Adviser: Marcos Inácio Marcondes.

Our objectives with this study were 1) evaluate the effects of lactation number, somatic cells count (SCC), season, production system, and breed on milk yield (MY), and fat and protein milk concentrations of cows in organic herds, 2) evaluate the effects of supplementing methionine (Met) and lysine (Lys) as rumen-protected AA on milk yield and composition of mid-lactating Holstein cows in a commercial dairy feeding a low-forage diet, and 3) gather information of feeding practices in Brazilian dairies and identify whether those practices are associated with herd milk production level. In the first study, a Wood model was used to fit an average lactation curve for MY and milk fat and protein from a data set containing individual monthly cow's milk test days from 14 organic dairies collected between 2012 to 2015. Overall, MY and milk fat and protein were affected by lactation number, breed, feed condition, season, and somatic cell count. Greater milk yield was found for cows in the fourth lactation, for Holstein cows, in the winter and spring season, and for cows under grazing feed conditions, and for cows with low SCC. In the second study, a total of 314 multiparous cows were randomly assigned to control [CON; 107 g of dry distillers grains (DDG)] or rumen protected Met and Lys (RPML; 107 g DDG + 107 g of RPML). Throughout 42 d of study, cows were grouped in a single dry lot pen and fed the same TMR diet twice daily. Milk components from a.m. and p.m. milkings were determined from samples collected at d 0, 14, 28, and 42 of the study. Treatments effects were evaluated at the cow-level considering milk yield and composition taken at baseline (1 wk before the experiment) as a covariate in the models. Clinical mastitis risk was assessed by Poisson regression. Plasma Met increased (26.9 vs. 36.0 µmol/L), and Lys tended to increase (102.5 vs. 121.1 µmol/L) with RPML supplementation. Cows supplemented with RPML had higher milk yield (46.0 vs. 45.4 kg/d); however, milk components yield and concentration were not affected by RPML supplementation. Although somatic cell count was not affected by RPML supplementation, the risk of clinical mastitis was 0.39 times lower for RPML than CON cows (95% CI: 0.17-0.90). Results suggest that RPML supplementation increased milk yield and decreased the risk of clinical mastitis in mid-lactation cows. In the third study, an online survey was performed to assess Brazillian dairy farmers' most common feeding practices in confined systems. The survey consisted of 38 questions divided into 4 sections to assess feeding practices performed in high milk-production pens. The questionnaire was mailed to 500 dairy producers, and 135 responses (27.6%) were returned. After data screening, the remaining 82 responses were analyzed. From the 82 responses, 56 (68%) and 26 (32%) were answered by the dairy manager and nutritionists, respectively. Dairies were categorized according to their 305-day milk production (kg) as low production (LP; <7,000; n = 27), medium production (MP; 7,000 to 10,000; n = 35), and high production (HP; >10,000; n = 20). Overall, herd size averaged 175 lactating dairy cows containing Holstein (n = 52; 63%), Holstein × Gyr (n = 22; 27%), and Jersey (n = 8; 10%) herds. High production cows were housed on compost barn (n = 42; 51%), dry lot (n = 24; 29%), and free-stall (n = 16; 20%). The HP and MP herds had a greater risk ratio to have a trough wash protocol than LP herds. The HP herds had a greater risk ratio to evaluate TMR physically effective fiber NDF (peNDF) than LP herds. The MP herds had a greater risk ratio to measure feed efficiency, check forage dry matter (DM), and evaluate corn processing compared to LP herds. The risk ratio was not different among the 3 herds groups regarding the use of mixer wagon, evaluating TMR DM, evaluating particle size distribution, calibration of the wagon scale, use of TMR stabilizers, feedstuffs composition analysis, feed after milk time, feed push-ups, feed bunk cleaned-up, and feed for refusals, have employee´s training protocol, group primiparous separately from multiparous, and have a colling system. Althougt most of risk ratio were similar between herds, the HP and MP herds had greater frequencies on the most feeding practices compated LP herds. In summary, survey results can be used to develop and disseminate target information on feeding practices and feed bunk in Brazilian dairies.

Keywords: Organic milk production. Rumen-protected amino acids. Feed bunk practices.

RESUMO

ABREU, Marcelo de Barros, D.Sc., Universidade Federal de Viçosa, fevereiro de 2022. **Associação de características animal, ambiental e de alimentação no desempenho produtivo de vacas leiteiras em sistemas de produção de leite orgânico, efeito da suplementação de aminoácidos protegidos no desempenho de vacas leiteiras no terço médio de lactação e práticas alimentares de manejo em fazendas leiteiras no Brasil.** Orientador: Marcos Inácio Marcondes.

Objetivou-se com este estudo 1) avaliar os efeitos do número de lactações, contagem de células somáticas (CCS), estação do ano, sistema de alimentação e raça na produção de leite e no conteúdo de gordura e proteína do leite de vacas em rebanhos orgânicos; 2) avaliar o efeito da suplementação de metionina (MET) e lisina (LIS) protegidos da degradação ruminal sobre a produção e composição do leite de vacas holandesas no terço médio de lactação, e 3) coletar informações sobre as práticas alimentares em laticínios brasileiros e identificar se essas práticas estão associadas ao nível de produção de leite dos rebanhos. No primeiro estudo o modelo de Wood foi usado para ajustar uma curva de lactação média para produção de leite e gordura e proteína do leite de um conjunto de dados contendo teste individuais mensais de vacas de 14 laticínios orgânicos coletados entre 2012 a 2015. No geral, produção de leite, gordura e proteína do leite foram afetados pelo número de lactação, raça, condição alimentar, estação do ano e CSS. Maior produção de leite foi observada para vacas Holandesas, para vacas na quarta lactação, nas estações de inverno e primavera, para vacas em regime de pastejo e para vacas com baixo CCS. Para o segundo estudo, 314 vacas multíparas foram aleatoriamente designadas para controle [CON; 107 g de grãos secos de destilaria (DDG)] ou MET e LIS protegidas no rúmen (RPML; 107 g DDG + 107 g de RPML). Ao longo de 42 dias de estudo vacas foram agrupadas em uma única baia de dry lot e foram alimentadas com dieta total duas vezes ao dia. A produção de leite e os casos clínicos de mastite foram registrados diariamente. Componentes de ambas foram determinadas a partir de amostras coletadas nos dias 0, 14, 28 e 42 do estudo. Os efeitos dos tratamentos foram avaliados no nível das vacas, considerando a produção e a composição do leite tomadas na linha de base (uma semana antes do experimento) como uma covariável nos modelos. O risco de mastite clínica foi avaliado por regressão de Poisson. Plasma MET aumentou (26,9 vs. 36,0 µmol/L), e

LIS tendeu a aumentar (102,5 vs. 121,1 µmol/L) com a suplementação de RPML. Vacas suplementadas com RPML tiveram maior produção de leite (45,4 vs. 46,0 kg/d); entretanto, a produção e concentração dos componentes do leite não foram afetados pela suplementação de RPML. Embora a contagem de células somáticas não tenha sido afetada pela suplementação de RPML, o risco de mastite clínica foi 0,39 vezes menor para RPML do que para vacas CON (IC 95%: 0,17-0,90). Os resultados do segundo estudo sugerem que a suplementação de RPML aumenta a produção de leite e diminui o risco de mastite clínica em vacas no terço médio de lactação. No terceiro estudo foi realizada uma survey para avaliar as práticas alimentares mais comuns utilizadas pelos produtores de leite brasileiros em sistemas de produção de confinamento. A pesquisa consistiu em 38 questões divididas em 4 seções para avaliar as práticas alimentares realizadas em baias de alta produção de leite. O questionário foi enviado a 500 produtores de leite e 135 respostas (27,6%) foram recebidas. Após a triagem de dados, as 82 respostas restantes foram analisadas. Das 82 respostas, 56 (68%) e 26 (32%) foram respondidas pelo gerente de fazenda e nutricionista, respectivamente. Os rebanhos foram categorizados de acordo com a produção de leite corrigida para 305 dias (kg) como: baixa produção (LP; <7.000; n = 27), produção média (MP; 7.000 a 10.000; n = 35) e alta produção (HP;> 10.000; n = 20) nível de produção de leite do rebanho. No geral, o tamanho dos rebanhos foi de 175 vacas em lactação, sendo 52, 22 e 8 rebanhos da raça Holstein, Girolando e Jersey, respectivamente. As vacas foram alojadas em compost barn (n = 42; 51%), dry lot (n = 24; 29%) e free-stall (n = 16; 20%). Os rebanhos HP e MP apresentaram maior risk ratio para lavagem de bebedouros que rebanhos de LP. Os rebanhos de alta produção tiveram maior risk ratio para avaliar FDN fisicamente efetiva (peNDF) da TMR em comparação com a LP rebanhos. Rebanhos de MP apresentaram maior risk ratio para mensurar a eficiência alimentar, verificar a MS da forragem e avaliar o processamento do milho em comparação aos LP rebanhos. Por outro lado, risk ratio não diferiu entre os três grupos de rebanhos para uso de vagão misturador, avaliação da MS da TMR, avaliação da distribuição de partículas da dieta, calibração da balança do vagão, uso de estabilizadores de TMR, análise de composição de alimentos, fornecimento da dieta após a ordenha, push-up a dieta, limpeza de cocho, alimentar para sobras, ter protocolo de treinamento de funcionários, agrupar primíparas separadamente de multíparas. Independentemente do nível de produção de leite, os rebanhos tiveram metas de sobras de 5% e

frequências de avaliação da eficiência alimentar (mensal), limpeza de cocho (7 × sem) e avaliação peNDF da TMR (mensal). A maior parte dos produtores de alta produção reportaram fornecer dieta três vezes ao dia, enquanto os produtores de MP e LP alimentam as vacas duas vezes ao dia. Quarenta e um por cento dos produtores de HP relataram flexão de ração cinco ou mais vezes por dia, enquanto apenas 15 e 31% dos MP e LP produtores relataram mesma frequência, respectivamente. Quarenta e quatro por cento dos produtores de HP relataram lavar bebedouro sete vezes por semana, enquanto apenas 26 e 17% dos produtores de MP e LP relataram a mesma frequência. Maior frequência de análise da composição da dieta foi relatada para a maioria dos MP (55%; mensal) produtores, seguido por HP (44%; mensal e <1 × mês) e LP (50%; ≤ 1x mês) produtores, respectivamente. Independentemente do nível de produção de leite, os rebanhos relataram avaliar a MS da forragem mensalmente e quando um novo silo é aberto. A maioria dos produtores de MP (38%) relatou avaliar o processo de grãos de milho em todos os vagões, enquanto a maioria da HP (54%) e LP (50%) relataram avaliá-lo apenas em alguns vagões. Em conclusão, os resultados da survey demonstraram que os produtores de rebanhos de HP e MP apresentaram maior frequência de práticas de manejo alimentar do que produtos de rebanhos de LP. No entanto, o risk ratio foi similar entre os grupos de rebanhos de diferentes níveis de produção. Resumidamente, estes resultados podem ser usados para desenvolver e disseminar informações-alvo sobre práticas de alimentação e de cocho beliches em fazendas brasileiros.

Palavras-chave: Leite orgânico. Aminoácidos protegidos. Manejo alimentar.

SUMMARY

1 GENERAL INTRODUCTION

The milk chain is one of the most important sectors of agribusiness worldwide. Even during the *Coronavirus* pandemic, the sector has shown strong resilience and keeping growth. In 2021, for instance, the total world milk production was forecast to reach 921.1 million tons, representing an increase of 1.6% compared to 2020 (FAO, 2021). In addition, the solid demand for milk products has driven an increase in the per capita milk products consumption, which is forecasted to reach 116.6 kg/year in 2021 (FAO, 2021). Besides world milk chain expansion and the increase of the dairy price index, dairy producers have tight profitability margins in the last years. This is likely because feedstuffs used to feed dairy cows have greater price increase than milk prices. Furthermore, dairy customers' concerns about food safety and health have risen significantly in recent years. This shift in customer preferences is reflected in the organic milk industry, which has grown substantially in the past decade (USDA NASS, 2019). However, despite organic milk production dating back to the 1940s, there is still a lack of knowledge on how breed, season, feed condition (grazing vs. no grazing), and somatic cell count (SCC) are associated with milk performance in this system (Brito and Silva, 2020).

On the other hand, the dairy community has worked on strategies to overcome those challenges and provide relevant information to the competitive dairy industry. Most improvements in the dairy industry are regarding nutritional, genetics, environmental. In protein nutrition, for example, dairy committees have established requirements for specific amino acids (AA) rather than just target dietary crude protein (NRC, 2001a; Van Amburgh et al., 2015). These advances are essential to improve animal nutrient utilization and increase animal production and health. Thus, the use of AA protected from ruminal degradation has appeared as a strategy to help nutritionists balance AA diets. Nonetheless, studies evaluating the use of rumen-protected methionine and lysine for cows fed no conventional protein source, and high by-products diet are scarce in the literature, deserving investigation.

Additionally, the number of studies evaluating management practices that improve animal production has widely increased in recent years (Miller-Cushon and DeVries, 2017). Thus, non-nutritional factors are becoming more important in the dairy sector, as improvements in milk production, efficiency, and animal welfare have been documented in the literature (Bach et al., 2008; Sova et al., 2014). In this sense, no guidelines or information regarding feed management practices are published in the Brazilian dairy industry. Therefore, gathering this information may be helpful to identify opportunities to optimize feed management and guide on-farm decisions on Brazilian dairy farms

Therefore, this thesis has 3 objectives regard dairy cattle production: 1) evaluate the association of breed, lactation number, season, feed condition, and SCC with milk yield and milk fat and protein content in organic dairy farms; 2) evaluate the effects of supplementing a rumen-protected methionine and lysine on the performance of lactating dairy cows; and 3) survey the feed management practices used in South and Southeast dairies in Brazil

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2 ARTICLE 1

Running head: Association of cow, feeding, and environmental conditions with milk performance in organic dairies

Effects of lactation number, feed condition, somatic cells count, and breed on milk yield and fat and protein milk concentrations in northeastern United States organic dairy farms

2.1 Abstract

Evaluation of milk yield (MY) and fat and protein milk concentrations are needed to help farmers make informed decisions on organic dairy management. We aimed to evaluate the effects of lactation number, somatic cells count (SCC), feeding condition, and breed on MY, and fat and protein milk concentrations in organic dairy herds. A data set containing individual monthly cow's milk test days from 14 organic dairies collected between 2012 to 2015 was used. Data were obtained from organic dairies located in Maine (n = 3), New Hampshire (n = 3), New York (n = 2), Pennsylvania (n = 3), and Vermont (n = 3). Milk test days were composited by Holstein (48.8%), Jersey (37.3%), and Holstein-Jersey crossbreed (13.9%) cows. The Wood model was used to fit an average lactation curve for MY and milk fat and protein. Overall, the MY lactation curve had an intercept of 22.0 kg/d, with a MY peak of 25.1 kg/d at 28 days in milk (DIM). When expressing as energy-corrected milk yield, the intercept was estimated as 27.2 kg/d, with a peak of 27.6 kg/d at 9 DIM. Milk fat concentration was estimated started as 4.40%, with a minimum milk fat of 4.10% at 41 DIM, while milk protein started as 4.13%, with a minimum of 3.10% at 70 DIM. Lactation number quadratically affected MY, with first and fourth lactation cows having the lowest and greatest average MY, respectively. Milk SCC showed an interaction effect with DIM on the predicted MY, and cows with SCC ≥ 200,000 cells/mL had greater milk drop at the end of lactation than cows with SCC of 50,000 cells/mL. Grazing cows had greater MY (20.3 kg/d) than confined cows (19.3 kg/d). Holstein cows showed the greatest MY (22.0 kg/d), followed by crossbreed (20.4 kg/d) and Jersey (17.0 kg/d) cows. Milk fat and protein were linearly and quadratically affected by lactation number, respectively. Feeding conditions affected milk fat but not milk protein concentration, with confined cows having greater milk fat concentration (4.35%) than grazing cows (4.16%). Jersey cows showed the greatest milk fat and protein (4.74; 3.58%), followed by crossbreed (4.14; 3.27 %) and Holstein (3.89; 3.10 %) cows, respectively. Overall, MY and milk fat and protein were affected by all variables, except feeding conditions, that did not affect milk protein.

Keywords: milk components, milk production, organic milk

2.2 Introduction

The adoption of organic dairy systems has substantially increased since the first Certified Organic Survey in 2008 (USDA NASS, 2010). In 2019, the number of certified organic herds and cows reached 3,100 and 363,404, respectively, up 54% and 66% from the 2008 Certified Organic Survey (USDA NASS, 2010, 2019). Additionally, milk has become the top organic commodity sales in the United States, with a revenue of $1,585 million, up 14% from 2016 (USDA NASS, 2017). Besides market demand, producers were encouraged to change from conventional to organic systems because of improved economic viability (Dalton et al., 2008; Pereira et al., 2013).

Milk production and components are crucial for a dairy farm's profitability, and a substantial part of dairy economic resilience is linked to producers' strategies choices such as breed, feeding condition, and health and herd management. Brito and Silva (2020) highlighted that organic milk producers are currently dealing with reduced pay prices, tight profit margins, and canceled contracts due to oversupply of organic milk, despite the growth of the organic dairy industry in the last decade.

The factors that affect MY and components in conventional herds are well characterized. For instance, it is well known that MY and components follow season (Salfer et al., 2019, 2020) and that they are also affected by breed (Stocco et al., 2017), lactation number (Mellado et al., 2010; Yang et al., 2013), and level of concentrate intake (Walsh et al., 2007). On the other hand, there are few studies that exclusively looked at factors that affect milk yield and components in organic herds. Most studies have focused on comparing MY and components between conventional and organic systems (Butler et al., 2011; Adler et al., 2013; Schwendel et al., 2015).

Roesch et al. (2005) concluded that cows on organic system had lower milk MY compared to conventional production.

However, production outcomes in organic systems might do not reflect the same results of conventional due to their specific rules on feeding, health practices, breeding, living conditions (Nauta et al., 2009; Clasen et al., 2020). Cows under organic systems are restrict to consume 30% of dry matter intake from pasture and remain grazing at least for 120 days in the grazing season. Differences on nutrition and handle and management practices between organic and conventional systems is associated to affect milk yield and components concentrations (Schwendel et al., 2015). Therefore, understanding factors that affect milk yield and components is essential to optimized animal and economic performance in organic systems (Rodríguez-Bermúdez et al., 2019).

Although the lactation number, feed condition, breed and SCC are well described for conventional systems, to the best of our knowledge, no study gathering these effects on organic systems is described in the literature. Thus, the objective of the present study was to evaluate the impact of breed, lactation number, feeding system (grazing or non-grazing), and SCC on MY and milk fat and protein using individual cow information from 14 organic certified dairy herds in the northeastern United States.

2.3 Material and methods

A cross-sectional study was performed to gather information on factors that affect MY and milk fat and protein concentrations in organic dairies herds. A dataset in excel spreadsheet format was obtained through Dairy Herd Information Association (DHIA), with individual cow's milk test-day through of lactation period from 2012 to 2015. The data set contained information from 14 northeastern organic dairies located in the states of Maine (n = 3), New Hampshire (n = 3), New York n =

2), Pennsylvania (n = 3), and Vermont (n = 3). All organic dairies used in the study used managed intensive rotational grazing (Hafla et al., 2016). From 2012 to 2014, the botanical composition was mostly composited by grasses, followed by legumes and weeds, respectively (Hafla et al., 2018). The majority of the grazing species (67%) were composited by cool-season grasses, including orchardgrass (*Dactylis glomerata* L.), perennial ryegrass (*Lolium perenne*), Timothy (*Phleum pratense* L.), Kentucky bluegrass (*Poa pratensis* L.), tall fescue (*Festuca arundinacea* S.), and festulolium (*Festulolium* spp.) (Hafla et al., 2016). Clovers made up approximately 26% of the grazing species, including red clover (*Trifolium pratense* L.) and white clover (*Trifolium repens* L.). Herbage (i.e., grazed forage) nutritive value averaged 19.5% CP, 51% NDF, 31.4% ADF, and 1.39 Mcal/kg NE_L (Hafla et al., 2016). During the grazing season, estimated proportion of herbage in the diet averaged 63.8% (DM basis). From the 14 farms included in this study, 2 fed a TMR with cows having access to pasture during the grazing season, 3 fed only forage (i.e., conserved forage, grazed herbage), and the remaining 9 dairies fed conserved forage and concentrate in addition to access to pasture in the summer months. Forages and concentrates included bailages, haylages, corn silage, wheat, roasted soybean, soybean meal, flaxseed meal, and linseed meal (Hafla et al., 2016, 2018).

The original dataset contained 14,245 milk test-day from 2,139 individual lactating Holstein (n = 757), Jersey (n = 578), and Holstein × Jersey (n = 213) crossbreed cows. Cows with less than 5 milk test days per lactation were removed from the data set. Additionally, cows should have the first test day before 60 DIM and the last test day after 150 DIM. This procedure was done to guarantee a good fit of the lactation curve for each cow. The final dataset contained 13,885 milk test-day and included 10 variables: cow id, breed, dairy identification code, state, test-day year, test-day date, production system (grazing or non-grazing), lactation number, calving date, DIM, MY, milk fat (%), milk protein (%), and SCC (cells/mL). Milk fat and protein yield and concentrations as well as SCC were measured, analyzed, and informed by the DHIA.

Data management and calculations were performed in the excel spreadsheet. Individual SCC values were Log10 transformed in an excel spreadsheet before statistical analysis.

Statistical analysis

The statistical modeling was done in 2 steps. First, we fitted an average equation for MY, milk fat (MF), or milk protein (MP) for all cows according to the model proposed by Wood (1967) using the NLIN procedure of SAS (Statistical Analysis System, version 9.4):

$$Y_t = a \times DIM^b \times exp^{-c \times DIM} \quad \text{(Eq 1)}$$

Where Y_t = MY (kg/d) or MF (%) or MP (%); a = scaling factor; DIM = days in milk, and b and c are constants. At this point, no random effect was used.

Second, a model was built to understand the deviation of each individual data point from our average curve using the GLIMMIX procedure of SAS (Statistical Analysis System, version 9.4). In this model, observed MY, milk fat concentration, and milk protein concentration were used as dependent variables, and the fixed effects of feeding system, breed, lactation number, and SCC were used as model predictors (independent variables). To account exclusively for the variation between the data point and the average curve, we also added the predicted value from Equation 1 as a fixed effect in the model as a covariate. Additionally, we added fixed effects of quadratic components of milk SCC and the milk SCC × predicted and SCC × DIM interactions. Animal was included as a repeated measure in the model (random effect), as well as farm and year. Residual degrees of freedom were corrected according to the Satterthwaite procedure. The following variance-covariance structures were tested: AR(1), ARH(1), CS, CSH, TOEP, TOEP 2, TOEPH, TOEPH(2), UN, UN(1), UN(2), and VC. The variance-covariance that provided the best fit based on the Akaike information criterion was retained in the final model. Selected variance-covariance matrices were ARH (1) for MY, TOEP (1) for milk fat and milk protein concentrations. For all analyses, a level of 0.05 was used as the critical probability level. The milk peak, week of the peak, and milk persistence were calculated using the equations present in Wood (1967).

2.4 Results

Predicted MY, milk fat and protein concentrations

We used the Wood (1967) model to estimate the lactation curve for MY (Equation 2) to fit standard lactation curves, with an begin increasing phase to a maximum production (Figure 1A and 1B).

$$MY\ (kg/d) = 22.0457_{\pm 0.6225} \times DIM^{0.05527_{\pm 0.008352}} \times exp^{-0.001889_{\pm 0.000090} \times DIM} \quad \text{(Eq 2)}$$

$$ECM = 27.2093_{\pm 0.6898} \times DIM^{0.01129_{\pm 0.007495}} \times exp^{-0.001318_{\pm 0.000080} \times DIM} \quad \text{(Eq 3)}$$

where: MY = MY (kg/d); and DIM = days in milk.

The estimated MY at begin of lactation was 22.0 kg/d (intercept of Eq. 2), while peak MY was estimated as 25.0 kg at 28 DIM (Figure 1A). We also used a reparameterization of Wood's (1967) model to estimate milk fat concentration (Eq. 4) and milk protein (Eq. 5) and generated a reversed shape curve, with the begin value decreasing to a minimum fat and protein concentration (Figure 1C and 1D).

$$Milk_{fat}\ (\%) = 4.4056_{\pm 0.09137} \times DIM^{-0.02562 \pm 0.005928} \times exp^{-0.00062 \pm 0.000057 \times DIM} \quad \text{(Eq 4)}$$

$$Milk_{protein}\ (\%) = 4.1344_{\pm 0.04693} \times DIM^{-0.08781 \pm 0.003268} \times exp^{-0.00125 \pm 0.000032 \times DIM} \quad \text{(Eq 5)}$$

Milk fat concentration at begin of lactation was estimated as 4.40% (intercept of Eq. 4), and the minimum milk fat concentration was estimated as 4.10% at 41 DIM (Figure 1C). Milk protein concentration at begin of lactation was estimated as 4.13% (intercept of Eq. 5), and the minimum milk protein concentration was estimated as 3.10% at 70 DIM (Figure 1D).

Effects on MY

The lactation number had a quadratic effect ($P < 0.003$) on MY (Figure 2A). Milk yield increased from the first to the fourth lactation, with an average daily MY of 16.7, 19.3, 21.1, 21.6 for first, second, third, and fourth lactation respectively. Additionally, MY decreased from fourth to the fifth lactation averaging 20.6 kg/d (Figure 2A). We observed an interaction between milk SCC and DIM ($P < 0.001$). Overall, cows with milk SCC equal or greater than 200 × 1000 cells/mL had a more pronounced decrease in MY at the end of lactation than cows with milk SCC of 50 cells/mL (Figure 2B). Feeding system also affected MY, with grazing cows showing greater MY (20.3 kg/d) than confined cows (19.3 kg/d; Figure 2C). We also observed a breed effect on MY, whereby Jersey cows had lower MY than Holstein × Jersey crossbred (17.0 vs. 20.4 kg/d), with Holstein cows showing the greatest MY (22.0 kg/d) across breeds (Figure 2D).

Effects on Milk Fat Concentration

Milk fat concentration decreased linearly with the increase in lactation number ($P < 0.001$), and averaged 4.31, 4.32, 4.24, 4.20, and 4.21%, for first, second, third, fourth, and fifth lactation, respectively (Figure 3A). We also observed an interaction between milk SCC and DIM (*P* < 0.003). Specifically, cows with milk SCC (× 1000) equal to 50 cells/mL had lower milk fat around the fifth week of lactation than cows with milk SCC equal to or greater than 200 cells/mL (Figure 3B). Feeding system further affected milk fat concentration, with confined cows having greater (P < 0.001) milk fat (4.35%) than grazing cows (4.16%; Figure 3D). Jersey cows had milk fat concentration 14.5% and 21.8% greater than Holstein × Jersey crossbred and Holstein cows, respectively (Figure 3E).

Effects on Milk Protein Concentration

Lactation number showed a quadratic effect (*P* < 0.001) on milk protein concentration (Figure 4A). Primiparous had lower protein concentration (3.29) that peaked in the second lactation, averaging 3.34%, and decreased after that, with values of 3.31, 3.30, and 3.33%, for third, fourth, and fifth lactations, respectively (Figure 4A). Primiparous Milk SCC affected milk protein concentration quadratically (*P* < 0.003; Figure 4B). Overall, milk protein content reached nadir around the 10th week of lactation regardless of milk SCC score. Feeding system did not affect milk protein concentration (*P* = 0.17; Figure 4D). Jersey cows had greater (P < 0.001) milk protein concentration (3.58%) than Holstein × Jersey (3.27%) crossbred and Holsteins (3.10%, Figure 4E), whereas averaged 9.5% and 15.3% higher than Crosses and Holstein (4.16%), respectively.

2.5 Discussion

This objective of this study was to evaluated the effects of lactation number, feeding condition, breed, and somatic cells count (SCC) on MY, and fat and protein milk concentrations in organic dairy herds. We used milk test days information from

2012 to 2015 to evaluate the effects of lactation number, milk SCC, feeding system, and breed on MY, and milk fat and protein concentrations. Descriptive statistics for the final data set used in the present study is presented in Table 1. Variables tested are discussed separately in the following sections.

Lactation number

Effects of lactation number on MY were investigated for the first time by Kincaid and Touchberry (1966). Since then, it has been well documented that cow's MY increases from the first to subsequent lactations, which decrease after cows reach the lactation peak (Ray et al., 1992; Roesch et al., 2005; Salfer et al., 2019). Increased DMI and the mammary gland development generally explain greater MY for multiparous than primiparous cows (Miller et al., 2006; de Souza et al., 2019). Thus, similar to our results, studies also reported a quadratic effect of lactation number on MY in either organic (Roesch et al., 2005; Horn et al., 2012) and conventional systems (Ray et al., 1992). However, in these studies (Roesch et al., 2005; Horn et al., 2012), MY peaked at fifth lactation, while in the present study cows peaked at fourth lactation. In the simulation of Horn et al. (2012), organic cows reached the annual profitability peak in the sixth lactation besides of peak of milk in the fifth lactation. Lee and Kim (2006) reported that an eduction of MY after third or more lactations is frequently associated with impaired immune system and consequently greater disease susceptibility in conventional milk systems (Lee and Kim, 2006). For instance, Adriaens et al. (2021) evidenced that cows in third or greater lactation had more negative deviation of milk production in the milk test, caused mainly by transition diseases and mammary gland infections, which lead to greater and non-recovery milk losses.

Similar to our results, studies under conventional management reported a decrease in milk fat concentration as lactation number increased (Yang et al., 2013; Salfer et al., 2019). In contrast, Schutz et al. (1990) reported a quadratic milk fat response from first to third and greater lactation for both Holstein and Jersey cows. Increased MY and udder infections are the most common reasons for decreased milk fat concentration as lactation number increases (Bondan et al., 2018).

It is well documented that cow increases the risk of health problems as the number of lactation increase, impacting milk protein synthesis (Lee and Kim, 2006; Zhang et al., 2016). Guo et al. (2021) reported that oxidative stress had a detrimental influence on milk protein production in Holstein cows. This happens because oxidative stress causes insulin resistance, which is a hormone linked to milk protein synthesis via the mTOR signal pathway (Rius et al., 2010). Thus, similar to our results, previous studies have also evidenced a decrease in milk protein concentration as the number of lactations increases (Yang et al., 2013; Salfer et al., 2019). However, in the present study, cows in their first lactation had lower milk protein concentration than cows in their second or third lactation. Therefore, we speculate that the protein requirements of primiparous were not fulfilled, which might be associated with the greater AA requirements once that primiparous cows are still growing. As a result, less AA is available for mammary gland protein synthesis (NRC, 2001).

Somatic cells count

The inflammatory response of the bovine mammary gland against infection pathogens drivers increases in milk SCC, which is usually related to decrease MY (Seegers et al., 2003; Dürr et al., 2008). Therefore, cows with milk SCC greater than

200,000 cells/mL during the first lactation month showed decreased MY and longevity in organic dairy systems (Fernandes et al., 2021). In our modeling (Figure 2B), cows with lower milk SCC (i.e., 50,000 cells/mL) had greater lactation peak and persistence than cows with SCC greater 200,000 cells/mL. These results are in concordance with previous studies in organic (Bennedsgaard et al., 2003) and conventional herds (Hand et al., 2012), where milk losses were directly associated with increased milk SCC and ranged from 0.35 to 1.09 kg/d for cows with milk SCC > 200,000 cells/mL (Hand et al., 2012). Milk losses were associated with parity rather than SCC by Gonçalves et al. (2018), with higher milk losses for second and third lactation cows than first lactation cows, which was also demonstrated by Dürr et al. (2008).

Increases in milk SCC have been reported to affect not only MY but also milk properties and components (Maréchal et al., 2011; Bobbo et al., 2016). However, there is no consensus on the effects of milk SCC on milk fat and protein concentration with studies indicating a negative (Harmon, 1994; Ballou et al., 1995; El-Tahawy and El-Far, 2010), positive (Bruckmaier et al., 2004; Silva et al., 2018), or no impact (Bobbo et al., 2016) of milk SCC on these 2 variables. In our study, cows with decreased milk SCC (i.e., 50,000 cells/mL) had lower milk fat concentration around 5 to 10 weeks of lactation compared to cows with elevated milk SCC. A similar pattern was observed for milk protein concentration; however, the difference between the SCC scores was less prominent than milk fat. This response might be attributed to dilution of milk fat concentration as cows with lower milk SCC produced more milk than those with greater milk SCC. On the other hand, milk protein concentration was less affected by changes in milk SCC.

Feeding system (Grazing vs. Confinement)

Effects of diets on MY and its components have been extensively explored in the literature (Kearney et al., 2004; Craninx et al., 2008). Studies have consistently demonstrated that cows under grazing systems produced les milk than cows under confinement systems, mainly due to the lower concentrate intake (White et al., 2002; Walsh et al., 2007). However, in our study, grazing cows produced more milk than cows in confinement. During the grazing season, cows received approximately 60% of the diet as high-quality herbage (19.5% CP; 51% NDF), which may have resulted in more digestible nutrients than diets offered in confinement (Hafla et al., 2016). Conserved forages used during confinement is typically more mature than herbage resulting in reduced digestibility. For instance, in a simulation with the same data set, cows (Holstein and Jersey) reach or exceed their protein requirements for producing 25 kg of milk consuming exclusively pasture during grazing season due to high levels of CP of the pasture under intensive management (Hafla et al., 2016). Furthermore, cows required additional energy supplementation to produce 25 kg or more milk during the grazing season; however, this study's cows averaged less than 25 kg in the grazing (19.8 kg/d) and non-grazing (18.8 kg/d) conditions.

Milk fat concentration was greater in confined cows compared to grazing cows. Early summer grass is low in fiber and high in non-structural carbohydrates, and it is also rich polyunsaturated fatty acids, which can reduce milk fat likely because of formation of intermediate of incomplete biohydrogenation such as trans 10 (Gottardo et al., 2017; Niero et al., 2021). Furthermore, the reduced fiber content and may impact cow rumination and, consequently, reduce rumen buffering. Also, heat stress may have affected the milk fat content once cows were grazing during late spring, summer, and early fall. Although grazing and confined cows had different milk fat content, milk protein content was similar.

Breed

Holstein cows are recognized as the greatest milk producers among the dairy breeds, while Jersey cows produce higher milk fat and protein concentrations (Palladino et al., 2010). Thus, as expected, Holstein cows had the greatest MY and peak MY followed by Holstein × Jersey crosses and Jersey. Jersey cows reached the peak MY 2 weeks earlier than Holstein (peak at week 5.6) and Holstein × Jersey crosses (peak at week 5.3). Holstein cows had greater milk persistence (80.5%) than Jersey (70.1%) and Holstein × Jersey crosses (71.8%) cows. These results are consistent with those of Schultze and Bright (1983) who found a similar milk persistence for Holstein (81.0%) and Jersey (77.0%) cows in conventional systems.

Milk fat and protein concentrations were greatest for Jersey cows followed by Holstein × Jersey crosses and Holstein. In conventional grazing systems, Palladino et al. (2010) have also demonstrated that Jersey cows had higher milk fat content (5.09%) than Holstein (3.79%) cows, with Holstein × Jersey crosses showing intermediate milk fat content (4.45%). Similarly, Schutz et al. (1990) found greater differences in milk fat than milk protein between Jersey and Holstein cows.

2.6 Conclusions

The present study used individual cow's milk test-day information to evaluate the effects of lactation number, parity, breed, and feeding system (grazing vs. confinement) on milk yield and milk fat and protein concentrations over the lactation in organic dairy herds. The Wood's equation was used to fit an average milk and components curve, and then a second model was built to understand the deviation of each data point from the average curve. Milk yield and milk fat and protein concentrations were affected by all independent variables, except for feeding system that did not affect milk protein concentration. Greatest milk yield was observed for cows in the fourth lactation, with milk yield peaking in winter and spring. In addition, grazing and Holstein cows had greatest milk yield, Milk fat concentration was greater for cows in the second lactation, with milk fat peaking in the fall and winter. Confined cows had greater milk fat concentration than grazing cows, with Jersey cows showing the greatest milk fat concentration. Milk protein concentration was greater for cows in the second lactation, with milk protein peaking in the fall, and Jersey also showing the greatest milk protein content. Cows with low milk SCC had greatest milk yield and lowest nadir for milk fat and protein concentrations. In addition, this study's findings can be used as a strategy on the onset of organic dairy systems.

2.7 References

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Table 1. Descriptive statistics from the data sets used from organic herds in the study

Item	Number of information	Percentage (%)
Breed,		
Holstein	6,954	48.8
Jersey	5,308	37.3
Crossbreed (Holstein × Jersey)	1,983	13.9
State,		
Maine	3,353	23.5
New Hampshire	2,843	20.0
New York	2,419	17.0
Pennsylvania	1,856	13.0
Vermont	3,774	26.5
Feeding system,		
Grazing	6,070	42.6
Non-grazing	8,175	57.4
Lactation number,		
1	4,272	36.9
2	2,763	23.9
3	1,840	15.9
4	1,387	12.0
5	912	7.9
6	409	3.5

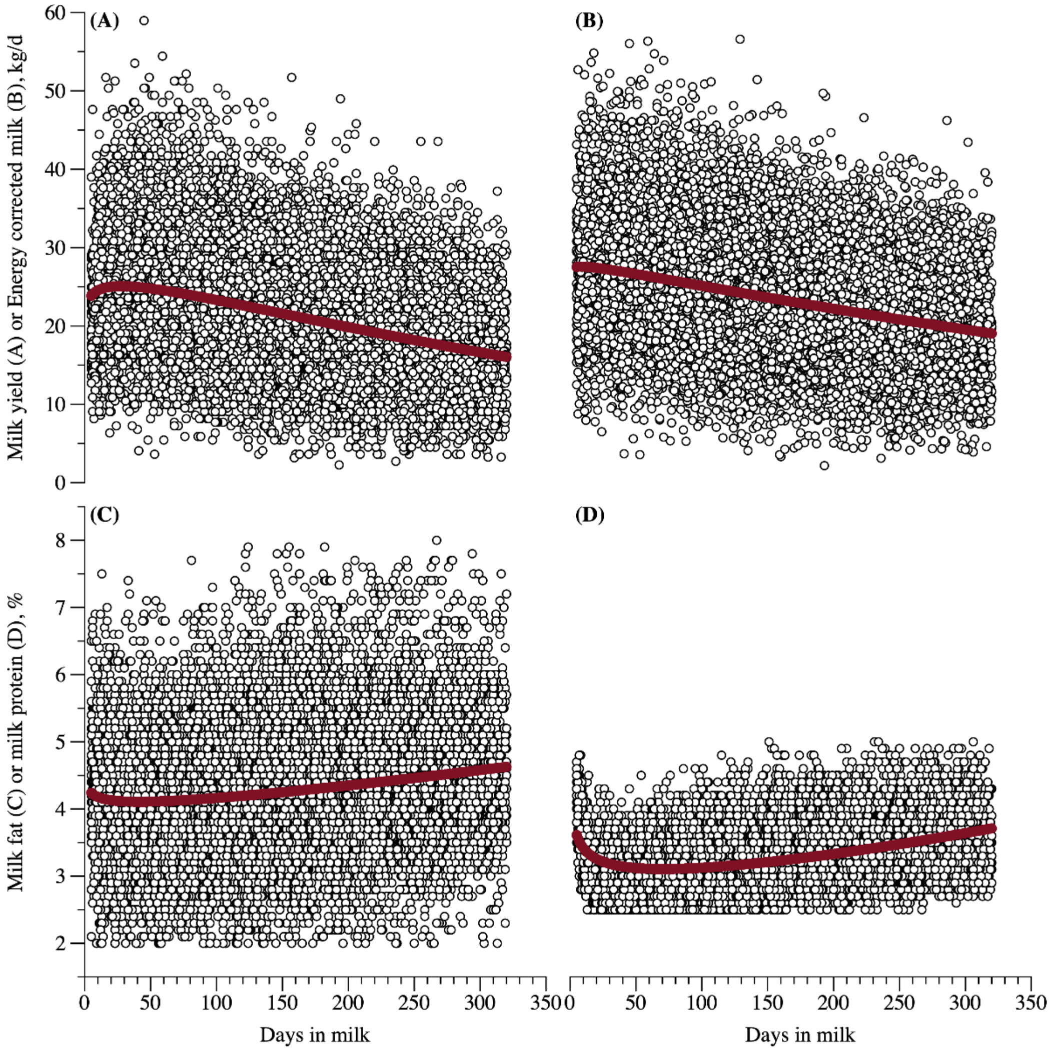

Figure 1. (A) Milk yield (kg/d), (B) energy corrected milk (kg/d), (C) milk fat concentration, and (D) milk protein concentration estimated using the equation $Y_t = a \times DIM^b \times exp^{-c \times DIM}$ Wood (1967) model.

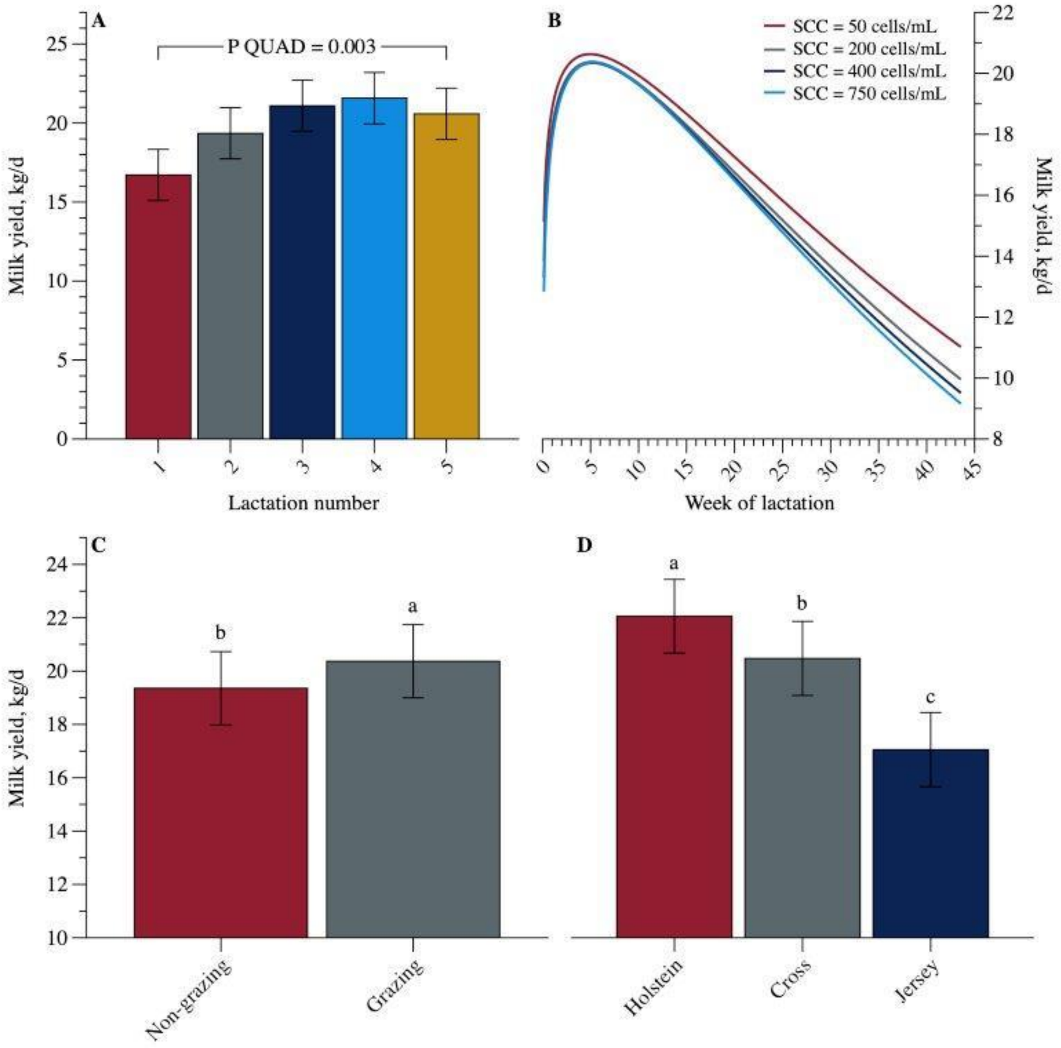

Figure 2. Effects of (A) lactation number, (B) somatic cells count (cells/mL), (C) feeding system, and (D) breed on milk yield (kg/d) estimations.

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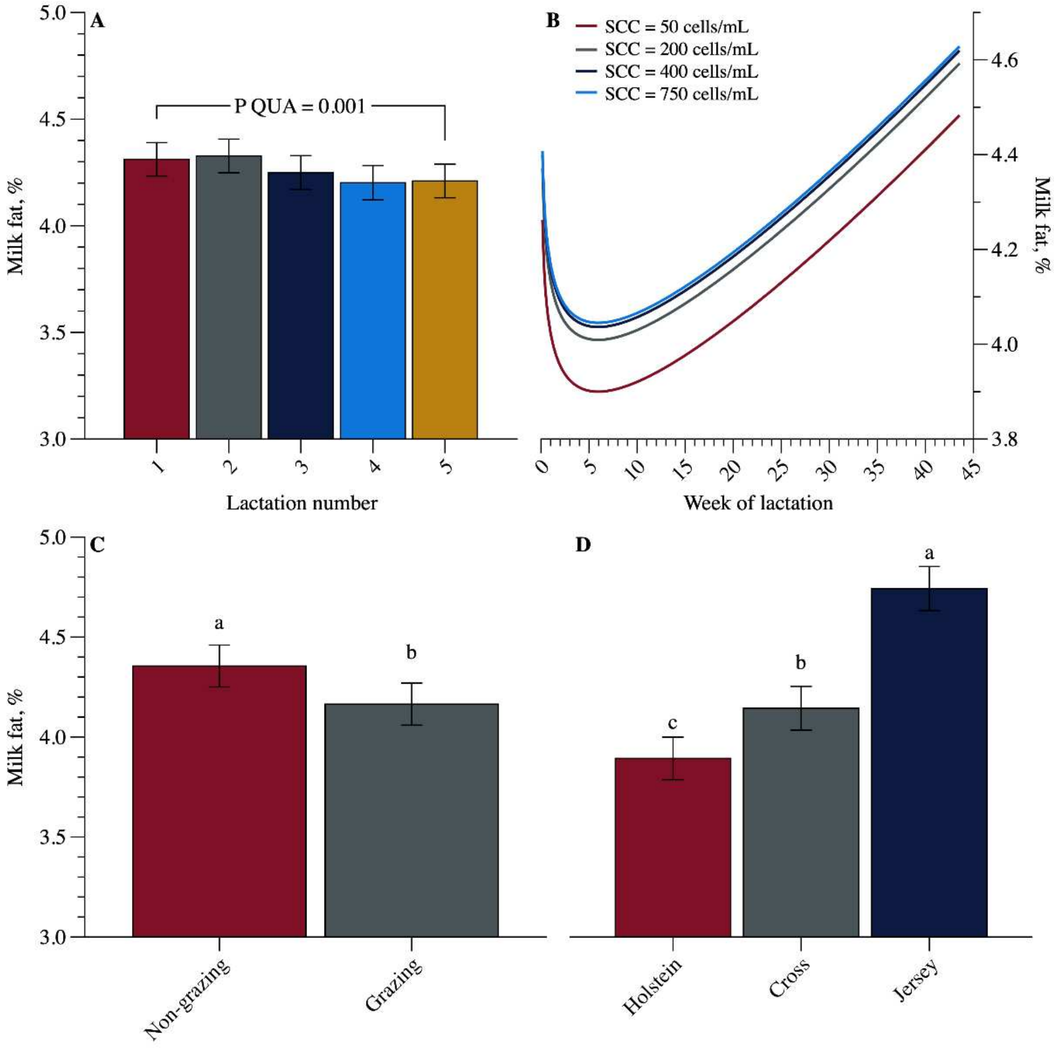

Figure 3. Effects of (A) lactation number, (B) somatic cells count (cells/mL), (C) season, (D) feeding system, and (E) breed on milk fat concentration (%) estimations.

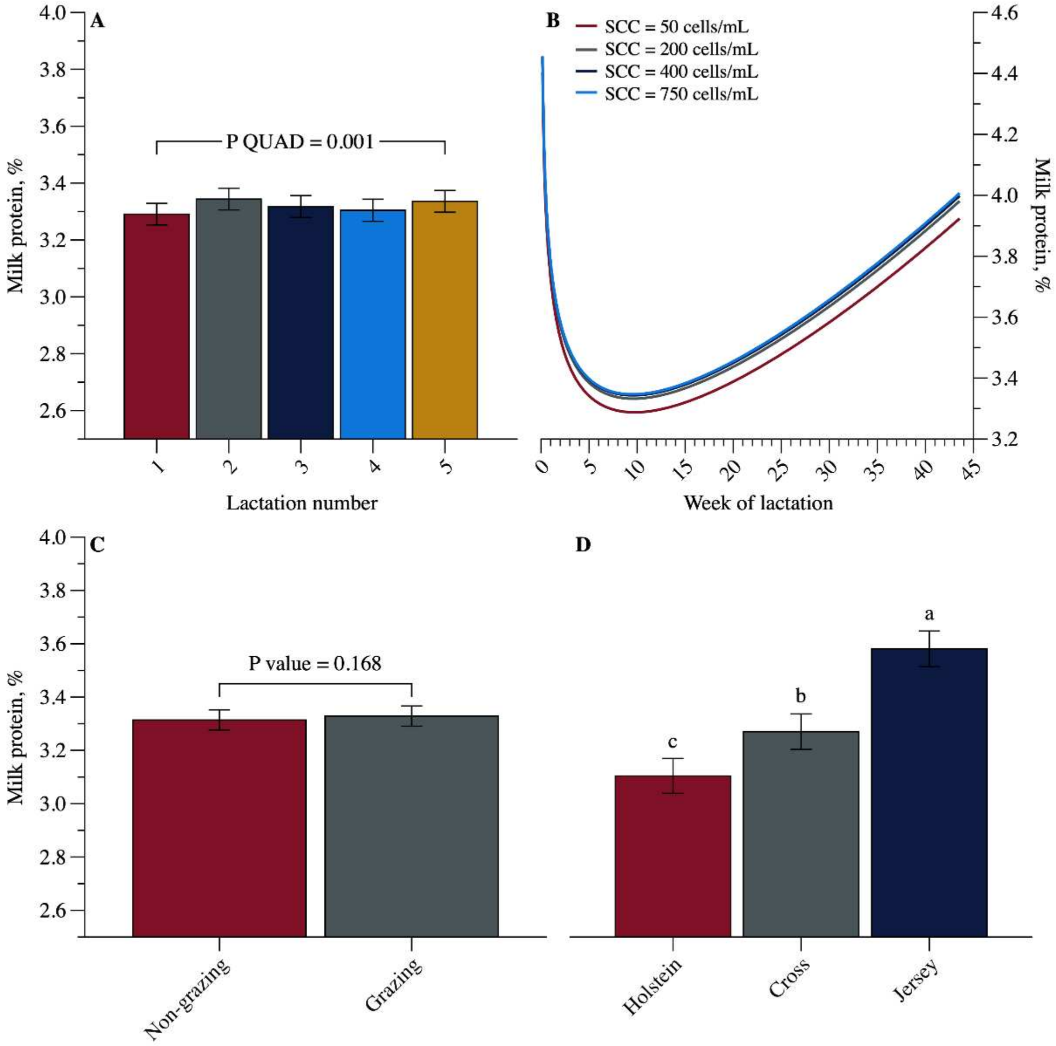

Figure 4. Effects of (A) lactation number, (B) somatic cells count (cells/mL), (C) season, (D) feeding system, and (E) breed on milk protein concentration (%) estimations.

3 ARTICLE 2

Running head: Rumen-protected amino acids supplementation in mid-lactation cows

Milk yield and mastitis risk after supplementing mid-lactation multiparous Holstein cows fed high by-product low-forage diets with rumen-protected Methionine and Lysine in a California commercial dairy

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3.2 Abstract

The objective of the present study was to evaluate the effects of supplementing Met and Lys as rumen-protected AA on milk yield and composition as well as on mammary gland health of mid-lactating Holstein cows from a commercial dairy feeding a low-forage diet. A total of 314 multiparous cows were randomly assigned to control [**CON**; 107 g of dry distillers grains (DDG)] or rumen-protected Met and Lys (**RPML**; 107 g DDG + 107 g of RPML). Throughout 42 d of study, all cows were grouped in a single dry lot pen and fed the same TMR twice daily. Treatments were top-dressed on the TMR immediately after morning delivery. Blood samples were taken from a subset of 24 cows per treatment for plasma AA, and 22 cows for plasma minerals and blood urea nitrogen (BUN) determination, respectively. Milk yield and clinical mastitis cases were recorded daily. Milk components from a.m. and p.m. milkings were determined from samples collected at d 0, 14, 28, and 42 of the study. Body condition score (BCS) was evaluated at d 0 and 42 of the study. Milk yield and components were analyzed by multiple linear regression. Treatments effects were evaluated at the cow-level considering milk yield and composition taken at baseline (1 wk before the experiment) as a covariate in the models. Clinical mastitis risk was assessed by Poisson regression. Plasma Met increased (26.9 vs. 36.0 µmol/L), and Lys tended to increase (102.5 vs. 121.1 µmol/L) with RPML supplementation. In addition, when reported as the percentage of essential amino acid (%EAA), cows supplemented with RPML increased plasma Lys compared to CON (7.9 vs. 9.0 %). Cows supplemented with RPML had higher milk yield (45.4 vs. 46.0 kg/d); however, milk components yield and concentration were not affected by RPML supplementation. Although somatic cell count was not affected by RPML supplementation, the risk of clinical mastitis was 0.39 times lower for RPML than

CON cows (95% CI: 0.17-0.90). Plasma Ca concentration was higher in RPML cows compared to CON (2.39 vs. 2.46 mmol/L); but other plasma minerals and BUN were non affected by RPML supplementation. No changes in BCS associated to RPML supplementation were observed. Results showed that RPML supplementation increasead milk yield and decreased the risk of clinical mastitis in mid-lactation cows. Further studies are needed to clarify the biological mechanisms for mammary gland responses to RPML supplementation.

Keywords: Rumen protected amino acid, lysine, methionine, mastitis

3.3 Introduction

In the mid-1970s, researchers identified Lys and Met as the first limiting AA for milk yield and milk protein secretion in lactating dairy cows (Schwab et al., 1976). Subsequent studies confirmed that both AA could be co-limiting depending on the source of protein and its quality (Rulquin, 1987). For dairy cows, the primary sources of AA for intestinal absorption are microbial protein and RUP. The AA profile of microbial protein resembles that of casein, the most abundant protein in milk (Schwab et al., 1976). However, the AA profile of the RUP fraction of most commonly used protein sources presents various degrees of Lys or Met deficiencies relative to casein (Santos et al., 1998). Furthermore, as milk yield increases, there is a large demand for RUP sources to meet EAA needs (NRC, 2001b); thus, most high-producing dairy cow diets would be inherently deficient in 1 or both of these EAA.

Over the last 4 decades, studies have been designed to understand the implications of supplementing these 2 main limiting AA in a rumen-protected (**RP**) form. For instance, cows fed MP deficient diets supplemented with RP-Met and RP-Lys had an increase in milk and protein yield (Wu et al., 1997; Socha et al., 2005; Wang et al., 2010) and milk protein concentration (Socha et al., 2005; Watanabe et al., 2006; Lee et al., 2019). Similarly, cows fed rumen-protected AA (**RPAA**) to meet the requirements also had positive responses on milk protein and fat yield and concentration, even when diets were estimated to supply adequate MP (Weiss, 2019; Swanepoel et al., 2020). Furthermore, balancing dairy cow diets to meet Lys and Met requirements through RPAA supplementation has been shown to improve the immune response (Osorio et al., 2013; Zhou et al., 2016; Batistel et al., 2018), reproductive efficiency (Toledo et al., 2017), and mammary gland health (Lee et al., 2019).

Most lactating dairy cow diets in California are formulated to include locally available by-products that can replace forage. Typical by-products used include almond hulls, whole cottonseed, dry distillers grains, canola pellets, and corn gluten feed (Heguy, 2021). This allows the formulation of more cost-effective diets, but it may compromise the AA supply (St-Pierre and Weiss, 2015). Evaluation of RP-Met and RP-Lys supplementation has often involved typical corn-based diets, but few efforts have been made to study AA balancing with RPAA in high by-product low-forage diets (Swanepoel et al., 2020). In 2018, California adopted the Federal Milk Marketing Order (USDA, 2018), which is based on milk component pricing. Thus, there is a great interest in evaluating feeding strategies that may improve milk yield and components cost-effectively. In this context, RPAA could be a viable strategy. We hypothesized that balancing high by-product low-forage diets to meet Met and Lys requirements using RPAA would increase plasma Met and Lys concentrations and positively affect performance and udder health. Our objective was to evaluate the effects of supplementing rumen-protected Lys and Met (**RPML**) on milk yield and composition as well as on mammary gland health of mid-lactation Holstein cows from a commercial dairy consuming a high by-product low-forage diet.

3.4 Materials and methods

All procedures in this study were approved by the Institutional Care and Use Committee at the University of California (protocol #22025).

Study Herd Management

The study was conducted on a commercial dry-lot Holstein herd in California from March to April 2020. The dairy was identified with the collaboration of a local nutritional consultant and selected based on their diligent feeding management practices, electronic daily milk yield records, and willingness to participate in the study. The farm milked 4,800 cows twice a day (at 0400 and 1400 h) in 2 double 40-stall parallel milking parlors. The herd had a 305-d mature equivalent milk of 11,200 kg/cow.

Study Design

A minimum of 122 mid-lactation cows per treatment was deemed necessary to detect a 1.4 kg milk yield difference at a 5% significance level and 80% power, with a standard deviation of 5.5 kg (Osorio et al., 2013; POWER procedure of SAS, Version 9.4, SAS Institute Inc., Cary, NC). Thus, a total of 314 multiparous Holstein cows were enrolled, allowing 30% additional cows per treatment to account for potential losses due to health issues and culling. All cows were housed in the same pen.

Study cows were randomly assigned to 1 of 2 treatments: control [**CON**; 107 g of dry distillers grain (DDG); n = 152] or rumen-protected Lys and Met (RPML; 107 g of DDG plus 107 g of RPML; n = 162) using a random number generator (rand function; Microsoft Excel, 2010, Microsoft Corp., Redmond, WA). The RPML treatment was a total of 214 g DM, whereas the CON was 107 g DM top-dressed on the same RMT. The RPML treatment included 104.8 g of Smartamine ML (44% L-Lys; 15% D-Met) and 2.2 g of Smartamine M (75% D-Met; Adisseo USA Inc., Alpharetta, GA) plus 107 g of DDG as a carrier.

Two months prior to study implementation, individual feedstuffs were sampled and analyzed to determine the nutrient composition and AA profile before designing the RPML supplementation strategy [wet chemistry: Cumberland Valley Analytical Services (Hagerstown, MD); AA with HPLC: Missouri Columbia Agricultural Experiment Station Chemical Laboratory (Columbia, MO); Supplemental Table S1].

Based on previous research by Whitehouse et al. (2017), the bioavailability of Met and Lys was 80%; thus, the RPML treatment was formulated to increase the metabolizable Lys (mLys) and Met (mMet) from 5.9% and 2.2% of MP to 7.0% and 2.6% of MP, respectively. Every other day, treatments were prepared off-site by weighing equal amounts of RPAA and DDG and mixing them for 1 min using a 1.44 m^3 wheelbarrow mixer (10N694, Westward Model 10N694). The RPML and CON treatments were stored in plastic buckets with lids and transported to the farm. The lack of RPAA damage during the mixing process was confirmed by a proper analysis at Adisseo (Commentry, France).

One week before starting the RPML supplementation, study cows were moved to a single dry-lot pen equipped with individual headlocks at the feed bunk and offered the to top-dressing treatments by receiving 107 g of DDG while locked at the feedbunk. During the study period, treatments were individually top-dressed immediately after the first-morning feed delivery as described by Toledo et al. (2017). Cows remained locked at the feedbunk for 10 min after top-dressing treatments, and researchers visually inspected the feedbunk for treatment leftovers and removed them prior to releasing the cows. No leftovers of the top-dressed supplement were observed, showing that cows consumed the supplemnts entirely. All cows were fed the same basal diet twice a day as TMR at 0600 and 1000h (Table 1). The basal diet was formulated by the dairy farm nutritionist using the NDS Professional ration formulation software (version 6.55; RUM&N, NDS Professional, Reggio Nell’Emilia, Emila-Romagna, Italy).

Data and Sample Collection and Laboratory Analyses

Blood Sampling and Analyses. Blood samples were collected from a randomly selected subset of 24 cows per treatment (rand function; Microsoft Excel, 2010, Microsoft Corp., Redmond, WA). Samples were collected 10 h after top-dressing treatment on d 0 and 14 to determine plasma AA and on d 0, 14, and 42 to determine plasma BUN and minerals (Ca, P, Mg, Na, K, Fe, Cu, and Zn). Blood samples were withdrawn into 10 mL evacuated tubes containing EDTA (Vacutainer, Becton Dickson, Franklin Lakes, NJ) via puncture of the coccygeal blood vessels. Samples were immediately placed into a portable refrigerator set at 4ºC and transported to the laboratory within 3 h after collection. At the lab, samples were centrifuged to collect plasma (1,900 × *g* at 4ºC for 20 min), split into 3 aliquots, and stored at -80ºC for AA analysis and -20ºC for BUN and mineral analyses. At the study completion, free plasma AA concentrations were determined using ion-exchange HPLC (Agriculture Experiment Station Chemical Laboratory, Columbia, MO). Plasma BUN was determined by a colorimetric method (Vitros 4600 Chemistry Analyzer; Iowa State University Laboratory, Ames, IA). Total plasma Ca, P, Mg, Na, K, Fe, Cu, and Zn concentrations were determined by Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES; Melton et al., 1990; California Animal Health & Food Safety Laboratory, University of California Davis, CA). The intra- and inter-assay coefficients of variation for plasma BUN were 2.4 and 3.8%, and for plasma total minerals were 2.0 and 4.4% for Ca, 2.2 and 3.7% for P, 2.3 and 3.5% for Mg, 2.2 and 2.2% for K, 2.2 and 2.3% for Na, 6 and 6.2% for Fe, 2.8 and 5.7% for Zn, 2 and 5.2% for Cu, respectively.

Milk Yield, Milk Composition, and BCS. Daily milk yield records were obtained from the herd management software (DairyComp305; Valley Agricultural Software, Tulare, CA). Individual milk samples (a.m. and p.m. milkings) were collected at d 0, 14, 28, and 42 into 60 mL tubes containing a 2-bromo-2-nitropropane-1,3 diol as a preservative (Capitol Vial Dairy Industry & Across Organic, Thermo Scientific^{TM}). Milk fat, true protein, lactose, total solids, MUN, and SCC were determined from those samples using an Fourier Transform Spectrometer/Flow Cytometer Infrared Analyzer (Bentley Instruments, Chaska, MN) by the Dairy Herd Improvement Association (Tulare, CA). Clinical mastitis cases were recorded by farm personnel whenever any clots in the milk strip test at any a.m. or p.m. milking. Following herd management protocols, cows with clinical mastitis were transferred to a hospital pen to receive antibiotic treatment and consequently removed from the study. The ECM and 3.5% fat corrected milk (FCM) yields were calculated according to Tyrrell and Reid (1965) and NRC (2001) as follows: ECM (kg/d) = [(0.323 × milk yield) + (12.95 × fat yield) + (7.20 × protein yield)] and 3.5% FCM (kg/d) = [(0.432 × milk yield) + (16.218 × milk fat yield)]. One trained researcher scored all cows at d 0 and 42 of the study to determine BCS, using a 5 points scale with 0.25 increments (Ferguson et al., 1994).

Feedstuffs. Individual feed ingredients were sampled once each week from the herd feed center. Dry matter was determined by drying at 60°C for 48 h in a forced-air ventilated oven (#1685; Sheldon Manufacturing, Ind, Cornelius, OR). Before analysis, all samples were ground to pass through a 1-mm screen (Wiley Mill; Thomas Scientific, Philadelphia, PA), composited in a 6 wk sample, and sent to Cumberland Valley Analytical Services (Hagerstown, MD) for analysis of N (990.03; AOAC, 2000), NDF (Van Soest et al., 1991), ADF (973.18; AOAC, 2000), starch (Hall, 2009), crude fat (2003.05; AOAC, 2000), ash (942.05; AOAC, 2000), and minerals (985.01; AOAC, 2000).

Statistical Analyses

All statistical analyses were performed using SAS (version 9.4; SAS Institute Inc., Cary, NC). Before data analysis, daily milk yield data was screened for outliers; observations at ± 5.0 SD from the cow's mean were considered likely to be acquisition errors (i.e. milk weights attributed to the wrong identification number), and thus, not included in the analysis. Normal distribution was evaluated using the UNIVARIATE procedure for all continuous outcomes. Descriptive statistics of baseline information collected a week prior study initiation were generated using the MEAN and FREQ procedures (Supplemental Table S2). Study outcomes include milk yield [calculated as a weekly average of the sum of both morning and afternoon daily milk weights (kg)]. Daily weighed means for milk components were calculated and used for data analysis. ECM, 3.5% FCM, and milk fat, protein, and lactose yields were calculated by multiplying milk components by averaged daily milk yield (7 d average prior to each test day). Other outcomes evaluated were MUN, SCC, plasma BUN, plasma minerals, plasma AA, BCS, and risk of clinical mastitis.

Milk yield, ECM, 3.5% FCM, milk protein yield and concentration, milk fat yield and concentration, MUN, milk SCC, plasma BUN, and plasma minerals) were analyzed as repeated measures using the MIXED procedure of SAS. The Kenward-Roger method was used to estimate the denominator degrees of freedom. Week was included in the REPEATED statement with cow within treatment as the subject of the statement and the variance-covariance structure with the best fit for each variable selected based on the lowest Akaike's information criterion. Baseline parameters and DIM were tested as a covariate and kept in the model when significant ($P \leq 0.05$). Parity was considered in the first model (2nd,3rd, and ≥4th); however, it did not reach significance in any model and was removed. All final models included the fixed effect of treatment, week of study, and their 2-way interaction. Plasma AA and BCS, were analyzed as described above without the effects of week and their interaction. Before analysis, SCC data were transformed using the LOG10 function; results are reported as Log_{10} SCC. All results are presented as LSM. Homoscedasticity and independence of the errors' assumption were assessed by examining model residuals plots.

The risk of clinical mastitis was analyzed by Poisson regression using the GENMOD procedure as proposed by Ospina et al. (2012). The model included the fixed effect of treatment, and parity was included as a categorical covariate (2nd,3rd, and ≥4th); however, it did not reach significance and was removed. The log link function and the LSMEANS statement with the exp option were used to obtain the treatment risk ratio (RR) and its Wald 95% CI. The RR represents the ratio of the probability of clinical mastitis for RPLM cows to the probability for CON cows. Overall model fit was assessed with the goodness-of-fit chi-squared test. Significance was declared at $P \leq 0.05$, and trend at $0.05 < P \leq 0.10$. Accompanying figures were built using GraphPad Prism software (version 9.1.0; GraphPad, San Diego, CA).

3.5 Results

A description of the 314 cows enrolled in the study is provided in Supplemental Table S2. The DIM and parity for the subset of cows selected for blood analysis were as follows: CON 125 ± 38 DIM (average ± SD) and parity 2nd (54%), 3rd (17%), and 4th (29%), and RPML 145 ± 43 DIM and parity 2nd (54%), 3rd (33%), and 4th (13%). Following herd management protocols, 47 cows were subject to early removal from the experimental pen (Supplemental Table S3). On average, those cows left the study at 24 ± 11 d after enrollment, and their milk yield and milk components data were kept and included in the analysis up to the removal date.

Plasma AA

Greater plasma concentration of Met (36.0 vs. 26.9 µmol/L; *P* < 0.001), Phe (61.3 vs. 57.0 µmol/L; *P* = 0.04), and Tau (73.4 vs. 65.7 µmol/L; *P* = 0.02) was observed for RPML compared to CON cows. Similarly, RPML cows tended to have higher plasma Lys (121.1 vs. 106.8 µmol/L; *P* = 0.07) compared to CON cows (Table 2). When expressed as percentage of EAA and total AA (**TAA**), RPML cows had higher Met [EAA: 2.7 vs. 2.0 % (*P* < 0.001); TAA: 1.5 vs. 1.1 % (*P* < 0.001)] and Lys [EAA: 9.0 vs. 7.9 % (*P* = 0.01); TAA: 4.8 vs. 4.2 % (*P* < 0.001)] compared to CON cows (Figure 1; Table 2).

Milk Yield, Milk Components, ECM, and FCM

Milk yield, milk components yield and concentration, and ECM and FCM yeilds are described in Table 3. Overall, RPML cows had a slightly higher milk yield compared to CON cows (46.0 vs. 45.4 kg/d; *P* = 0.05; Figure 2). There were no statistically significant treatment effects for milk components (Table 3).

Significant time effects were observed for all the evaluated outcomes (Table 3). Milk yield, ECM, 3.5% FCM, milk fat yield, milk protein yield, and lactose yield decreased with time (*P* < 0.001), while milk fat, protein, and lactose concentration increased with time during the study (*P* < 0.001). No interactions of treatment and time were observed for milk yield or component yield or concentration (Table 3).

Clinical Mastitis Risk, SCC, and BCS

Clinical mastitis was observed on both CON (12.5%; 19/152) and RPML (4.9%; 8/162) cows but the risk of clinical mastitis was 0.39 times lower for RPML cows than for CON cows (RR = 0.39; 95% CI = 0.17 to 0.90; $P = 0.03$). However, no treatment effects were observed for $\log_{10}$SCC (CON: 1.83; RPML: 1.83; $P = 0.92$). Log_{10}SCC increased with time ($P < 0.001$), but no interaction treatment by time was observed ($P = 0.74$; Table 3). The change in BCS was not affected by treatment (CON: -0.04; RPML: -0.04; $P = 0.82$).

Plasma Minerals and BUN

Higher plasma Ca concentration (2.46 vs. 2.39%; $P = 0.03$) was observed for RPML compared to control cows (Table 4); however, no differences were observed in concentrations of other minerals among treatments. Plasma concentration of Ca increased, and K tended to increase while plasma Fe decreased with time ($P < 0.001$; Table 4). There was a trend for a treatment by time interaction for plasma Na concentration; RPML cows had a higher plasma Na than control cows at the 14 d of study ($P = 0.08$; Table 4). Concentrations of BUN were not different between RPML and CON cows (12.6 vs. 12.7 mg/dL; $P = 0.92$).

3.6 Discussion

The recent adoption of the Federal Milk Order by California dairies (USDA, 2018) has led to a growing interest among producers and nutritionists to revise feeding strategies that favor milk components. Balancing diets for AA have been proposed as a strategy to improve dairy cows' performance; however, most studies have been conducted in the Midwest and Northeast of the United States with diets heavy in corn silage. Very little information is available using the unique California diets rich in affordable and locally sourced by-products that partially replace traditional forages. Therefore, this study was designed to evaluate the effects on production performance and udder health of increasing Lys and Met supply with RPAA in mid-lactation multiparous cows fed a high by-product and low-forage diet.

Plasma AA

In this study, the RPML diet was estimated to supply 21.3 g/d of Met and 1.4 g/d of Lys above requirements. However, CON diets were deficient in Met and Lys (–9.4 g/d of Met and –32.3 g/d of Lys requirements). Therefore, the differences in the supply of Met and Lys were reflected in the plasma AA profile. Plasma Met and Lys were 33% and 13% higher, respectively, in RPML than CON cows. These results suggest that top-dressing is a viable practice to administer RPAA. Accordingly, prior studies reported increases in plasma Met ranging from 19 to 79% after top-dressing RP-Met to mid-lactation cow diets (Piepenbrink et al., 1996; Lee et al., 2012; Toledo et al., 2017). Also, top-dressing RP-Lys to mid-lactating cows for 70 to 140 d increased plasma Lys from 2.7 to 19.5% (average 9.6%); however, this increase was significant in some studies (Christensen et al., 1994; Piepenbrink et al., 1996) and only numerical in others (Lee et al., 2012; Paz and Kononoff, 2014; Weiss, 2019). According to a meta-analysis, plasma Met concentration is linearly associated with duodenal Met supply from RPAA or infused sources (Patton et al., 2015). However, this linear relationship does not apply to Lys, as the mammary gland can uptake more Lys than is required for milk yield and protein synthesis (Patton et al., 2015; Martineau et al., 2017). Other plausible explanations for the lesser increase in plasma Lys could be associated with Lys lower pos-ruminal absorption or its greater demands for milk yield or other biological functions (Lapierre et al., 2009; Lapierre et al., 2012). Alternatively, the ruminal escape of the RPAA used in this study could be less than expected. Another factor that should be considered is sampling time, as it may have affected our results. Blood samples for plasma AA analyses were collected at approximately 10 h after top-dressing treatments; this sampling time was selected to capture the post-supplementation peak of plasma Met concentration, as reported by Toledo et al. (2017). However, it is unclear if there is an optimum time to detect a plasma Lys concentration peak in top-dressed cows.

Milk yield, Milk Components, ECM, and FCM

In this study, milk components and ECM were not affected by treatment; however, a slight increase in milk yield was observed for cows assigned to RPML. This modest increase in yield was not evident 1 week after treatment cessation [wk 7: CON (42.7 kg/d); RPML (42.8 kg/d)], suggesting an association between treatment administration and milk yield. Recent evidence from a meta-analysis showed a positive association between the supply of mMet (g/d) and milk and milk protein yield (Lean et al., 2018); nevertheless, the aforementioned study failed to uncover an association between production outcomes and mLys (g/d) supply. Furthermore, a meta-analysis and a systematic review conducted over a decade ago provided evidence of positive associations between milk and milk protein yield and RP-Met and RP-Met/Lys supplementation (Patton, 2010; Robinson, 2010).

Most studies on RPAA supplementation have been conducted on corn silage-based diets typical of the Midwest US. However, some studies have evaluated the effects of RP-Met or RP-Lys supplementation under California management conditions at different lactation stages. Supplementing early and mid-lactation cows with RP-Lys resulted in no treatment effects on milk or milk protein yield and negative effects on milk fat yield (Swanepoel et al., 2010). However, early lactation cows supplemented RP-Met showed a modest increase in milk protein and fat contents but a negative effect on milk yield (Swanepoel et al., 2015). More recently, Swanepoel et al. (2020) observed increased milk yield and milk protein after supplementing RP-Met to fresh and mid-lactating cows. Milk fat concentration was also higher for cows supplemented RP-Met but only during early lactation (Swanepoel et al., 2020). Similar to our study, previous Californian studies were high in by-products and low in forage. The main protein sources were canola meal (pellets solvent), dried distillers grain, and whole cottonseed. The variable production response across studies after RPAA supplementation on commercial California farms suggests that more efforts are needed to identify cost-effective strategies to ensure an adequate supply of EAA in dairy cow diets.

The lack of response in milk components and the minor increase in milk yield observed in our study after RPLM supplementation might be explained by one or multiple reasons. First, lower dietary deficiencies of Met and Lys in the control diet than initially predicted. Second, other dietary nutrients (i.e., EAA, energy) may be co-limiting milk yield and milk components synthesis, as suggested in Armentano et al. (1993) studies. Third, Met and Lys could have been diverted for physiological functions other than milk protein yield, such as the immune response. During our study, cows were exposed to unusually rainy weather, and the muddy pens increased the risk of mastitis. Fourth, in our study, all cows with clinical mastitis were removed earlier due to farm management. Clinical mastitis is negatively associated with milk yield and components (Hortet and Seegers, 1998), and in this study, the incidence of clinical mastitis was greater in CON group. Lastly, early-lactating cows appear to have greater milk yield and components after RPAA supplementation than mid-lactation animals as the ones used in this trial (Socha et al., 2005).

Plasma Minerals

There is limited literature evaluating the association between AA supply and plasma mineral concentration in dairy cows. A study in grazing cows showed higher concentrations of plasma Mg after supplementation of RP-Met and RP-Lys, but no effects on Ca were reported (Younge et al., 2000). One plausible explanation for the higher plasma Ca concentration for RPML could be an improvement in Ca absorption explained by the role of Lys in intestinal Ca absorption (Lee et al., 1983). For instance, in human patients with osteoporosis, oral Ca and Lys supplementation decrease urinary Ca excretion compared to patients supplemented only with oral Ca (Civitelli et al., 1992). In addition, previous studies have observed an association between lower blood Ca concentrations and clinical mastitis (El Zubeir et al., 2005; Yildiz and Kaygusuzoğlu, 2005). Thus, changes in Ca could potentially be explained by an indirect effect of RPAA supplementation on udder health, as explained in the next section. Future research is needed to clarify the effects of RPML supplementation on Ca absorption and retention, as well as its implication on cow health and performance.

Mastitis risk, SCC (Udder Health)

Few studies have evaluated the effects of RPAA supplementation on mammary gland health (Lee et al., 2019); to our knowledge, our study is the first to associate RPAA supplementation with the risk of mastitis. There is evidence that EAA are critical to the immune and inflammatory response. For example, Dai et al. (2020) observed that the supply of Met and Arg alleviated the pro-inflammatory responses of lipopolysaccharide-challenged bovine mammary epithelial cells. Similarly, dairy sheep supplemented with RP-Lys or RP-Met had down-regulated genes related to pro-inflammatory signaling (Tsiplakou et al., 2020). In addition, increased phagocytosis and oxidative functions of peripartum cows were observed after supplementing RP-Met (Zhou et al., 2016). Thus, it is plausible that a lower risk of mastitis for RPML cows may be associated with Met and Lys functions in the innate immune system response.

Additionally, higher plasma Ca concentration was observed on RPML cows, which may also favor cows' immune response. Plasma Ca concentration has been associated with innate immune response in peripartum dairy cows (Ducusin et al., 2003; Kimura et al., 2006; Martinez et al., 2014). Also, low blood Ca concentration at peripartum has been associated with delayed teat canal closure after milking (Barragan et al., 2018), higher SCS (Valldecabres and Silva-del-Río, 2021), and increased risk of clinical mastitis (Curtis et al., 1985; Hossein-Zadeh and Ardalan, 2011).

Supplementing cows with RPAA was not associated with milk Log_{10}SCC. Zhou et al. (2016) did not observe changes in SCC despite the increased immune response of cows supplemented with RP-Met. However, cows supplemented with RP-Met and RP-Lys during the first 22 days of lactation had lower SCC than unsupplemented cows (Lee et al., 2019). The lack of treatment effects on SCC in the present study could be partially explained by the study design; the sampling protocol for SCC determination may not have allowed for the detection of the increase in milk SCC associated with clinical mastitis, as cows with clinical mastitis left the study to receive antimicrobial treatment after the diagnose. The present study was not designed to characterize the role of EAA on udder health, but our results warrant further research.

3.7 Conclusions

Supplementing RP-Met and RP-Lys to mid-lactation Holstein cows reduced the risk of clinical mastitis but did not affect the Log_{10} SCC. Supplemental RPML increased plasma Ca, Met and numerically increased Lys, whereas plasma Lys increased as a proportion of total EAA. Supplementing cows fed a low forage high by-product diet with RPML resulted in a modest but significant increase in milk yield and did not affect the yield and concentration of protein and fat. Further studies should evaluate the effects of RPML supplementation on clinical mastitis and identify the role of Met and Lys in mammary gland health.

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Table 1. Ingredients and chemical composition for the basal diet, and the supply of metabolizable AA to mid-lactation multiparous Holstein cows by treatment.

Item	Basal diet, % of DM
Ingredient	
Alfalfa hay	12.1
Corn silage	7.9
Wheat Silage	3.5
Haylage	3.4
Oat hay	0.9
Rolled corn	18.6
Almond hull	17.7
Canola meal	12.5
Cottonseed whole	6.5
Wet distillers	3.5
Soy hull	4.0
Corn gluten	3.7
RFDGS[1]	2.5
Fat blend[2]	1.2
Mineral-vitamin mix[3]	2.0
Chemical composition[4]	--
DM	58.0
CP	16.9
aNDFom[5]	31.8
Starch	18.6
Sugar	9.6
Crude fat	4.8
Ash	7.8
Ca	0.7
P	0.4
Mg	0.4
K	1.6

	Treatment[6]	
Estimations[7]	CON	RPML
ME allowable milk, kg/d	40.2	40.2
MP allowable milk, kg/d	43.0	44.2
ME Mcal/d, supply	68.9	69.1
ME g/d, balance	-5.9	-6.5
MP g/d, supply	3020.5	3087.7
MP g/d, balance	-100.9	-76.8
Ruminal ammonia, % of requirement	179.5	180.5
Met, g/d	65.4	92.2
Lys, g/d	183.4	221.8

Met g/d, balance	-9.4	21.3
Lys g/d, balance	-32.3	1.4
Met, % MP	2.17	2.99
Lys, % MP	6.07	7.18
Met, g/Mcal ME	0.95	1.33
Lys, g/Mcal ME	2.66	3.21
Lys/Met ratio	2.8:1	2.41:1

[1] Reduced-fat dried distillers grains with solubles (Novameal., Novita, Brookings, SD

[2] Composed of 70.8% Alba Palmfat (Global Agri Resources Private Limited, Edison, NY) and 29.2% of EnerGII (Virtus nutrition, LLC, Corcoran, CA).

[3] Composed of 29.0% calcium carbonate, 26.4% of SQ-180 (Arm & Hammer Animal Nutrition), 11.3% white salt, 9.4% of OmniGen-AF (Phibro Animal Health Corporation),7.5% of PDS L45 TM (vitamin A 265,000 IU/kg, vitamin D_3 52,000 IU/kg, vitamin E 677 IU/kg), 7.5% of urea, 6.4% of Magnesium oxide 54%, 1.2% of Zinpro Avalia 4 (Zinpro Corp., Eden Prairie,MN), 1.1% of vegetal oil.

[4] Chemical analysis (Cumberland Valley Analytical Services; Hagerstown, MD).

[5] Organic matter amylase neutral detergent fiber.

[6] Treatments included: control (CON) = 0 g/d of rumen protected Met and Lys and RPML = 107 g/d of mix of Smartamine ML (97.9%) and Smartamine M (2.1%) top-dressed.

[7] Predicted by CNCPS v 6.55 using the NDS Professional, Reggio Nell'Emilia, Emila-Romagna, Italy, version 6.55; RUM&N.

Table 2. Effects of supplementing mid-lactation multiparous Holstein cows with rumen-protected Met and Lys (RPML) on plasma AA profile after 14 d of supplementation.

	Treatment[1]			
Item	CON	RPML	SE	*P*-value
EAA, mmol/L	--	--	--	--
Arginine	87.7	89.5	3.15	0.71
Histidine	61.8	61.9	1.28	0.97
Isoleucine	163.9	158.9	4.96	0.48
Leucine	277.0	267.5	7.30	0.36
Lysine	106.8	121.1	5.40	0.07
Methionine	26.9	36.0	1.76	<0.01
Phenylalanine	57.0	61.3	1.42	0.04
Threonine	102.5	98.6	3.69	0.46
Tryptophan	36.3	37.3	39.55	0.67
Valine	394.0	382.8	9.10	0.39
Lysine, % EAA	7.9	9.0	0.002	0.01
Methionine, % EAA	2.0	2.7	0.001	<0.01
Lysine, %TAA[2]	4.2	4.8	0.14	<0.01
Methionine, %TAA	1.1	1.5	0.05	<0.01
BCAA[3]	835.2	808.6	20.84	0.37
BCAA/EAA, %	63.82	61.31	0.53	<0.01
Total EAA	1313.9	1314.9	33.80	0.48
NEAA, mmol/L				
Alanine	244.8	243.3	6.98	0.88
Asparagine	49.3	47.3	1.67	0.39
Aspartic acid	9.6	9.6	0.41	0.98
Glutamic acid	45.3	46.4	1.10	0.50
Glutamine	232.4	228.7	5.81	0.65
Glycine	264.7	251.9	8.40	0.28
Proline	98.5	96.6	3.10	0.66
Serine	81.6	78.3	2.19	0.28
Taurine	65.7	73.4	2.64	0.02
Tyrosine	70.9	69.9	2.70	0.79
BCAA/TAA, %	33.9	32.7	0.54	0.12
Total NEAA	1162.8	1145.4	29.29	0.67

[1] Treatments included: control (CON) = 0 g/d of rumen protected Met and Lys) and RPML = 107 g/d of mix of Smartamine ML (97.9%) and Smartamine M (2.1%) top-dressed.

[2] Total AA

[3] Branched-chain AA

Table 3. Effects of rumen-protected Met and Lys (RPML) on productive performance and BCS changes in mid-lactation multiparous Holstein cows assigned to supplementation (RPML, n = 162) or control (CON, n = 152).

	Treatment[1]			*P*-value		
Item	CON	RPML	SE	Treatment	Time	Treatment × Time
Milk yield, kg/d	45.4	46.0	0.23	0.05	<0.01	0.42
ECM, kg/d	48.1	48.4	0.46	0.56	<0.01	0.70
3.5% FCM, kg/d	48.6	49.0	0.49	0.52	<0.01	0.70
Fat	--	--	--	--	--	--
Fat, %	4.00	4.00	0.03	0.92	<0.01	0.32
Fat yield, kg/d[2]	1.79	1.80	0.02	0.63	<0.01	0.48
Protein	--	--	--	--	--	--
Protein, %	3.09	3.11	0.01	0.25	<0.01	0.26
Protein yield, kg/d[2]	1.39	1.40	0.01	0.40	<0.01	0.58
Lactose	--	--	--	--	--	--
Lactose, %	4.82	4.82	0.01	0.98	<0.01	0.27
Lactose, kg/d	2.17	2.20	0.24	0.47	<0.01	0.93
Total solids, %[3]	11.92	11.90	0.56	0.82	<0.01	0.14
MUN, mg/dL	10.6	10.5	0.14	0.57	<0.01	0.48
Log_{10} SCC	1.83	1.83	0.02	0.93	<0.01	0.74

[1] Treatment included:: control = 0 g/d of rumen protected Met and Lys and RPML = 107 g/d of mix of Smartamine ML (97.9%) and Smartamine M (2.1%) as top-dressed.

[2] Milk component yields were calculated as ponderated weight of milk components (a.m. and p.m.) multiply by daily milk yield (7 d average prior to test day).

[3] Sum of fat, protein, and lactose concentrations per test day.

Table 4. Effects on plasma minerals and BUN of multiparous Holstein cows supplemented with a rumen-protected Methionine and Lysine source (RPML, n = 22; CON, n = 22).

	Treatment[1]			*P*-value[2]		
Item	Control	RPML	SE	Treatment	Time	Treatment × Time
Ca, mmol/L	2.39	2.46	0.02	0.03	<0.01	0.57
P, mmol/L	2.10	2.14	0.04	0.50	0.65	0.54
Mg, mmol/L	1.01	0.99	0.10	0.20	0.74	0.74
Na, mmol/L	136.61	137.50	0.67	0.35	0.14	0.08
K, mmol/L	20.76	20.51	0.41	0.50	0.06	0.64
Fe, µmol/L	26.24	25.07	2.08	0.72	0.05	0.75
Cu, µmol/L	13.67	13.75	0.30	0.80	0.87	0.34
Zn, µmol/L	13.96	13.92	0.36	0.97	0.82	0.74
BUN, mg/dL	12.6	12.7	0.39	0.92	0.17	0.77

[1] Treatments included: control = 0 g/d of rumen protected Met and Lys and RPML = 107 g/d of a mix of Smartamine ML (97.9%) and Smartamine M (2.1%) as top-dressed.

[2] Treatment effect of supplementing RPML (Control vs. RPML); Time = effect of time of supplementing RPML and Treat × Time = interaction between Treatment and time.

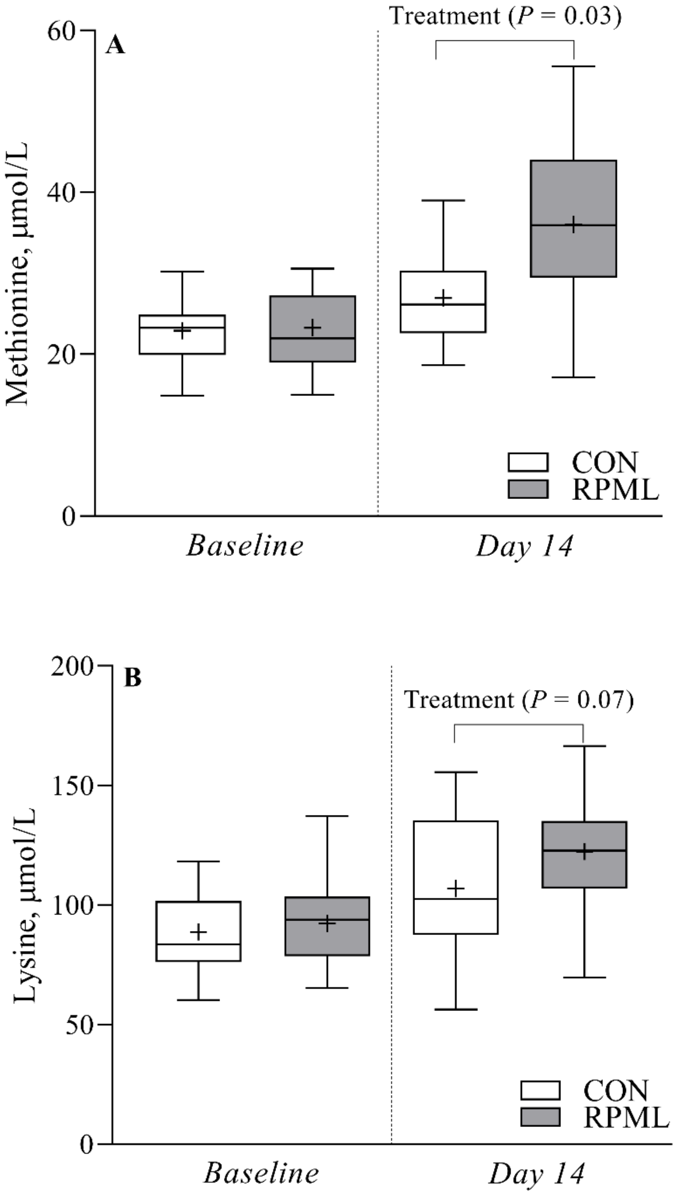

Figure 1. Effect of supplementing multiparous Holstein cows with either 0 g/d of rumen-protected methionine and lysine supplementation (□ CON; n = 24) or a RPML supplementation (■ RPML; n = 24) with 107 g/d of RPML top-dressed on TMR on plasma concentration (µmol/L) of Met (A) and Lys (B) during the baseline period and at 14 d after first receiving RPML. In the box plots, the boundaries of the box closest to and farthest from the horizontal axis indicate the 25th and 75th percentiles, respectively; the line within the box indicates the median; whiskers above and below the box indicate the 10th and 90th percentiles; points above and below the whiskers

indicate outliers outside the 10th and 90th percentiles, respectively; and + represents the mean.

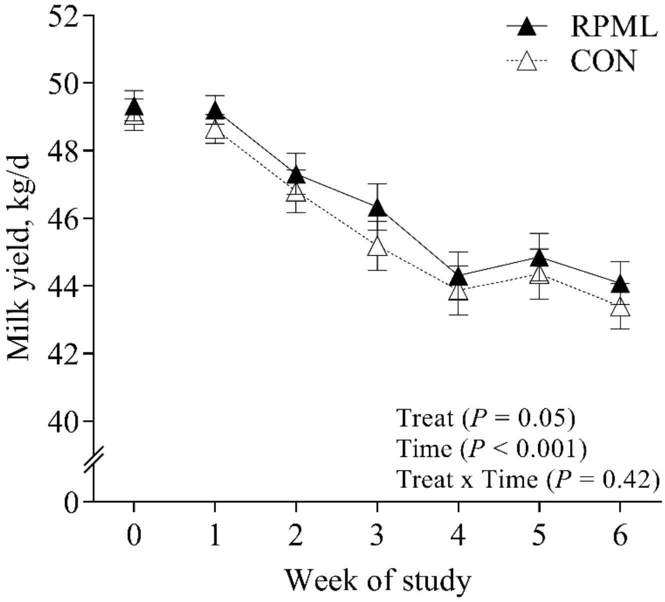

Figure 2. Effect of supplement multiparous Holstein cows with either 0 g/d of RPML (Δ CON, n = 152) or a rumen-protected Met and Lys supplementation (RPML, n = 162) with 107 g/d of Smartamine ML (97.1%) and Smartamine M (2.1%) as top-dressed (▲ RPML) on milk yield during 42 d. Fixed effects in the model were treatment (P = 0.05), time (P < 0.001), and treatment by time interaction (P = 0.42).

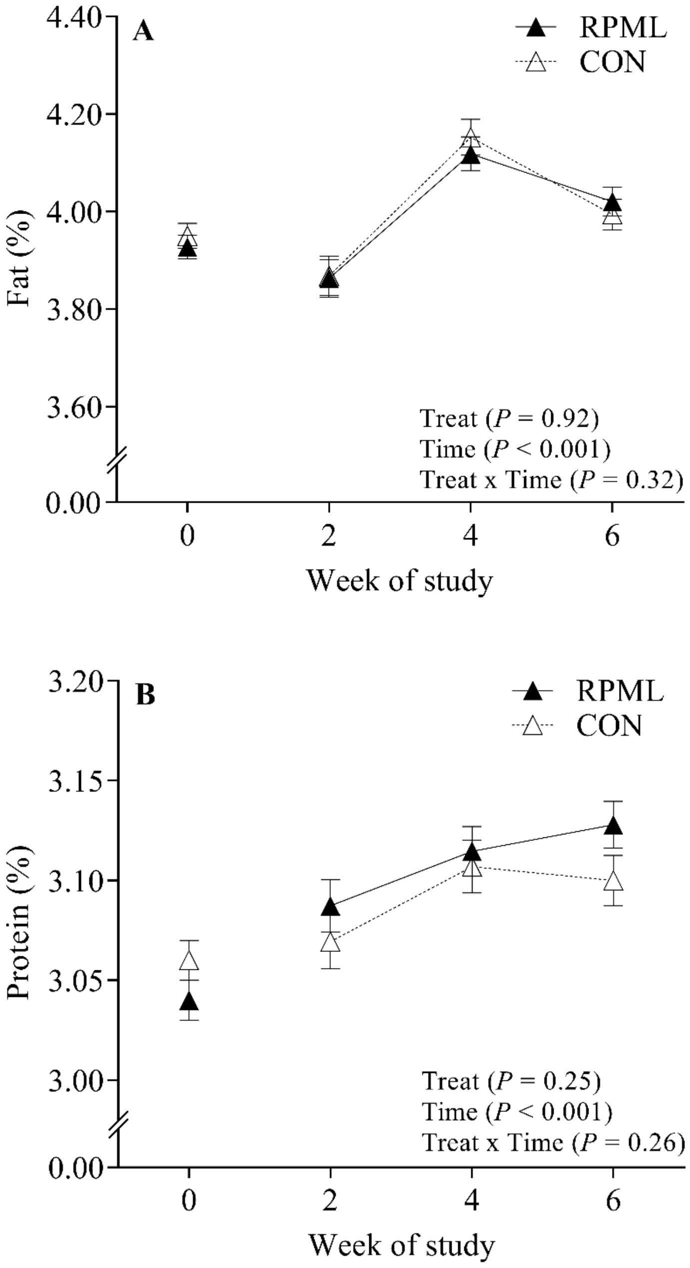

Figure 3. Effect of supplement multiparous Holstein cows with either 0 g/d of RPML (Δ CON) or a rumen-protected methionine and lysine supplementation (RPML) with 107 g/d of Smartamine ML (97.1%) and Smartamine M (2.1%) as top-dressed (▲ RPML) on milk fat (A) and protein (B) percentage over 42 days of study. Milk fat (P = 0.92) and protein (P = 0.25) was not affected by treatment (P > 0.05).

Supplemental Table S1. By-products crude protein and essential amino acid composition.

	Ingredient[1]					
Item	ALM	CAN	CGM	RFDGS	WDG	WCTS
% DM	--	--	--	--	--	--
CP	5.2	41.8	25.3	33.8	31.2	25.7
Met	0.05	0.79	0.40	0.59	0.56	0.37
Lys	0.14	2.19	0.78	0.89	0.96	1.17
Arg	0.12	2.38	1.16	1.38	1.37	2.82
Thr	0.14	1.67	0.91	1.22	1.08	0.79
Leu	0.21	2.84	2.14	3.75	3.22	1.50
Ile	0.12	1.65	0.76	1.34	1.16	0.85
Val	0.18	2.09	1.20	1.66	1.46	1.14
His	0.04	1.07	0.74	0.82	0.76	0.71
Phe	0.15	1.65	0.88	1.64	1.40	1.42
Try	0.05	0.48	0.13	0.26	0.23	0.33

[1] Ingredient: ALM = Almond hull; CAN = Canola meal; CGM = Corn gluten meal; RFDGS = Reduced fat distillers grains with solubles (novameal); WDG = Wet distillers; WCTS = Whole cottonseed.

Supplemental Table S2. Descriptive statistics for continuous (mean ± SD) and categorical (proportions) parameters of 314 multiparous Holstein cows at enrollment for control (CON, n = 152) and rumen-protected methionine and lysine supplementation (RPML, n = 162) cows included in the statistics.

	Treatment[1]		*P* - value
	Control	RPML	
Milk yield	49.1 ± 7.2	49.3 ± 6.6	0.73
DIM	129.8 ± 37.7	131.9 ± 41.0	0.63
Fat (%)	3.95 ± 0.52	3.93 ± 0.50	0.53
Protein (%)	3.06 ± 0.21	3.04 ± 0.21	0.19
Log_{10}SCC	1.73 ± 0.42	1.71 ± 0.42	0.57
Parity, (%)	--	--	0.04
2nd	45.1	55.5	--
3rd	25.4	25.0	--
≥4th	29.5	19.5	--
BCS at enrollment, %	--	--	0.57
≤3.0	16.4	13.9	--
3.0< BCS<3.5	69.6	73.6	--
≥3.5	14.0	12.5	--

[1] Treatments included: control = 0 g/d of of rumen protected Met and Lys and RPML = 107 g/d of mix of Smartamine ML (97.9%) and Smartamine M (2.1%) as top-dressed.

Supplemental Table S3. Reasons for incompliance of study supplementation period for multiparous Holstein cows assigned to control (CON, n = 152) or rumen-protected Met and Lys supplementation (RPML; n = 162) treatments.

	Treatment[1]		
Item	Control	RPML	Total
Enrolled cows	152	162	314
Mastitis	19	8	27
Lameness	2	1	3
Unknown[2]	7	9	16
Sold	1	--	1
Total number of cows removed	29	18	47
Cows completing experiment	123	144	267

[1] Treatments included: control = 0 g/d of rumen protected Met and Lys and RPML= 107 g/d of a mix of Smartamine ML (97.9%) and Smartamine M (2.1%) as top-dressed.

[2] Unknown: no description of the reason for removal was recorded.

4 ARTICLE 3

Running head: Feeding practices management in Brazilian dairies

Survey of feed management practices used in high milk-producing pens in South and Southeast dairies in Brazil

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4.1 Abstract

Objective: This study aimed to gather information on the feeding practices in confined Brazilian dairies and identify their relation with herd milk production.

Material and Methods: Thus, an online survey consisted of 38 questions was mailed to 500 dairy producers. From the 135 responses, 82 responses were utilized. Dairies were categorized according to their 305-d milk production (kg) as low (LP; <7,000; n = 27), medium (MP; 7,000 to 10,000; n = 35), and high production (HP; >10,000; n = 20).

Results and Discussion: The HP and MP herds had a greater risk ratio to have a trough wash protocol compared to LP herds. The HP herds had a greater risk ratio to evaluate TMR physically effective fiber NDF (peNDF) than LP herds. The MP herds had a greater risk ratio to measure feed efficiency, check forage dry matter (DM), and evaluate corn processing compared to LP herds. Any other feed practices had different risk ratio among the 3 groups. The feed refusals and the frequencies of feed eficiency, feedbuk clean-up, and TMR peNDF was similar among herds. The HP producers reported greater frequencies of feed, feed push-up, and washing the water troughts than MP and LP, respectively. Higher frequency of feed composition analysis was reported for most MP, followed by HP and LP, respectively. Regardless of milk production level, herds reported evaluating forage DM monthly and when a new silo was opened. Most MP producers reported evaluating corn kernel process during harvesting in all wagons, while most HP and LP reported evaluating it only in some wagons. In conclusion, this survey demonstrates that HP and MP herds' producers showed greater frequencies of feeding management practices than products of LP herds.

Implications and Applications: Most feeding practices had a similar risk ratio among herds. Furthermore, survey results can be used to develop and disseminate target information on feeding practices and feed bunk mamangement in Brazilian dairies.

Keywords: Brazilian dairies, feed bunk management, feedstuffs evaluation, total mixed ration

4.2 Introduction

Brazilian dairy production has growth substantionaly in the past years, reaching 35,5 billions of litters in 2020. Currently, Brazil ranks fourth in worldwide milk production, which represents a growth of 45% from 2006 to 2017 (FAO, 2020). This improvement is frequently attributed to intensification of genetics selection, cow confort, and management practices as well as by the transition from grazing to confined (Marcondes et al., 2020; Rocha et al., 2020).

Feeding a total mixed ration is a typical and operational form to reach cows requirements in confined system. However, dairy farms face a challenge to ensure that cows are consume the diet on a consist bases and convert it into milk. Its because non nutriotional aspects, as well as diet composition, are related to influence cows consumption and milk performance (Bach et al., 2008; Sova et al., 2014).

Particularly in recent years, researchers have shown a potential increase in milk production and efficiency by implementing suitable TMR and feed management practices (Sova et al., 2013; Schingoethe, 2017). For instance, feeding practices that increase lying time, time at the feed bunk, and decreased sorting, competition, and time standing are frequently related to improved cow production (DeVries et al., 2003, 2005; Crossley et al., 2017). Furthermore, Bach et al. (2008) demonstrated that feeding practices accounted for most of milk production variation among 47 herds feeding identical diet composition, therefore, highlighting the importance of feeding management in milk production.

Understanding the interaction between feeding strategies and animal performance is essential for producers worldwide, particularly those using confined systems. Thus, although the literature have extensively addressed the effects of feeding strategies on milk production of dairy cows (Bewley et al., 2017; Schingoethe, 2017), to the best of our knowledge, no study has summarized the adoption of those strategies in Brazilian dairies. Thus, the objective of this study was to describe the most common feed bunk practices in high milk production pens on Brazilian dairy operations and identify whether any feeding management is associated with herd level milk production (HLMP).

4.3 Materials and Methods

Survey Description

An online cross-section survey was designed to obtain data from Brazilian dairy producers' feeding and feed bunk strategies. The survey was written in Portuguese, and a introductory letter was provided to explain the survey's aim. A total of 500 online surveys were sent to Brazilian dairy producers by email and social media (e.g., Instagram). The producer's mail address was obtained from nutritional consultant contacts. On social media, producers were advertised through their chat page. Survey invitations were directed exclusively to dairy producers that housed high producing cows in confinement systems such as free stall, compost barn, dry lot, and/or tie-stall. Producershave the option to submit their responses anonymously to prevent bias; however, they could include their contact information to receive study results after the research conclusion. Responses were computed from December 2020 to February 2021.

The survey consisted of 38 multiple-choice questions with no enforced response and a single selection option. The questions were divided into 4 sections: (1) *general herd information,* (2) *TMR preparation and feedstuff evaluation,* (3) *feed bunk practices, and* (4) *high production cows management*. All questions were related to the management of cows housed in the highest milk production pen (Supplementary Table 1).

Questions were added into online questionnaire software (Qualtrics, Provo, UT), and personalized links were created to avoid the risk of double responses. Before sending to producers, a pilot survey was applied to 3 dairy producers and 4 nutritionists, and feedback on questions and content organization was obtained. Further, the time to completethe survey questionare was estimated between 10 and 15 minutes. The study questionnaire was reviewed and approved by the human research ethics committee of the Federal University of Viçosa, Viçosa, Minas Gerais, Brazil.

The housing systems were characterized as free-stall, compost bedded pack, or dry lots. The complete description of the housing systems was not accessed in the survey; however, a description of dairies in this same region was reported by Marcondes et al. (2020). Briefly, in free-stall, cows were managed collectively, and cows have individual bedding made of sand or mattresses, with a long alley. In compost bedded packs, cows had continued access to collective bedding (average of 15 m^2 per animal) composed of shavings or coffee hulls, and fans distributed above the bedding. In open dry-lots, cows were fed in unshaded concrete feeding areas and managed in large areas (55 m^2 per animal), covered by little vegetation, with a shading area of at least 4.00 m^2 per animal. Dairy farms' most common forage sources were corn silage and Tifton hay and ground corn, soybean, whole cottonseed, and citrus pulp as concentrate ingredients. It is worth mentioning that all dairy farms fed TMR. Herds were predominantly composited by a single breed, and 2 herds had more than 1 breed. In these herds, Girolando breed made more than 85% of the number of cows; therefore, these herds were categorized as Girolando herds.

Experimental Design and Statiscal Analysis

Responses from survey software were converted into Microsoft Excel® for data management. Answers were reviewed for typographical errors, coherence, and completeness. Surveys received in blank were excluded, and inconclusive responses were not included in the data analysis. Descriptive statistics were performed with the PROC MEANS and PROC FREQ of SAS 9.4 (SAS Institute Inc., Cary, NC). As a result, the percentages shown in the results only apply to producers who indicated engaging in such practice. To identify the relationship between practices with HLMP, the herd's milk productions were categorized according to the reported 305-d milk yield/cow as low production (LP; < 7,000 L/cow), medium production (MP; 7,000 to 10,000 L/cow), or high production (HP; > 10,000 L/cow). The herd categorization was determined based on the data set milk production distribution. We were unable to group herds in similar sizes because their average 305-d milk production did not follow a normal distribution among range options. Thus, the LP, MP, and HP groups were composited by 27 (33%), 35 (42%), and 20 (24%) herds, respectively. The question under feeding practices realization ("yes or "no" questions) were dichotomized, and 3 risk ratio comparations were performed to compare the levels of milk production (LP, MP, and HP). The risk ratio was estimated via Poisson regression through the GENMOD procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). The risk ratios were estimated using the MP or LP herds as reference in the comparations.

4.4 Results and Discussion

General Information

The survey response rate was 27.6%, with 138 respondents out of 500 survey invitations. From 138 recorded surveys, 21 blanks and 33 incomplete surveys were removed from the dataset. Incomplete surveys were those which producers started to answer the questionnaire but did not finish. The remaining 82 survey responses were used to perform the final analyses. Overall, the error survey rate was 7.1%, with a 95% confidence level (Custon Insight Inc., 2010). Descriptive statistics for each group of herds are presented in Table 1.

Overall, the average herds size was 171 ± 294 (mean ± SD) lactating dairy cows. Breeds were Holstein (**HOL**; n = 52; 63%), Holstein × Gyr (**HG;** n = 22; 27.0%), and Jersey (**JER**; n = 8; 10%). Respondent herds house their high producing lactating cows on compost barn (n = 42; 51%), dry lot (n = 24; 29%), and free-stall (n = 16; 20%). The producers' 305-day milk yield (kg) frequency responses were: < 6,000 (n = 14; 17%); 6-7,000 (n = 13; 16%); 7-8,000 (n = 11; 13%); 8-9,000 (n = 10; 12%); 9-10,000 (n = 14; 17%); 10-11,000 (n = 7; 9%); 11-12,000 (n = 5; 6%), 12-13,000 (n = 7; 9%); > 13,000 (n = 1, 1%). Recorded responses came from the states of Minas Gerais (n = 55; 67%), Santa Catarina (n = 9; 11%), Rio Grande do Sul (n = 7; 9%), Paraná (n = 5; 6%), São Paulo (n = 4; 5%), and both states of Espírito Santo and Rio de Janeiro (n = 1; 1.0%). From 82 respondents, 74 (90%) reported have a nutritionist (Table 1). Overall, most produceres reported being visited by a nutritionist monthly.

Total mixed ration preparation

It is well known that wagon mixers are the most common equipment used to mix the dietary ingredients and deliver a standardized diet throughout the feed bunk (Schingoethe, 2017). Most of the HP and LP herds reported using wagon mixer, while only 58% of the LP herds reported using wagon mixer. However, the risk ratio of the use wagon mixer was not different among herds (Figure 1). This lower adoption by the LP producers could be attributed to the lower number of lactating cows to justify the investment compared with larger herds (Schingoethe, 2017). In addition, most HP and MP herd respondents reported using vertical mixers with 75 and 58%, respectively, while 60% of the LP herds respondents used a horizontal mixer.

Most herds of this survey added ingredients to the mixer wagon in the following order: forages (hay and silages), concentrates, by-products, and minerals/vitamins regardless of HLMP (Table 2). Regardless of HLMP, producers reported TMR mixing time ranging from 5 to 10 min after the inclusion of the last ingredient (Table 2). The TMR DM evaluation was not associated with HLMP (Figure 1). Most producers targeted 50% TMR moisture (50% DM) regardless of the HLMP. The TMR DM has been shown to impact lactating cows' feeding behavior and intake (Felton and DeVries, 2010). Cows easily sort extremely dried diets (TMR DM > 55%) against longer particles, which is associated with an increased risk of subacute ruminal acidosis (Stone, 2004; Stauder et al., 2020). On the other hand, high moisture diets (< 45% DM) might limit DMI because of gut fill (Lahr et al., 1983). In the present study, the risk ratio to evaluate TMR DM was not associated with the HLMP. In addition, approximately 75% of the producers reported goals of 50 to 55% of DM in the TMR. This goal meets the optimal TMR DM ranges of 45 to 60% indicated by Schingoethe (2017).

Wagon mixer scale calibration was not associated with the HLMP (Figure 1), whereas only 42% (n = 25/82) of respondents indicated calibrating the wagon scale at least once a year (Table 2). Evaluating the accuracy of the wagon scale is vital to feed cows precisely (Sova et al., 2014). Studies conducted in commercial dairies demonstrated high variation between the formulated and fed diet, which can be attributed in part to load errors (Sova et al., 2014; Trillo et al., 2016). Calibration of wagon scale was similar among the HLMP whereas the most common frequency was monthly, regardless of herd classification. Because feed is the most expensive cost of a dairy farm, making an investment and taking the time to analyze the most critical equipment used to handle it is needed. In addition, incorrect ingredient weighing would substantially influence feed efficiency (FE) estimations.

In tropical countries such as Brazil, the high temperature provides conditions for moldy TMR, which has been associated with a reduction in feed nutritive value and animal performance (Kung, Jr., 2010). In addition, Felton and DeVries (2010) reported a negative impact of increased TMR temperature on cows' intake. Thus, the use of TMR stabilizers such as organic acids (OA) has been suggested to decrease TMR heating and nutrient degradation by slowing mold growth. In this survey, the use of TMR stabilizers was not linked to HLMP (Figure 1), with only 15% of the respondents reported using TMR stabilizers. In a study conducted in Brazil, Gheller et al. (2020) demonstrated that the addition of OA minimized the increase in TMR temperature and led to greater DMI and 3.5% fat-corrected milk (FCM) of cows fed those diets. Thus, producers and nutritionists should be encouraged to add stabilizers to the diet to improve TMR aerobic stability, which might impact animal performance. All TMR preparation practices had a similar risk ratio between HP and MP herds.

Feeding practices in high production pens

Delivery of fresh feed has been demonstrated as the most significant feed practice to stimulate cows' feeding intake (DeVries and Von Keyserlingk, 2005). Additionally, greater feeding frequency reduces sorting, allowing for more consistent diet quality throughout the day (Kononoff et al., 2003; DeVries and Von Keyserlingk, 2005). In the present survey, most HP producers reported feeding cows 3 times/d, while most MP and LM producers reported feeding cows 2 times/d (Figure 3A). In a study conducted by Hart et al. (2014), cows had the greatest DMI when feeding frequency was increased from 2 to 3 times/d. However, in that same study, milk yield was not affected by feeding cows 2 or 3 times daily, and the authors suggested that the extra DMI was used on the body reserves rather than milk production but was effective in increasing energy intake.

Feeding cows after milking helps to keep cows standing, which is one of the most effective preventive measures for lowering mastitis (Hogeveen et al., 2011). In a cross-sectional study, herds offered fresh feed after morning and evening milkings had a lower incidence of clinical mastitis than herds that did not offer feed after milking (Peeler et al., 2000). Additionally, DeVries et al. (2010) demonstrated that cows lying down 40 to 60 min after milking had lower odds of intramammary infection than cows lying down within 40 min after milking. In the present study, the risk ratio of feed cows after milk time was not associated with HLMP (Figure 2). Although information regarding clinical mastitis or somatic cells count was not assessed in this study, it is known that good milking practices and feed management affect mammary gland health (Schultze and Bright, 1983).

Cows usually push the diet out of their reach in the feed bunk due to their natural head movement during feeding. Consequently, feed push-up is commonly used for ensuring continuous feed availability in the feed bunk. Furthermore, a greater frequency of push-ups is linked to reducing feed sorting, more time spent eating, and uniform distribution of feeding time throughout the day (DeVries et al., 2005). The HLMP did not affect the feed push-up risk ratio in the present study. However, most of the HP producers (41%) reported feed push-up 5 or more times/d, whereas the majority of MP producers (47%) reported 3 or 4 push-ups/d, while most of the LP producers (50%) reported 1 or 2 push-ups/d (Figure 3B). In a study by Miller-Cushon and DeVries (2017), feed sorting, lying time, and milk production and components were not affected by an increase of feed push-up frequency from 3 to 5 times/d. However, because cows in that study were housed individually, the findings should be interpreted with caution because they may not reflect the genuine social interactions among cows housed in free stalls or compost bedded packs.

Lactating cows are frequently fed slightly more than their predicted DMI to allow cows to increase DMI, also called feeding for refusals. The lenght of time that feed provided is associated with feed sorting and milk production (Schütz et al., 2006). In the present study, all herds were equally associated with feeding for refusals. Furthermore, most producers reported feeding cows for 5% of refusals regardless of the HLMP (Figure 3C). A high control of TMR refusals must be encouraged to reduce feed waste. Feed efficiency is one of the most common indices used to evaluate milk production efficiency. At the herd level, FE is most commonly calculated as the 3.5% FCM yield produced per kilogram of DM consumed at the pen level. In the current study, the MP herds had a greater risk ratio to assess FE than LP herds, but MP dairies had a similar risk ratio compared to HP herds (Figure 2). In addition, HP and LP herds had a similar risk ratio to measure FE (Figure 2). Regardless of the HLMP, most producers (83%) evaluate FE monthly (Figure 3D).

Cleaning protocols of feed bunk and water trough are essential to avoid orts or water spoilage, impacting cows' milk production and health. In the present study, HP and MP herds had a greater risk ratio to have water troughs protocol than LP herds but had the same risk ratio to clean up feed bunk (Figure 2). In addition, HP producers had the highest frequency of water troughs clean-up compared to MP and LP (Figure 3F). The risk ratio of providing feed training to their employee's was equally between the 3 groups (Figure 2).

High production pens management

The HP pens' most common feed bunk space ranged from 70 to 80 cm regardless of the HLMP (Figure 4A). Producers reported stocking density of 100% in 56% of HP, 50% of MP, and 45% of LP (Figure 4B). Producers of the HP, MP, and LP herds reported 19, 4, and 5% of overstocking (>100%), respectively. The HP producers (52.9%) introduced cows to the high production pens daily, while MP (31.0%) and LP (39.3%) producers indicated a bi-weekly introduction of cows to high production pens.

Literature has extensively demonstrated differences in dairy cows' requirements and feeding behavior that justifies grouping primiparous and multiparous lactating cows in different pens (Grant and Albright, 2001; DeVries et al., 2011; Kalantari et al., 2016). In addition, Phillips and Rind (2001) demonstrated that when separated from multiparous cows, primiparous cows had increased milk yield compared to those grouped with multiparous. In the current study, grouping primiparous separated from multiparous was not associated with HLMP (Figure 2). However, HP and MP producers were more associated to group primiparous separated from multiparous for entire lactation, while LP producers reported in the close-up period (Figure 4C). In a survey by Contreras-Govea et al. (2015), grouping cows was associated with the number of lactating cows in the herd. Only 28% of producers with less than 200 lactating cows reported feeding more than 1 diet, compared to 66% of herds with 200 or more lactating cows reporting feeding 2 or more diets. Therefore, grouping primiparous separate from multiparous from calving to the peak of lactation could decrease the feed competition and increase milk production and well-being of primiparous cows. Although this separation might be limited by facilities space, the desire to make management simple, and the need for additional labor, dairy producers should be encouraged to evaluate cows' feed behavior and define a better grouping strategy.

High temperature and humidity environments, like those found in Brazil, are linked to economic losses due to decreased cow performance. In a review, Negrón-Pérez et al. (2019) demonstrated that cows under colling management have improved milk production and reproduction performance. In the present study, the risk ratio of having a colling system was not associated with HLPM (Figure 2). The reasons for not using colling systems were not asked in the survey; nonetheless, they may be linked to a lack of understanding of cooling systems' benefits and technical specifications and the high cost of cooling systems in Brazil. No differences were observed between HP and MP herds (Figure 2).

Feedstuffs evaluation

High production dairy cows demand large quantities of nutrients to support their production. In this sense, nutrient-deficient diets would reduce milk yield, lead to weight loss, impair reproduction, and impact disease resistance (Weiss, 1998). Thus, the characterization of the composition and physical ingredients is essential to meet cow's requirements through diet formulation and feed bunk management. In the present study, MP herds had a greater risk ratio of determining forage DM and evaluated corn processing than LP (Figure 1). The HP had a similar risk ratio to evaluated forage DM and evaluated corn processing compared to LP and MP herds (Figure 1). In addition, MP producers reported shipping feeds for laboratory analysis more frequently than HP, followed by LP herds (Figure 5A). Monthly forage DM assessment was the most frequent for HP (38%) and MP (58%) herds (Figure 5B). The MP producers reported a greater frequency of corn process evaluation (Figure 5D). Therefore, it is conceiveable that dairies conducting a more detailed evaluation of their ingredients might feed cows more accurately than others, leading to better production outcomes.

Cows fed adequate particle size (PS) and peNDF are associated with better ruminal function and optimized milk performance (Zebeli et al., 2012). Specifically, forage PS may alter dramatically before and after TMR preparation (Heinrichs et al., 1999). Thus, dairy farms and nutritionists should constantly check the PS distribution to ensure the delivery of a consistent TMR, In our study, the HP herds had a greater risk ratio of estimating TMR peNDF than LP herds (Figure 1). Particle size distribution and TMR peNDF risk ratio were similar between HP comparations. Furthermore, the most often reported assessment frequency of TMR peNDF was once a month, regardless of HLMP (Figure 5C). Although no information was provided, we believe that nutritionists conducted monthly evaluations because it corresponds to the frequency of nutritionist visits in Brazil. Furthermore, results from the PS evaluation are used to determine whether the diet peNDF > 8 mm. Studies have demonstrated that lactating cow's diets should have at least 19% of peNDF > 8 mm to promote adequate ruminal health and milk components production (Zebeli et al., 2012). The (NASEM, 2021) has recognized the utility of this methodology to evaluate the dietary fiber; however, authors concluded that inclusion in the recommendation is limited due to lack of standard methods to measure effective fiber of feeds and establish requirements. In the field, nutritionists not only evaluate the TMR particle size but also forages and silages peNDF at harvesting. Thus, as a guideline, these parameters should always be evaluated when new ingredients are added to the diet, forage DM changes, a new silo is opened, or increased sorting. None of the others feeding practices has differed between HP and MP herds (Figure 1).

4.5 Applications

We revealed a wide range of feeding and feed bunk practices among Brazilian dairies. The HP herds had greater risk ratio to analyse TMRpe and to have through wash protocol than low milk production herds. In addition, the HP herds had greater risk ratio to analyse corn process than MP. The MP herds had greater risk ratio to analyses forage DM, to measeure feed efficiency, to have a trough wash protocol than low milk production herds. However, the frequencies of feeding practices evaluations were quite variable between herds, and HP and MP herds reported more intensive feeding practices than LP herds. These findings emphasized a variation in technical knowledge access by milk producers at distinct levels of milk production and highlighted the opportunity for improvement on feed and feed bunk management practices in Brazilian dairies. Moreover, to support the success of management strategies, both governmental and private initiatives are required to promote knowledge transference between academia and dairy producers. Finally, this study suggests that adopting feeding practices and feed bunk management might be critical to optimizing the productive cow's response to the diet fed.

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Table 1. Descriptive characteristics of dairy farms according to the three groups of herd milk production level

	Herd milk production level			
Item	LP[1] (n=27)	MP[2] (n=35)	HP[3] (n=20)	Overall
Lactating cows, n average ± SD	135 ± 367	150 ± 146	273 ± 380	175± 299
Herd breed, n herds, %				
Holstein	12 (33)	21 (34)	19 (95)	52 (63)
Holstein x Gyr	9 (45)	12 (60)	1 (5)	22 (27)
Jersey	6 (22)	2 (6)	-	8 (10)
Housing system, n herds, %[4]				
Compost Barn	8 (30)	22 (63)	12 (60)	42 (51)
Dry lot	16 (58)	7 (20)	1 (5)	24 (29)
Free stall	3 (12)	6 (17)	7 (35)	16 (20)
Milkings, n herds, %[4]				
2	21 (79)	16 (46)	8 (40)	45 (55)
3	6 (21)	19 (54)	12 (60)	37 (45)
Have nutritionist, n herds, %[4]	20 (75)	35 (100)	19 (95)	74 (90)

[1] Low production; [2] Medium production herd; [3] High production herd; [4] Percentage of dairies that have a nutritionist; [4] Housing system and number of milkings in the high milk production pens

Table 2. Characteristics for the three groups of herd milk production level on nutritionist visit frequency and TMR preparation questions (number of respondents in parenthesis) in high milk production pens

	Herd milk production level, %		
Item	LP (n=27)[1]	MP (n= 35)[2]	HP (n= 20)[3]
Nutritionist visit frequency, %			
Weekly	36 (5)	19 (6)	24 (4)
Biweekly	-	47 (15)	12 (2)
Monthly	50 (7)	34 (11)	59 (10)
>Monthly	14 (2)	-	6 (1)
Mixing type[4],			
Horizontal	60 (3)	42 (5)	25 (1)
Vertical	40 (2)	58 (7)	75 (3)
Mixing time[5], min			
≤ 5	17 (1)	36 (4)	50 (3)
< 5 to ≤ 10	50 (3)	36 (4)	17 (1)
< 10 to ≤ 15	33 (2)	18 (2)	17 (1)
< 15 to ≤ 20	-	9 (1)	17 (1)
TMR moisture goal[6], %			
<50	5 (1)	12 (3)	7 (1)
50 ≤ to ≤55	95 (19)	88 (22)	93 (13)
>55	-	-	-
Particle size distribution, frequency			
Weekly	-	-	9 (1)

Biweekly	14 (1)	27 (4)	18 (2)
Monthly	57 (4)	40 (6)	45 (5)
>Monthly	29 (2)	33 (5)	27 (3)
Scale revision, frequency/yr			
1x	-	25 (3)	29 (2)
2x	43 (3)	25 (2)	29 (2)
≥3	57 (4)	50 (6)	43 (3)

[1] Low production herds; [2] Medium production herds; [3] High production herds; [4] Considering only those using wagon mixer; [5] Time after last ingredient load; [6] Total mixed ration.

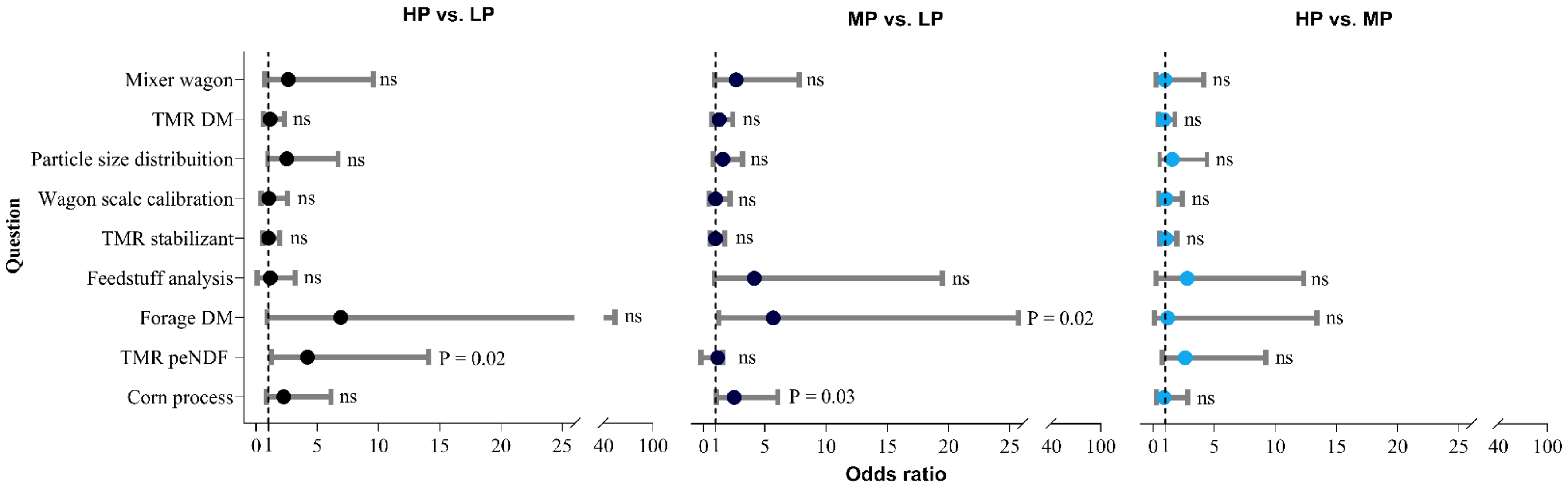

Figure 1. Risk ratio for each feeding practice. Herds classified as low milk production (305d milk yield; <7,000 L/cow) were used as the reference group when compared with high and medium production herds. Herds classified as medium milk production (305d milk yield; 7,000 to 10,000 L/cow) was the reference when compared with high milk production herds. The dot represents the risk ratio, and the gray bars represent the confidence interval. The dashed line represents the group reference.

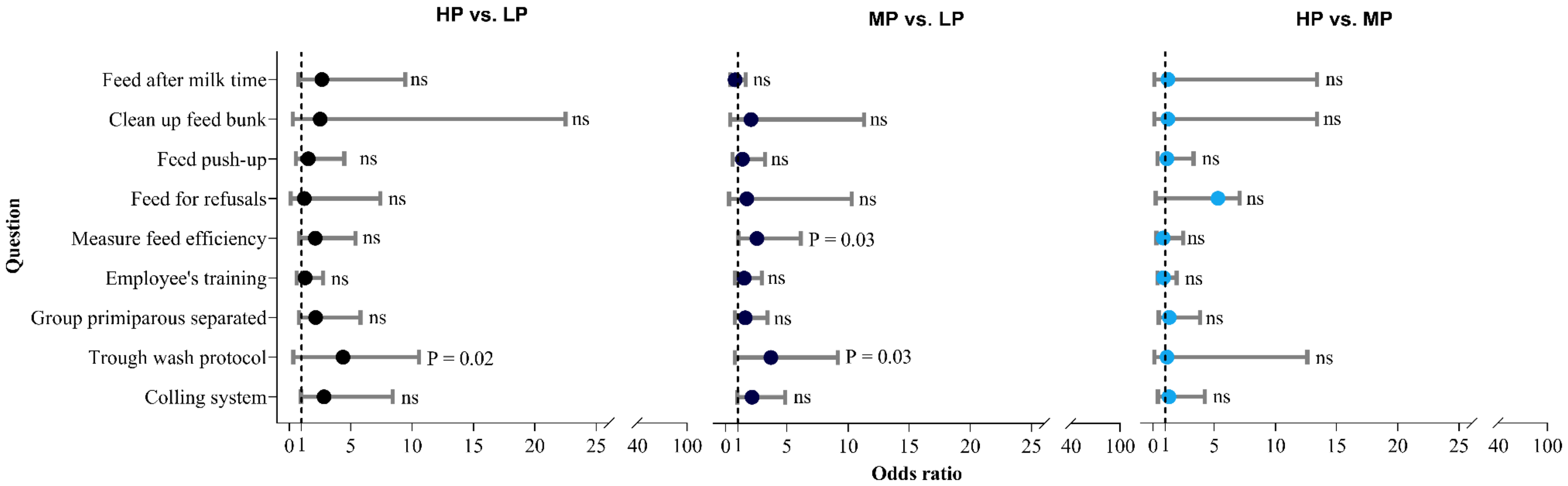

Figure 2. Risk ratio for each feeding practice. Herds classified as low milk production (305d milk yield; <7,000 L/cow) were used as reference group when compared with high and medium production herds. Herds classified as medium milk production (305d milk yield; 7,000 to 10,000 L/cow) was the reference when compared with high milk production herds. The dot represents the risk ratio and the gray bars represent the confidence interval. The dashed line represents the group reference.

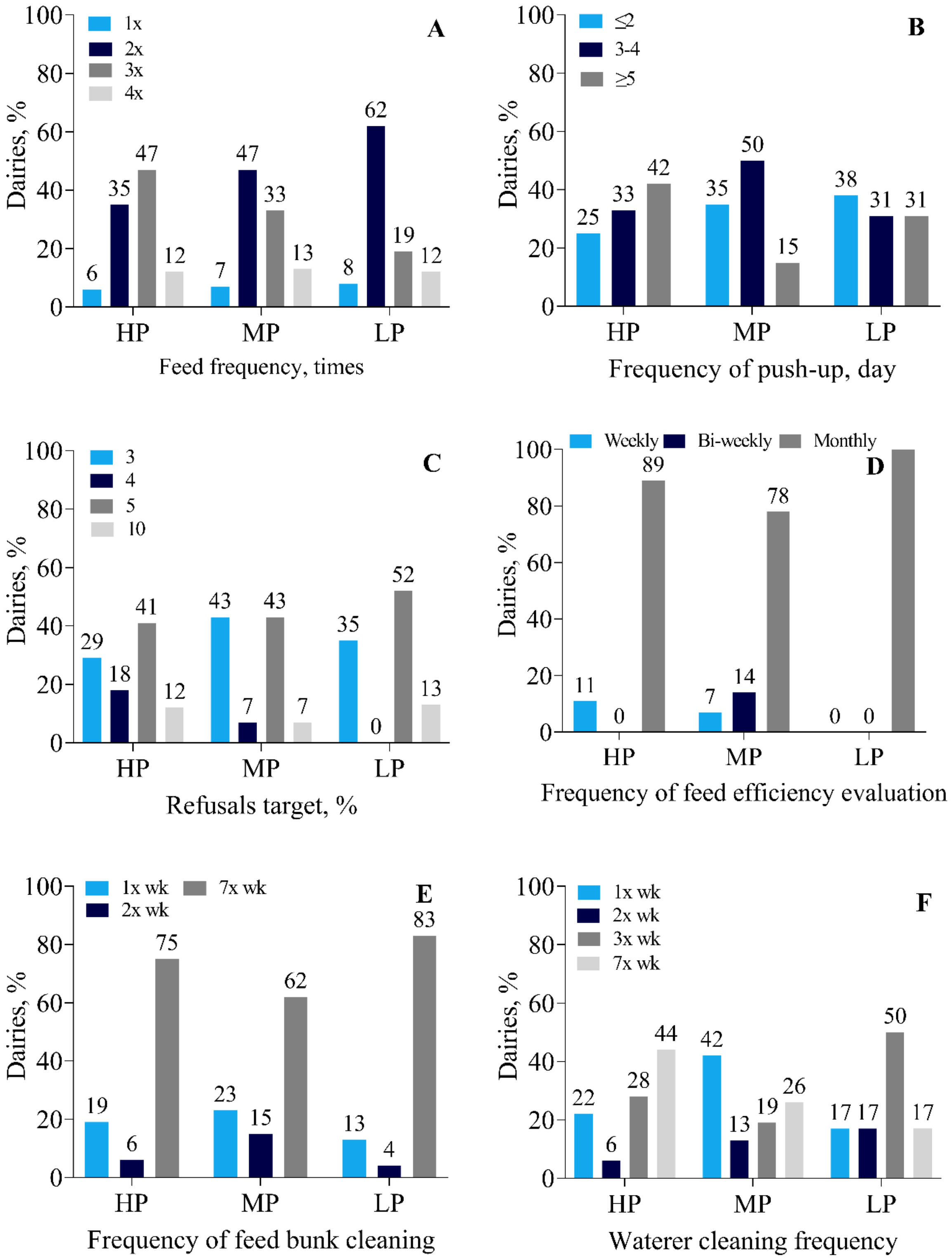

Figure 3. Frequency of feed cows (A), feed push-up (B), feed refusals target (C), feed efficiency (D), feed bunk cleaning (E), water trough cleaning (F) on high milk production pens on high production herds (HP), medium production herds (MP), and

low production herds (LP). Numbers on the top of the bars represent the percentage of the respondents.

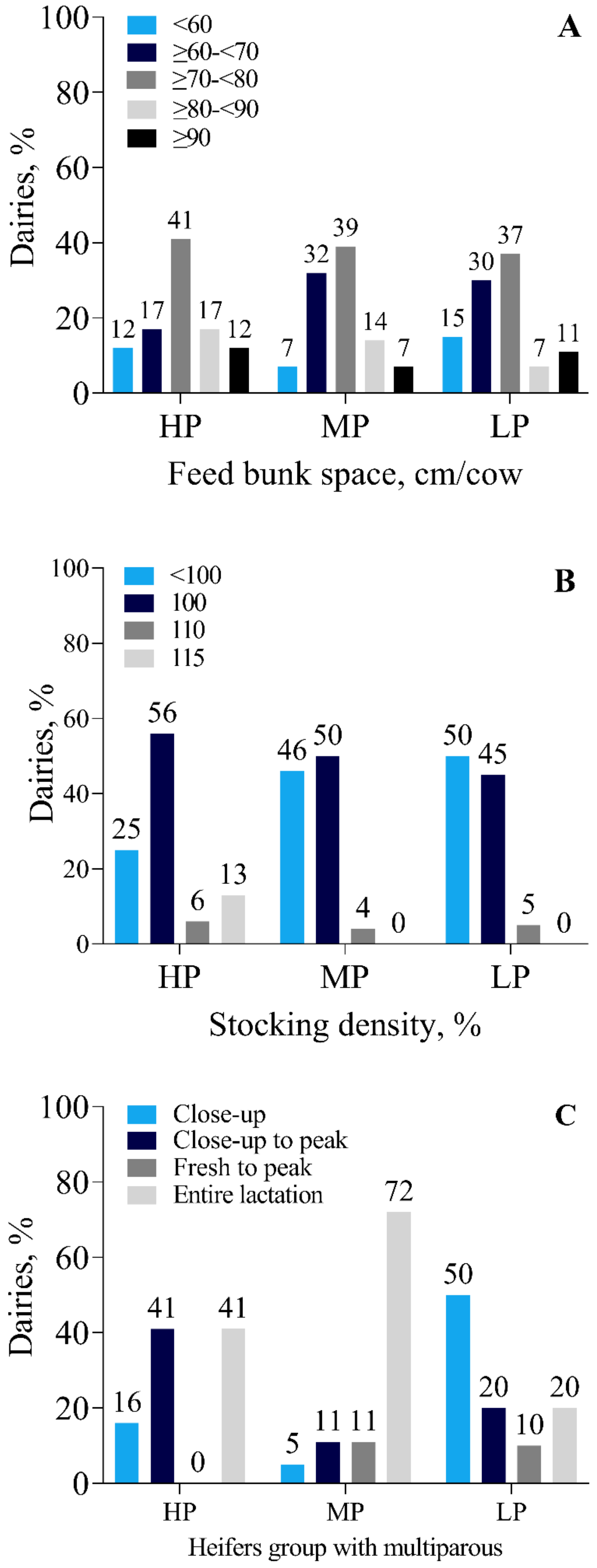
A
Dairies, %
100
80
60
40
20
0
<60
≥60-<70
≥70-<80
≥80-<90
≥90
12
17
41
17
12
7
32
39
14
7
15
30
37
7
11
HP
MP
LP
Feed bunk space, cm/cow
B
Dairies, %
100
80
60
40
20
0
<100
100
110
115
25
56
6
13
46
50
4
0
50
45
5
0
HP
MP
LP
Stocking density, %
C
Dairies, %
100
80
60
40
20
0
Close-up
Close-up to peak
Fresh to peak
Entire lactation
16
41
0
41
5
11
11
72
50
20
10
20
HP
MP
LP
Heifers group with multiparous

Figure 4. Feed bunk space (A), Stocking density (B), and primiparous group (C) on high milk production pens on high production herds (HP), medium production herds (MP), and low production herds (LP). Numbers on the top of the bars represent the percentage of the respondents.

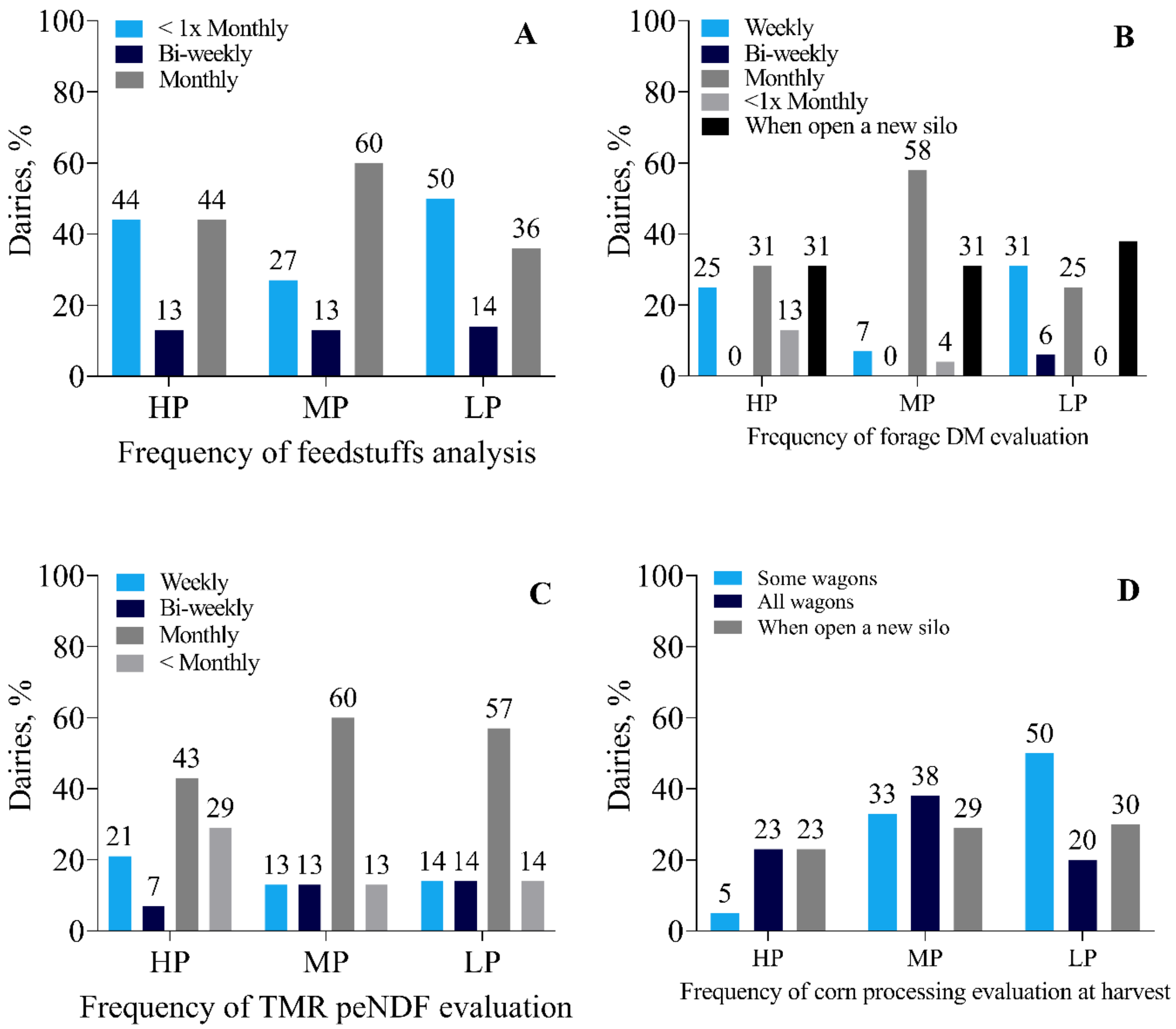

Figure 5. Frequency of feed cows (A), feed push-up (B), feed refusals target (C), feed efficiency (D), feed bunk cleaning (E), water trough cleaning (F) on high milk production pens on high production herds (HP), medium production herds (MP), and low production herds (LP). Numbers on the top of the bars represent the percentage of the respondents.

Supplementary Table 1. Survey questions with their respective answers' options.

Questions	Answer option
General farm and herd information	-
What state is the farm localized in?	PR[1], RS[2], SC[3], ES[4], MG[5], RJ[6], or SP[7]
What is the main breed used?	Holstein, Holstein x Gyr crosses, Jersey, other
What was the average number of lactating dairy cows in the past year?	Open question
What systems are your high-production dairy cows housed in?	Free-stall, compost barn, dry lot, and tie-stall
Do you receive a nutritionist visit?	Yes/No
How often does the nutritionist visit your dairy?	≤ Weekly, bi-weekly, monthly, >monthly
What was the 305-d milk production of your cows in the last year?	≤6, 6 < to ≤ 7, 7 < to ≤ 8, 8 < to ≤ 9, < 9 to ≤ 10 , < 10 to ≤ 11, 11 < to ≤ 12, < 12 to ≤ 13, or > 13 × 1,000L
TMR preparation and feedstuff evaluation:	-
Do you use a wagon mixer?	Yes/No
What is the mixing type?	Vertical/horizontal

Questions	Answer option
What order are the ingredients loaded in the wagon?	Open question
What is the mixing time (min.) after loading the last ingredient?	≤5, 5 < to ≤ 10,10 < to ≤ 15, < 15 to ≤ 20
Do you check TMR dry matter?	Yes/No
What is the TMR dry matter target (%)?	<50, 50 ≤ to ≤ 55, > 55
Do you evaluate TMR particle using a PSPS? How often?	Yes/No; Weekly, biweekly, monthly, > monthly
Do you calibrate the mixer wagon scale? How often?	Yes/No; Open
Do you use any TMR stabilizers? (e.g. organic acids)	Yes/No
Do you send feedstuffs samples for laboratory analysis? (yes/no) How often?	Yes/No; Open
Do you evaluate TMR physically effective NDF?	Yes/No; Open

Questions	Answer option
(yes/no) How often?	
Do you evaluate corn silage particle size at harvest? (yes/no) How often?	Yes/No; Open
Feed bunk practices:	-
How many times a day is the TMR fed?	1, 2, 3, 4
Do you do feed push-ups? How many times a day?	Yes/No; 1, 2, 3 4, ≥ 5
Do you clean up the feed bunk?	Yes/No
Do you feed for refusals? What percentage?	Yes/No; Open
Do you measure feed efficiency? How often?	Yes/No; Weekly, bi-weekly, monthly
High production cows management:	-
What is the feed bunk space?	Open question
What is the actual pen stocking density?	Open question
Do you feed the high-production cows group immediately after milk time?	Yes/No

Questions	Answer option
Do you group primiparous separated from multiparous cows?	Yes/No
Do you have a water trough wash protocol?	Yes/No
Do you have a cooling system?	Yes/No

5 GENERAL CONCLUSIONS

In the first study, the association of cow, feeding, and environmental conditions with milk performance in organic dairies was evaluated. Overall, cows managed under an organic milk system had milk yield and milk fat and protein affected by lactation number, breed, feed condition, season, and somatic cell count. In the second study, the effects of supplement mid-lactation cows with rumen-protected methionine and lysine were investigated. In this study, supplemented cows had greater milk and a lower risk of mastitis than no supplemented cows. Additionally, the supplementation of the amino acid did not affect the milk components. In the third study, information regarding feeding practices in high milk production pens of Brazilian dairies was gathered. In this study, herds were split in three groups according to the level of milk production to evaluate with any practice was linked to herd milk production level. Overall, herds had a similar risk ratio to perform most of the feeding practices. However, high and medium milk production herds had greater feeding practices frequencies than low milk production herds. Results presented in this thesis contributed to better understanding 1) Factors affecting milk performance in organic systems, 2) Milk performance of mid-lactation cows supplemented with rumen-protected methionine and lysine, and 3) The feeding practices adopted by Brazilian dairies producers in confined systems.