DAIRY FARM MANAGEMENT: SECURING ANIMAL HEALTH, WELL-BEING AND PRODUCTIVITY

EDITED BY: Richard Van Vleck Pereira, Sharif Shafik Aly, Vinicius Machado, Georgios Oikonomou and Ane Nødtvedt

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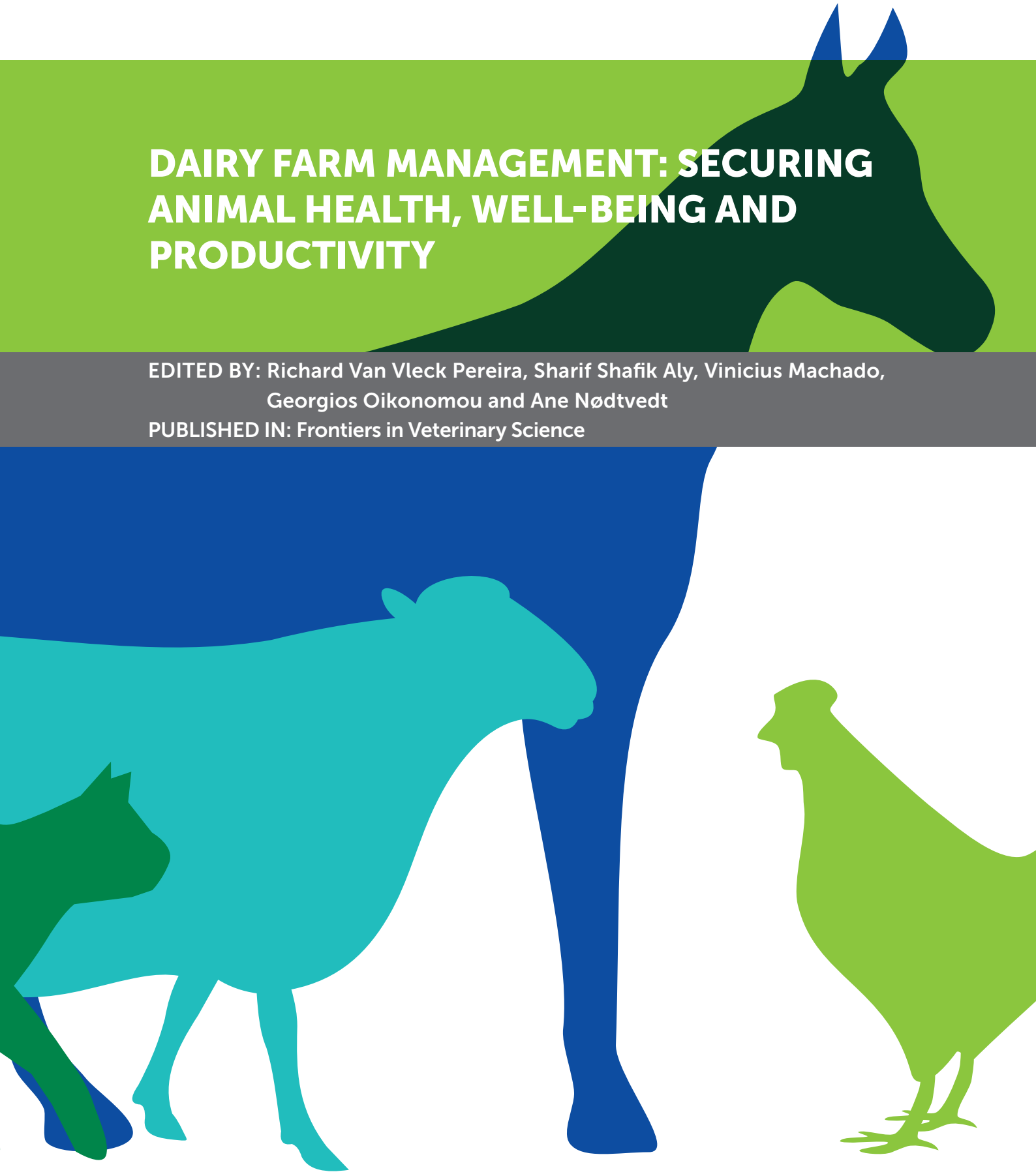

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DAIRY FARM MANAGEMENT: SECURING ANIMAL HEALTH, WELL-BEING AND PRODUCTIVITY

Topic Editors:
Richard Van Vleck Pereira, University of California, United States
Sharif Shafik Aly, University of California, United States
Vinicius Machado, Texas Tech University, United States
Georgios Oikonomou, University of Liverpool, United Kingdom
Ane Nødtvedt, Norwegian University of Life Sciences, Norway

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Farmer and Veterinary Practices and Opinions Related to the Diagnosis of Mastitis and Metabolic Disease in UK Dairy Cows

Francesc X. Donadeu[1]*, *Natalie L. Howes*[2], *Cristina L. Esteves*[1], *Martin P. Howes*[2], *Tim J. Byrne*[2] *and Alastair I. Macrae*[1]

[1] *The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian, United Kingdom,* [2] *AbacusBio International Ltd., Easter Bush, Midlothian, United Kingdom*

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Edited by:
Ane Nødtvedt,
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Reviewed by:
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Dennis Makau,
University of Minnesota Twin Cities, United States

***Correspondence:**
Francesc X. Donadeu
xavier.donadeu@roslin.ed.ac.uk

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Production diseases are highly prevalent in modern dairy herds, resulting in lost productivity and reduced animal welfare. Two important production diseases are mastitis and metabolic disorders. The availability of robust diagnostic tools that can detect animals at early stages of disease is crucial to prevent the high costs associated with lost productivity and the treatment of clinically and/or chronically diseased animals. Despite a variety of diagnostic methods being available to farmers and veterinarians, the incidence of these diseases in UK dairy herds has not changed over the last decade, underscoring the need for improved approaches for early disease detection. To this end, we administered a questionnaire to farmers and veterinarians to understand current diagnostic practices in the UK dairy cow sector, and to gather opinions on the suitability of currently available diagnostic tests in order to identify specific areas where improvement in diagnostic technologies and/or practices are needed. Data from a total of 34 farmers and 42 veterinarians were analyzed. Results indicated that most farmers surveyed used a combination of methods to diagnose mastitis and metabolic disorders, the most popular of which were visual inspection and milk recording somatic cell count data for mastitis, and body condition score and milk ketone testing for metabolic disorders. These preferences were not always in line with veterinarian recommendations of different diagnostic tools. Moreover, veterinarians indicated they were not satisfied with currently available diagnostic tools or how these were implemented by farmers. Both farmers and veterinarians recognized there was substantial room for improvement of current diagnostic tools, particularly in regard to the need to detect disease early. A majority of respondents preferred new diagnostic tests to be suitable for use with milk rather than blood or urine samples, and to yield results within 24 h. Finally, both groups surveyed identified economic cost as the most important barrier for the future uptake of new diagnostic technologies. The information obtained should guide the future development of diagnostic approaches that meet both the expectations of farmers and veterinarians, and help bring about a reduction in the incidence of production diseases in UK dairy herds.

Keywords: cattle, dairy, mastitis, metabolic disease, diagnostic tools

INTRODUCTION

Based on recent Agriculture and Horticulture Development Board figures (1) the UK dairy industry comprises 1.9 million dairy cows producing nearly 14 billion liters of milk every year, with a total of 13,000 active dairy farmers. Conservative estimates for the dairy industry worldwide are 300 million cows producing 600 million tons of milk every year on 120 million dairy farms. In the UK alone, milk production is worth £8.8bn at wholesale level, making up almost 20% of total agricultural output.

Keeping milk production profitable for farmers in the context of national and global economies critically depends on dairy herds maintaining good cow health. Production diseases can result from intensive dairy cow management in modern farm systems. Because of their high incidence in dairy herds, production diseases substantially limit milk production and threaten the sustainability of the dairy industry in the UK and globally (2–4). Production diseases include mastitis, infertility, lameness, and several metabolic disorders, and occur with highest frequency during the period around calving when physiological stress associated with the high energy requirements of gestation and lactation are at their greatest, thus compromising immunity and resistance to disease (5, 6). In the case of metabolic disorders (including ketosis, ruminal acidosis, hypocalcaemia, and hypomagnesaemia), although clinical disease incidence is relatively low compared to mastitis (<10 vs. 40%), subclinical cases are highly prevalent (>30%), and predispose affected cows to other production diseases as well as reducing milk production (7). In this context, the availability of robust diagnostic tools that can detect animals at early stages of disease, particularly in the case of mastitis and metabolic disease, is crucial to prevent the high costs derived from lost productivity and treatment of clinically and/or chronically diseased animals (3).

A variety of approaches are available for the early detection of mastitis and metabolic disease (8, 9). Somatic cell or bacterial counting, either in individual samples or bulk milk, or ion conductivity tests are routinely used for mastitis. For metabolic disease, body condition scoring and/or quantification of fat/protein ratios, metabolite levels (ketone bodies, fatty acids) or minerals in blood and/or milk are commonly used to establish individual or herd-wide prevalence or susceptibility to the disease. Yet the actual ability of these approaches to identify the very early stages of disease or predict likelihood of disease in healthy herds is limited, and concerns related to high cost or labor requirements may limit the uptake of some approaches. The fact that the incidence of mastitis and metabolic disease in UK herds has not changed over the last decade (3) underscores the need for novel, accurate and cost-effective methods for early disease detection. New approaches are being tested, e.g., quantification of inflammation related proteins in blood or milk (8) and composite approaches for automated systems (10), although they do not always meet the conditions allowing efficient and affordable implementation in modern farming systems. Up-to-date information on diagnostic practices and preferences by key stakeholders in dairy cow health, i.e., farmers and veterinarians, is essential to guide current and future efforts to develop successful diagnostic approaches for dairy cows.

With this in mind, we wished to gain insight into the needs of the dairy industry in relation to existing technology for the diagnosis of mastitis and metabolic disease in cows. To do this, we distributed a questionnaire among farmers and veterinarians to understand current diagnostic practices in UK dairy farms, and to reveal existing opinions on the suitability of currently available diagnostic tests and the specific areas where improvement is needed.

METHODS

Two questionnaires, one for farmers and one for veterinarians, were prepared using SurveyGizmo (11). The questionnaires were prepared using our team's combined expertise in animal science, farm animal medicine, agribusiness consultancy and dairy farming. Each questionnaire included separate questions on Mastitis diagnosis, Metabolic disorder diagnosis (including Ketosis, Hypocalcaemia, Acidosis, Fatty liver disease and Hypomagnesaemia) and Barriers to technology uptake (see Appendix in **Supplementary Material**). Questions were written to maximize information obtained from respondents without pre-empting/biasing their response. Restrictive settings were used that ensured each question was answered before the respondent could move onto the next question. Where multiple Likert scales were provided in succession within one question, it was ensured that a minimum of three Likert variables were answered before the respondent could progress to the next question. The two questionnaires were appropriately pre-tested "in-house" before being released online.

Online links to the farmer and veterinarian questionnaires were sent by e-mail to a total of 500 farmer and 600 veterinary contacts, respectively, maintained by the Dairy Herd Health and Productivity Service (DHHPS) at Edinburgh's Royal (Dick) School of Veterinary Studies (R(D)SVS). The DHHPS provides veterinary diagnostic and consultancy services throughout the UK. Contacts across the UK that had used DHHPA services at least once over the past 36 months were used. Moreover, questionnaires were made publicly available on twitter, requesting that only participants in the UK complete the survey. In all cases, questionnaires were available for completion online from 7th to 28th February 2019. Approval was obtained from the Human Ethical Review Committee at the R(D)SVS before the questionnaires were released.

After completion, each individual questionnaire was manually screened for any obvious signs of falsification and to ensure that the partially completed questionnaires contained information worthy of analysis (e.g., more than just demographic info). Acquired knowledge, for example of the relationship between herd sizes and various management practices, was used to assess authenticity of responses. Two questionnaires were excluded from the outset. One farmer questionnaire was excluded because the farmer was based in Kenya, and a veterinary questionnaire was excluded because the respondent was a nutritionist, not a veterinarian. Questionnaire data were analyzed as follows. SurveyGizmo was used to obtain number of responses, percentages and mean (± SE) score values, whereas 95%

confidence intervals (CI) for percentages were calculated in Minitab 17 (Minitab LLC) using the One sample proportion test. All Figures were prepared using GraphPad 8.0 software.

RESULTS

Respondent Demographic Information

A total of 61 out of 500 dairy farmers responded to the survey. Of those, 34/500 (6.8%) responded to all or almost all (≥70%) of the questions, and were included in the data analyses. Respondent distribution based on location, role on a farm, herd size, calving system and feeding system are shown in **Figure 1**. A total of 59 out of 600 veterinarians contacted undertook the questionnaire, of which 42/600 (7.0%) responded to all or almost all (≥70%) of the questions. Out of the 42 veterinarians, 23 were located in England (54.8%), 11 in Scotland (26.2%), seven in Wales (16.7%) and one in Republic of Ireland (2.4%).

Mastitis Diagnosis

Seventeen out of 34 farmers surveyed (50.0%) reported that their average somatic cell count (cells/ml) was <150,000, and 14/34 (41.2%) reported average cell counts between 150,000 and 200,000. Only 3/34 (8.8%) of respondents reported counts above 200,000.

Results of the questionnaire highlighted that 32 out of 34 farmers (94%) used visual identification to identify mastitis, usually in combination with other methods (**Figure 2**, **Table 1**). Only 4/34 farmers (12%) used a single diagnostic method (visual identification) to diagnose mastitis (**Table 1**). Visual identification was most commonly used with SSC data from routine individual milk recording (10/34 farmers, 29%), whereas a further 14 farmers (41%) used these two approaches together with either or both of California mastitis test (CMT) and conductivity test. Only 2/34 farmers (6%) reported not using visual identification, using instead SSC data from routine individual milk recording in combination with CMT. On the other hand, veterinarians surveyed recommended multiple methods to identify mastitis in their client's dairy cows, in particular visual identification, individual milk recording and CMT (**Figure 2**). When asked how often the whole herd was checked for mastitis (**Table 2**), 22/34 farmers (65%) responded that the whole herd was checked daily, whereas

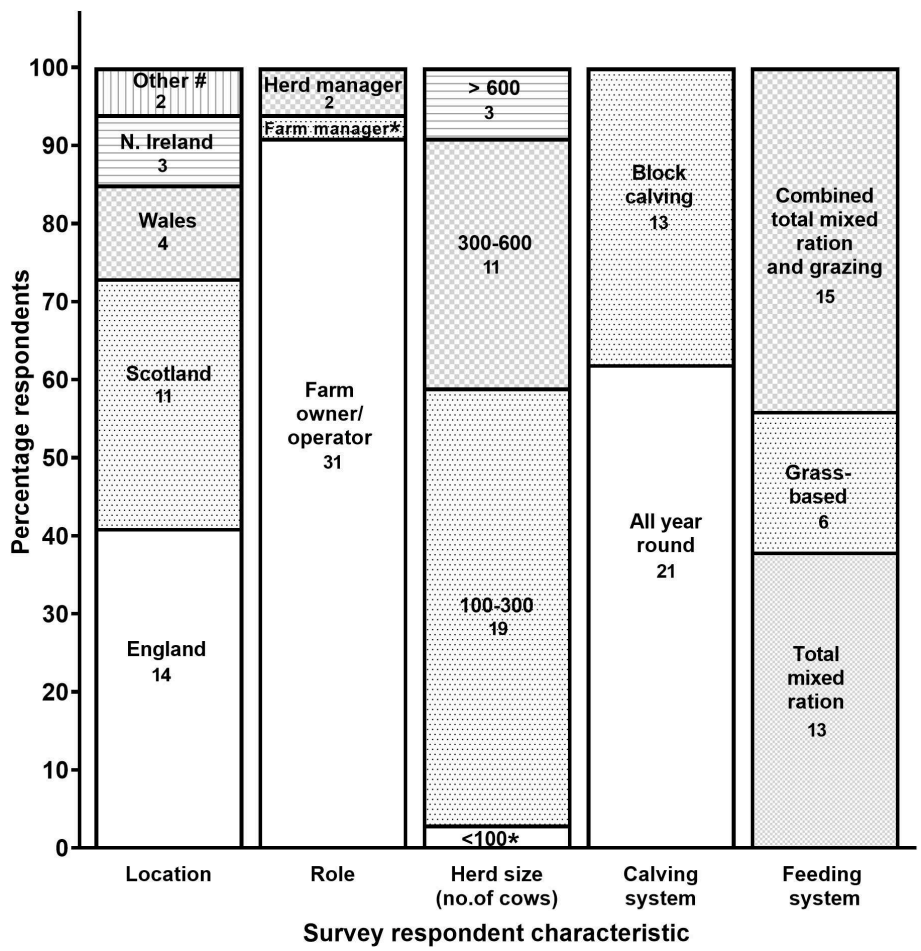

FIGURE 1 | Characteristics of respondents and farms that participated in the farmer questionnaire ($n = 34$). Actual numbers of respondents for each category are also shown. #Includes Guernsey and Republic of Ireland, *One respondent only.

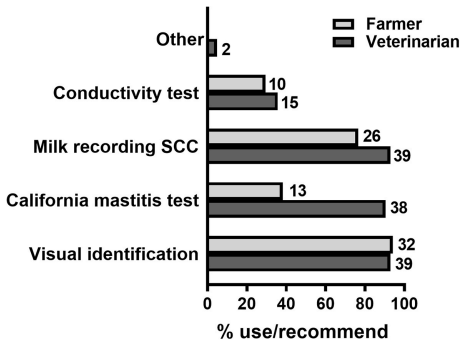

FIGURE 2 | Methods and practices used for the diagnosis of mastitis. Percentages of farmers using/veterinarians recommending each method listed are shown by horizontal bars. Number of respondents are shown next to each bar. *N* = 34 farmers, 42 veterinarians.

TABLE 1 | Number of methods used to diagnose mastitis (*N* = 34 respondents).

Number of diagnostic methods	No. respondents	% respondents	% respondents (95% CI)
1	4	11.8	3.3–27.4
2	16	47.1	29.8–64.9
3	11	32.3	17.4–50.5
4	3	8.8	1.9–23.7

TABLE 2 | Frequency with which the entire herd (all cows in one milking) was checked for mastitis (*N* = 34).

Frequency	No. respondents	% respondents	% respondents (95% CI)
Daily	22	64.7	39.6–72.2
Weekly	1	2.9	0.1–13.5
Monthly	4	11.8	2.9–24.2
Annually	1	2.9	0.1–13.5
When there was a high bulk somatic cell count	3	8.8	1.9–23.7
Never	2	5.9	0.7–19.7
Other	1	2.9	0.1–15.3

only 2/34 farmers (6%) responded that they never checked the whole herd for mastitis at one time. When queried who was responsible for identifying most cows with mastitis on farm (**Table 3**), questionnaire respondents identified farm workers/milk harvesters (15/34 or 44%) and herd managers (10/34 or 29%) as finding the most mastitis. Moreover, 30/34 farmers surveyed (88.2%) stated that they treated more clinical that subclinical cases of mastitis.

Farmers were then asked to rate several characteristics of current mastitis detection methods from 1 to 5 (1 = strongly disagree, 3 = neither agree/nor disagree, 5 = strongly agree;

TABLE 3 | Individual/system on farm responsible for identifying the most mastitis (*N* = 34).

Individual/system	No. respondents	% respondents	% respondents (95% CI)
Farm manager	5	14.7	4.9–31.1
Herd manager	10	29.4	15.1–47.5
Milk harvester/farm worker	15	44.1	27.2–62.1
Automated detection system	3	8.8	1.9–23.7
Other	1	2.9	0.1–15.3

Figure 3A). Respondents agreed most (mean, 4.2 ± 0.1) with "Current tests are informative for decision making," whereas "Current tests detect issues early" was rated lowest (mean, 3.5 ± 0.2), just above neutral. Conversely, when asked, veterinarians felt in general that the current veterinary services and methods available for detecting mastitis were inadequate and were not correctly utilized/implemented by farmers (**Figure 3B**). In addition, when asked to rate the need for improvement in current diagnostic methods, both veterinarians and farmers believed substantial improvement was needed particularly in the current tests' ability to identify an animal's susceptibility to mastitis, quantify the chance of reinfection and identify subclinical mastitis (**Figure 3C**). In regard to a test capable of identifying animals predisposed to mastitis, 41/42 veterinarians (97.6%) acknowledged they would promote such a test in order to reduce antibiotic use. Moreover, when asked if they would be willing to treat more animals for subclinical mastitis to reduce the number of clinical mastitis cases, 27/42 veterinarians (64.3%) suggested that they would be willing to promote this method compared to 15/42 (35.7%) who would be against it.

Farmers and veterinarians were also asked about the characteristics of an ideal mastitis test. Given the choice between different sample sources for testing, farmers and veterinarians rated a milk test as the top preferred choice followed by a blood test (**Figure 4**). In addition, a majority of both farmers (24/34 or 71%) and veterinarians (25/42 or 60%) preferred mastitis assay results to be available within 24 h (**Table 4**).

Metabolic Disorder Diagnosis

Farmers and veterinarians had strikingly different perceptions of the impact of metabolic disorders on UK dairy herd health. Whereas, 40/42 veterinarians (94.7%) believed that the prevalence of metabolic disorders was a major issue on farm, only 9/34 farmers (27.3%) had the same opinion. Moreover, when asked to rank the prevalence of different metabolic disorders in UK herds, both farmers and veterinarians ranked Ketosis first, followed by Hypocalcaemia, Acidosis, Fatty liver disease and Hypomagnesaemia.

Of the different approaches available to assess metabolic health in cows, body condition scoring was used by the largest number of farmers surveyed (25/33 or 75.8%; **Figure 5**). Most farmers

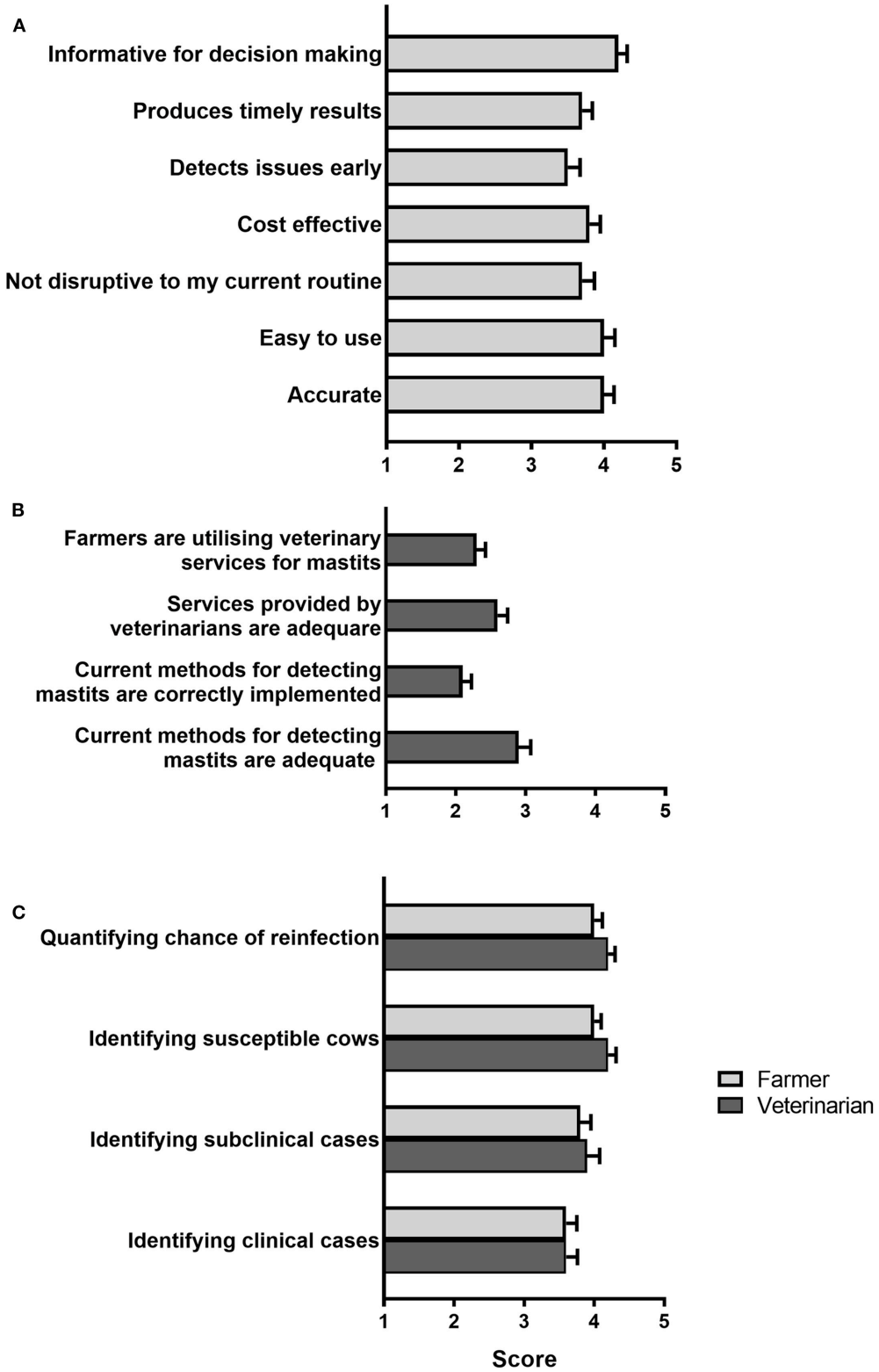

FIGURE 3 | Opinions on current approaches to diagnose mastitis. **(A)** Farmer rating of the characteristics of current diagnostic methods in response to the statement "Current diagnostic tools for mastitis are…" **(B)** Veterinarian rating of different statements related to current diagnostic approaches. **(C)** Rating of the need for improvement of different aspects of current diagnostic tests. In all cases, respondents were asked to rate their agreement with each statement provided from one (strongly disagree) to five (strongly agree). Mean (± SE) scores are shown. $N = 34$ farmers, 40 veterinarians.

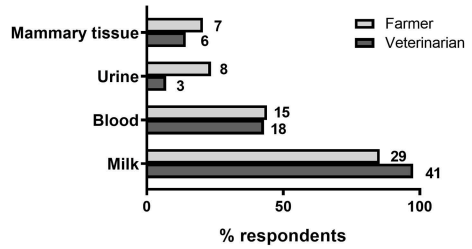

FIGURE 4 | Mastitis test preferences. Percentage of farmer and veterinarian respondents indicating the suitability of each of *four* sampling sources for a mastitis test. Number of respondents are shown next to each bar. *N* = 34 farmers, 42 veterinarians.

TABLE 4 | Turnaround time preferences for a mastitis test (*N* = 34 farmers, 42 veterinarians).

Turnaround time		No. respondents	% respondents	% respondents (95% CI)
Same day	Farmers	9	26.5	12.9–44.4
	Veterinarians	11	26.2	13.9–42.0
Overnight	Farmers	15	44.1	27.2–62.1
	Veterinarians	14	33.3	19.6–49.5
2–3 days	Farmers	9	26.5	12.9–44.4
	Veterinarians	17	40.5	25.6–56.7
5–7 days	Farmers	1	2.9	0.1–13.5
	Veterinarians	0	0	-

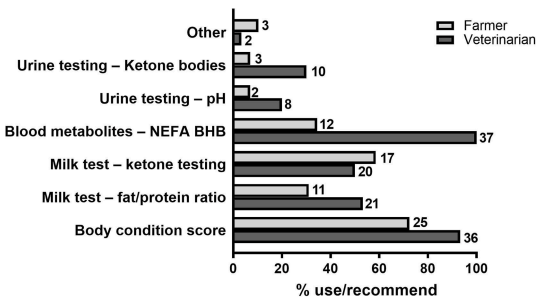

FIGURE 5 | Methods and practices used for diagnosis of metabolic disorders. Percentage of farmers using/veterinarians recommending each method listed. Number of respondents are shown next to each bar. *N* = 33 farmers, 37 veterinarians.

used a combination of approaches (**Table 5**), with 20/33 farmers (57.6%) using body condition scoring together with one or several of the milk, blood or urine tests indicated in **Figure 5**, of which milk ketone analysis was the most popular as it was used by 17/33 farmers (51.5%). Two farmers (6%) indicated they used liver biopsy and daily milk yield records, respectively, as additional tests to identify metabolic disease. In addition, 34 of 36 veterinarians surveyed (94.7%) recommend the use of blood metabolites in combination with animal body condition score to identify metabolic disorders in dairy cows, and 20/36 (56%)

TABLE 5 | Number of methods used to diagnose metabolic disorders (*N* = 33 respondents).

Number of diagnostic methods	No. respondents	% respondents	% respondents (95% CI)
1	9	27.3	13.7–46.7
2	15	45.5	29.1–65.2
3	2	6.1	0.8–20.8
4	7	21.2	9.0–38.9

TABLE 6 | Individual/system on farm responsible for identifying the most metabolic disorder cases (*N* = 32).

Individual/system	No. respondents	% respondents	% respondents (95% CI)
Farm manager	9	28.1	13.7–46.7
Herd manager	15	46.9	29.1–65.3
Milk harvester/farm worker	5	15.6	5.3–32.8
Automated detection system	1	3.1	0.1–16.2
Other	2	6.3	0.8–20.1

recommended also using milk tests for ketones and fat/protein ratios for that purpose (**Figure 5**).

When queried who was responsible for identifying most cows with metabolic disease on farm (**Table 6**), questionnaire respondents identified herd managers as responsible for identifying most diseased cows (15/32 or 47%) in almost half of the farms. Moreover, 19/31 farmers (61.3%) believed they detected predominantly clinical cases of metabolic disease. This figure was consistent with that obtained from surveyed veterinarians, 29/38 (76.3%) of which indicated they detected a higher proportion of clinical than subclinical metabolic disorder cases.

Farmers rating (1–5) of different characteristics of current metabolic disease detection methods showed a somewhat positive opinion (mean score between 3.5 ± 0.2 and 4 ± 0.1 in all cases; **Figure 6A**). On the other hand, as was the case for mastitis tests, veterinarians believed that farmers were not fully utilizing their services and were not implementing current practices correctly on farm (**Figure 6B**). Indeed, both farmers and veterinarians believed substantial improvement was needed for current metabolic disease tests to identify subclinical disease, and quantify an animal's susceptibility as well as chances of disease recurrence (**Figure 6C**).

Regarding opinions on the characteristics of an ideal metabolic disease test, farmers rated a milk test as their top preference choice followed by a blood test, whereas veterinarians preferred blood to milk (**Figure 7**). In addition, just over half of farmers (20/33 or 61%) and veterinarians (25/38 or 66%) would prefer metabolic disease assay results to be available within 24 h (**Table 7**).

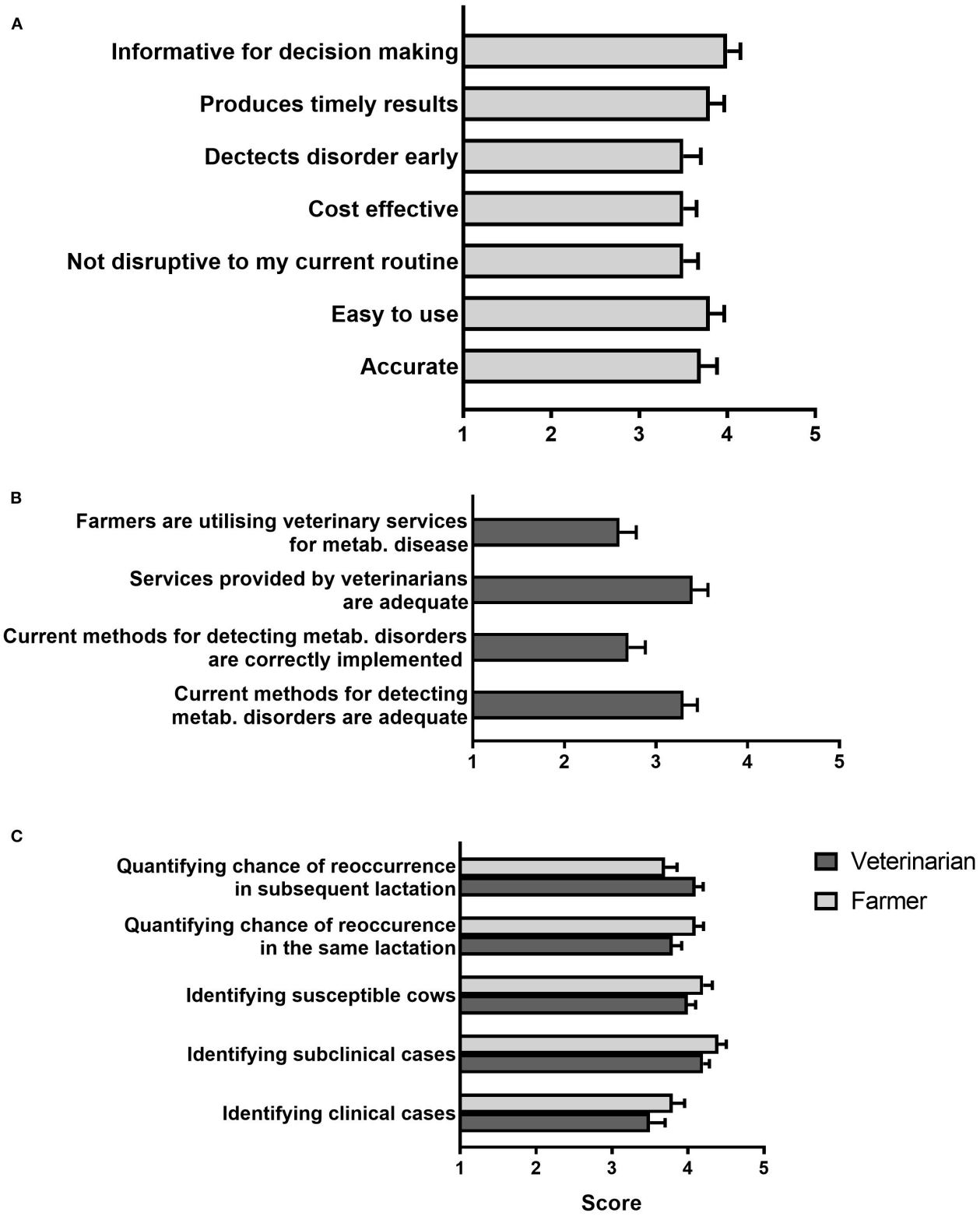

FIGURE 6 | Opinions on current approaches to diagnose metabolic disease. **(A)** Farmer rating of the characteristics of current diagnostic methods in response to the statement "Current diagnostic tools for metabolic disease are…" **(B)** Veterinarian rating of different statements related to current diagnostic approaches. **(C)** Rating of the need for improvement of different aspects of current diagnostic tests. In all cases respondents were asked to rate their agreement with each statement from one (strongly disagree) to five (strongly agree). Mean (± SE) scores are shown. $N = 33$ farmers, 36 veterinarians.

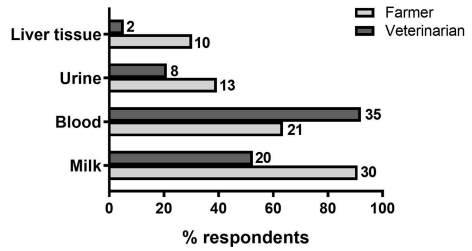

FIGURE 7 | Metabolic disease test preferences. Percentages of farmer and veterinarian respondents indicating the suitability of each of *four* sampling sources for a diagnostic test. Number of respondents are shown next to each bar. *N*= 33 farmers, 38 veterinarians.

TABLE 7 | Turnaround time preferences for a metabolic disease test (*N* = 33 farmers, 38 veterinarians).

Turnaround time		No. respondents	% respondents	% respondents (95% CI)
Same day	Farmers	9	27.3	13.3–45.5
	Veterinarians	15	39.5	24.0–56.6
Overnight	Farmers	11	33.3	18.0–51.8
	Veterinarians	10	26.3	13.4–43.1
2–3 days	Farmers	12	36.4	20.4–54.9
	Veterinarians	12	31.6	17.5–48.7
5–7 days	Farmers	1	3.0	0.1–15.7
	Veterinarians	1	2.6	0.1–13.8

Barriers to Technology Adoption

When asked to rank different potential barriers to the uptake of new diagnostic technologies (**Figure 8A**), farmers identified high upfront costs and high ongoing costs as the biggest barriers. No improvement in performance, need for significant changes to infrastructure and no reduction in operating costs were also important factors. All other factors rated closer to neutral (mean score between 2.5 ± 0.2 and 3.4 ± 0.2) on the 1 (no barrier) to 5 (major barrier) scale, with displacement of lower skilled roles and the requirement to learn new skills rated as the smallest barriers to overcome.

In comparison, veterinarians regarded high upfront cost as the only major barrier (median score $= 4.0 \pm 0.1$) to the uptake of new technology in their practices, with all other potential barriers rated closer to neutral, as shown in **Figure 8B**.

DISCUSSION

This study provided new information and opinions on current dairy herd diagnostic practices in the UK. Respondents were primarily selected from an updated UK-wide list of farmer and veterinarian users at the R(D)SVS dairy herd health services, that is representative of each of the two professional sectors in the UK. The percentages of farmer and veterinary contacts that actually completed each survey (about 7% each, see Results section) were slightly below the response rate (10–15%) typically expected with this type of surveys (www.surveygizmo.com), providing a margin of error (90%) of 12.3 and 13.8%, respectively. Moreover, respondent profiles in terms of herd size, calving and feeding systems, and geographical distribution (**Figure 1**) were representative of the wider UK dairy industry (1). Participants were self-selected volunteers who actively use information technologies (as these were online questionnaires). Voluntary respondents in a survey typically tend to be members of the sample populations (UK farmer and veterinary communities in this case) that are more concerned about the topic under survey and have also stronger opinions about it (12). Consequently, these groups are expected to be more willing to implement changes, or at least consider doing so, in order to improve dairy husbandry and health practices and profitability, as well as more likely to adopt new diagnostic practices and technology and, in the case of veterinarians, to recommend them to their clients. The above limitations, including potential biases, should be taken into account when interpreting the results of this study and implementing the suggested recommendations.

The combined results of the two questionnaires clearly indicated that in general surveyed farmers do not make full use of available diagnostic approaches for mastitis and metabolic disease, and in addition highlight a need for improved diagnostic tools that can better identify animals at early stages of disease. Addressing these two aspects will be key to successful implementation of early intervention strategies that can effectively reduce the current incidence of clinical disease and associated production losses incurred by dairy farmers.

In relation to mastitis, of the four diagnostic procedures considered, only CMT and conductivity tests, when used routinely on farm, may allow for prompt detection of pre-clinical disease, enabling effective reduction of clinical mastitis cases through early intervention measures (8). Yet just above 2/3 of farmers surveyed (23/34 or 68%) use either of these two techniques for diagnosing mastitis on farm, while most (26/34 or 76%) use SCC from milk recording data. The relatively low uptake of CMT, a simple and low-cost approach that can be used independently of automated milking systems, is in contrast with the high percentage of veterinarians that recommend it. Thus, encouraging wider use of CMT by farmers may in general be effective itself in reducing the incidence of mastitis in UK farms.

Similar to mastitis, most farmers surveyed (24/33 or 73%) use a combination of diagnostic approaches to assess metabolic status in their cows. In the majority of those cases (26/33 or 79.2%), these include body condition scoring and metabolite analyses in milk or blood. In contrast, only a small proportion of farmers (5/33 or 15.2%) favored the use of urine samples for diagnostic testing, in agreement with veterinarian preferences. Quantification of blood metabolites is considered the gold standard for the diagnosis of hyperketonaemia, and available blood-based tests have shown to have higher accuracy than cow-side tests using milk or urine (9, 13). Yet despite blood metabolite testing being the most widely recommended of all diagnostic approaches (100% of veterinarians surveyed), only 12/33 farmers (36.4%) indicated they routinely use this approach for diagnosing metabolic disease, instead being more in favor of milk sample

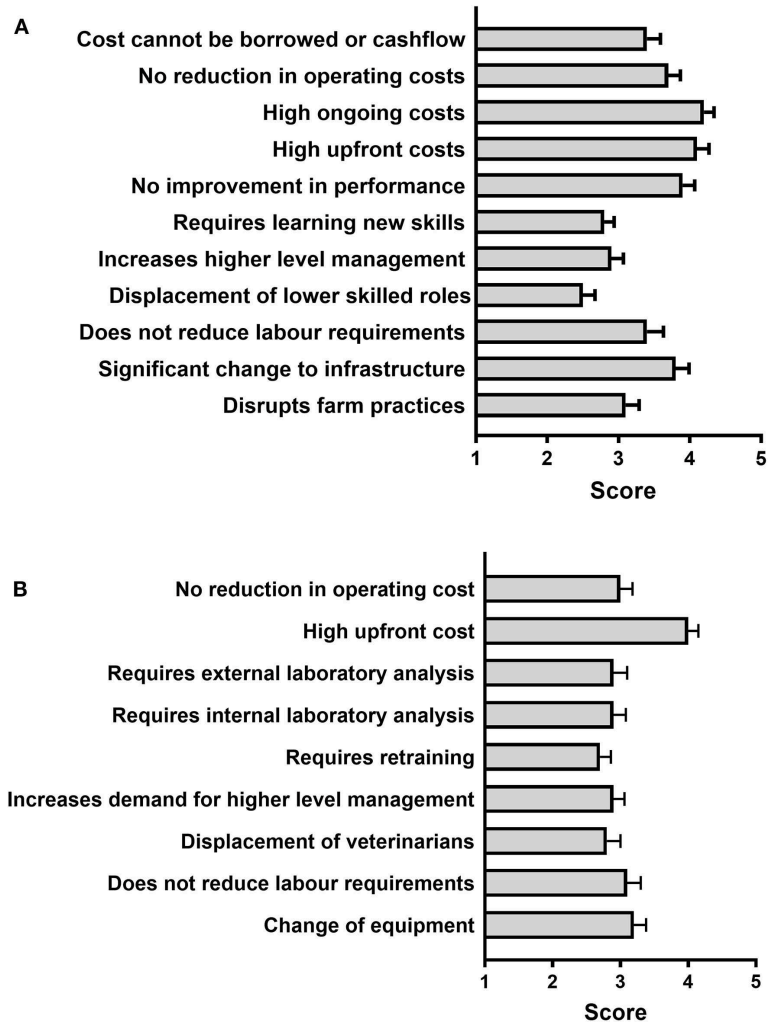

FIGURE 8 | Farmer **(A)** and Veterinarian **(B)** rating of different potential barriers to new technology uptake in their farms/practices. Respondents were asked to rate each statement given from one (no barrier to adoption) to five (major barrier to adoption). Mean (± SE) scores are shown. *N* =33 farmers, 37 veterinarians.

testing. Thus, assay simplicity and low cost, as is the case for cow-side milk-based assays, is a primary determinant of farmers' choice for a diagnostic test for their herd.

Although farmers had a moderately positive opinion of current tests for the diagnosis of mastitis and metabolic disorders, they also believed there is substantial room for improvement, especially in regards to the ability of available tools to detect disease early. This is in agreement with the reduced number of subclinical cases compared to clinical cases detected in the surveyed farms, as shown by a majority of respondents stating that they predominantly treat clinical over subclinical cases for both mastitis (88.2% of farmers) and metabolic disease (61.3% of farmers, see Results section). On the other hand, in general veterinarians did not believe that tools and veterinary services currently available for the diagnosis of metabolic disorders, and especially mastitis, are adequate, including the limited ability of available tools to detect subclinical disease, in agreement with farmers' opinions. Importantly, veterinarians were also concerned that farmers do not make full use of veterinary services and that current diagnostic methods are not appropriately implemented on farm. Based on these opinions, a need for tests that are farmer-friendly (preferably cow-side using milk samples) and able to identify animals at early disease stages and/or at risk of disease should guide future research and test development efforts in dairy cow diagnostics. In addition, as would be expected, economic concerns including implementation and running costs, as well as cost effectiveness in the context of farm operations, topped the list of factors seen by farmers as potentially limiting the uptake of new diagnostic technology. From the point of view of the animal health diagnostics sector, the development of commercially-viable kits using new technologies that meet all farmer's requirements, particularly cost expectations, will be a challenge.

Questionnaire results highlighted several discrepancies between farmer and veterinarian opinions, specifically in relation to the impact of metabolic disorders on herd health and productivity, and the recommended vs. actual use of specific diagnostic tools for mastitis and metabolic disorders. Based on this finding, appropriate farmer education on the benefits and advantages of the different diagnostic approaches available would

facilitate decision-making by farmers based on solid clinical evidence. This is essential to bring about an effective reduction of the incidence of clinical mastitis and metabolic disease in UK farms, and prevent major industry losses in terms of milk production and animal welfare.

In summary, this study highlighted current diagnostic practices related to dairy herd mastitis and metabolic disorders in the UK farms surveyed. Responses from the farmers and veterinarians surveyed revealed major gaps in both available technology and its application on-farm to effectively diagnose disease in cows. The results indicate a need for new and/or improved diagnostic tools able to accurately detect disease early, whilst at the same time being farmer-friendly (e.g., suitable for use in milk) and affordable. Together with appropriate farmer education on the importance of early diagnosis and the best approaches available, this information should guide the development of diagnostic kits that meet both the expectations of farmers and veterinarians, and assist in bringing about a reduction in the incidence of production disease in UK dairy herds.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Approval was obtained from RDSVS Human Ethical Review Committee.

AUTHOR CONTRIBUTIONS

FD, NH, MH, TB, and AM designed the study. NH and MB carried our the survey. FD, NH, and CE analyzed the survey data. FD, NH, CE, and AM wrote the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00127/full#supplementary-material

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CONCEPTUAL ANALYSIS
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Using Biosecurity Measures to Combat Respiratory Disease in Cattle: The Norwegian Control Program for Bovine Respiratory Syncytial Virus and Bovine Coronavirus

Maria Stokstad[1], Thea Blystad Klem[2], Mette Myrmel[3], Veslemøy Sunniva Oma[1], Ingrid Toftaker[1], Olav Østerås[4] and Ane Nødtvedt[1]*

[1] Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, Oslo, Norway, [2] Norwegian Veterinary Institute, Oslo, Norway, [3] Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, Oslo, Norway, [4] Section for Research and Development in Primary Production, Tine SA, Oslo, Norway

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Anette Ella Boklund, University of Copenhagen, Denmark

***Correspondence:**
Maria Stokstad
maria.stokstad@nmbu.no

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Bovine respiratory disease (BRD) cause important health problems in all cattle husbandry systems. It contributes substantially to the use of antimicrobial substances and compromises animal welfare and the sustainability of the cattle industry. The existing preventive measures of BRD focus at the individual animal or herd level and include vaccination, mass treatment with antimicrobials and improvement of the animal's environment and general health status. Despite progress in our understanding of disease mechanism and technological development, the current preventive measures are not sufficiently effective. Thus, there is a need for alternative, sustainable strategies to combat the disease. Some of the primary infectious agents in the BRD complex are viruses that are easily transmitted between herds such as bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCoV). This conceptual analysis presents arguments for combatting BRD through improved external biosecurity in the cattle herds. As an example of a population-based approach to the control of BRD, the Norwegian BRSV/BCoV control-program is presented. The program is voluntary and launched by the national cattle industry. The core principle is classification of herds based on antibody testing and subsequent prevention of virus-introduction through improved biosecurity measures. Measures include external herd biosecurity barriers and regulations in the organization of animal trade to reduce direct and indirect transmission of virus. Improved biosecurity in a large proportion of herds will lead to a considerable effect at the population level. Positive herds are believed to gain freedom by time if new introduction is avoided. Vaccination is not used as part of the program. Dissemination of information to producers and veterinarians is essential. We believe that reducing the incidence of BRD in cattle is essential and will lead to reduced antimicrobial usage while at the same time improving animal health, welfare and production. Alternative approaches to the traditional control measures are needed.

Keywords: bovine respiratory disease, winter dysentery, disease control, population-based, prevention, BRSV, BCoV

INTRODUCTION

Bovine respiratory disease (BRD) is a worldwide health concern in cattle and is one of the most common diseases in calves and young stock in all production systems. The disease is multifactorial and develops in complex interaction between factors associated with the host, the pathogens and the environment. The existing preventive measures therefore include a wide range of strategies. Despite advances of newer and better therapeutic and preventive medications, as well as efforts to improve management and optimize the environment to prevent BRD, the morbidity and mortality rates have not declined. A recent review of evolving views on BRD control measures concludes that blanket vaccination and mass treatment provides inconstant control for BRD and highlights the need to reappraise the use of these measures (1). Our question is, however, if there are alternative strategies to antimicrobial treatment and vaccines that could be effective in reducing the impact of BRD in a sustainable cattle production.

This conceptual analysis presents arguments for combatting BRD at the population level through improved external biosecurity in cattle herds. The rationale for such a program will be given by describing the current impact of BRD, the effect of the current preventive measures and the likely effect of additional biosecurity improvements. Bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCoV) are two important causative agents in BRD. The Norwegian control program for BRSV and BCoV is presented as an example of a novel and alternative strategy to prevent and reduce BRD. Challenges of such a program and relevant differences between Norway and other areas are also discussed.

CURRENT IMPACT OF BRD

BRD is a common disease in cattle worldwide, both in feedlots and non-feedlot husbandry systems (2, 3). In US feedlot cattle, BRD is the most frequently reported illness (4). In Norway, it is the most commonly diagnosed disease and the most common cause of mortality in calves (5, 6).

BRD has negative effects on the animals' life and the producers' economy. It is a major cause of morbidity, mortality and economic loss in both the beef and dairy cattle industries (7, 8). Fatalities, treatment costs, and handling of sick animals contribute to the economic losses in the acute stage of an outbreak. Considerably higher number of animals are usually found to have lung-lesions at slaughter, compared to the number of clinical BRD cases in a herd. This indicates that observed clinical cases represent only part of the problem (9). The long-term consequences are also less recognized, but reduced feed conversion, growth rate and performance might contribute considerably to the total economic losses. A long-term reduction in weight gain (7 months) was seen following a BRD outbreak among bulls in Norway (10). Calves with BRD have also been found to produce less milk when they reach first lactation (11). National studies from the UK have estimated costs associated with BRD amounting to £80 million annually for the cattle industry (12). The only scientific publication where the national-level economic effect of BRD has been estimated is from France, where an epidemiology- and productivity model was used to link BRD incidence with productivity in the different cattle industry sectors (13). The authors found that eradication of BRD in beef calves would increase the whole beef sector's productivity by 4.7–5.5%, but that the benefits from eradication would differ between enterprises. For example, young bull and veal feedlot enterprises were estimated to increase in productivity by 8.7–12.8% while the breeding farms would gain less (5.1–6.0%) (13).

Antimicrobial usage in animals may affect both public health and the environment (14). BRD and mastitis are the two main causes of antimicrobial usage in cattle worldwide, and accounts for the main quantity of antimicrobials used. Respiratory disease is the most common reason for metaphylactic antibiotic therapy in the US. 71% of feedlot cattle receive antimicrobials in feed, and 13.4% are treated with injectable antimicrobials to prevent or treat BRD (4). A wide variety of antimicrobials are used, usually broad-spectrum antibiotics including those recommended for human use only (4). In Denmark, BRD accounts for 79% of antimicrobials used in veal calves and young bulls (15). Also in Norway, BRD is the main reason for therapeutic antimicrobial usage in both dairy calves and in the beef industry (16). Reduction of BRD would significantly reduce the total use of antimicrobials in the cattle industry and by that reduce the risk of antimicrobial resistance development.

Livestock contribute to the total human-induced greenhouse gas emissions, with cattle production accounting for the majority (60%) of the livestock sector's emissions (17). Practices that improve production efficiency, such as better health management, are examples of interventions that reduce greenhouse gas emissions from livestock (17). BRD is a major production–limiting disease in both the dairy and beef industry (4, 8, 18), hence reduction of BRD is a relevant intervention to reduce the emissions from the livestock sectors. Delabouglise et al. (13) also concluded that enhancing BRD control, particularly in beef breeding farms, would substantially increase the productivity of the French cattle industry, reduce its environmental impact and satisfy consumers' demand (13).

For BRD, the severity of clinical signs, the high incidence of chronic cases, and the high mortality and morbidity estimates underscore the importance of limiting BRD to improve animal health and welfare. Freedom from disease is a fundamental aspect of animal welfare.

TODAYS' PREVENTIVE STRATEGIES–ARE THEY SATISFACTORY?

The multifactorial nature of BRD and the global differences in production systems of beef and dairy cattle have led to a variety of prevention strategies. Common for all strategies are attempts to either improve the animal environment, strengthen the general health and host immunity and/or minimize animal exposure to the relevant pathogens. Vaccination and preventive antimicrobial medication are the most common preventive measures, with the aim to keep a low infection pressure and/or helping the host to

combat infection. All preventive measures focus on the single animal or herd as the unit of interest.

Mass Medication With Antimicrobials

Mass medication involves administering antimicrobials to groups of animals, either as preventive/prophylactic treatment or as metaphylaxis. Murray et al. (1) concluded in a review that mass medication provides inconsistent control of BRD and poses a serious concern regarding the effect on emergence of antimicrobial resistance. A meta-analysis of randomized, controlled clinical trials concluded that antimicrobial prophylaxis and metaphylaxis demonstrated moderate, yet highly variable reductions in the relative risk of BRD morbidity (19). The most substantial reductions of relative risk were from critically important broad-spectrum antimicrobials. However, metaphylactic treatment with macrolides were found to have no effect on incidence of BRD in a controlled trial (20). In addition, a high prevalence of multidrug resistant *Mannheimia haemolytica* has been found in cattle after metaphylaxis and treatment for BRD (21). Baptiste and Kyvsgaard (19) also concluded that BRD prophylaxis/metaphylaxis represents a major driver of antimicrobial consumption for highly variable short-term gains in terms of absolute risk reduction of morbidity and mortality. The use of mass medication can hardly be seen as in accordance with the current strategy to prevent antimicrobial resistance through prudent use of antibiotics recommended by the World Health Organization, United Nations, Food and Agriculture Organization and World Organization for Animal Health (14). It is therefore necessary to promote control of BRD without the use of antimicrobial mass-medication.

Vaccination

The use of vaccines to reduce the impact of BRD in dairy and beef cattle is common practice worldwide, although the practice lacks convincing scientific support. The development of effective vaccine programmes has been challenging (1). The short duration of immunity provided by vaccines against mucosal viral infections and the need to vaccinate immunologically immature calves in the presence of maternal antibodies have led to suboptimal effect of vaccines and challenge the cost-benefit of its use (1, 22). The effect of vaccination on herd immunity depends on the efficacy of the vaccine, but also on vaccine management such as the proportion of animals vaccinated and the timing of vaccination (23). Several authors have reviewed the vaccine efficacy of BRD vaccines, with conflicting results in calves and feedlot cattle (24, 25), and a systematic review and meta-analysis assessing the effect of commercially available BRD vaccines showed no significant difference in the risk of BRD in vaccinated calves, compared to controls (26). Despite years of research and advances in vaccine development, the use of vaccines has not provided the wanted effect against BRD.

Management to Maintain Good Animal Health and an Optimal Environment

Improvement of the environment can favor healthy development of animals with a robust immune system. Management factors including excessive handling, commingling, and movement of animals increase the risk of BRD due to stress and immunosuppression (3, 27). An important management factor is a good routine for adequate intake of colostrum (8, 28). Annual and seasonal variation in mortality rates due to BRD have been documented, with increased rates during winter (3, 29). This has been partially explained by higher animal density during confined housing, poor ventilation and inclement weather (12). Studies from Scandinavia have found that reduction of the animal density and age span in group-pens along with 1 week of isolation of new-borns from adult cows may prevent BRD (30, 31). Nevertheless, maintenance of good health alone does not result in sufficient reduction of BRD (8), and despite education and consulting of producers on optimal management strategies, it may be difficult to achieve the desired results.

CAN INCREASED HERD BIOSECURITY PREVENT BRD?

Altogether, optimizing management for improved animal robustness against infections, vaccination and mass medication contribute to reduction in the occurrence of BRD. However, despite improvements in our understanding of pathogenesis, the pathogens involved, vaccine technology and means of prevention and treatment, BRD remains one of our most important cattle health concerns in intensive cattle production. The effect of the current preventive measures is not satisfactory, and time is ripe for a novel approach. Can improved biosecurity provide a solution to the problem?

Biosecurity is a set of management and physical measures designed to reduce the risk of introduction, establishment and spread of animal infections or diseases to, from and within an animal population (32). National level biosecurity implies that restrictions on import of live animals and biological products are in place to protect a population from introduction of new infectious agents. External biosecurity refers to measures aiming at preventing introduction of disease into herds. Internal biosecurity relates to limiting transmission of infectious agents between animals or groups within a herd. For BRD, internal biosecurity measures have been reviewed with a focus on factors that limit pathogen exposure within the herds such as vaccination, housing, ventilation and control of other diseases (33).

In the following, herd level biosecurity will refer to external biosecurity at the herd level, which so far has received little, if any, attention regarding BRD. The general herd level biosecurity is relatively low in modern cattle production, also compared to other livestock species such as poultry and swine (34, 35). Few biosecurity measures are usually undertaken, resulting in a constant risk of disease transmission between farms. Implementation of biosecurity measures is hampered by factors such as cost, perceived usefulness, workload and lack of clarity as to how and why measures should be undertaken (34, 36–39). Improved herd level biosecurity can be implemented in single herds, or on a regional or national level. To justify efforts to control BRD through improved herd level biosecurity, the following questions need to be addressed: is BRD a transmissible

disease between herds? If so, is it possible to stop the transmissible infectious agents at the farm gate? And can these agents be eliminated from infected herds?

Is BRD a Transmissible Disease Between Herds?

BRD is a multifactorial disease, and can be caused by a specter of pathogens, often in combination. Viral pathogens such as BRSV, bovine herpesvirus 1, bovine parainfluenza virus 3, bovine viral diarrhea virus and BCoV can cause disease directly, and/or predispose animals to bacterial infections (40–44). Most of these primary BRD pathogens are highly contagious viruses that can easily spread between herds (29, 44), either directly through live animal contact/movement, or indirectly through contaminated environment or fomites brought between herds. The most common bacterial agents are *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni* (44–46). These bacteria appear to have lower transmissibility, and bacterial disease in several animals is therefore most likely a result of exposure of animals to the same risk factors, such as virus infection and/or suboptimal environment, at the same time (47). *Mycoplasma bovis* can also contribute to BRD, either as primary or secondary pathogen. Live animal movement seems to be the primary means by which *M. bovis* is transmitted between herds (48).

Variations in the potential for between-herd-transmission between bacterial and viral pathogens affects how effective herd biosecurity is at reducing risk of introduction. The effect of increased biosecurity will therefore vary depending upon which pathogens are present in the area of interest, and their relative contribution to BRD development. The effect of increased biosecurity on the risk of introduction is likely larger for virus than bacterial components of BRD.

Several important BRD pathogens are absent or eradicated in Norway, such as BVDV, bovine herpesvirus 1 and *M. bovis* (49). This highlights the impact of two other viruses in the BRD complex; BRSV and BCoV. Both are highly prevalent in the Norwegian cattle population (31, 50) as they are in most parts of the world, both in intensive and extensive husbandry systems (51, 52). BRSV has been reported responsible for 60% of the BRD epidemics observed in dairy herds (42, 53, 54) and up to 70% in the beef herds (40, 41). In Norway, BRSV has been reported as the main etiological agent causing BRD outbreaks (55). BCoV causes BRD (56) in addition to winter dysentery (contagious acute diarrhea in adult cattle) and diarrhea in calves (52), which further increases the negative consequences of BCoV (57).

Both BRSV and BCoV can be easily transmitted between herds, and epidemics with rapid spread between herds within a region have been reported (57, 58). Modes of transmission are either directly through live animal contact (59) or indirectly via contaminated personnel or utensils brought between herds (60). Herds with limited or no purchase of cattle may also experience outbreaks of BRD, most likely due to introduction of infectious agents by indirect routes, and/or that the causative pathogen was already circulating in the herd (61). Indirect transmission depends upon the stability of the viruses outside the host, which is generally short for enveloped RNA viruses such as BRSV and BCoV. However, there are uncertainties regarding the stability of both viruses. Under laboratory conditions, BCoV remained infective for 2 weeks under cool and moist conditions (62). For both BRSV and BCoV, temporary carriage of virus on fomites has been shown: infective BCoV was detected on fomites (clothes, boots and equipment) 24 h after exposure to virus-shedding calves, while for BRSV, only viral RNA, and no infectious virus, was detected (60). The same study found that personnel in contact with virus-shedding calves carried both BCoV and BRSV RNA on nasal mucosa, but none were positive for infective virions. It was therefore concluded that transmission of virus via human nasal mucosa is likely limited (60). Airborne transmission for BRSV and BCoV has been shown indoors (63) but is most likely restricted to droplet and aerosol spread. Airborne transmission across longer distances, i.e., between farms, has not been described and is likely of limited importance. Transmission of virus from other species to cattle has never been demonstrated and is likely to be of minor importance under normal circumstances.

In conclusion, BRSV and BCoV can be easily transmitted between herds via live animal movements or indirectly via contaminated fomites brought between herds. Airborne transmission and transmission from other animal species such as wildlife, is less likely.

The high impact of BRD in Norway despite freedom from several of the well-recognized pathogens indicates the importance of BRSV and BCoV as key contributors to BRD. Because they are easily transmitted between herds, it can be argued that BRD is a transmissible disease between herds.

Is It Possible to Stop the Viruses at the Farm Gate?

Because purchase of cattle is an important risk factor for introduction of respiratory pathogens (54, 59), closed herds could be a means to prevent BRD. However, breeding enough replacement animals might not be practical or possible in all systems. Other measures to prevent introduction via live animals to a herd includes purchase of known virus-free animals, routines for safe loading and transportation of animals and isolation of arriving animals. Examples of measures to avoid introduction by people or fomites are establishing infection control sluices including routines for changing boots and clothing upon entering a herd (64), and to avoid bringing contaminated equipment between herds. Safe loading of animals can also prevent indirect transmission.

A recent study from Belgium identified both BRD in general, and especially BRSV infection, as main adult cattle diseases where biosecurity measures should be prioritized (65). Toftaker et al. (59) showed that the odds of being positive for one virus were approximately five times larger if a herd was positive for the other virus, indicating some common risk factors for BRSV and BCoV. Ohlson et al. (64) found a clear association between higher herd biosecurity levels and lower prevalence of herd infection. Implementation of external herd biosecurity routines, such as control sluices, and measures for safe trade are likely to

reduce transmission between herds. It would reduce the risk of introduction to the herd where it is implemented, but also the risk of further spread from that herd.

How Can the Viruses Be Eliminated From Infected Herds?

If introduction of BRSV and BCoV to herds can be avoided, the next question is; what happens with the already infected herds? When BRSV and BCoV cause acute infections in individual animals, the viruses replicate locally in the respiratory epithelial cells and are shed in exhaled air and nasal secretions (43, 51). BCoV also infects enterocytes and is excreted in feces (43). Experimental studies have shown shedding of BRSV from day three to nine post infection (46), and from day two to ten for BCoV (66, 67). Viral RNA can be detected for an extended period (67, 68), but might not represent infective virus. Both infections give short-lived immunity (69–71). Introduction of virus to a herd usually results in rapid spread and high within-herd prevalence. This is particularly seen during the winter season (50, 72). Depending on factors such as herd size, management and the immunity of the herd, viruses may continue to circulate due to subclinical infections in naïve animals and/or reinfections with viral shedding in seropositive animals (43, 73).

Some data indicate that persistence of BRSV and BCoV in individual animals is possible. Infective BRSV has been isolated from lymph nodes 71 days post infection (74). BCoV persistence has been demonstrated in cell culture (75). Long-term PCR positivity in calves has been shown in one experimental study, but transmission potential was not confirmed by virus isolation/sentinel trials nor was sequencing of virus done to exclude new infection (76). The epidemiological role of such persistence in individual animals is somewhat unclear, but transmission of reactivated virus to susceptible animals has never been shown.

In a longitudinal study, repeated sampling of dairy herds showed that 32–42% of the herds changed their BRSV antibody status from positive to negative based on pooled calf sera during a 6-month time period (50). Similar results have been found for both BRSV and BCoV in Swedish dairy herds (77). This indicates rapid self-clearance of virus from herds without specific interventions. Molecular epidemiology supports this view—virus varies both temporally and spatially between outbreaks, suggesting that outbreaks are caused by introduction of new virus rather than through reactivation or the existence of carrier animals (78–80). This implies that with the current herd size and management conditions in the Nordic countries, herds can self-clear from virus if new introduction is avoided.

POSSIBLE ALTERNATIVE APPROACHES TO COMBAT BRD AT A REGIONAL, OR NATIONAL LEVEL

Given the substantial impact of BRD and unsatisfactory results of existing preventive measures, alternative strategies to combat BRD are urgently needed. Stakeholder interest is of fundamental importance to succeed in animal disease control. Furthermore, a herd level control program requires a cost efficient and reliable method for classification of herds as well as adequate disease monitoring.

Producer Attitudes to Regional Disease Control Efforts

A systematic approach to BRD control implies systematic measures to reduce the incidence of the transmissible infectious agents, and in the Norwegian example, for BRSV and BCoV infection on herd level, implemented on a sectoral, regional or national level. Is this achievable? The answer depends on cultural and structural conditions of the cattle industry in the area of interest. Introduction of virus could, in principle, be prevented in any herd. However, a synergetic effect can be acquired if measures are implemented by most of the herds in an area or country. How successful a herd's biosecurity measures are also depend upon the infectious pressure from the outside. If this is reduced due to better biosecurity in surrounding herds, the benefit will be mutual. A central, joint organization to run a program through is therefore an advantage. In Norway, the largest dairy company (TINE SA) is a co-operative owned by the producers and 96% of the dairy herds report to the Norwegian dairy herd recording system, where membership is voluntary (81). This probably contributes to high compliance in voluntary control efforts established by the industry.

A producer's willingness to implement management strategies or disease control programs has been found to be influenced by individual values and beliefs, by other producers, the industry or the government (82). Earlier positive experiences with disease-control makes it easier to introduce new projects. For example, the Norwegian producers likely have a stronger willingness to participate in joint disease-control efforts, also for non-reportable diseases, due to the successful elimination of bovine virus diarrhea from the cattle population in 2006 (83). This program was established in 1992 by the dairy- and breeding organizations, in collaboration with the animal health authorities. Ringworm due to *Trichophyton verrucosum* has nearly been eliminated due to an eradication program that combined vaccination and zoosanitary measures (84). Cost-benefit analyses of previous national control programs in the dairy cow and goat sectors have proven that the efforts paid off (85, 86). Motivation is crucial and necessary in order to succeed in implementing measures that requires extra effort.

Herd Classification and Disease Monitoring

In order to monitor the disease situation at the population level, a suitable classification system for herds is needed. Different sample material can be used, and a diagnosis can be made on individual animal level or at the group/herd level. Generally, infection with BRSV and BCoV can be diagnosed by detection of virus, viral antigen, or viral RNA in tissues, secretions, or excretions of infected animals (43, 78, 87). Antiviral antibodies are usually detected by commercial ELISA tests and there is a good agreement between titers in serum and milk for both viruses (88).

During viral shedding, nasal swabs (BRSV/BCoV) and feces (BCoV) can be used for antigen detection or for genome

detection by RT-PCR (87). Antibody detection can also be used during outbreaks but requires paired acute and convalescent samples. Serological investigations are used for retrospective diagnostics and screening studies (prevalence), often at the herd level. Because animals are seropositive for many years after infection (66, 89), seropositivity is a slow-changing indicator which indicates previous exposure to virus, but not necessarily presence of virus.

The herd-sensitivity and herd-specificity of a diagnostic test is influenced by the basic performance of the test, the within-herd prevalence and the number of animals tested. Misclassification can arise as a result of imperfect test performance or changes in status after testing. Imperfect test performance could also be due to a suboptimal test-regime with regard to which, and how many, animals are tested.

The interpretation of testing will depend on the age and number of animals sampled. Bulk tank milk serology can provide an estimate of herd-level seroprevalence of BRSV and BCoV (90). The method is cheap, but the result only reflect that there has been virus present in the herd during the last years. Sampling of a group of younger animals has also been used, with the assumption that the selected animals are representative for their age group in the herd e.g., pooled milk samples from primiparous cows. As they usually are 2–3 years old, the sample will reflect a herd's infection-history 2–3 years prior (77). Serum from a group of calves under 1 year of age will indicate virus circulation within the last year, if calves young enough to have maternal antibodies are excluded. Classification of herds based on serological analysis of a group of animals is therefore possible, and the different options have pros and cons with regard to cost and value. We reiterate that the gap between seropositivity and virus presence is considerable. A seronegative herd is, on the other hand, a good indicator of a virus-free herd, and in the context of a control program, finding the free herds might be most important.

THE NORWEGIAN BRSV AND BCoV CONTROL PROGRAM

The recently launched Norwegian BRSV and BCoV control program is presented as an example of a national level control program based on systematic improvement of external biosecurity at the herd level. The program contains no vaccination or mass-treatment. A brief description of cattle production in Norway is included for context, followed by an outline of the chosen method for herd classification and the applied biosecurity measures.

Milk production in Norway is extensive and based on small, mostly family run-farms. The number of dairy herds is around 8,300, with an average herd size of 27 cows in 2018. For members of the Norwegian dairy herd recording system, production data is available to advisors and veterinarians. Many producers rear their own heifers and keep bull-calves for slaughter, which means that young stock and adult cows are often kept in the same or nearby facilities. The number of beef herds is 3,600. These are predominantly suckler-cow herds with an average number of 23 cows, which rear their calves until slaughter (16). There is no tradition for specialized beef production, but over the last decade, several cow-calf operations with beef-breeds (or beef-crosses) and a few fattening units have been established.

In a nationwide study of 134 randomly selected Norwegian dairy herds, Gulliksen et al. (31) found 31.2% of the calves in 71.1% of the herds to be positive for antibodies to BRSV, while the same numbers for BCoV were 39.3% and 80.7%, respectively (31). Toftaker et al. (59) found the prevalence of seropositive herds in bulk tank milk to be 46.2% for BRSV and 72.2% for BCoV in two counties in the western part of Norway. Large variations were found in prevalence across the study region, with high risk clusters as well as overall geographic trends. Negative herds were found in close proximity to positive herds (59). About 40% of the herds were positive for antibodies to both viruses, while 22% were negative for both.

The control program for BRSV and BCoV was initiated by a joint cattle industry and launched in Norway in 2016. The goal of the program is to reduce the occurrence of BRSV and BCoV in the national cattle herd. A key feature is to classify all herds according to BRSV/BCoV antibody status (sero-positive or -negative) and protect animals in both positive and negative herds from infection through herd biosecurity measures. Vaccination or antimicrobial treatment is not included in the implementation plan, and vaccination against BRD is usually not practiced. Knowledge of herd status is assumed to motivate producers to implement the recommended measures for prevention of virus introduction. Furthermore, sero-negative herds who can document specific additional biosecurity measures are eligible for financial incentives.

The costs of the Norwegian control program are shared between producers and the industry. The dairy industry financed the initial screening of dairy herds and the meat industry financed testing of beef herds. After that, the cost of one testing per year per herd is covered by the industry. A project leader is employed by the industry partners and responsible for information flow and education of veterinarians, producers and others within and outside the organizations.

Classification of Herds

The principle of herd classification in the control program is illustrated in **Figure 1**. Specifically, dairy herd classification is based on serological examination of (1) bulk tank milk, (2) pooled milk samples from first-parity cows, or 3) pooled serum from young stock. If testing of bulk tank milk indicates sero-positivity, producers are encouraged to test pooled milk samples from four first-parity cows. If this yields a positive result, testing of pooled serum from young stock is recommended. Only homebred, unvaccinated animals above 180 days of age (to avoid maternal antibodies) are tested in (2) and (3). If four animals are not available, three and two may be used. Beef herds are tested using young stock only. The system is the same regardless of housing conditions or size.

All samples are tested with a new multiplex immunoassay for BRSV and BCoV antibodies (MDV-Enferplex BCV/BRSV multiplex from Enfer Scientific, Naas, Ireland). The sensitivity and specificity for the bulk tank application of the test have been

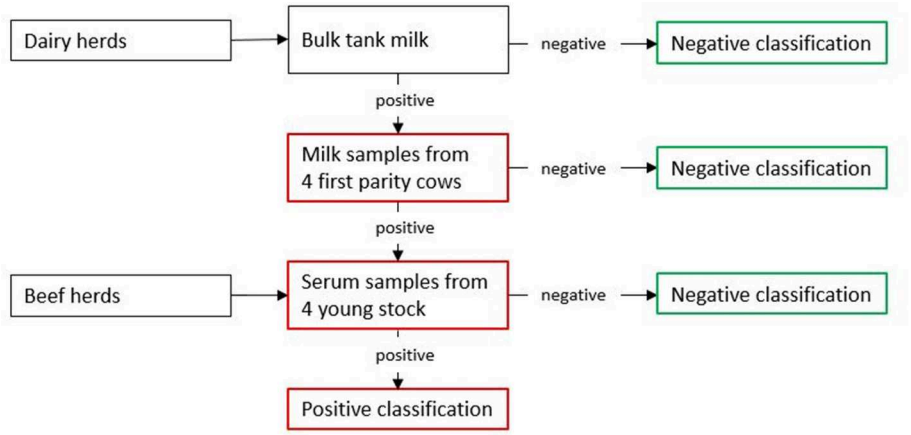

FIGURE 1 | Classification of herds in the Norwegian control program for bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCoV). Samples of milk and serum are analyzed for antibodies against BRSV and BCoV using a BCoV/BRSV multiplex immunoassay.

estimated to 94.4 and 90.6 for BRSV and 99.9 and 93.7 for BCoV, respectively (90).

All producers automatically receive free material for collection of samples. Untested herds and herds with inconclusive results are classified as positive in the program. A negative status is valid for 1 year, and the producers are automatically reminded when new testing is recommended. Purchase of animals from positive herds automatically leads to positive status.

Biosecurity Measures

The recommended biosecurity measures aim at protecting herds from introduction of virus via direct (live animals) and indirect (people and fomites) routes. All producers are encouraged to avoid live animal contact between negative and positive herds by purchasing animals or sharing pastures with animals only from sero-negative herds. Live-animal trade is organized by the producer organizations, both for replacement animals and for animals shipped to slaughter. Since the launch of the control-program, separate transport vehicles have been used for animals from negative and positive herds. Farmers are encouraged to build suitable loading areas for shipment of live animals. Furthermore, improved external biosecurity is encouraged by implementing restricted human access into herds. There is a legal requirement to provide sluices where veterinarians, AI technicians, advisors, claw trimmers, service people and others can change to protective clothing and footwear provided by each herd. Advisory support from the program is provided to ensure a feasible design of these sluices. In general, the advice is for the herd to provide clothing and footwear for visitors, washing facilities with cold and hot water and suitable storage areas for equipment.

To encourage compliance with the control program, herds can acquire a "Healthy herd status" by fulfilling a set of specific criteria. These criteria include having a sero-negative status for both BRSV and BCoV. In addition, the herd needs a veterinary certificate confirming high external biosecurity through the implementation of a physical barrier sluice. A loading-area for shipment of live animals to and from the herd is also required, to enable the truck driver to access the animals without entering the barn. A "Healthy herd" status is rewarded with an increase of approximately 10% in price when selling young-stock and breeding animals.

A final measure to be mentioned is the establishment of a "hot-line," where producers report episodes of diarrhea or respiratory disease by phone. This is done to enable rapid discovery of possible outbreaks, and a notification leads to warning of relevant personnel (e.g., field practitioners and milk truck drivers) such that necessary precautions can be taken to avoid further disease transmission and increase the vigilance in the area.

DISCUSSION

We have presented arguments for biosecurity-based control of BRD and outlined the ongoing Norwegian control program for BRSV and BCoV. We argue that successful population-level disease control is possible through external herd level biosecurity measures but that several conditions must be met.

Generally, the requirements for initiating a control program will differ according to biological factors (species affected, zoonotic potential, reservoir, population structure and basic characteristics of the infectious agents etc.), possible control measures (movement control, stamping–out, isolation, vaccination etc.), availability of technical tools (diagnostics tests, treatment) and socioeconomic considerations (91). Lindberg and Houe (92) concluded that for successful control of bovine viral diarrhea virus (BVDV), three elements are necessary: basic biosecurity, elimination of virus from infected herds and monitoring to evaluate progress and detect new infections/reinfections. Despite considerable biological differences between BVDV and BRSV/BCoV, the same three same elements are also fundamental in the control of BRSV and BCoV.

The first element, biosecurity, is the primary focus in the control program. The aim is to reduce risk of introduction of virus both through live animals, people and fomites. A critical point is the efficacy of the recommended protective measures. This may differ according to management system and herd structure. For example, large herds have been shown to have more visitors and thereby more indirect contacts compared to smaller herds (34). This can partly explain why large herd size is a frequently reported risk factor for herd level positivity to both BRSV and BCoV (58, 59, 64, 93, 94).

The effect of the recommended protective measures also depends upon the compliance to these, where the motivation among stakeholders, veterinarians and producers is crucial. It is also an ongoing measure that needs to be nourished over time. Basic education, as well as a continuous flow of updated information, is necessary. Knowledge about the occurrence of the infections is useful to motivate action. The impact of BRD is well-recognized among farmers and veterinarians in Norway, and they are usually aware that BRSV and BCoV are the primary pathogens, and that BCoV also causes winter dysentery. This probably makes it easier to motivate the producers for control of BRSV and BCoV in Norway compared to countries with other Specters of BRD pathogens. For BRSV and BCoV, the documented varying prevalence, and presence of negative herds in high-prevalence areas (50, 59), shows the Norwegian producers that it is possible to stay negative also if neighbors are positive. For regions with higher prevalence of BRSV and BCoV, an important step forward would be to perform an antibody-screening with a classification method that gives a recent picture, for example investigation of first-parity cows or young stock before concluding that all herds are positive. For countries with severe problems due to other BRD pathogens such as BVDV, *M. bovis* and IBR, it is probably wise to focus on these pathogens first. However, the preventive measures will generally have positive effect on the transmission of many other infections.

The second element, elimination of virus from infected herds, receives little attention in the program as self-clearance is regarded likely. This is probably more effective in small herds, and the small average herd size in Norway is therefore an advantage. In larger herds, naïve cattle in sufficient numbers might be available all the time, and both acute, subclinical infections and possible persistent infections are more likely. Altogether, control might be more challenging in areas where herds are larger, and more intense monitoring might be necessary. Nevertheless, biosecurity-based control might still succeed if new introduction of virus is avoided, as it will most likely be a question of time before virus cease to circulate also in larger herds.

The third element, monitoring of progress, is based on the feasibility of the classification of herds, and the frequency of the testing. There is a need for herd-level diagnostic tools that accurately classify the herds in a cost-efficient manner. Serological investigations will result in an overestimation of prevalence, as earlier discussed. In the Norwegian test-regime the small average herd size might cause few first-parity cows or calves to be available, consequently reducing the herd-sensitivity. The within-herd prevalence is to some degree unknown and probably variable between herds, and within groups in the herds, which further complicates the matter. In the control program, the test-result is valid for a full year. The probability of virus introduction after classification is considerable, particularly in herds that purchase animals. An updated herd-classification based on the combination of bulk milk tank testing, herd size, information on animal movements and geographical location has been shown to provide a more accurate estimate of herd status (95) and could potentially improve progress of the program.

Altogether, herd size influences all the three fundamental elements discussed here. It is also where the Norwegian situation differs considerably from most European countries. Our average dairy herd comprises 27 cows and suckler-cow herds 23, and there is an absence of feed-lots as well as few and small fattening herds. In addition, herd size might also influence the time until a new infection is detected. In Norway, the number of animals tested is the same regardless of size. In herds with many animals a control program with a more intense diagnostic test regime regarding both number of animals tested and frequency of testing, might be necessary. Herd sizes are increasing in Norway, which coincides with an increase in the recorded number of infectious diseases (96). Infection control in areas with larger herds is therefore likely to be more challenging, but even more necessary and rewarding if successful.

Stakeholders and producers are obviously concerned with the costs related to a control program; is it worth it? The financial losses due to BRSV and BCoV in Norway were analyzed by the industry prior to onset of the program. This included the available knowledge of the viruses' effect on BRD and winter dysentery, and the costs of running a control program were weighted against the impact (not published). It was concluded that controlling BRSV and BCoV would be cost-efficient and should be prioritized. There are several uncertainties in such an analysis. In a study from France, the authors assumed that a reduction of BRD incidence between 20 and 50% was a realistic outcome to expect from improvements in farm biosecurity (13), but further studies that link epidemiology and livestock productivity in a larger scale is needed.

The situation in Norway with few transmissible and notifiable diseases highlight the large impact of BRSV and BCoV. Control of these highly contagious viruses require a systematic approach, and a cooperative culture with a common goal. Previous experience from systematic eradication and control of other diseases might have contributed to a culture for disease control through prevention and joint efforts in Norway. Successful control of BRSV and BCoV here could motivate to action also in other countries. Effects on public health is a profound reason for animal disease control. The expected benefits is considerable regarding the usage of antimicrobials and antimicrobial resistance, in agreement with the present OIE strategy (90). Another expected "by-product" of the control program is the likely reduction of infections caused by other pathogens transmitted via the same routes, both endemic and

emerging pathogens. The Norwegian BRSV and BCoV control program indicates a way forward in how to achieve improved animal health and welfare.

CONCLUDING REMARKS

Antimicrobial resistance is a major public health threat. Growing concerns regarding the environmental impacts of livestock calls for new and innovative measures to prevent endemic diseases, and thereby improve the sustainability of the cattle industry. An alternative strategy to combat BRD is urgently needed. We believe it is both desirable and possible to control BRSV and BCoV, and subsequently reduce BRD, through biosecurity measures. The Norwegian initiative represents a new way of thinking that will likely have wider implications. The ultimate goal is improved animal health, welfare and a reduction in antimicrobial usage in the cattle sector as well as a more effective production.

AUTHOR CONTRIBUTIONS

Development of concept was done during a walk-shop where all authors contributed ideas and input. Drafting of the manuscript was led by MS, with contributions and critical review from all authors. All authors have read and approved the final product.

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Performance of Online Somatic Cell Count Estimation in Automatic Milking Systems

Zhaoju Deng[1], Henk Hogeveen[1,2], Theo J. G. M. Lam[1,3], Rik van der Tol[4] and Gerrit Koop[1]*

[1] Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands, [2] Chair Group Business Economics, Wageningen University and Research, Wageningen, Netherlands, [3] GD Animal Health, Deventer, Netherlands, [4] Farm Technology Group, Wageningen University and Research, Wageningen, Netherlands

Somatic cell count (SCC) is one of the most important and widely used mastitis diagnostics. For detecting (sub)clinical mastitis, online SCC related measurements are more and more used in automatic milking systems (AMS). Sensors such as an automated online California Mastitis Test (O-CMT) allow for high frequency screening of high SCC cows within a herd, which makes it potentially powerful to identify episodes of mastitis. However, the performance of O-CMT measurements, as compared to SCC determined in the laboratory (L-SCC), has only scarcely been described. The aims of this study were (1) to assess the agreement between the O-CMT measurement averaged over different time windows and the corresponding L-SCC measurements; (2) to determine the optimal time window for averaging O-CMT as compared to L-SCC; (3) to explore the added value of time-series of frequent O-CMT measurements in individual cow udder health monitoring compared to L-SCC measurements. Data were collected from 50 farms in 6 different countries that were equipped with AMS using O-CMT measurements and also performed regular L-SCC testing. We found that the overall concordance correlation coefficient (CCC) between O-CMT and L-SCC was 0.53 but differed substantially between farms. The CCC between O-CMT and L-SCC improved when averaging O-CMT over multiple milkings, with an optimal time-window of 24 h. Exploration of time series of daily O-CMT recordings show that this is an effective screening tool to find episodes of high SCC. Altogether, we conclude that although O-CMT agrees moderately with L-SCC, because of its high measurement frequency, it is a promising on-farm tool for udder health monitoring.

Keywords: somatic cell count, online-California mastitis test, udder health monitoring, on-farm screening tool, automatic milking machine, mastitis, dairy cow

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****Correspondence:***
Zhaoju Deng
zhaoju.deng2014@gmail.com

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INTRODUCTION

Mastitis is one of the main diseases in dairy cattle and leads to economic losses, usage of antimicrobials, and reduced animal welfare (1, 2). Udder health monitoring programs including regularly measured somatic cell count (SCC) have been widely used as a first step to improve udder health (3). These monitoring programs create awareness of udder health problems which, combined with mastitis prevention plans, motivate farmers to change on-farm udder health management to decrease the incidence of mastitis (4, 5).

The SCC of composite cow milk, as part of a dairy herd improvement (DHI) program, is a key indicator for udder health monitoring in current practice (6) and is generally measured using flow cytometry-based laboratory techniques (7). This routinely measured SCC in the laboratory (L-SCC) has long been the standard for udder health monitoring (8). The collection and shipping of samples for SCC measurement, however, is costly and time consuming and therefore generally DHI testing is done only every 3–6 wk. More frequent measurements would allow for earlier diagnosis, but requires an on farm test that can be performed at low per sample costs. The online California Mastitis Test (O-CMT) measurement is an automated sensor for mastitis monitoring in dairy farms with an automatic milking system (AMS).

The principle of the O-CMT sensor evaluated in our study is based on an automated CMT by taking a fixed volume of well-mixed composite milk from a cow milking. The milk is mixed with a fixed volume of reagent, after which the viscosity of the mixture is measured. The measured viscosity is transformed into a value, expressed in cells/mL, based on a calibration curve (9). The O-CMT is not comparable to L-SCC in terms of test characteristics, missing data, calibration and quality control, but due to frequent measurements, it may serve as a useful on farm screening tool. Although a single O-CMT measurement may not be precise, averaging multiple O-CMT recordings within different time windows may be helpful in gaining precision. Thus, we assume frequently measured O-CMT averaged over a certain time window may yield a better correlation to L-SCC.

Until now, a number of comparisons between SCC measured on farm and L-SCC have been published (10–15). Due to the characteristics of the gelling process of the mixture, the agreement between O-CMT and L-SCC was found to be poorer in low SCC ranges (< 200,000 cells/mL; 9), while higher ranges of SCC (> 500,000 cells/mL) show a fair to good correlation (12). Hence, the performance of the O-CMT likely depends on the udder health situation of the farm. However, the performance of O-CMT in a large number of herds with varying udder health status is unknown and thus the practical value of this frequent O-CMT measurements in the field is unclear. Therefore, the aims of this study were (1) to assess the agreement between O-CMT measurements in different time windows and L-SCC measurements under field conditions; (2) to determine the optimal time window for averaging O-CMT as compared to L-SCC; (3) to explore the added value of time-series of frequent O-CMT measurements in individual cow udder health monitoring compared to L-SCC measurements.

MATERIALS AND METHODS

Data Collection

Routinely collected O-CMT data from January 1st, 2015 to April 29th, 2016 from AMS farms having an O-CMT sensor system produced by Lely Industries N.V. (Maassluis, the Netherlands) and the DHI milk production recording data from the same farms over the same period were provided by Lely Industries N.V. Details of the data collection can be found in Jensen et al. (16). The data consisted of the rough, non-validated data that farmers also use. In all datasets, country and farm identifications were transformed to non-traceable identifications by Lely because of privacy concerns. The AMS data consisted of country and farm identification, cow identification, parity, calving date, date and time of milking and O-CMT measurement. The default measurement frequency of O-CMT was every third milking. When a cow had a high SCC (>200,000 cells/mL), the measurement frequency became higher. Farmers were advised to calibrate the sensor twice per year using standardized cow milk sample provided by Lely. The DHI data consisted of farm identification, cow identification, DHI test date and L-SCC. The L-SCC were tested in different laboratories, depending from which country the farm was. Because of the position of those laboratories in the milk payment scheme, the laboratories were certified (ISO 13366-1) to ensure the quality of measurements. This study was carried out in accordance with the commitments contained in the Basel Declaration and adhered to the General Data Protection regulations of the European Union. As no animal experiments were performed, no ethical approval was required for this study.

Data Preparation

In the dataset we observed a small proportion (0.009%) of O-CMT being from milkings with an extremely low milk yield (<1 kg). Incomplete milkings with O-CMT might occasionally be present in our dataset. The raw dataset contains 7,427,010 records and was cleaned as follows:

1) records (n = 95,669) with composite milk yield per milking <1 kg were deleted;
2) records (n = 153,735) within 7 days after calving were deleted because of the confounding effect of early lactation on the SCC;
3) records with no O-CMT values (n = 4,527,244) or O-CMT = 1,000 cells/mL (n = 39,455) were deleted; The latter records were deleted, because the sensor automatically transforms all O-CMT ≤ 1,000 cells/mL to 1,000 cells/mL;
4) records from cows on L-SCC test dates when no L-SCC was available (n = 730) or with L-SCC ≤ 1,000 cells/mL (n = 377) were deleted;
5) records (n = 358,985) from cows with an L-SCC < 2, 000 cells/mL were deleted;
6) records (n = 2,693) from farms with ≤ 100 L-SCC measurements were deleted;
7) records from 7 days before until 7 days after the L-SCC test dates were selected for each cow. Within these 15 days (7 days before and after DHI test date plus the DHI test day) period, only records with valid O-CMT value for every day were selected, which resulted in 1,816,144 deleted records.

The resulting cleaned dataset used for further analyses. After cleaning the dataset, all SCC values were $\log_{10}$-transformed for the purpose of obtaining approximate normal distribution.

Abbreviations: SCC, somatic cell count; O-CMT, online California mastitis test; L-SCC, somatic cell count determined in the laboratory; O-CMT 24 h, average of multiple online California Mastitis Test measurements within a 24 h time window; IMI, intramammary infection; DHI, dairy herd improvement.

Assessment of Repeatability of O-CMT

Before the evaluation of agreement between the two tests, we assessed the repeatability of the O-CMT measurements. We defined an episode as being the period 48 h before and after an L-SCC test date. Consequently, the records within 48 h before and after the L-SCC test dates for each cow were selected from the cleaned dataset. Only episodes with ≥ 1 O-CMT measurements for every day were selected. A linear mixed regression model was constructed using the O-CMT measurements as dependent variable and episode, herd and cow as random effects. That way we were able to estimate the variance in O-CMT within each episode for each cow from every herd. Consequently, the intraclass correlation coefficient (ICC), calculated from the four variance components (episode, cow, herd, and the residual) extracted from this linear mixed model, represents the repeatability of O-CMT measurements (which equals to 1—the underlying "true" variation and the measurement error of O-CMT measurements within the episode).

Agreement Between O-CMT and L-SCC

Concordance correlation coefficient (CCC) between two continuous measurements is one of the most commonly used methods to evaluate the agreement between two tests (17). In this study, we calculated the CCC between O-CMT and L-SCC to evaluate the measurement performance of single O-CMT and its averages calculated over multiple time windows.

Single Comparison

For the single comparison between O-CMT and L-SCC, all L-SCC and a randomly sampled O-CMT record per cow on the DHI test dates were selected. We first examined the CCC between the selected O-CMT records and the corresponding L-SCC records using the Bland-Altman plot (18) to display the relationship between O-CMT and L-SCC. Meanwhile, we calculated the CCC between these O-CMT and L-SCC records.

Because DHI test results only had a test date and no time stamp, for each DHI test date, there were possibly multiple O-CMT records. All of these O-CMT records were used in the CCC calculation with equal weight in determining the optimal time window that would result in the highest CCC between average of multiple O-CMT and L-SCC.

Time Window for Averaging Multiple O-CMT

To determine which time window, using the average of O-CMT measured within, resulted in the highest correlation between the O-CMT and the L-SCC, 7 time windows centered around the DHI test dates were created. Time windows were constructed as multiples of 24 h before and after the center of the DHI test date, leading to 7 time windows (spanning 24, 48, 72, 96, 120, 144, and 168 h). We first selected the records within the 168 h time window (168 h before and after the DHI test date) for each cow and each DHI test date from the dataset. The records within the 168 h time window for each L-SCC record of each cow were regarded as an episode. For each episode, the number of O-CMT measurements per day was counted. Episodes were included when they were from farms that had at least 100 episodes with ≥ 1 O-CMT measurement(s) on every day within the episode. For each episode, the average of O-CMT for each of the 7 time windows was calculated.

To calculate the CCC, a linear mixed model was applied using the lme function in the nlme package [version 3.1–142; (19)]. To calculate the overall CCC of all farms, the test method (binary variable: O-CMT or L-SCC) was included in the model as fixed effect; random herd and random cow effect were also included in the model. To calculate the individual farm level CCC, test method and individual cow were used as fixed effect and random effect, respectively, by using the epi.ccc function in epiR package [version 1.0–11; (20)]. The CCC between the average of multiple O-CMT within different time windows and L-SCC were calculated for 3 different ranges of L-SCC (L-SCC within 1,000–9,999,000 cells/mL, 100,000–1,500,000 cells/mL (the performance range of L-SCC), 200,000–9,999,000 cells/mL).

In addition to identify the optimal time window, we tried to find potential factors associated with the individual herd level CCC at the optimal time window using the available data. A linear regression model was built using the individual herd CCC as dependent variable and herd average parity, monthly herd geometric mean of L-SCC and monthly herd milk yield as independent variable. A full model, as well as a model using backward selection based on AIC, were fitted. All analyses were performed in R version 3.6.2 (21).

Case-Wise Evaluation of O-CMT and L-SCC Measurements

The time window which resulted in the highest CCC in the previous analysis was used for calculating the moving averages for multiple O-CMT measurements over a longer time period. Four different O-CMT 24 h patterns were selected, which were representative of SCC patterns observed in field data. These selected O-CMT patterns were plotted along with the L-SCC measurements in the same time frame. In this way, the practical relevance of frequent O-CMT measurements in detecting high SCC episodes due to (sub)clinical manifestations of mastitis was illustrated.

RESULTS

Descriptive Statistics

The descriptive statistics of the final dataset for the calculation of CCC between O-CMT and L-SCC are provided in **Table 1**. In total, 434,371 records from 4,829 cows at 50 farms in 6 countries were used in the analysis. Large differences in herd size were seen between farms and countries, with farms from country 2 and country 3 on average being larger than other farms. Overall, O-CMT values were higher than L-SCC values. All the herd average L-SCC values were below 200,000 cells/mL and only farms from country 2 had a herd average O-CMT higher than 200,000 cells/mL.

Assessment of Repeatability of O-CMT

A total of 144,048 records from 14,504 episodes and 4,829 cows at 50 farms in 6 countries were used for the estimation of the repeatability of O-CMT measurements. The estimated ICC was 0.58, which suggests that 42% of the variance within the

TABLE 1 | General descriptive statistics of the farms in the cleaned dataset for SCC measured online (O-CMT) and SCC measured in the laboratory (L-SCC) and the data for calculating the correlation between both.

Country	Total number of farms	Average number of cows per farm	Number of records	Farm geometric mean of L-SCC (×1,000 cells/mL)	Farm geometric mean of O-CMT on L-SCC test dates (×1,000 cells/mL)	Farm average CV[a] of L-SCC	Farm average CV of O-CMT on L-SCC test dates per cow	Concordance correlation coefficient between L-SCC and single O-CMT one L-SCC test dates per farm
1	1	36	1,787	75	195	0.38	0.32	0.518
2	19	114 (12–475)	9,644 (485–34,644)	165 (49–336)	213 (83–339)	0.45 (0.37–0.53)	0.53 (0.46–0.61)	0.658 (0.241–0.851)
3	9	150 (69–278)	11,231 (4,302–29,613)	141 (75–262)	198.2 (132–313)	0.44 (0.36–0.52)	0.52 (0.43–0.62)	0.592 (0.437–0.778)
4	1	59	12,170	74	102	0.7	0.58	0.479
5	13	68 (17–161)	8,800 (579–28,623)	155 (39–315)	165 (79–309)	0.49 (0.37–0.61)	0.62 (0.51–0.72)	0.578 (0.341–0.759)
6	7	46 (28–62)	3,142 (1,955–4,959)	92 (45–141)	146 (97–230)	0.41 (0.34–0.49)	0.40 (0.35–0.46)	0.421 (0.136–0.533)
Total	50	97 (13–282)	8,693 (480–32,723)	145 (38–317)	186 (78–326)	0.46 (0.19–0.79)	0.53 (0.29–0.86)	0.525 (0.142–0.787)

A total of 2,361,484 records from 7,788 cows in 52 farms in 6 countries were included in the cleaned dataset. The numbers with parenthesis are averages and parenthesis are 2.5–97.5% quantile.

[a]*Coefficient of variation.*

FIGURE 1 | Bland-Altman plot displays the difference between single $\log_{10}$-transformed online CMT (O-CMT) values and $\log_{10}$-transformed laboratory measured SCC (L-SCC) against the average of both measurements on the DHI test days. Most of the records are within the limits of agreement. Overall, the differences between the two measurements are decreasing.

episode was due to the O-CMT measurement. However, it was not possible to distinguish the "true" variation between O-CMT measurements from measurement error of the O-CMT.

Concordance Correlation Coefficient Between L-SCC and O-CMT

Single Comparison

In total, 29,008 O-CMT records of 4,829 cows in 50 farms from 6 countries could be linked to 29,008 valid L-SCC measurements on the same day.

Figure 1 shows the Bland-Altman plot of the $\log_{10}$-transformed single O-CMT compared with the L-SCC measurement. The Bland-Altman plot suggests that the correlation between O-CMT and L-SCC is non-linear. The difference between these two measurements decreases in the high SCC area.

Figure 2A displays a scatter plot of the L-SCC and the randomly selected O-CMT measurement on each DHI test

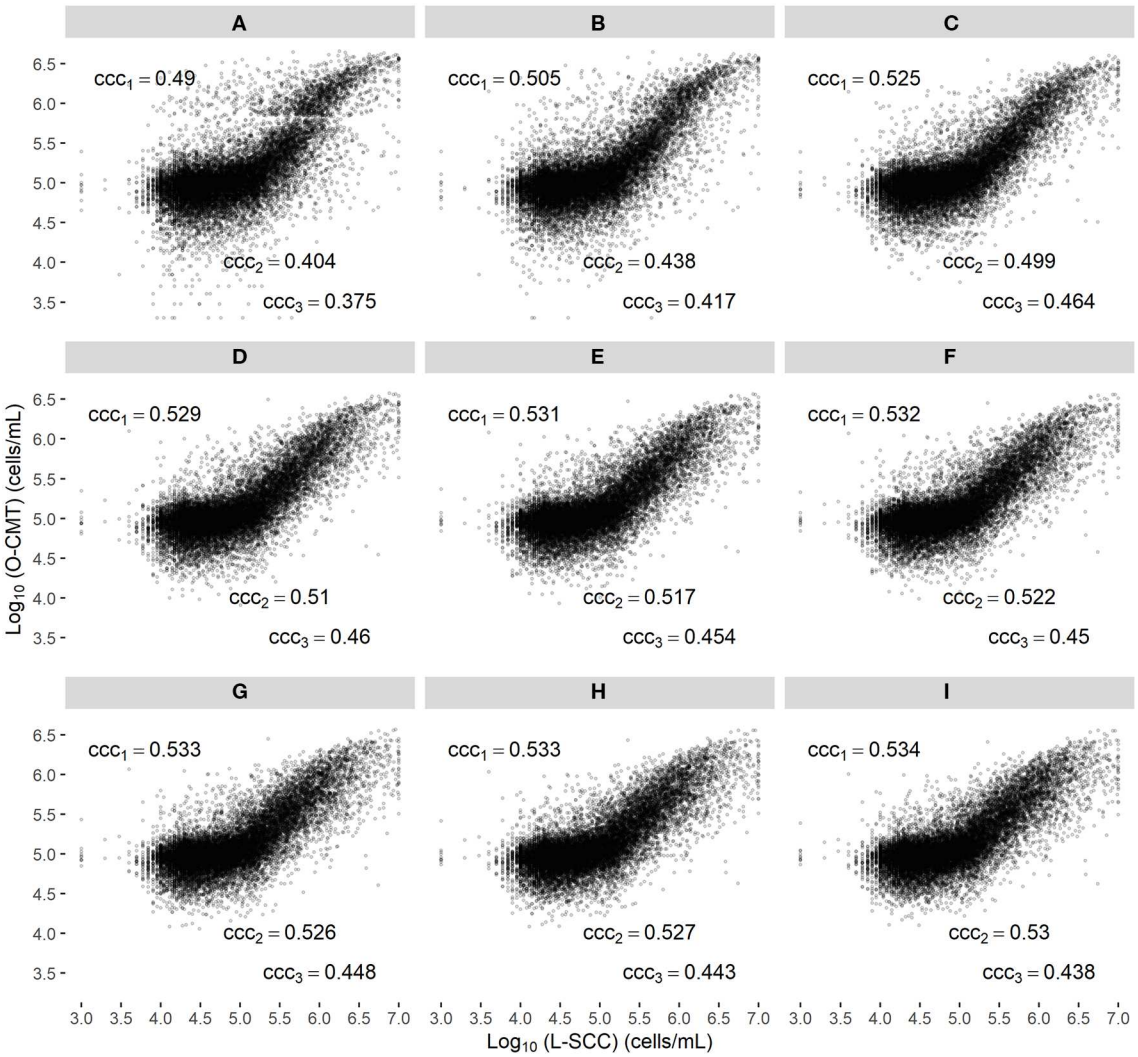

FIGURE 2 | Scatter plot of the log_{10}-transformed online CMT (O-CMT) values for randomly sampled one O-CMT records on DHI test dates against log_{10}-transformed laboratory measured SCC (L-SCC) **(A)** and the average of multiple O-CMT within different time windows against L-SCC (**B–I**, corresponding to time windows from 0 to 168 h, increasing by steps of 24 h); ccc_1 represents the overall concordance correlation coefficient between log_{10}-transformed O-CMT and log_{10}-transformed L-SCC, ccc_2 is the concordance correlation coefficient with L-SCC within the range of 100,000–1,500,000 cells/mL and ccc_3 is the concordance correlation coefficient with L-SCC in range of 200,000–9,999,000 cells/mL. Farms with ≥ 100 DHI tests with valid SCC results measured by O-CMT and L-SCC were included.

date per cow, and gives the CCC across several L-SCC ranges (1,000–9,999,000 cells/mL, 100,000–150,000 cells/mL, 200,000–9,999,000 cells/mL), showing that the agreement between L-SCC and O-CMT is better in the higher SCC regions but not necessarily with a higher CCC. The overall CCC between L-SCC and the average of O-CMT measurement within a 24 h time window was 0.53 (95% CI: 0.14–0.79).

Time Window for Averaging Multiple O-CMT

Figures 2B–I show that the CCC between averaged O-CMT within different time windows and L-SCC increased from **Figures 2A–C** (the 24 h time window) for all the 3 SCC ranges. The CCC in the 3 SCC ranges only increased marginally, when the time window was further expanded (**Figures 2D–I**). Therefore, we considered 24 h as the optimal time window to average the multiple O-CMT measurements in this study.

We found substantial variation in CCC between O-CMT 24 h and L-SCC between farms. The farm-level CCC was positively related to the farm's geometric mean L-SCC (**Table 2** and **Figure 3**).

Figure 4 gives the number of O-CMT records per L-SCC record in different SCC ranges for the 7 time windows. It is obvious that the number of O-CMT measurements does increase with longer time windows. Moreover, it is also visible that more O-CMT measurements are made when O-CMT is higher (> 200,000 cells/mL). A 0 h time window averages about 2 O-CMT values, whereas a 24 h time window contains on average about 5 O-CMT records.

TABLE 2 | Correlation between online SCC estimation and the SCC measured in the laboratory in different studies.

Study	SCC estimation method	Country	Number of farms	Number of AMS or SCC sensors[a]	Number of cows	Number of records	Correlation
Casura et al. (10)	CMT[b]	NA[c]	1	NA	298	2,331	0.57
Leslie et al. (11)	CMT	Canada	1	2	140	1,000	0.71
Kamphuis et al. (12)	CMT	New Zealand	1	2	200	456	0.76
Mollenhorst et al. (13)	CMT	Netherlands	3	6	191	3,191	0.47
Neitzel et al. (14)	CMT	Germany	1	7	165	1,357	0.2–0.57
Sørensen et al. (15)	Flow cytometry	Denmark	7	>16	2,325	713,326	0.93[d]
Current study	CMT	6 countries	50	113	4,829	434,671	0.53[e]

[a]*Automatic milking system or online somatic cell count sensor.*
[b]*SCC estimated based on the California mastitis test principle.*
[c]*Not found.*
[d]*The square root of R squared from regression using log-transformed L-SCC as dependent variable and log-transformed O-CMT as independent variable.*
[e]*Concordance correlation coefficient between average of online-SCC within a 24 h time window and the SCC measured in laboratory.*

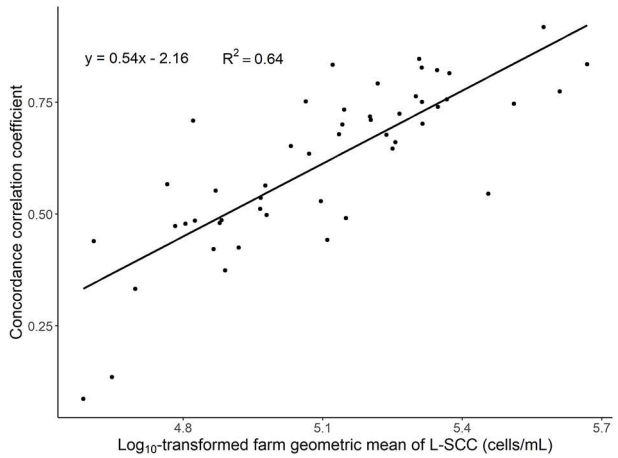

FIGURE 3 | Scatter plot of concordance correlation coefficient between the average of multiple $\log_{10}$-transformed online measured CMT (O-CMT) values over a 24 h time window and $\log_{10}$-transformed laboratory measured SCC (L-SCC) against the $\log_{10}$-transformed geometric mean herd SCC per farm on 50 farms. The regression line has a beta estimate of 0.54 and the R-squared is 0.64.

Case-Wise Comparison of O-CMT With L-SCC Measurements

Figure 5 displays 4 different SCC patterns from 4 different cows that were representative of our data. Overall, the O-CMT 48 h patterns were corresponding to the L-SCC patterns for each cow, **Figure 5A** shows a healthy udder before 130 DIM, with indication of two short (new) intramammary infection (IMI) occurring around 134 and 162 DIM, and of a chronic persistent IMI starting around 190 DIM; **Figure 5B** shows an IMI in early lactation that seemed to have cured between 64 and 180 DIM with indications of a new IMI in late lactation; **Figure 5C** presents an udder with a chronically persistent IMI with large variation in day-to-day O-CMT 48 h; **Figure 5D** indicates a healthy udder with a brief IMI in the late stage of lactation.

DISCUSSION

In this study, we aimed to evaluate the performance of O-CMT measurements in comparison to L-SCC. The value of O-CMT measurement is an estimation of SCC within ranges instead of an exact measurement of SCC (9). Hence the O-CMT values should be interpreted with caution. The overall CCC between O-CMT within a 24 h time window and L-SCC in 50 farms was 0.53 (95% CI: 0.14–0.79). The CCC increased most when averaging O-CMT over a 24 h time window. Our results suggest that frequent O-CMT measurement is a valuable on-farm tool for monitoring udder health of individual cows, despite the fact that a single O-CMT measurement may be less accurate than a single L-SCC measurement.

The data we used in this study consisted of rough, non-validated data, representative of how the data arises in practice. The samples from the O-CMT differed from the samples for the L-SCC. Besides that, it is clear that there is a lower level of quality control for the O-CMT measurements, for instance by non-optimal calibration procedures, in comparison to the L-SCC measurements. This may jeopardize the agreement between the two tests. Therefore, a direct comparison between the measurement systems in order to establish the preciseness of the O-CMT measurement is impossible with our data. However, by comparing the O-CMT measurements with the L-SCC measurements on milk from the same cow on the same day, we were able to provide insight in the practical usability of the O-CMT measurements.

Prior to the correlation analysis, we evaluated the repeatability of the O-CMT measurements within a 48 h time window assuming that the underlying SCC of a cow was stable within this 48 h time window (5 days). The repeatability, as represented by the ICC, was 0.58. Since natural daily variation in SCC exists, we consider the repeatability of O-CMT measurement to be acceptable within the period of 5 days.

We found an overall CCC between O-CMT and L-SCC of 0.53, which is in line with previous studies, that found values somewhat higher or lower than our estimate (**Table 3**). Previous studies, however, only used a small number of farms to assess

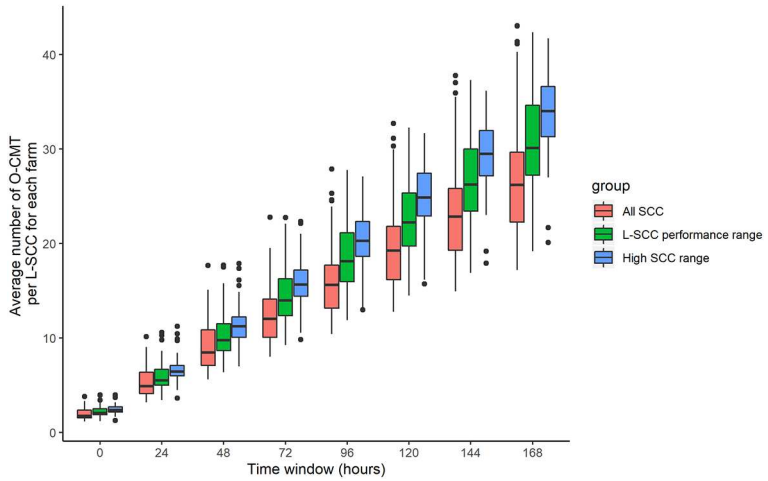

FIGURE 4 | Farm average number of online CMT (O-CMT) values per SCC value measured in the laboratory (L-SCC) for all O-CMT and for L-SCC performance range (100,000–1,500,000 cells/mL) as well as high SCC range (> 200,000 cells/mL) separately for different time windows.

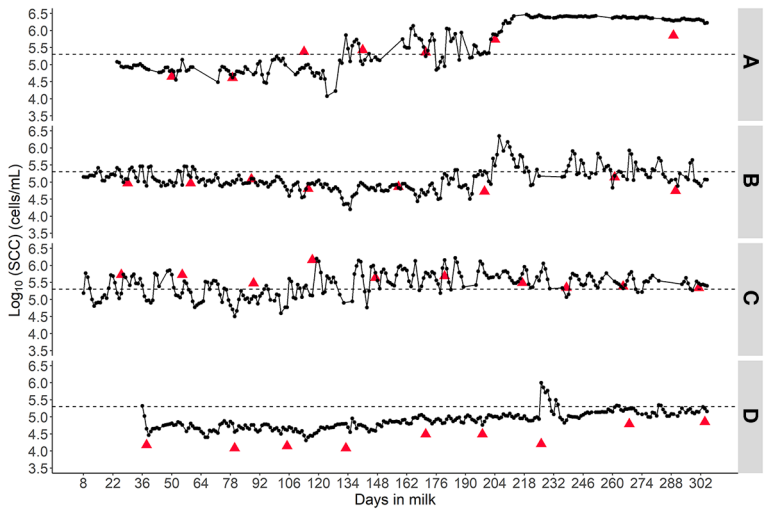

FIGURE 5 | Four different SCC patterns to demonstrate the value of frequently measured online SCC in individual cow udder health monitoring. **(A)** Indicates a chronic intramammary infection; **(B)** suggests an infected udder that cured followed by a re-infection; **(C)** displays a cow likely with chronic IMI that shows a fluctuating SCC pattern and **(D)** probably is a healthy udder with one brief high SCC episode. The triangles represent laboratory measured SCC results and the dots connected by a line represent the online CMT measurements averaged over a 24 h time window. The dashed horizontal line represents 200,000 cells/mL.

these correlations. In our data, we found a large variation in CCC between farms. This between-farm variation was largely explained by the farm level L-SCC (**Figure 3**), likely due to the fact that the correlation is higher in the higher SCC ranges. In other words, the CCC might depend on the prevalence of high SCC cows on farms. As displayed in **Figure 1**, the difference between O-CMT and L-SCC was deceasing as the herd average L-SCC increases. There are several other reasons for the fact that the CCC between O-CMT and L-SCC differs between farms. First, although the sensor are "factory calibrated" and farmers are advised to perform the calibration twice per year, not all farmers may actually have done this. Neitzel et al. (14) reported a significant difference between sensor devices in measuring the O-CMT and showed that the Pearson's correlation coefficient between O-CMT and L-SCC was higher after calibration. These differences in calibration between farms or sensors will likely have led to an underestimation of the true overall correlation between both SCC measurement methods relative to using well-calibrated sensors.

Although CCC between O-CMT and L-SCC was rather not sufficient, we consider there are several reasons for this imperfect agreement between O-CMT and L-SCC. First, the O-CMT evaluated in our study uses a different technique, based on a CMT derived method to quantify the O-CMT whereas L-SCC

TABLE 3 | Estimates from linear regression model using the herd level concordance correlation coefficient between online CMT and SCC measured in laboratory as dependent variable and the herd average of monthly geometric somatic cell count (SCC_{herd}), herd average parity ($Parity_{herd}$), as well as herd average monthly milk yield (Milk $yield_{herd}$) as independent variables.

Variable	Estimate	
	Full model	Backward selection model
Intercept	−2.33	−2.16
SCC_{herd}	0.55	0.54
$Parity_{herd}$	0.05	
Milk $yield_{herd}$	0	

Backward selection using AIC was applied for model selection. The full model included all the independent variables and the final model only with the variable remained in the model after model selection.

actually counts the number of cells using flow cytometry. The online sensor has an algorithm that transforms the viscosity of the gel formed by DNA and test reagent, to an O-CMT value based on calibration against L-SCC. Thus, by definition, the indirectly measured single O-CMT is less accurate than a single L-SCC measurement. Second, the performance range of L-SCC (the range in which its accuracy is guaranteed) is 100,000–1,500,000 cells/mL (22) while we noticed that more than half of the L-SCC measurements in our dataset were <100,000 cells/mL. Measurements outside the range in which the two tests perform well-contributed substantially to the imperfect correlation between these two measurements (**Figure 2A**). The scatter plots in **Figure 2** display weak S-shape, suggesting that the algorithm that transforms viscosity to an SCC value can be further optimized to better correlate to the L-SCC reference test. By adapting the transformation, the association between O-CMT and L-SCC can be made more linear, which should result in a higher (linear) correlation between the two. Lastly, although we did not evaluate that in this study, farmers may not re-fill the CMT reagent in time. Field experience learns this occurs and thus it may also affect the correlation between O-CMT and L-SCC.

With the availability of novel on-farm milk quality sensors, quality control of such measurements also has to be implemented on-farm. For decades, laboratories have calibrated their methods and compared their results, for instance by the use of ring trials. In contrast with these highly controlled laboratory systems, there is no systematic quality control system in place for automated on-line milk quality measurements. Since these on-farm milk quality systems become more and more important, it would be good if quality control programs for on-farm milk quality systems would be developed.

The L-SCC in our dataset were measured in different laboratories. Potentially there may be differences in L-SCC measurement between laboratories. However, data quality control in the laboratories for L-SCC measurements was assumed to be good because these laboratories are also involved in quality-based milk payment schemes and work under ISO certification (ISO13366-1). Meanwhile, by using a random herd effect in linear mixed models, potential laboratory effects were corrected for in the statistical modeling.

In **Figure 2**, we showed that the overall CCC between O-CMT and L-SCC in the range of 1,000–9,999,000 cells/mL, increased mostly at a 24 h time window. The overall CCC between O-CMT and L-SCC was increasing only slightly with longer time windows. There seems to be an optimum time window for averaging O-CMT, and we suggest 24 h as the optimal time window, in which the random error present in single measurements is strongly reduced, but the capacity to monitor infection dynamics over time is still acceptable.

The number of milkings with an O-CMT measurement per L-SCC measurement is substantially higher for high L-SCC (> 200,000 cells/mL) than for all SCC range, because of the algorithm that prescribes to measure O-CMT every milking after a high measurement is recorded, while the sensor only measures O-CMT every third milking in low SCC cows.

Figure 5 illustrates that the O-CMT measurements present the same trend as L-SCC, while giving more information on short high SCC episodes. This information is missed by L-SCC, given that DHI test is normally performed every 3–6 weeks, which limits the power of L-SCC in detecting high SCC episodes. Thus, O-CMT seems more valuable in individual cow udder health monitoring. In addition, there may be pathogen species that cause specific SCC patterns. De Haas et al. (23) found that clinical mastitis caused by *Escherichia coli* was significantly associated with a short peak in SCC while *Staphylococcus aureus* was significantly associated with longer increased SCC, whilst no clear patterns were found for *Streptococcus dysgalactiae* or *Streptococcus uberis*. Compared to traditional methods (e.g., bacteriological culturing), the use of frequent O-CMT measurements can serve as a cheap and fast on-farm screening method for mastitis. It is fully automated and can be executed for almost every milking. These characteristics make O-CMT and other on-line SCC measurement methods a suitable tool for on-farm individual udder health monitoring. The measurements may also be used to identify subclinical mastitis cases that warrant further diagnostics such as bacteriological culture to explicitly identify the mastitis-causing pathogens. Further research to link the O-CMT patterns to pathogen species would be useful and highly relevant to develop tailor-made treatment plans to further optimize treatment strategies and reduce antimicrobial usage. Our results show added value of O-CMT measurement, but to further quantify the added value of O-CMT in detecting high SCC episodes, more work is needed. Specifically, work should be carried out on algorithms to mine these intensively measured O-CMT for early detection of high SCC as well as to quantify long term udder health related effects (such as incidence rate of clinical mastitis, milk production, total antimicrobial usage) and the economic value of the use of O-CMT measurements.

CONCLUSION

The overall concordance correlation coefficient between O-CMT and L-SCC of all farms was 0.66, and increases when the farm level SCC is higher. The average of multiple O-CMT

measurements over a 24 h time window was found to provide an optimum between correlation between O-CMT and L-SCC and the capacity to capture udder health dynamics. The O-CMT measurement shows to be a promising on-farm tool for individual cow udder health monitoring, specifically because of its high measurement frequency.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the commitments contained in the Basel Declaration and adhered to the General Data Protection regulations of the European Union. As no animal experiments were performed, no ethical approval was required for this study.

AUTHOR CONTRIBUTIONS

ZD performed data analysis and wrote the manuscript. GK, TL, HH and RT designed the study. All authors interpreted the data, edited and approved the final manuscript.

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Conflict of Interest: TL was employed by the company GD Animal Health.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Evaluation of Heat and pH Treatments on Degradation of Ceftiofur in Whole Milk

*Adriana Garzon[1], Pramod Pandey[1], Lisa Tell[2], Sharif S. Aly[1,3], Robert Poppenga[4] and Richard Pereira[1]**

[1] Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, Davis, CA, United States, [2] Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, Davis, CA, United States, [3] Veterinary Medicine Teaching and Research Center, University of California, Davis, Tulare, CA, United States, [4] California Animal Health and Food Safety Laboratory, Davis, CA, United States

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***Correspondence:**
Richard Pereira
rvpereira@ucdavis.edu

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Waste milk feeding practices have been implicated as a potential source for disseminating antimicrobial resistant bacteria among animals and the environment. Two interventions that have shown potential for degrading antimicrobial drugs in milk are heat and pH treatment. The aim of this study was to evaluate the effect of heat and pH treatments on the degradation of ceftiofur and ceftiofur free acid equivalents in milk at concentrations previously found in waste milk on dairy farms by spiking saleable pasteurized whole milk with ceftiofur sodium. Three heat treatments of ceftiofur sodium spiked milk were evaluated for their ability to degrade ceftiofur: 63°C for 30 min (LTLT), 72°C for 15 s (HTST) and 92°C for 20 min (HTLT). Two pH treatments of ceftiofur sodium spiked milk were evaluated: pH 4.0 (LpH) and pH 10 (HpH). Control samples spiked with ceftiofur sodium were kept at room temperature and samples collected at corresponding times for heat and pH treatments. Four treatment replicates were performed for each treatment group. Ceftiofur was quantified in milk samples using liquid chromatography mass spectrometry (LC-MS/MS) and ceftiofur free acid equivalents (CFAE) were measured using high-performance liquid chromatography (HPLC). HTLT resulted in a degradation of 35.24% of the initial concentration of ceftiofur. Ceftiofur degradation did not differ between control and the remaining two heat treatment groups (LTLT and HTST). HpH resulted in degradation of the 95.72 and 96.28% of the initial concentration of ceftiofur and CFAE, respectively. No significant changes in degradation of ceftiofur or CFAE were observed for control or LpH treatments. In conclusion, our study results were that alkalinizing milk to pH 10 and heating milk to 92°C for 20 min degraded ceftiofur and CFAE in spiked simulated waste milk demonstrated promising potential as treatment options for degrading ceftiofur and CFAE in waste milk, and further research is needed to evaluate the viability for implementation of these treatments in dairy farms.

Keywords: antibiotics, β-lactams, drug residue, inactivation, waste milk

INTRODUCTION

Antimicrobials are undoubtedly one of the most important tools for preventing and treating diseases. Decreasing the rate of selection for drug resistance is of importance to both human and veterinary medicine. Non-saleable milk, also known as waste milk, is milk withheld due to pharmaceutical residues from lactating cows receiving drugs for therapeutic reasons. To reduce production losses due to waste milk, 30.6% of dairy farms in the U.S. feed waste milk to preweaned calves (1). Feeding calves waste milk has also been associated with antibiotic residues violations (2, 3). Slaughter withdrawal intervals recommendations for veal calves fed colostrum from cows receiving antibiotics during the dry period have been estimated (4). The disposal of waste milk with pharmaceutical residues can be laborious and costly to dairy farmers and could still represent a potential source for selection of resistance in the environment (5). There is therefore a need for approaches that would allow the sustainable use of waste milk without the selection of antimicrobial resistance or other unwanted outcomes.

A study by Pereira et al. (6), evaluated the impact of feeding waste milk spiked with residual concentrations of ampicillin, penicillin, ceftiofur, and tetracycline, according to the most prevalent drugs previously identified in waste milk on New York dairies (7). By the end of the trial, calves fed with milk spiked with antimicrobials had significantly higher proportions of *E. coli* resistant to one of six different antimicrobials, as well as multidrug resistant (MDR) *E. coli* (resistant to 3 or more drugs) compared to control calves fed milk without added antimicrobials (71% MDR treatment vs. 13% MDR control, $P < 0.0001$). Decreasing drug residues in milk could avoid the deleterious impacts of feeding waste milk on selection of drug resistance.

Degradation of β-lactam antibiotics in aqueous solutions is influenced by temperature and pH (8, 9). Ceftiofur beta-lactam is unstable in aqueous base (pH 10.0) and acid (pH 3.0) solutions (8). Acidification of milk to a pH between 4.0 to 4.5 and fed to preweaned calves is a practice that has become common in recent years in the US, with the objective of lowering the milk pH to a point where it is unsuitable for bacterial growth and survival without undesirable health side effects on calves (10). The impact of acidification of waste milk on drug residue degradation is currently unknown. Heat treatment of waste milk to reduce bacterial counts could potentially be an option for antimicrobial degradation. Roca et al. (11) reported degradation of 41.2% of cephapirin (a first-generation cephalosporin) in milk, after samples were heat at 63°C for 30 min, and further degradation of different beta-lactam drugs occurred when samples were exposed to higher temperatures (100°C). The aim of this study was to evaluate the effect of heat and pH treatments on the concentration of ceftiofur and ceftiofur free acid equivalents (CFAE) in milk, added at concentrations previously observed in waste milk on dairy farms.

MATERIALS AND METHODS

Spiked Milk Samples

Saleable pasteurized homogenized whole milk (3.25% fat content) was spiked using stock solutions of ceftiofur, as previously described (6). Briefly, 60 mg of ceftiofur sodium powder (93.6% purity, Sigma-Aldrich, St. Louis, MO) was diluted in 93.6 ml of distilled water (Millipore Corp., Bedford, MA) with 0.96% of dimethyl sulfoxide (Cell Signaling Technology, Danvers, MA, USA) added to increase the solubility of ceftiofur, to a stock concentration of 600 μg/ml, which was used to spike a volume of 3 l of milk, to a final concentration of 200 ppb for heat treatment trials and 400 ppb for pH trials. Stock solutions were stored in individual vials at −80°C until used. Concentrations of ceftiofur targeted in milk batches were based on previously reported concentrations of ceftiofur in waste milk on dairy farms in the US (7, 12).

A total of four repetitions with new milk batches were conducted for each heat treatment and pH assay. This number of repetitions was based on reported references for heat and pH stability of antimicrobials, where we estimate an 80.5% statistical power to identify a significant difference between samples after treatment when compared to the control group ($\alpha = 0.05$, standard deviation = 0.22, difference to detect = 0.18) (8, 11, 13).

Heat Treatment

Three heat treatments were evaluated, where two temperature and time combinations were based on pasteurization treatments used for waste milk on dairy farms: low temperature—long time (**LTLT**), where samples were heated to 63°C (145°F) and held at that temperature for 30 min; high temperature—short time (**HTST**), where samples were heated to 72°C (161°F) for 15 s; and high temperature—long time (**HTLT**), where samples were heated to 92°C (197.6°F) and held at that temperature for 20 min. A control group was maintained at room temperature, and samples from this group were collected at the corresponding times for the three heat treatment samples.

The same initial milk batch spiked with ceftiofur was divided in four aliquots and used for each heat treatment group to reduce between treatment group variations for each repetition. Collected samples were stored at −80°C until drug quantification. Four replicates were performed for each treatment group. Outline of heat treatment procedure is displayed in **Supplemental Figure 1**.

pH Treatment

Two pH treatments were evaluated: low pH group (**LpH**), prepared by adding diluted formic acid to milk and gently stirring until a pH of 4.0 was achieved; and high pH group (**HpH**), prepared by adding sodium hydroxide to milk samples and gently stirring until a pH of 10.0 was achieved. The pH was measured using a pH meter (basic pH meter 840087, Sper Scientific ltd., Scottsdale, AZ). A control milk group (pH ∼6.5–6.7) kept at room temperature was used as a control sample, and samples from the control group were collected at the same time points as samples for the pH treatment groups.

Similar to heat treatment protocol, the same initial milk batch was divided in three aliquots after spiking with ceftiofur and used for each pH treatment and control group. Samples collected were stored at −80°C until drug quantification. Four replicates were performed for each treatment group. All milk treatment groups were gently stirred before samples were collected at each time point, as well as every 6 h after beginning of testing. Outline of pH treatment procedure is displayed in **Supplemental Figure 2**.

Chromatographic Analysis

Ceftiofur was quantified in samples using liquid chromatography mass spectrometry (LC-MS/MS) at the California Animal Health & Food Safety toxicology laboratory (Davis, CA). This approach only quantified ceftiofur, and not desfuroylceftiofur. Sample analysis was performed using a LC-MS/MS method described in the Food and Drug Administration (FDA) LIB# 4443 (14). The limit of quantification (LOQ) of the assay was 100 ppb of ceftiofur in milk. Samples below the limit of detection after treatment were analyzed using 10 ppb of ceftiofur as final concentration.

Concentrations of ceftiofur free acid equivalents (CFAE) were measured using high-performance liquid chromatography (HPLC). CFAE was quantified only for samples from the high pH treatment group, due to significant ceftiofur degradation in the high pH trial. The method has been described in a previous study (15). Briefly, dithioerythritol was used to cleave any macromolecules bound to desfuroylceftiofur in milk and to convert the parent drug and metabolites to desfurylceftiofur. The sample was then run through a C18 solid phase extraction (SPE) column (Thermo Scientific, Rockwood, TN, USA) and derivatized with iodoacetamide to create desfuroylceftiofur acetamide. After elution from the C18 SPE column, further clean-up was done on a strong cation exchange (SCX) SPE (Agilent Technologies, Santa Clara, CA, USA). The HPLC analysis was done isocratically (mobile phase was 7% acetonitrile, 1% acetic acid, with 90 mg heptane sulphonic acid/liter, and pH = 4.0) on a Nova-Pak C18, 4μm, 3.9 X 150 mm (Waters Corporation, Milford, MA, USA) with UV detection at 240 nm. The standard curve was made in milk with a range from 0.01 to 1.0 μg/ml. Quality control samples were spiked to obtain a final concentration of 200 ppb for heat treatment trials and 400 ppb of Ceftiofur for the pH trials, with average recovery rate of 94%.

Statistical Analysis

Assumption of normality for ceftiofur and CFAE concentration from pH treatment trials was tested using Shapiro-Wilk W test, and assumption of homogeneity of variance was tested using Levene's test using JMP. If assumptions were maintained, analysis was conducted using JMP Pro 14 (SAS Institute, Cary, NC). To evaluate the effect of pH treatment over time on the degradation of ceftiofur and CFAE, multivariate mixed models were fitted to the data using the GLIMMIX procedure of SAS. Two models were generated where the dependent variables were ceftiofur and CFAE. Independent variables offered to the model were treatment (e.g., control, LpH, and HpH), sampling time points and the interaction between treatment and time points. The effect of individual sample identifier as well as trial number was controlled in all the models as a random effect. Because samples from HpH resulted in multiple ceftiofur concentrations in milk below the limit of detection (10 ppb), a more conservative approach was used to evaluate the data where samples with a ceftiofur concentration below the 10 ppb detection level, were labeled as having a ceftiofur concentration of 10 ppb. Tukey-Krammer pairwise comparison between all different treatment groups and time points was conducted. When either Shapiro-Wilk W test or Levene's test was rejected, the non-parametric Dunn All Pairs for joint ranks test in JMP was used to evaluate if there was a significant difference in the ceftiofur concentration in milk between pH treatment groups and control samples for each time point using pairwise approach. The Dunn All Pairs for joint ranks test was chosen because it has been shown to be a better choice because it has been shown to be a more powerful test for detecting differences between extreme treatments, and because joint ranking procedure have been shown to have slightly higher power than the pairwise ranking, reducing the risk of type 2 errors (16).

Assumption of normality for ceftiofur concentration from temperature treatment trials was tested using Shapiro-Wilk W test, and assumption of homogeneity of variance was tested using Levene's test using JMP. To evaluate the effect of heat treatment over time on the degradation of ceftiofur, multivariate mixed models were fitted to the data using the GLIMMIX procedure of SAS. Independent variables offered to the model were treatment (e.g., control, HTLT, LTLT, and HTST), sampling time points and the interaction between treatment and time points. When either Shapiro-Wilk W test or Levene's test was rejected, the non-parametric Dunn All Pairs for joint ranks test in JMP was used to evaluate if there was a significant difference in the ceftiofur concentration in milk between temperature treatment groups and control samples for each time point using pairwise approach. A $P \leq 0.05$ was considered statistically significant for analysis conducted in this study.

RESULTS

Heat Treatment Group

The results of the heat treatment assay are displayed in **Figure 1**. The least square means (**LSM**) for the initial concentration of ceftiofur for the heat treatment was 128.5 ppb (95% confidence interval 121.07–135.96). Data for ceftiofur concentration for heat treatment rejected the Shapiro-Wilk W test, and the Dunn All Pairs for joint ranks test was used for this analysis.

Control sample collected from pool of milk following spiking and mixing of milk at room temperature, prior to any heat treatment, did not significantly differ from control samples collected at timepoints 15 s, 20 min, and 30 min (**Supplemental Table 1**). There was a significant degradation of ceftiofur when samples were heated at 92°C and held to that temperature for 20 min (HTLT) compared to the control group ($P < 0.016$) (**Supplemental Table 1**), with the final LSM for ceftiofur at 83.22 ppb (CI 95% 76.62–89.81). The degradation of ceftiofur in milk did not significantly differ between the control, HTST and LTLT, with LSM observed at 129.9 (CI 95% 124.29–135.68), 127.56 ppb (CI 95% 120.96–134.15) and

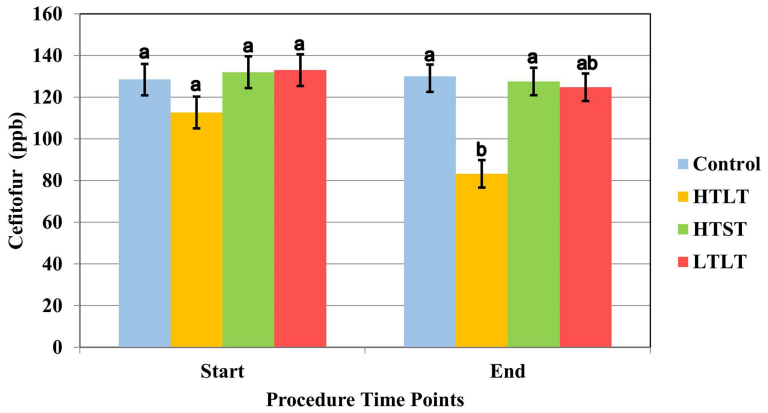

FIGURE 1 | Least square mean of ceftiofur (LSM ± SD) upon target temperature (start) was reached and at the end of each heat treatment protocol for milk samples heated at 63°C for 30 min (**LTLT**), 72°C for 15 s (**HTST**) and 92°C for 20 min (**HTLT**) and control. Letter reflect the results for Dunn All Pairs for joint ranks non-parametric test, and different letter indicate a significant difference between treatment group within each time point.

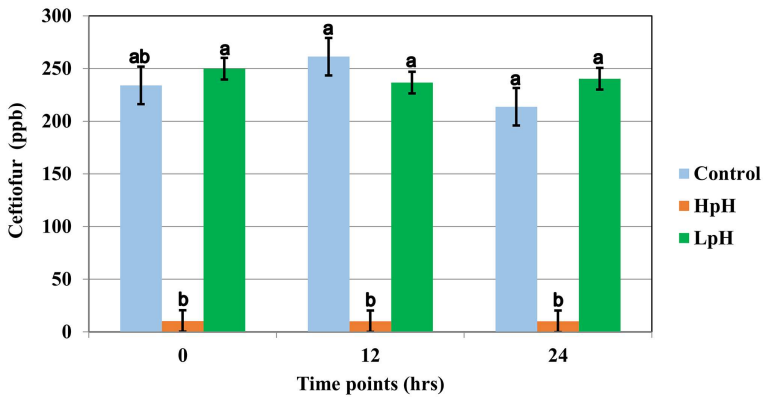

FIGURE 2 | Least square mean of ceftiofur (LSM ± SD) following spiking milk (Ct), upon target pH, and 12 and 24 h after target pH was reached for milk samples reaching a pH = 10 (**HpH**), milk samples reaching a pH = 4.0 (**LpH**) and control sample. Letter reflect the results for Dunn All Pairs for joint ranks non-parametric test, and different letter indicate a significant difference between treatment group within each time point.

124.78 ppb (CI 95% 118.18–131.37), respectively (**Figure 1** and **Supplemental Table 1**).

pH Treatment Group

Ceftiofur concentration for treatment group HpH was below detection levels for 8 of 12 samples at timepoint "0," which was collected immediately after adding sodium hydroxide to milk samples and gently stirring until a pH of 10.0 was achieved. For HpH group, all samples collected at timepoints 12 h and 24 h were below the detection limits (10 ppb for ceftiofur). Data for ceftiofur concentration for pH treatment rejected the Shapiro-Wilk W test, and the Dunn All Pairs for joint ranks test was used for this analysis.

The results of the pH treatment assay are displayed in **Figure 2**. The LSM for the initial concentration of ceftiofur for pH treatment was 234 ppb (CI 95% 216.19–251.80). The LSM concentration of ceftiofur was 213.75 ppb (CI 95% 195.94–231.55) for normal pH and 240.33 ppb (CI 95% 230.04–250.61) for low pH but declined to 10 (CI 95% −0.28–20.28 ppb) ppb immediately after sodium hydroxide was added and pH 10 was achieved, resulting in a significant degradation of ceftiofur when compared to the control group ($P < 0.0001$) (**Figure 2** and **Supplemental Table 2**).

Control sample collected from pool of milk following spiking and mixing of milk at room temperature, prior to any pH treatment, did not significantly differ from control samples collected at timepoints 12 h and 24 h (**Supplemental Table 2**).

Quantification of Ceftiofur Free Acid Equivalents

The results of the high pH treatment assay on the concentration of CFAE in ceftiofur spiked whole milk are shown in **Figure 3**. Neither normal variance nor equal variance assumptions for the use of a liner regression model were rejected for the CFAE dataset. The mean initial concentration of CFAE was 286.5 ppb (CI 95% 252.40–320.59). The concentration of CFAE in samples decreased to a mean of 113.58 ppb (CI 95% 82.1–144.84) after

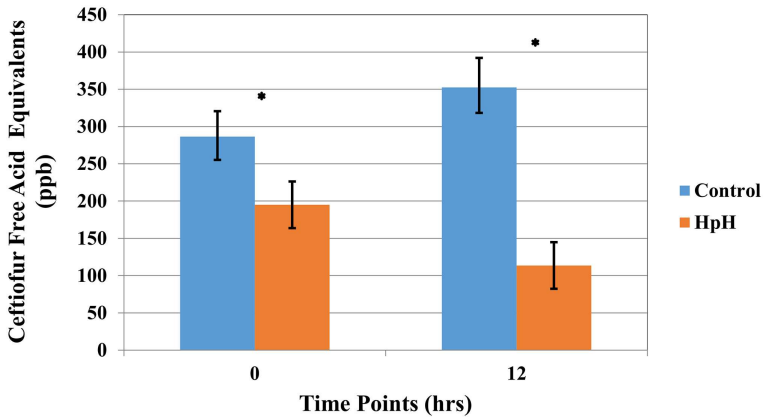

FIGURE 3 | Least square mean of ceftiofur free acid equivalents (CFAE) (LSM ± SD) following spiking milk (Ct), upon target pH, and 12 h after target pH was reached for milk samples reaching a pH = 10 (**HpH**) and control sample. Asterisk represents time points where a significant difference was observed between HpH and control for that same time point.

milk was alkalized to pH 10, resulting in a significant degradation of ceftiofur when compared to the control group ($P < 0.0001$) (**Figure 3** and **Supplemental Table 3**).

DISCUSSION

Heating ceftiofur spiked milk at 92°C for 20 min resulted in a significant reduction in ceftiofur and CFAE concentrations when compared to the control treatment. Similar to our findings, Zorraquino et al. (17) evaluated heat treatment of five cephalosporin drugs, not including ceftiofur, and observed an inactivation of over 90% of cephlosporin drugs tested when milk samples were heat treated at 92°C for 20 min, and between 6 and 18% degradation when heat treated at 60°C for 30 min. A difference compared to our study is that their study did not measure drug concentrations using chromatographic methods but instead a bioassay based on the inhibition of *Geobacillus stearothermophilus* var. *calidolactis*. A potential concern with heating milk at 92°C is the possible effect on nutrient content. Higher temperatures have been shown to decrease the percentage of soluble whey proteins in milk due to denaturalization (18). Our results support that further research should be conducted to evaluate the viability of introducing an approach that uses temperatures higher than those traditionally used in the dairy industry.

No significant degradation of either ceftiofur or CFAE was observed using the HTST or LTLT treatments when compared to the control group, indicating that time as well as heating temperature are critical factor for effective ceftiofur degradation. HTST and LTLT are common practices for pasteurization of waste milk fed to calves with the goal of lowering bacterial contamination (19). A study by Li et al. (20) evaluated the effect of temperature on the degradation of ceftiofur in aqueous solutions with or without addition of recycled water derived from a beef farm. Samples were incubated at 15, 25, 35, and 45°C. Ceftiofur hydrolysis rate in deionized water without wastewater increased from 0.1 to 5.4 × 10^{-3} h^{-1} as temperature increased from 10 to 45°C, which represented a hydrolysis rate increase of 3.8 times by each 10°C increased in temperature. A difference in our study was the effect of all other components in milk that can results in a different degradation dynamic then that observed in water. Half-lives of cephalosporins other than of ceftiofur in milk, have been shown to be between 32 and 90 min at 70°C, and 40 to 127 min at 60°C (11). Horton et al. (21) reported that for complete degradation of cefquinome, a fourth generation cephalosporin, milk required a heat treatment at 50°C for more than 72 h, resulting in a 86% degradation after 48 h of incubation ($t_{½}$ = 30.9 h). Our results indicated that hydrolysis of cephalosporins at 63°C and 72°C may require longer time than standard pasteurization protocols currently being used by the dairy industry.

Treatment of ceftiofur spiked milk using a pH of 10 resulted in a significant and prompt degradation of ceftiofur and CFAE, although the latter occurred at a slower pace. A study by Horton et al. (21) observed similar results, with increasing milk pH to 10.0 resulting in a reduction of cefquinome concentration, a fourth-generation cephalosporin, below the limit of detection (<125 μg/kg) within 8 h. A potential concern with alkalinizing milk is the potential effects on nutrients, as well as safety as a food product for calves. Increasing milk pH to 10.0 has been demonstrated to decreased casein micellar size and milk turbidity, that did not return to the initial levels after milk pH was adjusted to a normal milk pH, indicating a permanent alteration of casein micelles (22). This permanent change in caseins structure may affect the nutritional value of waste milk. Another possible aspect that may influence the applicability of alkalization of milk is palatability as well as the effect on bacteria growth, which to our knowledge, has not been estimated. Further studies are needed to evaluate the effect of alkaline treatment on milk quality as well as approaches to adjust final pH and supplementation of additional nutrients in the milk before feeding calves.

Acidification of milk to a pH of 4.0 did not result in a significant degradation of ceftiofur. Other studies have indicated that acid-catalyzed hydrolysis had a negligible effect on degradation of other β-lactams (23, 24). In a study by Mitchel et al. (24), hydrolysis rates of three β-lactam antibiotics were evaluated using acetate and borate buffers at pH 4.0–9.0, incubating samples at 25, 50, and 60°C. The calculated half-lives of cefalotin (first-generation cephalosporin), cefoxitin (second-generation cephalosporin) and ampicillin at pH 4.0 and 25°C were 5.2, 9.3, and 3 days, respectively. First and second generation cephalosporins may differ in degradation pathways to third generation cephalosporins (e.g., ceftiofur), which could affect hydrolysis rate and half-lives of the components. Gilbertson et al. (23) observed similar results to our study when evaluated ceftiofur degradation on acetate (pH 5.0), phosphate (pH 7.0), and borate (pH 9.0) buffers incubated at 22 and 47°C.

In the Gilbertson et al. (23) study the reported half-lives of ceftiofur at 22°C were 100.3, 8 and 4.2 d at pH 5.0, 7.0 and 9.0, respectively. Even though, half-lives of antibiotics between both studies were considerably different (23, 24), they were still longer than the time evaluated in our study, which explain pH 4.0 did not increase ceftiofur degradation in milk samples. One difference between both studies and ours is that they evaluated antibiotic degradation using buffers solutions instead of milk, which may influence the degradation rate of antibiotics. Acidification of waste milk is a preservation method used to inhibit bacterial growth and survival without affects its nutritional value (25). Lowering milk pH to 4.0 using formic acid has shown to reduce coliform and aerobic bacterial growth in milk replacers (26) and raw bulk tank milk (27), as well as decrease diarrhea episodes in calves in compare with pasteurized and untreated waste milk (28). Acidified milk is fed by 1.7% of farms in the United States (29), and if successful, may represent a cost-effective strategy to treat antimicrobial residues in milk. Furthermore, our study provides novel information to clarify that waste milk acidification as a bacteria inhibition process cannot be assumed to have an effect on degradation of ceftiofur residues.

Desfuroylceftiofur is the main metabolite product of ceftiofur hydrolysis (30). Free desfuroylceftiofur is an active metabolite with the intact cephalosporin part of the molecule responsible for biological activity. Desfuroylceftiofur is the marker residue for ceftiofur, with a tolerance level in milk of 0.1 ppm. The marker residue is the residue whose concentration is in a known relationship to the concentration of total residue in edible tissue (31). An approach to measure both free desfuroylceftiofur and conjugated ceftiofur is to quantify the ceftiofur-free acid equivalents (CFAE) (32). In our study, given the significant degradation of ceftiofur observed when milk pH was increased to 10.0, CFAE concentrations were also evaluated to determine if ceftiofur was just being converted to another microbiologically active metabolite.

CONCLUSION

Heat and pH and treatments might be alternative cost effective on-farm strategies that could increase the degradation of antimicrobials on waste milk. Adding sodium hydroxide to ceftiofur spiked milk until pH 10 was achieved increased the degradation of ceftiofur and CFAE in milk. Heating ceftiofur spiked milk to 92°C for 20 min also decreased ceftiofur concentrations in spiked milk samples but to a much lesser extent. Further studies to evaluate the possibility of using these approaches on farms are needed, including palatability, adjusting final treatment products to allow safe consumption of milk by calves, and evaluating if these alternative methods reduce the potential for antimicrobial resistance when feeding antibiotic contaminated waste milk to calves.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

RPe, PP, and SA contributed conception and design of the study. RPo, PP, RPe, and LT contributed to conducting the experiment and analytical testing of samples. RPe and AG performed the statistical analysis. RPe and AG wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00288/full#supplementary-material

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A Prospective Cohort Study on the Development of Claw Horn Disruption Lesions in Dairy Cattle; Furthering our Understanding of the Role of the Digital Cushion

*Bethany E. Griffiths, Philippa J. Mahen, Rachel Hall, Nikolaos Kakatsidis, Nick Britten, Kerry Long, Lara Robinson, Hannah Tatham, Rebecca Jenkin and Georgios Oikonomou**

Department of Livestock and One Health, Institute of Infection, Veterinary & Ecological Sciences, University of Liverpool, Liverpool, United Kingdom

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Saraya Tavornpanich, Norwegian Veterinary Institute (NVI), Norway

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***Correspondence:**
Georgios Oikonomou
goikon@liv.ac.uk

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Claw horn disruption lesion (CHDL) is the collective term used to describe non-infectious foot lesions such as sole ulcers (SU), sole hemorrhage (SH), and white line disease (WLD) that commonly affect dairy cattle. The potential role of the bovine digital cushion, an anatomical structure located under the pedal bone and composed mostly of adipose and connective tissue, in the aetiopathogenesis of CHDL has recently been the subject of several studies. The aim of this prospective cohort study is to identify risk factors associated with the development of CHDL and to add further evidence regarding the role of the digital cushion. In order to achieve that we collected data from 500 lactations; 455 dairy cows from 3 farms were enrolled in this study. Data were collected from each animal on three occasions: 3–4 weeks before expected calving date, 1 week post calving, and 8–10 weeks post-calving. At each occasion, sole soft tissue thickness (the combined depth of the digital cushion and corium, SSTT) was measured using B-mode ultrasonography. At 8–10 weeks post-calving foot trimming was undertaken and the presence of CHDLs was recorded. Univariable analysis was undertaken between variables of interest, before multivariable regression models were constructed. Mixed effects multivariable linear regression models were created to describe the changes in SSTT and associations with various explanatory variables. Multivariable logistic regression models with the presence of SU, SH, or WLD as an outcome were also built. SSTT was shown to decrease from calving to early lactation (EL). Primiparous animals were found to have smaller SSTT, than multiparous animals. Animals with greater BCS had greater SSTT. Cows with a SU in early lactation had lower SSTT both at pre-calving and calving inspections comparing to cows without a SU. Cows that developed mastitis within 30 days of calving had approximately four times higher odds of developing SU compared to cows that did not develop mastitis. Our study advances our understanding of animal level risk factors associated with the development of CHDL and highlights the importance of the periparturient period.

Keywords: dairy cattle, lameness, claw horn disruption lesion, digital cushion, mastitis

INTRODUCTION

Lameness is one of the greatest challenges facing the dairy industry, given the severe negative impacts on animal welfare (1), fertility (2), and milk yield (3). It is associated with substantial economic losses (4) and is still highly prevalent within dairy herds (5). Claw horn disruption lesion (CHDL) is the collective term used for non-infectious lameness causing lesions such as sole ulcers (SU), sole hemorrhage (SH), and white line disease (WLD) that commonly affect dairy cattle (6). Lesions which make up CHDL are thought to be different presentations of a similar disease process (7), with SH preceding SU (8–10). Although widely recognized as a significant issue within the dairy industry their aetiopathogenesis is yet to be fully elucidated. Current research suggests these lesions are the result of contusions of the corium under the third phalanx (11). The insult causes hemorrhage and necrosis of the keratinocytes within the sole corium, reducing the ability of the cow to synthesize new claw horn in affected feet and resulting in CHDLs (6, 8).

A cow's suspensory apparatus is rudimentary in comparison to horses (12). To compensate, cows have a better developed digital cushion (DC) which supports a significantly larger proportion of their body weight. This structure was first studied in 1999 by Kofler et al. (13), and described further by Räber et al. (12). A thinner DC has been associated with increased risk of developing CHDL (14, 15) and it has been hypothesized that the DC becomes thinner as cows mobilize body fat after calving (16). A recent study by Newsome et al. (17) showed that digital cushion thickness (DCT) decreased during the periparturient period but this thinning could not be entirely explained by fat mobilization alone.

Furthermore, the process of parturition (calving) has been associated with increased laxity in the connective tissue supporting the distal phalanx within the claw (18), quite possibly due to the hormonal effect of relaxin or estrogen. Systemically induced inflammation around calving may also compromise the suspensory apparatus via activating matrix metalloproteinases, which in turn play a central role in the degradation of its connective fibers. In addition, proinflammatory mediators associated with direct stimulation of lipolysis (19) could also lead to fat mobilization from the DC and a reduction of the latter's protective properties. We have previously shown that early signs of local inflammation were indeed associated with reduced DCT in the beginning of lactation and before the development of detectable CHDL (20).

Given the severe, wide ranging negative effects, coupled with the high prevalence of cows afflicted with CHDL, research is required to further elucidate their aetiopathogenesis; this could translate to better prevention strategies. Research has associated the depth of the DC with the development of CHDLs however most studies examined cows at a single time point. The main objective of this prospective cohort study is to add further evidence as to how the DCT changes over the peri-parturient period and its association with the development of CHDL. The importance of other animal level risk factors is also investigated.

METHODS

Farm Recruitment and Ethics

The study was approved by the University of Liverpool Veterinary Research Ethics Committee (Reference VREC269). Data from 500 lactations were collected from 455 Holstein cows on three commercial dairy farms in the North-West of England and North Wales. The study was conducted between December 2014 and December 2015 on one of the three collaborating farms. The study was then continued on all three collaborating farms during the period between January 2017 and September 2017. The farms were selected due to their proximity to the Institute of Veterinary Science (University of Liverpool) and their willingness to collaborate with our research group.

Farm Characteristics

On farm 1, the milking parlor and one third of the waiting area floor was rubber matting on concrete. All other walkways were grooved concrete. Cows were housed in cubicle sheds. Concrete based cubicles were lined with various mattress types (rubber, gel) and bedded with sawdust. Pen passageways were grooved concrete and were automatically scraped two to three times an hour. Dry cows were housed in sheds with a deep straw lying area and a grooved concrete loafing area. Youngstock were housed in cubicles during the winter months, and at pasture in the summer month.

On farm 2, the milking parlor was concreted and no matting was present in the parlor or the collecting yard. All other walkways were grooved concrete. Cows were housed in cubicle sheds. High yielding cows had access to concrete based cubicles with mats and shallow sand. Low yielding and freshly calved cows had access to deep sand bedded cubicles. Pen passageways were grooved concrete and automatically scraped two to three times an hour. Dry cows were housed on pasture during the summer and on deep sand bedded cubicles during winter. Youngstock were housed on concrete based cubicles with straw from weaning until first service.

On farm 3, matting was present in the parlor. All other walkways were grooved concrete, with matting present on the exit of the parlor and down the main race. Cows were housed in deep sand bedded cubicle sheds. Pen passageways were grooved concrete and scraped three times a day with a tractor. Dry cows were housed on a separate unit with deep sand bedded cubicles. Youngstock were housed on a separate unit with cubicles.

The diets for all the cows were formulated according to NRC guidelines. All cows were scheduled for routine foot trimming, at drying off and at ∼60 days in milk for farm 1 and farm 3. Farm 2 cows were scheduled for routine foot trimming at drying off. Lame cows received additional foot trimming as necessary by farm staff. Footbaths, consisting of 4% copper sulfate twice weekly and 3% formalin once weekly, were located in the exit lane of the milking parlor at farm 1, whilst cows in Farm 2 were footbathed three times a week in 3% formalin on exit of the milking parlor. Farm 3 used a 3% formalin footbath once daily, located in the exit lane of the milking parlor.

Data Collection

Data were collected from each animal on three occasions: 3–4 weeks from the expected calving date, 1 week post-calving and 8–10 weeks post-calving, referred to as pre-calving, fresh, and early lactation (EL), respectively. On each occasion mobility was assessed, using the AHDB 0–3 scale scoring method (21) by observing the cow walking on a flat surface. Body condition score (BCS) was assessed using the Penn State method; scores were between 1 and 5 in 0.25 increments (1= very thin, 5 = obese) (22).

Cows were restrained in a foot trimming crush for measurement of DCT using an Easi-Scan ultrasound machine (sonographic B-mode, IMV Imaging, Bellshill, UK) equipped with a linear probe 5–8 MHz set at 5 MHz. The ultrasound machine settings were kept unchanged throughout the study. All measurements of DCT were undertaken at the midline, on the lateral claw of the hind left foot. To measure the DCT the foot was cleaned and loose horn was removed with a hoof knife, as described by Kofler et al. (13). Sole contact with the transducer was made using ultrasound gel (Ultrasound Gel, Henry Schein) and a gel standoff (Flexi gel standoff, IMV Imaging, Bellshill, UK). After freezing the image on the ultrasound monitor (Easi-Scan Ultrasound Remote Display, IMV Imaging, Bellshill, UK), measurements were taken to the nearest millimeter. Digital cushion thickness (DCT) was measured just dorsally to the tuberculum flexorum of the pedal bone at the typical SU site. The distance from the inner margin of the sole (identified as a thin echogenic line) to the distal edge of the pedal bone (identified as a thick echogenic line) was assessed. The anatomical area of the DC targeted for ultrasonography was the middle pad (11). The DCT measured here, is better described as the sole soft tissue thickness (SSTT), as both the DC and corium are included within the measurement taken.

When data were collected at 8–10 weeks post-calving (EL), both hind feet were trimmed using the Dutch five step method (23) and any visible foot lesions were recorded before SSTT was measured. The recorded lameness causing foot lesions were digital dermatitis, SU, WLD, SH, and interdigital hyperplasia. Cases were defined following the ICAR Claw Health Atlas definitions (24).

All other factors included in analysis, including calving date, age in days, parity, and important health information such as incidence of retained fetal membranes, milk fever, ketosis, mastitis, metritis, endometritis, and displaced abomasum, were obtained from the farms' management software.

Statistical Analysis

Data were analyzed using JMP Pro 14 (SAS Institute Inc., Cary, NC). Univariable analyses were undertaken between variables, before multivariable regression models were constructed. Parity was fitted in all models as a categorical variable with 3 levels (1 for animals in their first parity, 2 for animals in their second parity, and 3 for animals in their third or greater than third parity). SSTT was used as a continuous variable but was also turned into a categorical variable with 3 levels (3 terciles with 1 including the cows with the lowest SSTT measurements and 3 for those with the greatest SSTT measurements); this allowed for a more straightforward interpretation of logistic regression models outputs. Similarly, BCS was also turned into a categorical variable with 3 levels (level 1 for BCS <2.5, level 2 for BCS from 2.5 to 3, and level 3 for BCS >3).

In order to describe changes in SSTT and its association with CHDL a mixed effects multivariable linear regression model was used. The continuous dependent variable was SSTT and the following independent variables were originally offered to the model: Body condition score, calving season (Spring, Summer, Autumn, Winter), study (1 for data collected between December 2014 and December 2015, and 2 for data collected between January 2017 and September 2017), parity, time point of measurement (pre-calving, fresh and EL), assessor, and presence or absence of CHDL (SU, WLD, SH). These variables were offered to the model either because they were found to be associated with SSTT in univariable analyses ($P \leq 0.20$) or because they were of particular interest for this study (CHDL). Cow id nested within farm was fitted in the model as a random effect to account for within animal clustering of SSTT measurements. The covariance structure used was that of compound symmetry. Associations between explanatory variables were also investigated to identify collinearity between variables. Interaction terms of interest that were offered to this model were: time point of measurement by presence or absence of CHDL. Variables and their interactions were removed from the model manually and in a stepwise manner (with the variable with the highest P-value removed at each step), and only variables with $P < 0.10$ (F-test) were kept in the final model. If an interaction term was found to be significant, then the main effects were kept in the final model whether they were significant or not. The restricted maximum likelihood approach was taken when fitting the model. Rows with missing data were not included in the analysis. When two variables were both found to be significant but also strongly associated to each other (this was the case for assessor and study, with three assessors only participating in Study 1 and one assessor only participating in Study 2) the variable that led to a higher adjusted R^2 was kept in the final model. Residuals by model predicted values, studentized residuals, and residuals normal quantile plots were visualized in order to evaluate the model's goodness of fit and that assumptions of normality and homoscedasticity were met. Leverage plots (partial-regression residual leverage plots) for all fixed effects included in the model were also visualized. For categorical explanatory variables results are presented as least squares means ± standard error of the mean. Pairwise comparisons of least squares means were made using the Tukey-Kramer Honestly Significant Difference (HSD) test.

Logistic regression models with presence of SU, WLD, or SH at the EL inspection as an outcome were also built. Variables with a $P \leq 0.20$ in the univariable analyses were offered to these multivariable logistic regression models. Variables were removed from the models manually and in a stepwise manner (with the variable with the highest P-value removed at each step), and only variables with $P < 0.10$ (likelihood ratio test) were kept in the final model. Explanatory variables originally offered in the model with SU as an outcome were: SSTT terciles at fresh and EL, calving season (Winter, Spring, Summer, and Autumn), study (1 for data collected between December 2014 and December 2015,

and 2 for data collected between January 2017 and September 2017), incidence of clinical mastitis the first 30 days post calving, BCS group at EL, and parity. Explanatory variables originally offered in the model with SH as an outcome were: SSTT terciles at EL, calving season, study, and parity. Explanatory variables originally offered in the model with WLD as an outcome were: calving season, study, BCS group at pre-calving and EL, and parity. Farm was included in all the logistic regression models whether or not it was found to be significant. The Lack of Fit test was used to evaluate models goodness of fit and the likelihood ratio test was used to determine the overall significance of the models. The predictive ability of each one of the final three logistic regression models was assessed with receiver operating characteristic analysis and the calculated area under the curve. Results from logistic regression models are presented as Odds Ratios. *P*-values and 95% confidence intervals (CI) for calculated Odds Ratios are Wald based estimates. All comparisons between different levels of categorical explanatory variables are for the odds of developing CHDL (SU, SH, WLD) vs. the odds of not developing CHDL.

RESULTS

Four hundred and fifty-five cows were enrolled, 45 of them for two consecutive lactations, totaling 500 lactation enrolments. Of the 1,500 ultrasound assessments due, 137 were missed [50 at pre-calving, 42 at fresh and 45 at early lactation (EL)]. Reasons for missing assessments were failure of sorting gate, termination of measurements due to animal stress presenting a risk for animal or researcher safety, removal from herd, and injury preventing foot trimming. Five assessors carried out the ultrasound assessments (assessor 1; $n = 517$ assessments, assessor 2; $n = 519$, assessor 3; $n = 57$, assessor 4; $n = 94$, assessor 5; $n = 170$; unrecorded; $n = 6$). The median time of assessment relative to calving was −17 days for pre-calving, 6 days for fresh and 67 days for EL. **Table 1** shows summary data for the study population and assessments. More calvings occurred in spring than other seasons (spring $n =$ 266, summer $n =$99, autumn $n =$53, winter $n =$82). There were 144 parity one animals, 134 parity two, and 222 parity three and over. Median BCS of the population decreased from pre-calving to fresh to EL (pre-calving 3.25, fresh 3, EL 2.5). Twenty animals had a case of mastitis in the first 30 days of lactation. From the 462 foot trims/examinations carried out at the EL time point, 52 cases of sole ulcer (SU) were found, 105 cases of sole hemorrhage (SH) and 80 cases of white line disease (WLD). Descriptive statistics results are presented in **Table 1.**

Results obtained from Univariable analyses with SSTT and presence of SU, SH, or WLD as outcome variables are presented in **Supplementary Tables 1–4**.

Results obtained from multivariable mixed effects linear regression analysis with SSTT as an outcome are presented in **Table 2**. Time point ($P = 0.003$), parity ($P < 0.0001$), BCS ($P = 0.031$), time point by SU interaction ($P = 0.036$), and assessor ($P < 0.0001$) were statistically significantly associated with SSTT. The statistically significant time point by SU interaction highlighted that changes of SSTT across time points were different between animals that developed a SU in EL and the ones that did not. There was no significant difference between SSTT at pre-calving and fresh in cows that did not develop SU (adjusted means 8.73 ± 0.14 and 8.65 ± 0.12 mm, respectively), whereas SSTT at EL was statistically significantly lower than the other two time points (8.27 ± 0.13 mm). In cows with SU, SSTT was at its lowest immediately after calving (however this was only a numeric difference that was not statistically significant). Parity one animals had significantly thinner SSTT than parity two or three and over animals (8.55 ±0.11, 9.30 ± 0.11, and 9.54 ± 0.10 mm, respectively). Animals with higher BCS had higher SSTT, with an estimated increase in SSTT of 0.3 mm for every one point increase in BCS. Model's adjusted R^2 was 0.35.

TABLE 1 | Summary data for study populations.

Enrolled lactations (cows)	Farm 1	312 (267)
	Farm 2	75 (75)
	Farm 3	113 (113)
	Total	500 (455)
Missing ultrasound assessments	Pre-calving	50
	Fresh	42
	Early lactation	45
Total ultrasound assessments	1,363	
Time of assessments relative to calving (days) Median (range)	Pre-calving	−17 (−131–0)
	Fresh	6 (0–12)
	Early lactation	67 (37–97)
Season at calving	Spring: 266	
	Summer: 99	
	Autumn: 53	
	Winter: 82	
Parity	1: 144	
	2: 134	
	≥3: 222	
Study*parity	Study 1	Parity 1: 54
		Parity 2: 44
		Parity ≥3: 86
	Study 2	Parity 1: 90
		Parity 2: 90
		Parity ≥3: 136
Body condition score median (range)	Pre-calving	3.25 (2.25–4.5)
	Fresh	3 (1.75–4)
	Early lactation	2.5 (1.5–3.5)
Mastitis in first 30 days of lactation (primary cases)	20	
Claw horn disruption lesions ($n = 462$)	Sole ulcer: 52	
	Sole hemorrhage: 105	
	White line disease: 80	
Number of assessments per assessor	1: 517	
	2: 519	
	3: 57	
	4: 94	
	5: 170	

TABLE 2 | Results from multivariable mixed effects linear regression model for outcome sole soft tissue thickness (SSTT) (mm).

Explanatory variable	Category	Adjusted mean	Standard error	Tukey's HSD	*P*-value
Parity	1	8.55	0.11	A	<0.0001
	2	9.30	0.11	B	
	≥3	9.54	0.10	B	
Time point*sole ulcer	Pre-calving, no SU	8.73	0.14	A	0.036
	Pre-calving, SU	8.54	0.27	ABC	
	Fresh, no SU	8.65	0.12	AB	
	Fresh, SU	8.02	0.25	BC	
	Early lactation, no SU	8.27	0.13	C	
	Early lactation, SU	8.45	0.25	ABC	
Assessor	1	7.81	0.11	C	
	2	8.39	0.12	B	
	3	8.97	0.23	AB	<0.0001
	4	9.17	0.19	A	
	5	8.40	0.15	B	
BCS	Continuous variable	Estimate 0.30	0.14		0.031
Random effect		Variance Component			Percentage of total variance
CowID (nested within farm)		0.43	0.09		16.26
Residual		2.22	0.10		83.74

HSD, honestly significant difference. Levels within a variable with different letters are statistically significantly different (P < 0.05).

Results obtained from multivariable logistic regression analysis with SU, SH, or WLD as an outcome are presented in **Table 3**. The multivariable logistic regression model for outcome SU retained BCS at EL, mastitis in the first 30 days of lactation, SSTT tercile at fresh and parity, along with the forced variable farm. The odds for having a SU were higher for animals with a BCS of <2.5 at EL than those with a BCS of 2.5–3.0 (OR 3.59, 95% CI 1.78–7.26, $P = 0.0004$). Animals that had a case of mastitis in the first 30 days of lactation displayed higher odds of having a SU than those that did not get mastitis (OR 3.97, 95% CI 1.31–12.09, $P = 0.015$). Animals with a SSTT at fresh of 8–9.5 mm and those with SSTT at fresh <8 mm had higher odds of developing a SU than those in with SSTT >9.5 mm (OR 2.20, 95% CI 1.02–4.73, $P = 0.044$, and 2.40, 95% CI 0.92–6.23, $P = 0.074$, respectively). The numeric difference between animals with SSTT at fresh <8 mm and those with SSTT>9.5 mm was marginally not statistically significant. Animals in their second parity had lower odds of getting SU than animals in their third or greater than third parity (OR 0.39, 95% CI 0.16–0.97 $P = 0.043$). This model's AUC was 0.77.

TABLE 3 | Results from multivariable logistic regression models for outcomes SU, SH, and WLD.

Outcome	Explanatory variable	Level	OR	95% CI	*P*-value
Sole Ulcer	BCS category at early lactation	<2.5	3.59	1.78–7.26	0.0004
		>3.0	0.58	0.07–4.65	0.60
		2.5–3.0		Reference	
	Mastitis in first 30 days of lactation	Yes	3.97	1.31–12.09	0.015
		No		Reference	
	SSTT tercile at fresh	<8 mm	2.40	0.92–6.23	0.074
		8–9.5 mm	2.20	1.02–4.73	0.044
		>9.5 mm		Reference	
	Parity	1	0.88	0.38–2.05	0.77
		2	0.39	0.16–0.97	0.043
		3		Reference	
	Farm	1	1.79	0.73–4.34	0.20
		2	2.53	0.86–7.41	0.09
		3		Reference	
Sole hemorrhage	Parity	3	0.56	0.32–0.97	0.0006
		2	0.30	0.15–0.59	0.039
		1		Reference	
	SSTT tercile at early lactation	<8 mm	1.86	1.05–3.29	0.034
		8–9.5 mm	2.21	1.21–4.04	0.010
		>9.5 mm		Reference	
	Season	Winter	1.42	0.69–2.92	0.35
		Summer	2.52	1.29–4.93	0.007
		Autumn	1.12	0.48–2.64	0.78
		Spring		Reference	
	Farm	1	3.76	1.53–9.22	0.004
		2	3.20	1.20–8.54	0.02
		3		Reference	
White line disease	Parity	3	7.68	3.16–18.66	<0.0001
		2	4.44	1.72–11.47	0.002
		1		Reference	
	Farm	1	4.00	1.75–9.19	0.001
		2	1.48	0.50–4.37	0.47
		3		Reference	

Presented Odds Ratios (OR) are for each level against the reference category for the odds of developing SU, SH, or WLD; P-values and 95% confidence intervals (CI) are Wald based estimates.

In the model with SH as an outcome variable (**Table 3**), parity, SSTT tercile at EL, season, and farm remained as significant. Animals in their second parity and animals in their third or greater than third parity had lower odds of developing SH comparing to primiparous animals (OR 0.30, 95% CI 0.15–0.59, $P = 0.039$; OR 0.56, 95% CI 0.32–0.97, $P = 0.0006$). Animals with a SSTT in the bottom (<8 mm) or middle tercile (8–9.5 mm) at EL had higher odds of having SH than those in the top tercile (>9.5 mm) (OR 1.86, 95% CI 1.05–3.29, $P = 0.034$; OR 2.21, 95% CI 1.21–4.04, $P = 0.010$, respectively). Animals that calved in summer had higher odds of developing SH than those calved in spring. This model's AUC was 0.73.

Only parity and farm were retained as significant in the model for WLD. Parity two and parity three or greater than three animals had higher odds of developing WLD than those in parity one (OR 4.44, 95% CI 1.72–11.47, $P = 0.002$; OR 7.68, 95% CI 3.16–18.66, $P < 0.0001$, respectively). This model's AUC was 0.72.

DISCUSSION

We measured SSTT at three time points from pre-calving to EL and found it to be at its thinnest in EL, in line with previously published work (16). Primiparous animals were found to have thinner SSTT compared to multiparous animals. Cows displaying a SU in EL were shown to have numerically thinner SSTT both at pre-calving and calving than those without SU. Body condition was shown to be positively correlated with SSTT. Cows that developed mastitis within the first 30 days of calving had almost four times higher odds of developing a SU in EL comparing to cows that did not develop mastitis. Variables found to have an effect on the development of SU, SH, and WLD included parity, with parity two and parity three or greater animals showing lower odds of developing SH, whilst showed higher odds of developing WLD. Those animals with a SSTT in the middle tercile around calving were at higher odds of developing SU compared to those in the top tercile, whilst animals with a SSTT in the bottom or middle tercile at EL were at higher odds of developing a SH than those in the top tercile.

SSTT in our study was observed to be at its thinnest in EL. This is similar to previously published results (16), where SSTT was at its lowest from 30 to 120 days post-calving; however this study was cross sectional and unable to provide strong evidence regarding the change in SSTT over a lactation. This nadir at EL could be due to cows experiencing a negative energy balance, resulting in partial fat mobilization from the SST (12), as suggested by Bicalho et al. (16). A recent prospective cohort study by Newsome et al. (17) observed the thinnest point to be around calving, with an increase in thickness observed from calving to EL. The authors suggested that this could be due to increased laxity of the suspensory apparatus associated with calving, causing the distal phalanx to compress the SST.

The inconsistency regarding when in lactation the SSTT nadir occurs between our study and the Newsome et al. (17) study could be due to the difference in the fat pad targeted. A recent short communication by Hiss-Pesch et al. (25) reported that the fat pads within the digital cushion are not uniform and that fat could be mobilized from them at different rates. It could also be due to different farm management systems or differences in genetics. The farms used by Newsome et al. (17) featured automated milking systems, whereas all three farms within the current study used conventional herringbone or rotary milking parlors. This will affect the time cows spend on their feet and could potentially be associated with post-calving changes in SSTT (26). Work undertaken by Oikonomou et al. (27) described a heritability estimate of 0.33 for SSTT, therefore genetics may also play a role in the inconsistences presented by these two studies. Contrary to the Newsome et al. (17) study where the assessor measuring SSTT was blinded to stage of lactation, we assessed SSTT knowing whether the cows were at pre-calving, fresh, or EL stage. Therefore, unconscious bias in our measurements cannot be precluded and is another possible explanation for the observed discrepancy between the two studies. This is further discussed in the "study limitations" section of our discussion.

Our results show that cows within the middle tercile for SST thickness immediately after calving had approximately four times higher odds of developing SU than cows in the upper tercile. This finding was similar to that presented in 2009 by Bicalho et al. (16). Toholj et al. (14) also showed that cows with a SSTT below 3 mm had four times higher odds of developing a SU than cows with a SSTT above 3 mm. Furthermore, cows within both of the lower terciles for SSTT at EL were at greater odds of developing SH compared to those cows in the upper tercile, a finding supported by Newsome et al. (28). Our findings support the hypothesis that the time around calving is important in the development of CHDL's.

Cows that developed a SU in EL had lower SSTT during the pre-calving period than cows which did not develop SU and experienced a greater thinning of the SST around parturition. Newsome et al. (17) was able to show that cows which develop a SU or severe SH had thinner SSTT, yet thinning of the SST was not significantly associated with the development of CHDLs. Bicalho et al. (16) showed that cows with lesions, regardless of parity, had significantly thinner SSTT, whilst thin SSTT were associated with cows that had CHDLs in the same lactation, and cows that go on to develop CHDL's in the subsequent lactation (15). Previous work revealed that cows affected by lameness and CHDLs undergo new bone growth at the plantar and palmar aspect of the distal phalanx (29). These exostoses may reduce the DC capacity to protect cells within the corium from being contused resulting in further inflammation, and further development of exostoses and CHDLs. Another possible explanation for our findings is that the relaxation of the suspensory apparatus described by Tarlton et al. (18) to occur around calving period could be exacerbated in cows developing SU; the reasons behind this remain unknown but could be associated with the animals' genetic make-up. Several regions within the genome have recently been identified as being significantly associated with SSTT at calving (30).

Body condition score and SSTT were significantly associated, in agreement with previous studies (15–17). In EL, when body condition was at its lowest, SSTT was also at its thinnest. An increase of one condition score was associated with an increase in SSTT of 0.3 mm. This represents a smaller magnitude of effect than the results presented by Bicalho et al. (16) however is larger than those results presented by Newsome et al. (18). We also found that cows with a BCS of <2.5 at EL had higher odds of having SU in EL compared to cows with a BCS of 2.5 to 3. It has been shown by multiple studies that low BCS is associated with the development of lameness but can also a result of it (31, 32); cows with a low BCS at parturition had 9.4 times higher odds of developing lameness throughout lactation compared with better conditioned cows (33).

Parity was shown to be significantly associated with both the SSTT and the odds of developing SU, SH, and WLD. Primiparous cows were found to have significantly thinner

SST compared to multiparous cows, which is supported by the existing literature (12, 16, 17). Our results have shown that primiparous animals had higher odds of developing SH compared to multiparous animals, but had lower odds of developing WLD. This finding was again supported by previous findings (16, 34). One hypothesis is that the composition of the developing digital cushion has a somewhat protective function (35), together with the reduced forces going through the foot of comparatively lighter animals (12, 36) around calving when the insult is expected to have occurred, especially in primiparous animals where calving is expected to be more challenging as they are added to the herd and undergo parturition for the first time. Additionally, given these are naïve animals which are unlikely to have experienced CHDL's, exostosis is unlikely to significantly affect the development of these lesions. Therefore, SH occurs rather than a SU or WLD, given SH is thought to be a precursor or result from a milder insult (10). Animals in their third or greater than third parity were at higher odds of developing SU comparing to animals in their second parity. The effect of exostosis, digital cushion composition, increased force through feet and the stress of calving could increase the risk of animals in their third or greater than third parity forming SU and WLD over SH. However, animals in their third or greater than third parity did not have a significantly higher SU incidence than primiparous animals and this suggests a "non-linear" association between parity and incidence of SU, with second parity animals having the lowest incidence. This contradicts previous findings that suggested that SU incidence is lower in primiparous animals (37). The reason behind this finding is unclear; a possible explanation is that animals in their second parity may benefit from a more developed DC (comparing to primiparous animals) but are yet to experience the increased risk associated with multiple calvings and chronic inflammation that may be more evident in animals in their third or greater than third parity.

Cows that developed mastitis within 30 days of calving had almost four times higher odds of developing SU compared to those cows that did not develop mastitis. Clinical mastitis in the early lactation period has been linked with lameness (38), however not with CHDLs specifically. This highlights the potential role of early lactation systemic inflammation in the development of CHDL. The effect of systemic inflammation on the suspensory apparatus has been hypothesized to lead to CHDL. However, our study cannot clearly show such cause and effect relationships. Another likely explanation of our findings could be that cows with mastitis spend longer periods of time standing because of the discomfort associated with the disease and this predisposes them to the development of SU. An unknown common link associated with the aetiopathogenesis of both early lactation mastitis and SU development is another plausible explanation of our findings.

Our study has limitations that need to be taken into consideration when interpreting our findings. Multiple assessors were used for the measurement of SSTT. This has been accounted for in our model with SSTT as an outcome but not in the models where SSTT was an explanatory variable. When assessing SSTT, the assessor was not blinded to the stage of lactation and although no conscious bias was present the possibility of unconscious bias cannot be precluded. We have used a different dataset (collected as part of a larger, ongoing study) including repeated SSTT measurements on 136 cows in order to further investigate this (data not shown). These measurements were taken by the same assessor who was blinded to stage of lactation (or any other relevant information). Analysis of this dataset confirms our findings regarding the associations between parity and SSTT, and the association between SSTT at calving and presence of CHDL in EL. On the other hand, in this analysis, SSTT is not at its lowest at EL, but immediately after calving [similarly to the study by Newsome et al. (17)]. This would indeed suggest that an element of unconscious bias in the presented here study cannot be precluded. A larger scale study with measurements taken by the same, blinded, assessor would potentially help in clarifying this issue. Claw horn disruption lesion information was analyzed by animal in this study and not by claw, as undertaken by Newsome et al. (17), and no distinction was made between animals displaying these lesions on the studied claw and animals displaying these lesions on a different claw. Given inflammation is suggested to play an important role in the SSTT of cows affected with CHDLs, an important improvement in this study would be to include CHDL information by claw. Finally, another limitation of our study has to do with the fact that we only measured SSTT on lifted feet (similarly to the majority of studies on SSTT). Bach et al. (39) recently showed that measurements of SSTT on weight bearing feet yielded different results to the measurements taken on lifted feet. This study was only conducted on 10 animals so must be interpreted with caution but does however suggest that had we been able to measure SSTT on weight bearing feet our results could have been different.

CONCLUSION

This prospective cohort study found that SSTT significantly decreased from calving to EL and that SSTT at calving was associated with the development of SH and SU. The results presented are in general in line with some of the previously published literature. Parity was found to be significantly associated both SSTT and the development of SH, SU, and WLD. We have also shown an association between early lactation mastitis and the development of SU.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by University of Liverpool Veterinary Research Ethics. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

Data collection was undertaken by BG, NK, NB, LR, HT, RJ, RH, KL, and GO. Statistical analysis was undertaken by GO and assisted by PM. The manuscript draft was written by BG with significant contributions from PM and GO. GO conceived and designed the study and provided with funding. All authors have approved the final version of this manuscript and agree to be accountable for all aspects of the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00440/full#supplementary-material

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A Randomized Controlled Trial of Teat-Sealant and Antibiotic Dry-Cow Treatments for Mastitis Prevention Shows Similar Effect on the Healthy Milk Microbiome

Filippo Biscarini[1*†‡], *Paola Cremonesi*[1†], *Bianca Castiglioni*[1], *Alessandra Stella*[1], *Valerio Bronzo*[2], *Clara Locatelli*[2] *and Paolo Moroni*[2,3]

[1] *Institute of Biology and Biotechnology in Agriculture, National Research Council (CNR), Milan, Italy,* [2] *Università degli Studi di Milano, Dipartimento di Medicina Veterinaria, via dell'Università 6, Lodi, Italy,* [3] *Quality Milk Production Services, Animal Health Diagnostic Center, Cornell University, Ithaca, NY, United States*

Lactating cows are routinely treated at dry-off with antibiotic infusions in each quarter for the cure and prevention of pathogenic intramammary infection, which remains the most common disease in dairy herds. This approach is known as blanket dry-cow therapy, usually effective for the prevention and cure of infections, but has been shown to potentially contribute to the emergence and spreading of antibiotic resistant bacterial strains. Exploring the use of non-antibiotic treatments coupled with selective dry-cow therapy is necessary to reduce the risk of antibiotic resistance and potential interference with milk microbiome balance. The impact of selective dry-cow therapy on the physiological milk microbiome needs to be carefully evaluated. In this small-scale trial, five healthy (no mastits, SCC $<$200,000 $\mathrm{cells\ mL^{-1}}$) second-parity cows from dry-off to 5 days after calving were sampled. For every cow, each quarter received a different treatment: (i) bismuth salnitrate (internal teat sealant, OrbSeal®, Zoetis, Italy), front right quarter; (ii) cephalonium dihydrate (Cepravin®, MSD, Italy), rear right quarter; (iii) benzathine cloxacillin (Cloxalene dry, Ati, Italy), rear left quarter. No treatment was applied to the remaining quarter (front left) which served as experimental control. For 16S rRNA gene sequencing, bacterial DNA was extracted from 5 ml of milk samples, amplified using the primers for the V3–V4 hypervariable regions and sequenced in one MiSeq (Illumina) run with 2 $\times$ 250-base paired-end reads. Bacteriological results confirmed that the quarters were all healthy. The phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria* were the most abundant for all treatments and controls at all three timepoints, accounting for over 80% of the entire milk microbiota composition. No significant differences were found between treatments and controls in terms of the major alpha and beta diversity indexes, revealing that antibiotic, and non-antibiotic treatments for selective dry-cow therapy did not alter significantly the milk microbiome of dairy cows. The milk microbiota composition showed a clear evolution over the lactation cycle, and the overall changes in the milk microbiota diversity over the lactation cycle were mainly independent of treatments.

Keywords: dairy cows, prophylaxis, selective dry-cow therapy, antibiotics, milk microbiome, teat sealant, cephalonium, cloxacillin

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****Correspondence:***
Filippo Biscarini
filippo.biscarini@cnr.it

†These authors have contributed equally to this work

‡Currently seconded to the European Research Council Executive Agency (ERCEA), Brussels, Belgium. The views expressed here are purely those of the writer and may not in any circumstances be regarded as stating an official position of the European Commission

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1. INTRODUCTION

Intramammary infections (IMI) are still the disease class with the largest prevalence in dairy cattle farms worldwide [e.g., 24.8% of cows reported to be affected in the USA in 2013; (1)]. Given the high prevalence and the considerable estimated cost per case [$325–426; (2)], it has a substantial impact on the profitability of dairy farms. The main underlying pathogens involved in the aetiology of bovine mastitis include Gram-negative (e.g., *Escherichia coli*) and Gram-positive (e.g., *Staphylococcus aureus*) bacteria (3). Consequently, antibiotics have historically had a major role in the treatment of clinical and subclinical forms of mastitis in dairy cattle (4). The different means for therapy and prevention of IMI are implemented in mastitis control programmes that are adopted on a large scale by commercial dairy farms. The most common mastitis control protocols include blanket dry-cow therapy (BDCT), which relies on the antibiotic treatment of every cow during the dry period, and selective dry-cow therapy (SDCT), which targets those animals and specific mammary quarters that are infected and need to be treated (5, 6). The dry period is a critical component of the milk production cycle for two main reasons: (i) high cure rates for IMI can be achieved (7, 8), and (ii) the rate of new IMI is greater in the periparturient period than at any other point during lactation (9). Growing concerns and evidence on the development of antibiotic-resistant bacterial strains and their spread to other livestock species and humans, with potential zoonotic risks, are pushing the investigation and adoption of alternative strategies (10–12). Non-antibiotic solutions include probiotics, bacteriocins, bacteriophages, teat sealants, lactoferrin, herbal compounds, and vaccinations (4, 13–15). For dairy herds with a low prevalence of contagious mastitis and a consistently low somatic cell count (SCC), SDCT is a preferable alternative approach to mastitis control. Internal teat sealants (ITS) are a class of non-antimicrobial products that has proven to be just as efficacious as dry-cow therapy (DCT) in the prevention of IMI during the dry period. ITS may provide just a physical barrier or also inhibit bacterial growth (16). The use of an ITS in a SDCT program ensures that all healthy quarters have some form of protection against dry-period IMI. Studies have found that SDCT is better than BDCT in the prevention and treatment of IMI during the dry period and can reduce the use of antimicrobials by 21% (6, 17, 18).

Evidence has been accumulating on the role of the udder microbiomes (teat canal and milk) on the mammary health: their dysbiosis has been hypothesized as a predisposing factor for mastitis (19), in line with recent views that challenge Koch's "one microbe–one disease" paradigm in favor of the more complex concept of the pathobiome as etiologic agent (20). Mastitic quarters have been found to show higher bacterial load and lower diversity compared to healthy quarters (21–23). Previous works on the effect of mastitis treatments on the teat-canal and milk microbiomes involved mastitic cows treated with antibiotics or healthy cows under DCT with antibiotics and teat sealant. Results showed that the udder microbiomes change with infection and over time but appear to be resilient to therapeutic and prophylactic antimicrobial treatments (23). Derakhshani et al. (24) assessed the use of a penicillin-novobiocin formulation together with teat sealant; Bonsaglia et al. (25) evaluated the effect of a third-generation cephalosporin (ceftiofur) combined with teat sealant, and of teat sealant alone, on the milk microbiome. It remains to be determined whether or not other classes of antimicrobials may have a long-lasting effect on the composition of the udder microbiome as a whole and, specifically, of the milk microbiome.

Considering that 3rd and 4th generation cephalosporins are currently not recommended for veterinary use according to EU guidelines (26), it is important to evaluate other types of antimicrobials used in DCT and their effect on the bovine milk microbiome, relative to antibiotic-less prophylactic strategies and untreated controls. In this small-scale trial, we sampled healthy cows under DCT and implemented a within-subject experimental design based on udder quarters: each quarter received a different treatment: cephalonium dihydrate (first-generation cephalosporin), benzathine cloxacillin, and bismuth subnitrate (internal teat sealant); the last quarter was left untreated and served as experimental control. We hypothesize that antibiotic and non-antibiotic treatments for SDCT do not alter significantly the milk microbiome of healthy dairy cows: this would further support the replacement of antibiotics with teat-sealant for SDCT. We followed the microbiome research terminology proposed by Marchesi and Ravel (27).

2. MATERIALS AND METHODS

2.1. Ethics Statements

This study was conducted on a single commercial dairy farm in Romano di Lombardia (Bergamo, Italy), thanks to its long-standing relationship with the University of Milan. The study was reviewed and approved by the Ethics Committee for Animal Welfare of the University of Milan (authorization n. 88/2019).

2.2. Animals, Treatments, and Sampling Time

Five Holstein-Friesian cows were selected for this study from a 140 lactating-cows dairy farm in Northern Italy, with 1 year average bulk tank somatic cell count (SCC) of $159 \cdot 10^3$ cells mL^{-1} and herd milk production average of 37 L d^{-1}. These were all second-parity cows without any symptoms of clinical mastitis and SCC $<$ 200,000 cells mL^{-1} per lactation based on DHIA data (Dairy Herd Information Association), as per the study inclusion criteria. Cows had freestall housing with cubicles bedded with pelleted straw for lactating animals and straw during the dry period (duration in the range 54–62 days). The herd was also prescreened using bulk tank culture to determine whether cows were confirmed negative for *Mycoplasma* spp. The animals were followed over a period of 12 weeks, and sampled at three time points: dry-off, calving (colostrum) and 5 days in milk (5 DIM). Drying-off was abrupt. The animals remained healthy for the entire sampling period, without signs of clinical mastitis. During the experimental period, cows were fed ad libitum with a silage-free mixed ration using alfalfa hay, straw, and supplemented minerals and vitamins. After parturition, cows were milked twice a day (3 a.m., 3 p.m.) in a double-6 herringbone parlour.

During the dry-off period, in each cow three of the four quarters were treated with: (i) bismuth subnitrate (internal teat sealant, Orbeseal®, Zoetis, Italy), front right quarter; (ii) cephalonium dihydrate (Cepravin®, MSD, Italy), rear right quarter; and (iii) benzathine cloxacillin (Cloxalene dry, Ati, Italy), rear left quarter. No treatment was applied to the remaining quarter (front left) which served as experimental control. Cepravin is a first-generation semi-synthetic cephalosporin antibiotic (cephalonium dihydrate) with activity against aerobic Gram-positive and a few community-acquired Gram-negative bacteria. Cephalonium is used in veterinary medicine and has broad-spectrum activity. Cloxalene is benzathine cloxacillin, suited for dry-off and for the treatment of subclinical Gram-positive associated mastitis susceptible to cloxacillin [e.g., *S. aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, non-aureus *Staphylococci* (NAS), *Trueperella pyogenes*]. It is also used to prevent mammary infections that may arise during the dry period or around calving and early lactation. From each quarter milk samples were collected at dry-off (T1), the day of calving (T2, colostrum), and 5 DIM (T3): milk samples were collected before the afternoon milking. Sampling was carried out following the best practices for 16S rRNA-gene sequencing experiments (28). The sample size (5 cows, 4 quarters, 3 timepoints) was determined as a trade-off between ethics constraints (the fewer animals used, the better) and statistical power calculations (80% power to detect an effect of 0.41–0.44 standard deviations with 0.05 false positive -α- threshold). Milk microbiome studies of comparable size have been reported (24, 29, 30).

2.3. Milk Samples Procedures and Somatic Cell Count

Before milk sample collection, teat ends were carefully cleaned and disinfected with chlorhexidine and 70% alcohol in accordance with National Mastitis Council (NMC, 2017) recommendations for aseptic collection of milk samples. First streams of foremilk were discharged, and then approximately 10 mL of milk was collected with a sterile technique from each teat into sterile vials. These vials were previously identified with herd, cow number, quarter, and date. Samples were transported at 4 °C to the laboratory and frozen at −20 °C until bacteriological assays and SCC tests were performed. The SCC was estimated on a per-quarter basis with an automated somatic cell counter (Bentley Somacount 150, Bentley Instrument, Chaska, MN). Milk samples were split in two aliquots, one for bacteriology and one for sequencing. The same procedure was performed at all timepoints.

2.4. Bacteriological Analysis

Bacteriological milk cultures were performed at the University of Milan following published procedures recognized by the NMC (2017). From each sample, 10 µL of milk were spread on blood agar plates (5% defibrinated sheep blood). Plates were incubated aerobically at 37 °C and examined after 24 and 48 h. Colonies were provisionally identified based on size, Gram stain, morphology, and hemolysis pattern. Representative colonies were then subcultured on blood agar plates and incubated again at 37 °C for 24 h to obtain pure cultures. Catalase-negative Gram-positive cocci were identified as *Streptococci* and species were differentiated by further biochemical tests (growth in 6.5% NaCl broth, esculin hydrolysis, fermentation of ribose, sorbitol, sucrose, and inulin). Coagulase tube test was used to differentiate catalase-positive gram-positive cocci as *S. aureus* or NAS. Gram-negative isolates were identified using colony morphology, Gram-staining characteristics, oxidase test, indol test, and inoculation in Simmons citrate (Laboratorios Conda, Madrid, Spain), motility indol ornithine, and biochemical reactions on MacConkey (Oxoid, Basingstoke, UK). Microorganisms other than bacteria were confirmed by microscopic appearance. Samples where three or more pathogens grew were considered contaminated.

2.5. 16S rRNA-Gene Sequencing

For each quarter, 5 mL of milk were centrifuged by using a DNA extraction method based on the combination of a chaotropic agent, guanidium thiocyanate, with silica particles, to obtain bacterial cell lysis and nuclease inactivation (31). DNA quality and quantity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The isolated DNA was stored at −20 °C until use. Bacterial DNA was amplified using the primers described in literature (32) which target the V3–V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25 µL volume per sample. A total of 12.5 µL of Phusion High-Fidelity Master Mix 2x (ThermoFisher Scientific, Walthem, MA, USA) and 0.2 µL of each primer (100 µM) were added to 2 µL of genomic DNA (5 ng μL^{-1}). Blank controls (i.e., no DNA template added to the reaction) were also performed. No DNA extraction negative controls have been run. A first amplification step was performed in an Applied Biosystem 2,700 thermal cycler (ThermoFisher Scientific). Samples were denatured at 95 °C for 3 min, followed by 25 cycles with a denaturing step at 98 °C for 30 s, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. Amplicons were cleaned with Agencourt AMPure XP (Beckman, Coulter Brea, CA, USA) and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportion and sequenced in one MiSeq (Illumina) run with 2 × 250-base paired-end reads. The 16S rRNA gene sequences obtained from this study were deposited in the EMBL-EBI European Nucleotide Archive (ENA) repository with the accession number PRJEB38332.

2.6. Bioinformatics Processing

Demultiplexed paired-end reads from 16S rRNA-gene sequencing were first checked for quality using FastQC (33) for an initial assessment. Forward and reverse paired-end reads were joined into single reads using the C++ program SeqPrep (34). After joining, reads were filtered for quality based on: (i) maximum three consecutive low-quality base calls (Phred < 19) allowed; (ii) fraction of consecutive high-quality base calls (Phred > 19) in a read over total read length ≥ 0.75; (iii) no "N"

-labeled bases (missing/uncalled) allowed. Reads that did not match all the above criteria were excluded. All remaining reads were combined in a single FASTA file for the identification and quantification of OTUs (operational taxonomic units). Reads were aligned against the Greengenes closed reference sequence collection release 13.8, with 97% cluster identity (35), applying the CD-HIT clustering algorithm (36). A predefined taxonomy map of reference sequences to taxonomies was then used for taxonomic identification along the main taxa ranks down to the genus level (domain, phylum, class, order, family, genus). By counting the abundance of each OTU, the OTU table was created and then grouped at each phylogenetic level. Records belonging to OTUs with total counts lower than 10 in fewer than 2 samples were filtered out. All of the above steps, except the FastQC reads quality check, were performed with the QIIME 1.9 open-source bioinformatics pipeline for microbiome analysis (37). The command lines and parameters used to process 16S rRNA-gene sequence data are detailed in Biscarini et al. (38).

2.7. Alpha and Beta Diversity

The milk microbial diversity was assessed within- (alpha diversity) and across- (beta diversity) samples. All indices (alpha and beta diversity) were estimated from the complete OTU table (at the OTU level), filtered for OTUs with more than 10 total counts distributed in at least two samples. Besides the number of observed OTUs directly counted from the OTU table, within-sample microbial richness and diversity were estimated using the following indices: Chao1 and ACE (Abundance-based coverage Estimator) for richness, Shannon, Simpson, and Fisher's alpha for diversity (39–44), Simpson E and Pielou's J (Shannon's evenness) for evenness (45). The across-sample milk microbiota diversity was quantified by calculating Bray-Curtis dissimilarities (46). Prior to the calculation of the Bray-Curtis dissimilarities, OTU counts were normalized for uneven sequencing depth by cumulative sum scaling [CSS; (47)]. Among groups (teat sealant, cephalonium, cloxacillin, and control) and pairwise Bray-Curtis dissimilarities were evaluated non-parametrically using the permutational analysis of variance approach [999 permutations; (48)]. Details on the calculation of the mentioned alpha- and beta-diversity indices can be found in (38) (S2 Appendix).

2.8. Statistical Analysis

As a consequence of the chosen experimental design, data were hierarchically structured with treatments nested within individuals, and measurements repeated over time. Therefore, observations could not be assumed to be independent from each other, but were correlated within individual cows. This was taken into account in the linear models used to analyse between-group (treatments, timepoints) differences in terms of SCC, alpha diversity indices and OTU counts; SCC data were not normally distributed and have been log-transformed prior to the analysis. The model had the following form:

$$y_{ijk} = \mu + cow_j + [treatment|timepoint]_{k(j)} + e_{ijk} \qquad (1)$$

where y_{ijk} is the $log(SCC)$, alpha diversity index value or OTU counts for record i from cow j with treatment or timepoint k; μ is the intercept, cow_j is the systematic effect of the individual cow, $[treatment|timepoint]_{k(j)}$ is the effect of treatment or timepoint k nested within cow j and e_{ijk} is the residual. $Var(y) = \mathbf{\Sigma} + \mathbf{I}\sigma_e^2$, where $\mathbf{\Sigma}$ is a block diagonal matrix, with 1s on the diagonal and the covariances $\boldsymbol{\sigma}_{ij}$ between records within cows in the off-diagonal block elements; $\mathbf{I}$ is the identity matrix and $\boldsymbol{\sigma}_e^2$ is the residual variance. To test the interaction between treatments and timepoints, model 1 was expanded as follows:

$$y_{ijkz} = \mu + cow_j + treatment_{k(j)} + timepoint_{z(j)} + (\text{treatment x timepoint})_{kz(j)} + e_{ijkz} \qquad (2)$$

where terms were as in model 1 with the addition of the interaction terms $(\text{treatment x timepoint})_{kz(j)}$, again nested within individual cows. Besides correctly accounting for not independent nested observations, multilevel models as those in Equations (1) and (2) have the property of increasing the power of analysis through lower between-subject variability (each subject is its own control, fewer degrees of freedom).

TABLE 1 | Median somatic cell count (cells mL^{-1}) per treatment and time-point.

		Timepoint		
Treatment	**N (x time)**	**Dry-off**	**Calving**	**Early milk**
Cephalonium	5	4.30×10^4	1.38×10^6	2.00×10^4
Cloxacillin	5	1.39×10^5	1.39×10^6	4.60×10^4
Teat sealant	5	9.50×10^4	2.03×10^6	7.40×10^4
Control	5	1.34×10^5	1.56×10^6	1.89×10^5

2.9. Software

Reads from 16S rRNA-gene sequencing were processed with the QIIME pipeline v. 1.9 (37), used also to estimate most diversity indices. The ACE index and sample-base rarefaction were estimated using own *Python* (https://github.com/filippob/Rare-OTUs-ACE.git) and *R* (https://github.com/filippob/sampleBasedRarefaction) scripts. Plots were generated using the ggplot2 *R* package (49). Additional data handling and analysis was performed with the *R* environment for statistical computing (50).

3. RESULTS

3.1. SCC and Culture-Based Bacteriology

At the onset of the experiment the median quarter SCC per group was in the range 43,000 (cephalonium)–139,000 (cloxacillin) cells mL^{-1}. At the end of the experiment, SCC increased in the control group (+41.0%), and decreased with the teat sealant (−22.1%), cephalonium (−53.5%), and cloxacillin (−66.9%) treatments (**Table 1**). These differences were however not statistically significant. A physiological marked SCC increment was observed at calving (colostrum) across all groups (up to 2,000,000 cells mL^{-1}). Results from culture-based bacteriology showed that the milk samples used in this study were all negative to culture. No differences have been observed along the sampling period and among the quarters with different treatments.

TABLE 2 | Distinct OTUs included in the dairy cow core milk microbiome (100% of the samples).

Phylum	Class	Order	Family	Genus	Avg counts
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Corynebacteriaceae*	*Corynebacterium*	347.45
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Propionibacteriaceae*	*Propionibacterium*	1201.24
Firmicutes	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*	166.46
Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	277.52
Firmicutes	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Lactococcus*	28.40
Firmicutes	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*	1901.83
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	*Bradyrhizobium*	621.75
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Achromobacter*	46.87
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Enhydrobacter*	1373.12
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	3280.24
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*		2702.20
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*		220.06
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*			10240.48

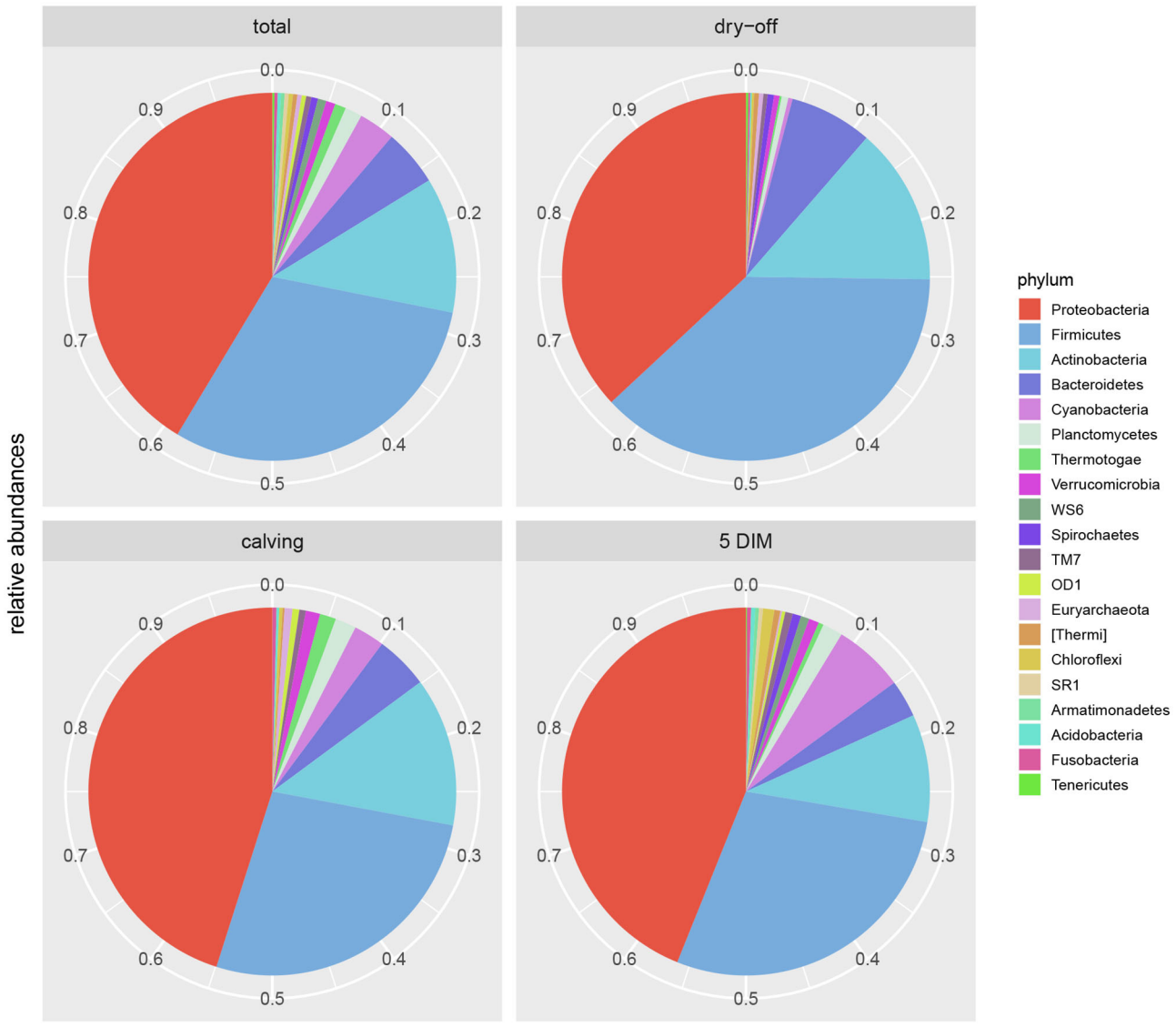

FIGURE 1 | Pie-charts of phylum relative abundances in the dairy cow milk microbiome over time. All data, and time point breakdown.

3.2. Sequencing Metrics

Sequencing the V3–V4 regions of the bacterial 16S rRNA-gene produced a total of 10,707,392 reads (joined R1-R2 paired-end reads), with an average of 178,456 reads per sample (5 cows × 4 quarters × 3 time-points = 60 samples). After quality filtering, 2,543,623 sequences were removed, leaving 8,163,769 sequences for subsequent analyses (76% average retention rate, maximum 85%, minimum 61%). The average number of sequences per treatment and time-point is reported in **Table S1**: this varies from a minimum of 93,474 (± 23,020) in the cephalonium group at dry-off to a maximum of 176,831 (± 122,987) in the cephalonium group at calving. The initial number of OTUs identified was 11 603; after filtering out OTUs with less than 10 counts in at least 2 samples, 4,495 distinct OTUs were left. To check whether sequencing depth and sample size were adequate to characterize the composition of the bovine milk microbiota, sequence-based and sample-based rarefaction curves were generated from the OTU table before filtering (11,603 OTUs). Sequence-based rarefaction curves were obtained from the QIIME pipeline; the sample-based rarefaction curve was produced with *ad hoc R* functions. The observed number of OTUs detected was plotted as a function of the number of reads (up to 40,922) in each sample and of the number of samples (**Figure S1**). Both curves tend to plateau asymptotically toward a maximum, indicating that sequencing depth and the number of samples were adequate to characterize the milk microbiota in the present study. Deeper sequencing or the addition of any other samples would likely not increase significantly the number of new OTUs discovered.

3.3. Core Milk Microbiome

Results from culture-based bacteriology confirmed that there were no milk samples either patently contaminated or from infected quarters. Therefore, results from 16S rRNA-gene sequencing from all samples could be used to characterize the core milk microbiome in dairy cows. Nevertheless, we can not positively exclude that a fraction of the bacterial taxa detected from 16S rRNA-gene sequencing at very low abundances in our milk samples could be the result of sporadic contamination. OTUs were grouped taxonomically from the phylum to genus level (phylum, class, order, family, genus). The 4,495 OTUs with more than 10 counts across samples clustered into 23 distinct phyla, 51 classes, 95 orders, 221 families, and 542 genera. Taxa with relative abundance < 0.1% were not considered. Considering OTUs shared by 99% of the samples, the dairy cow core milk microbiota comprised only a small portion of the total detected OTUs (**Table 2**), restricted to three phyla (*Proteobacteria*, *Firmicutes*, *Actinobacteria*). The core milk microbiome featured the genera *Corynebacterium*, *Propionibacterium*, *Staphylococcus*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Bradyrhizobium*, *Achromobacter*, *Enhydrobacter* with a relative majority of the families *Pseudomonadaceae*, *Alcaligenaceae*, and *Streptococcaceae*. In terms of relative abundances, **Figure 1** reveals that most of the reads belonged to the phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria*, which accounted for over 80% of the entire milk microbiota. A complete list of the bacterial groups at phylum, family, and genus level as well as their relative abundances is reported in **Table S2**.

3.4. Development of the Milk Microbiome Over Time (Dry-Off, Calving, 5 DIM)

Figures 1, **2** show the relative abundance of phyla and genera in the milk microbiome, overall and over time (dry-off, calving, 5 DIM). *Firmicutes* were found to be the most abundant phylum in the milk microbiome during dry-off, with *Proteobacteria* running up (39.3 and 36.7%, respectively), while at calving and 5 DIM milk sampling this was reverted, with *Proteobacteria* (47.9 and 46.2%) more abundant than *Firmicutes* (28 and 29.2%). The third and fourth most abundant phyla were *Actinobacteria* (13.9, 13.5, 10.8%) and *Bacteroidetes* (7.9, 5.5, 4.2%), at all timepoints. This has consequences on the *Firmicutes* to *Bacteroidetes* ratio (F:B), which is lower at dry-off (10.6) and higher at later time points (18.3, 22.3). **Table 3** reports the 61 OTUs, at the various taxonomic levels, which are significantly differentially abundant over time. The top significantly different OTUs are the genera *rc4-4*, *Saliniccocus*, *Dorea*, *Ruminococcus*, and *YRC22*, the families *Peptococcaceae* and *RF16*, the orders *RF39* and *Chlorophyta*, the phylum *Tenericutes*. In all cases, the largest difference in counts was observed at dry-off vs calving and 5 DIM. Indexes of richness (observed number of OTUs, Chao1, ACE), evenness (Simpson E, equitability -a.k.a. Shannon's evenness) and combinations thereof (Shannon's and Simpson's diversity indices, Fisher's alpha) describe the diversity of the milk microbiota. Results per timepoint are reported in **Figure 3** and **Table 4**: all comparisons between time points, except for Simpson E, were statistically significant. **Figure 4** shows the first two dimensions from the (non-metric) multi-dimensional scaling of the Bray-Curtis dissimilarity matrix.

3.5. Effect of Mastitis Treatments

Figure 5 reports the barchart of the relative abundances of phyla in the milk microbiome by dry-off treatment (cephalonium, cloxacillin, teat sealant, and control). The top three most represented phyla were the same in all treatments: *Proteobacteria* (43.0, 38.4, 46.4, 49.4%), *Firmicutes* (31.5, 33.7, 29.7, 31.1%), *Actinobacteria* (12.8, 11.9, 14.5, 10.8%). The fourth most common phylum was *Bacteroidetes* in milk samples from cephalonium (7.44%), cloxacillin (7.49%), and control (3.84%) quarters, *Cyanobacteria* in teat-sealant quarters (3.58%). The average F:B ratio was highest with teat sealant (31.6), followed by cloxacillin (16.4), controls (12.4), and cephalonium (7.8). Only five OTUs were significantly differentially abundant between mastitis treatments (**Table 5**): the phylum *Tenericutes* (p-value = 0.031), the class *Mollicutes* (p-value = 0.017), the order *Acholeplasmatales* (p-value = 0.040), the family *Yaniellaceae* (p-value = 0.036) with its genus *Yaniella* (p-value = 0.036). In all cases, the highest average number of counts was observed in quarters treated with cloxacillin. Overall comparisons of alpha diversity indices between treatments were not significant (**Table 4**). However, teat-sealant treated quarters showed a decrease in all diversity

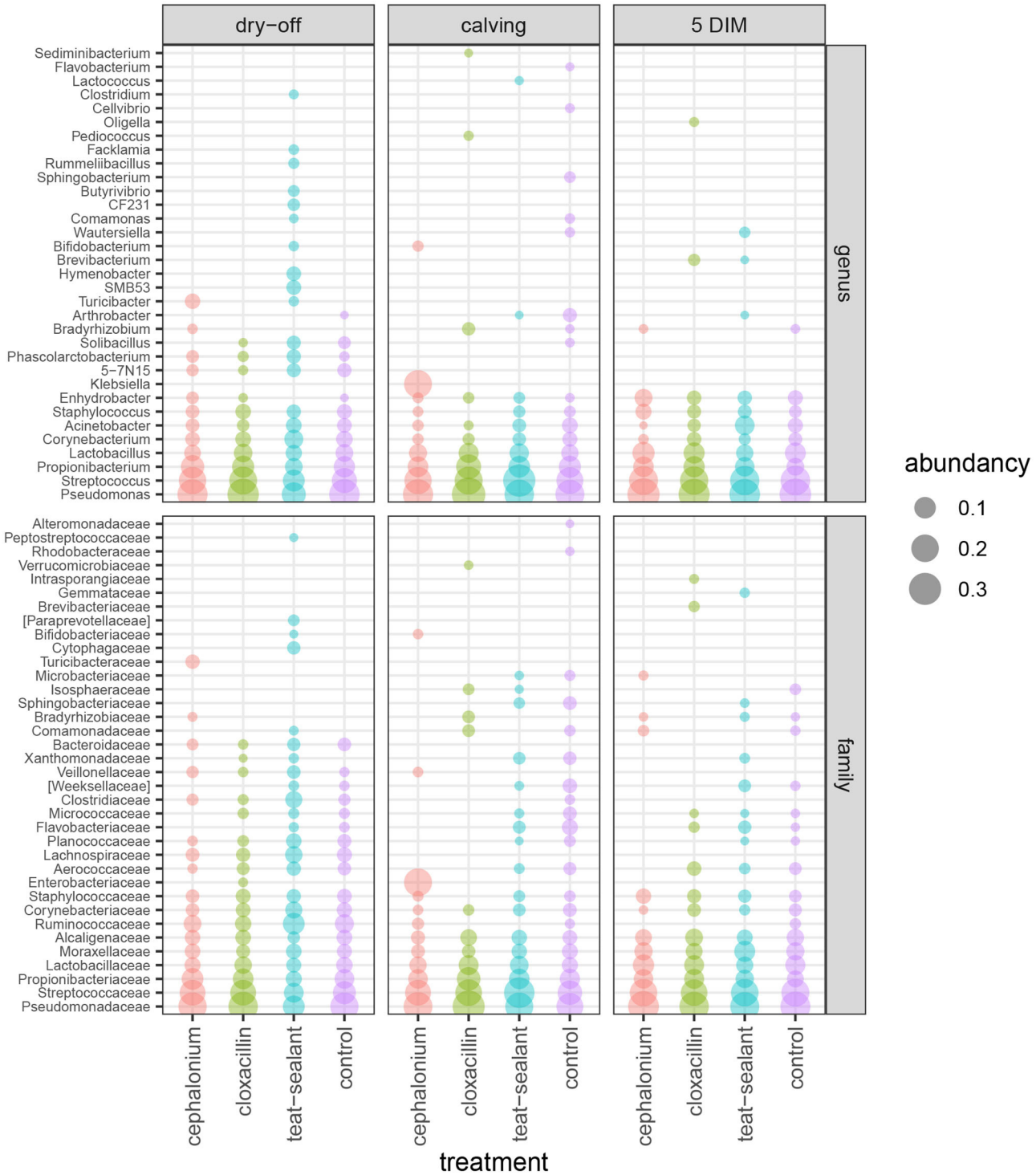

FIGURE 2 | Bubble chart of relative abundances for the families and genera identified in the bovine milk microbiome from 60 samples taken from differentially treated quarters at three timepoints (n = 5 lactating cows). Only taxa with relative abundance ≥ 1% are shown.

indices over time, when adjusting for variability at baseline (**Figure 4**, right pane). The first two dimensions from the (non-metric) multi-dimensional scaling of the Bray-Curtis dissimilarity matrix show extensive overlap between treatments, with no significant clustering (p-value from Permanova is 0.157).

TABLE 3 | OTUs with significant differential abundance between time points (alpha ≤ 0.05).

Taxon	OTU	Dry-off	Calving	5 DIM	*p*-value
Phylum	Tenericutes	57.80	1.30	17.30	1.39e-03
Phylum	Cyanobacteria	270.50	2586.15	8457.60	2.56e-02
Class	Mollicutes	49.45	0.95	17.30	1.66e-03
Class	Clostridia	12480.30	3593.15	2885.95	9.03e-03
Class	Verruco-5	24.70	1.95	0.00	1.93e-02
Class	4C0d-2	119.00	25.40	0.05	2.01e-02
Class	Chloroplast	148.40	2506.65	8450.05	2.37e-02
Class	Bacteroidia	3646.05	755.60	756.25	3.14e-02
Order	RF39	15.40	0.75	2.50	5.37e-05
Order	Chlorophyta	1.10	545.95	1268.30	3.18e-04
order	Clostridiales	12454.70	3304.15	2732.80	9.55e-03
Order	Aeromonadales	325.10	17.20	32.25	1.75e-02
Order	Rhodospirillales	42.05	277.95	599.15	1.88e-02
Order	WCHB1-41	24.70	1.95	0.00	1.93e-02
Order	YS2	119.00	25.40	0.05	2.01e-02
Order	Bacteroidales	3646.05	755.60	756.25	3.14e-02
Order	Anaeroplasmatales	16.90	0.05	0.00	3.74e-02
Order	Streptophyta	107.45	1959.00	7178.40	3.83e-02
Family	Peptococcaceae	75.85	7.65	5.00	2.52e-05
Family	RF16	185.40	18.05	27.20	1.35e-03
Family	Clostridiaceae	1740.15	709.35	384.25	1.86e-03
Family	S24-7	189.95	40.20	3.80	4.12e-03
Family	Ruminococcaceae	4620.30	881.80	733.45	9.30e-03
Family	Lachnospiraceae	2374.00	327.95	450.35	9.47e-03
Family	Moraxellaceae	2245.30	2558.45	6109.85	1.36e-02
Family	Rhodospirillaceae	31.35	267.65	592.85	1.74e-02
Family	RFP12	24.70	1.95	0.00	1.93e-02
Family	p-2534-18B5	15.80	1.40	0.15	2.10e-02
Family	Carnobacteriaceae	160.35	95.05	353.90	2.13e-02
Family	Peptostreptococcaceae	460.85	91.25	144.20	2.17e-02
Family	Succinivibrionaceae	323.00	7.05	28.40	2.40e-02
Family	Rikenellaceae	349.95	61.75	85.25	3.15e-02
Family	Bacteroidaceae	1261.20	329.30	244.50	3.16e-02
Family	Anaeroplasmataceae	16.90	0.05	0.00	3.74e-02
Genus	rc4-4	70.10	6.90	0.05	6.46e-06
Genus	Salinicoccus	34.90	0.00	0.50	4.63e-05
Genus	Dorea	267.80	26.40	25.30	4.91e-05
Genus	Ruminococcus	275.20	50.10	21.95	7.89e-04
Genus	YRC22	82.90	8.50	3.05	7.93e-04
Genus	Succinivibrio	145.50	4.25	18.50	1.54e-03
Genus	Alloiococcus	78.75	10.10	7.20	2.30e-03
Genus	GW-34	26.20	0.00	0.80	2.66e-03
Genus	Mogibacterium	13.90	4.20	0.65	5.32e-03
Genus	[Clostridium]	121.20	22.10	37.10	7.77e-03
Genus	SMB53	768.40	336.95	183.80	8.80e-03
Genus	Limnohabitans	0.45	2.20	26.45	9.91e-03
Genus	Helcococcus	14.75	0.05	0.00	1.38e-02
genus	Polaromonas	0.00	2.30	29.70	1.45e-02
Genus	Epulopiscium	91.05	21.25	1.00	1.46e-02
Genus	Phascolarctobacterium	989.60	288.30	132.85	1.79e-02

(Continued)

TABLE 3 | Continued

Taxon	OTU	Dry-off	Calving	5 DIM	*p*-value
Genus	Roseburia	51.90	3.10	15.80	2.16e-02
Genus	Butyrivibrio	448.50	108.40	160.55	2.24e-02
Genus	Clostridium	159.15	63.90	41.00	2.28e-02
Genus	Propionicimonas	38.55	1.00	0.20	2.98e-02
Genus	Coprococcus	153.25	28.35	40.10	3.03e-02
Genus	5-7N15	1084.85	225.20	220.15	3.16e-02
Genus	Anaerostipes	92.35	4.85	26.75	3.36e-02
Genus	Ruminobacter	165.80	0.50	5.15	4.32e-02
Genus	[Ruminococcus]	67.35	12.60	20.10	4.47e-02
Genus	Erythrobacter	5.90	27.90	2.65	4.63e-02
Genus	Rummeliibacillus	370.80	84.35	135.55	4.90e-02

Taxonomic level, specific OTU, counts at dry-off, calving, and 5 DIM, p-value.

4. DISCUSSION

In this paper, the effect on the milk microbiome of different treatments for mastitis prevention applied during the dry-off period has been investigated. Specifically, the antibiotics cephalonium and cloxacillin have been tested against a non-antibiotic treatment based on the application of an internal teat sealant, on a quarter by quarter basis. Untreated quarters were included in the experimental design as controls. Exploring non-antibiotic alternative options for the prevention of IMI at dry-off in dairy cows is a current research topic of paramount importance in the reduction of widespread antibiotic use in livestock, thereby contributing to alleviate issues related to antibiotic-resistant bacterial strains in veterinary and human medicine (11, 51).

The most interesting results on how the milk microbiome is altered in response to mastitis-prevention treatments are hereby discussed, together with insights into the general composition of the milk microbiome in dairy cows, and its development over the physiological status of lactating animals.

4.1. The Milk Microbiome in Response to Treatments

Two antibiotic (cephalonium and cloxacillin) and one non-antibiotic (teat sealant) treatments were compared in this study for their effect on the milk microbiome in Holstein-Friesian dairy cows. The most abundant phyla were consistently *Proteobacteria*, *Firmicutes*, and *Actinobacteria*, in this order, across treatments and controls. No significant differences were found between treatments and controls in terms of the major alpha and beta diversity indexes and OTU abundances (only 5 OTUs significantly different between treatments, **Table 5**). This is in line with similar findings from studies on DCT treatments and the milk microbiome: in clinically healthy Holstein-Friesian cows, Derakhshani et al. (24) found no differences in alpha diversity indices before and after BDCT treatment (combination of penicillin G and novobiocin, plus teat sealant), except for Chao1 (higher richness before BDCT than after), although in their study the effect of treatment was confounded with time (dry-off, calving). Bonsaglia et al. (25) also found no significant effect on the milk microbiome of DCT with either antibiotic (ceftiofur) plus teat sealant or teat sealant alone. The use of ITS does not lead to higher infection rates compared to antibiotics in DCT and at the same time appears to be neutral with respect to the milk microbiome (no differences between antibiotics, ITS, and controls): this justifies the replacement of antibiotics with ITS for DCT, which helps reduce the use of antimicrobials in dairy farms.

To reduce confounding from individual variability at the first sampling time (dry-off), alpha diversity indices were adjusted for baseline effect by removing the average values at dry-off (**Figure 3**, right pane): teat-sealant quarters appear to have lower adjusted diversity (except for Simpson's indices and equitability) compared to the two antimicrobial treatments and controls at calving and 5 DIM. Bonsaglia et al. (25) also found lower Chao1 and Shannon indices at DIM 7 with teat sealant compared to the combination of antibiotic plus teat sealant, though not significant. Bismuth subnitrate products not only act as a physical barrier, but also show inhibitory effect on bacterial growth (16): this can partially explain the efficacy of bismuth-based formulations in the prevention of intramammary infections over the dry period. Other products have been tested as teat sealants for their physical-barrier action, like wax plugs or intramammary polystyrene devices, but were unsuccessful in the long-term protection of cows against IMI and mastitis (52–54).

Contrary to expectations, antibiotic treatments did not cause a marked reduction of the milk microbiome diversity and bacterial counts, as reported also by previous publications (23, 25). This may be related to the specificity of the chosen antimicrobials, which targeted pathogens while leaving the rest of the microbiome practically unaltered [e.g., reduction of *Enterobacteriaceae* upon treatment with ceftiofur in the study of (23)]. Additional factors that can explain any differences between the results reported here and those found in literature include the study design, the time of sampling, the status of cows enrolled in the experiment, the libraries used for 16S rRNA-gene sequencing.

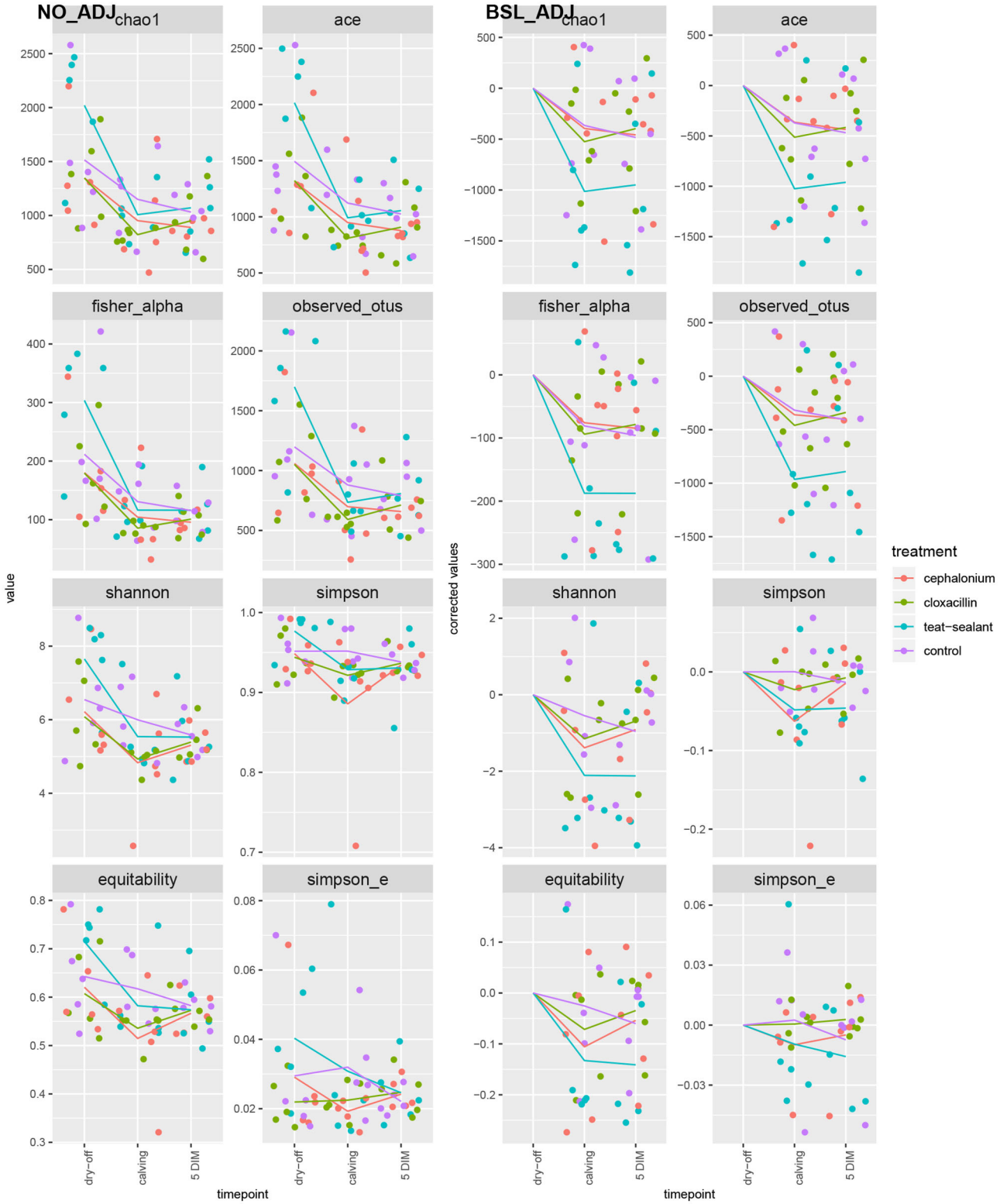

FIGURE 3 | Alpha diversity indices over time and per treatment. Raw values on the left pane (NO_ADJ), values adjusted for baseline on the right pane (BSL_ADJ). Solid lines are the average values per treatment and timepoint.

TABLE 4 | Alpha diversity indices by timepoint and treatment (*p*-value from linear mixed model).

Indices	Dry-off	Calving	5 DIM	Cephal.	Cloxac.	t-sealant	Controls	Timepoint	Treatment	Treattime
Chao1	1557.57	982.90	985.66	1062.76	1040.18	1366.56	1232.00	5.51e-06	6.36e-02	4.18e-01
Fisher alpha	218.75	109.21	107.07	126.70	121.98	178.70	152.66	6.91e-07	7.06e-02	3.62e-01
Observed otus	1251.65	726.20	741.85	804.93	784.80	1080.73	955.80	5.85e-06	6.41e-02	3.10e-01
Shannon	6.62	5.32	5.45	5.45	5.47	6.24	6.04	2.94e-04	8.80e-02	5.67e-01
Simpson	0.96	0.92	0.93	0.92	0.93	0.95	0.95	3.23e-02	3.03e-01	4.55e-01
Equitability	0.65	0.56	0.57	0.57	0.57	0.62	0.61	2.33e-03	1.14e-01	5.46e-01
Simpson e	0.03	0.03	0.02	0.02	0.02	0.03	0.03	3.81e-01	3.33e-01	7.28e-01
ACE	1536.77	954.79	957.43	1034.17	1011.04	1348.66	1204.79	4.74e-06	5.43e-02	3.73e-01

4.2. Core Milk Microbiome and Lactation Cycle

Table 2 and **Figures 1**, **2** offer a description of the milk microbiome in Holstein Friesian cows and of how it evolves over the lactation cycle. The core milk microbiome was defined as OTU shared by 99% of the samples (all): among genera usually associated with the milk milieu (*Lactobacillus*, *Lactococcus*, *Propionibacterium*), this includes also bacterial taxa commonly regarded as mastitis pathogens (*Staphylococcus*, *Pseudomonas*, *Streptococcus*). Similar findings have been reported in previous studies on the bovine milk microbiome (55). The most abundant taxa detected in the milk microbiota are the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Planctomycetes*, the families *Pseudomonadaceae*, *Streptococcaceae*, *Propionibacteriaceae*, *Lactobacillaceae*, *Moraxellaceae*, *Alcaligenaceae*, *Ruminococcaceae*, and the genera *Pseudomonas*, *Streptococcus*, *Propionibacterium*, *Lactobacillus*, *Corynebacterium*, *Acinetobacter*. *Proteobacteria*, *Firmicutes*, and *Actinobacteria* accounted for over 80% of the entire milk microbiota. These results are in agreement with the composition of the healthy milk microbiome previously reported in literature [see (55) for a review]. The milk microbiome from cows with clinical or subclinical mastitis is known to have lower alpha diversity and a different composition (22, 23). In Gram-negative mastitis, for instance, there is a higher relative abundance of *Proteobacteria* in the milk microbiome, specifically, of *Enterobacteriaceae* [over 60%, (23)]: in the present study, the relative abundance of *Enterobacteriaceae* was $<$ 1% at all timepoints (except for control quarters at calving, where it went up to $\sim$ 20%).

The milk microbiome showed a clear evolution over the lactation cycle -dry-off, calving (colostrum) and 5 DIM- as indicated by the distinct clustering of Bray-Curtis distances, which showed progressive separation from dry-off to calving and then to 5 DIM, and by the significantly different diversity indices between timepoints. In total, 61 OTU showed significant differences in abundance over time. As already reported by Derakhshani et al. (24), the family *Clostridiaceae* and the genus *Butyrivibrio* were significantly overrepresented in pre-DCT milk (dry-off, **Table 3**). In most cases (50 out of 61), these OTU were more abundant at dry-off (beginning of the experiment) than at subsequent timepoints (**Figure 6**). The transition from colostrum to mature milk comes along with shifts in the composition of mammary secretions, and some milk components, like milk oligosaccharides, can affect the composition of the milk microbiome (55). In humans, the milk microbiota composition has been reported to be related to host factors like BMI (body mass index) (56): in cattle, body condition (e.g., as measured by BCS: body condition score) is known to change profoundly from dry-off to early lactation, as a consequence of the major physiological changes associated with parturition and the onset of milk production, and it is therefore plausible that it can likewise influence the milk microbiome.

All alpha diversity indices differ significantly between timepoints (except Simpson's E), while the interaction term (timepoint $\times$ treatment) was never significant (**Table 4**, alpha

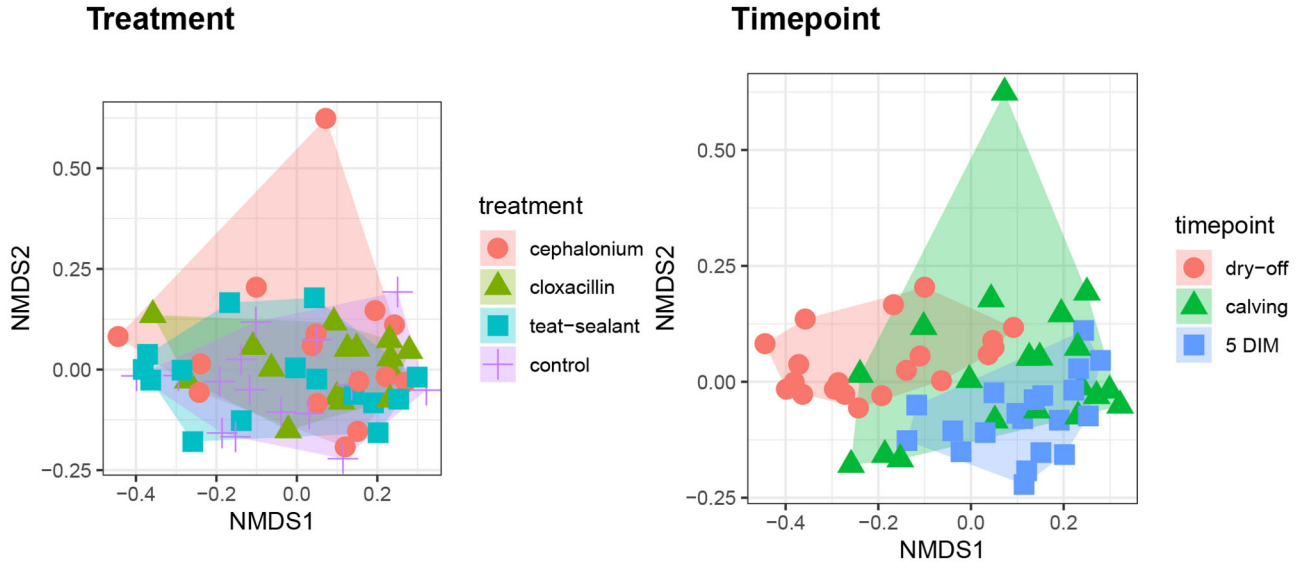

FIGURE 4 | First two dimensions from the (non-metric) multi-dimensional scaling of the Bray-Curtis dissimilarity matrix. Samples were grouped by experimental units: by treatment on the left pane, by timepoint on the right pane. PERMANOVA among treatments p-value = 0.157, PERMANOVA among timepoints p-value = 0.001 (using 999 permutations).

TABLE 5 | OTUs with significant differential abundance between treatments (alpha ≤ 0.05).

Taxon	OTU	Cephalonium	Cloxacillin	Teat-sealant	Controls	*p*-value
Phylum	Tenericutes	11.93	59.87	12.33	17.73	3.06e-02
Class	Mollicutes	10.87	54.00	10.07	15.33	1.70e-02
Order	Acholeplasmatales	6.13	31.27	2.20	3.20	4.04e-02
Family	Yaniellaceae	129.40	192.87	24.93	21.93	3.64e-02
Genus	Yaniella	129.40	192.87	24.93	21.93	3.64e-02

Taxonomic level, specific OTU, counts in quarters treated with cepravin, cloxalene, teat-sealant or controls, p-value.

diversity) indicating that overall changes in the milk microbiota diversity over the lactation cycle were independent of treatments. However, the fact that significantly different OTUs were more abundant at the beginning of the experiment (dry-off) may hint at a possible effect of treatments on the depletion of specific microbial taxa, in addition (or in combination) to the physiological influence of the lactation cycle.

When looking at phyla, a shift from *Firmicutes* to *Proteobacteria* as the most abundant phylum was observed between dry-off vs calving and 5 DIM. This is reflected in the evolution of the *Firmicutes*:*Bacteroidetes* (F:B) ratio, which increased with time. The F:B ratio has been used to describe the shift in the gut microbiota associated with aging in humans (57), where it has been reported to increase with time, as found in the present study but on a different timespan. More importantly, the F:B ratio in the gut microbiota is known to play a role in adipogenesis: in studies on obesity in mice and humans, it has been related to higher blood and tissue fat (58, 59), although cause/effect remains unresolved. In Holstein-Friesian cows, Jami et al. (60) observed a strong positive correlation between the F:B ratio in the rumen microbiota and milk-fat yield: this latter finding is mirrored in this study, where a higher F:B ratio has been found in the milk microbiome at the onset of milk production (calving and 5 DIM), when a sharp increase in fat anabolism in the mammary gland takes place. A yet unresolved but interesting question is whether the parallel association between increased F:B ratio and milk yield in both the rumen and milk microbiota is linked to the role of common metabolic pathways in the biosynthesis of fatty acids or, on the other hand, points to interconnections between the two microbial communities.

4.3. Implications for the Dry-Cow Therapy

Selective dry-cow therapy (SDCT) consists of treating with antimicrobials only cows with IMI, while non-antibiotic treatments are used on healthy cows. Since 80% of the antibiotics used in dairy farming are used to treat mastitis (23, 61), the adoption of SDCT over BCDT is bound to have a large global effect and can help reduce the spread of antimicrobials resistance (62). Teat sealants are among the non-antibiotic treatments commonly used for SDCT, and it is relevant to assess their impact on the milk microbiome relative to BDCT. Bonsaglia et al. (25)

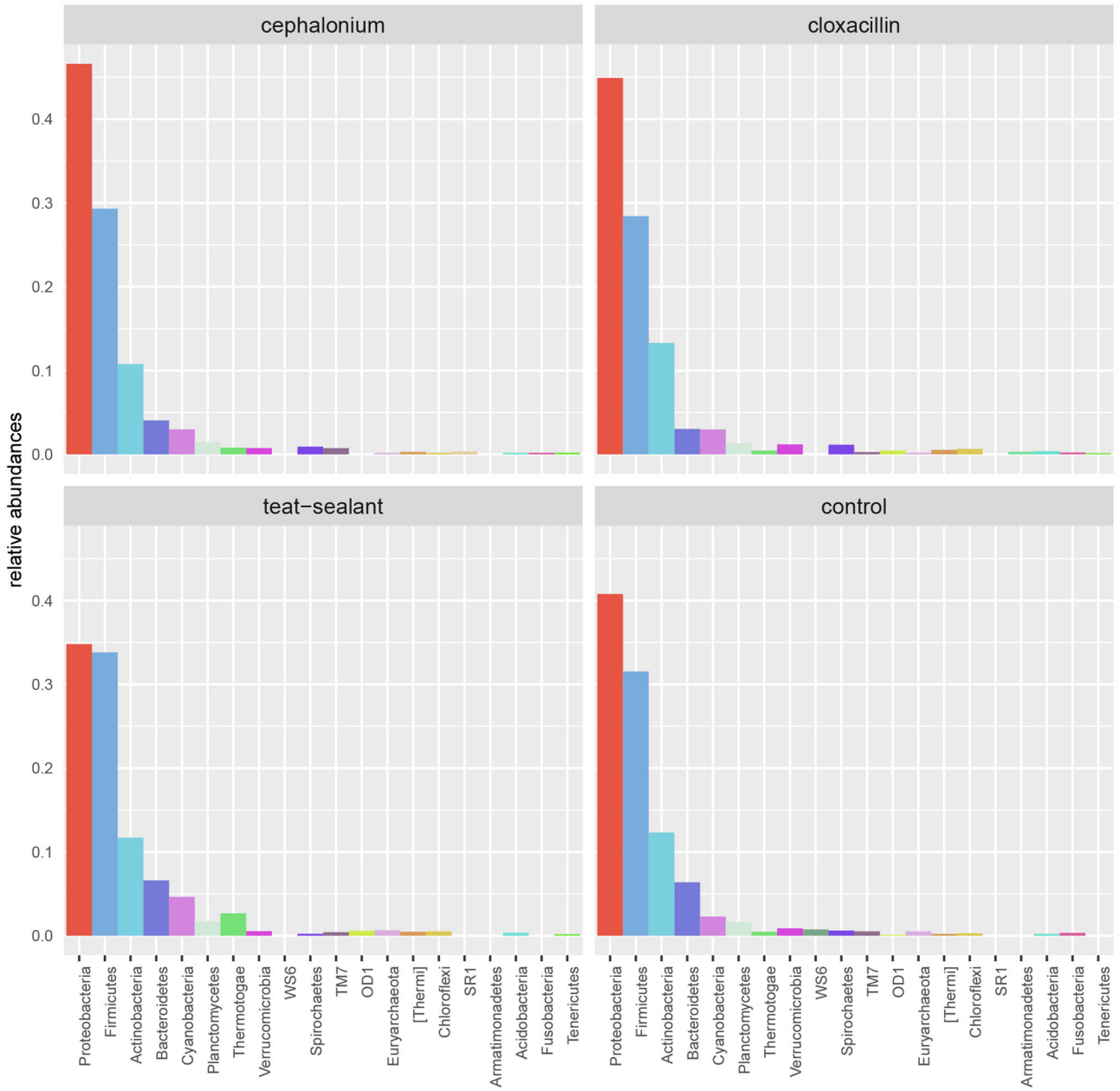

FIGURE 5 | Bar-charts of phylum relative abundances in the dairy cow milk microbiome between mastitis treatments: cephalonium, cloxacillin, teat sealant, controls.

already suggested that cows screened as negative for mastitis during lactation can be managed at dry-off with teat sealant alone without detrimental effects on milk microbiome and bacterial load at first week postpartum. Similar results have been found in the present study, where antimicrobials were directly compared to teat sealant alone rather than in combination, with the added value of testing a first-generation cephalosporin rather than, as did previous works (23, 25), third-generation cephalosporins which are currently not recommended for veterinary use in EU. Our study included cows with low SCC (<200,000 cells mL^{-1}) along the whole lactation and without IMI before dry-off, and we found no differences in the prevalence of IMI after calving between quarters treated with different DCT antibiotics and quarters treated only with ITS. ITS play a key role in the success of SDCT programs and their use is highly recommended to achieve good results (7, 63). Importantly, we found no significant differences in the milk microbiome between DCT treatments with antibiotics or ITS. It is however important to be aware of the potential limitations of the present study, which include: (i) the sample size (5 cows, although the statistical power has been increased by adopting a nested quarter-based design); (ii) results are directly applicable only to Holstein-Frisian second-parity

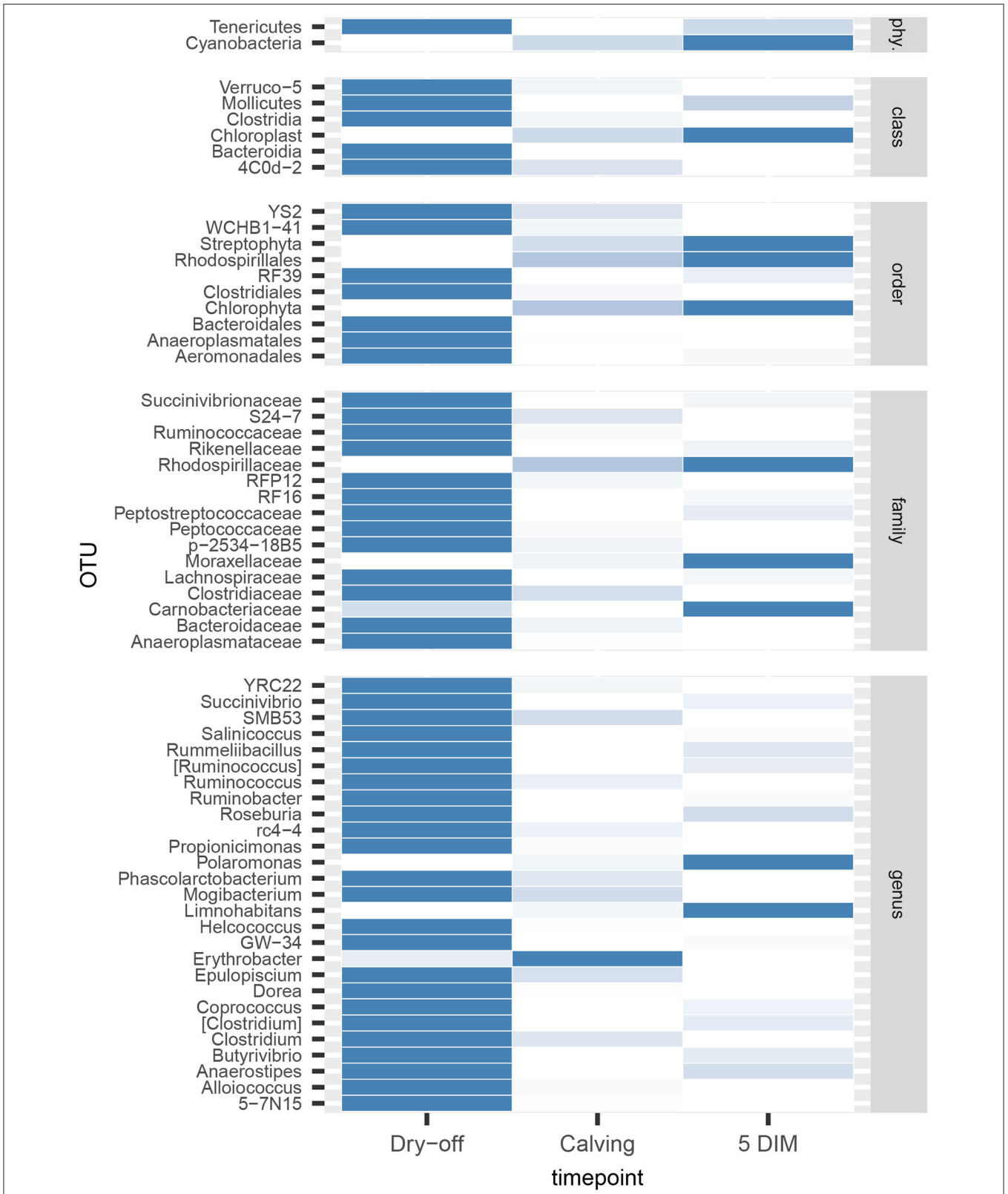

FIGURE 6 | Heatmap of bacterial counts at different timepoints for OTUs found to be significantly different over the lactation cycle. phy.: phylum.

cows; (iii) cows were sampled from a single intensive-farming herd in Northern Italy.

Summarizing, the milk microbiomes of healthy dairy cows prophylactically treated with either antibiotics or teat sealants did not show significant differences within 5 DIM from calving. Combined with the analogous efficacy for mastitis prevention and the reduction in the use of antimicrobials, this further supports the adoption of teat sealants as replacement of antibiotic prophylaxis (BDCT) in healthy cows.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena/browser/view/PRJEB38332.

AUTHOR CONTRIBUTIONS

PM and VB: experimental design and supervision. VB: sampling of biological material. CL and VB: bacteriology. PC and BC: sequencing. FB and AS: bioinformatics. FB: statistical analysis. PC, BC, FB, and PM: writing of the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00581/full#supplementary-material

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The Effect of Metaphylactic Use of Tildipirosin for the Control of Respiratory Disease in Long-Distance Transported Dairy Calves

*Maria Luiza Celestino[1], Leticia Fernandes[1], Paulo Roberto Menta[1], Daniela Paiva[1], Thiago Lauro Ribeiro[1], Thiago Silva[1,2], Todd R. Bilby[3], Rafael C. Neves[1,4], Michael A. Ballou[1] and Vinicius S. Machado[1]**

[1] Department of Veterinary Sciences, College of Agricultural Sciences and Natural Resources, Texas Tech University, Lubbock, TX, United States, [2] Department of Animal Science, School of Animal Science and Food Engineering, University of São Paulo, São Paulo, Brazil, [3] Merck Animal Health, Madison, NJ, United States, [4] Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Purdue University, West Lafayette, IN, United States

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***Correspondence:**
Vinicius S. Machado
vinicius.machado@ttu.edu

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The objective of this study was to evaluate the efficacy of two metaphylactic strategies using tildipirosin for the control of bovine respiratory disease (**BRD**) in dairy calves transported to a heifer raising facility within their first week of life. A total of 2,100 calves were enrolled in the study. Animals were transported for ∼1,715 km, from dairies located in Minnesota to a calf raising facility located in New Mexico, where they were housed in individual hutches until weaning. Three days after arrival, calves were randomly allocated into three groups: (1) META1: single subcutaneous (SQ) injection of tildipirosin (Zuprevo™, Merck Animal Health) at enrollment at 4 mg/kg; (2) META2: SQ injection of tildipirosin at enrollment and 17 days later; (3) CON: untreated controls. The BRD incidence was 11.4, 10.8, and 9.4% for calves enrolled in the CON, META1, and META2, respectively ($P = 0.44$). Lung lesions diagnosed through ultrasonography was found in 21.0, 21.0, and 21.8% of calves enrolled in CON, META1, and META2, respectively ($P = 0.99$). Mortality tended to be greater for CON calves in comparison to META2 calves (1.5 vs. 0.6%, $P = 0.06$), but did not differ between calves enrolled in CON and META1 groups (1.5 vs. 1.2%, $P = 0.55$). Growth was not affected by metaphylaxis. The average daily gain for calves enrolled in CON, META1, and META2 was 517, 518 and 525 g, respectively ($P = 0.25$). Blood analysis revealed that some of the markers of inflammation assessed were influenced by metaphylaxis. At 27 days after enrollment, META2 calves had decreased concentrations of haptoglobin, serum amyloid A, and aspartate aminotransferase, compared to CON calves ($P < 0.05$). Additionally, CON calves had increased concentrations of globulins and lower albumin to globulin ratio than META2 calves at the end of the weaning period ($P < 0.05$). In conclusion, tildipirosin metaphylaxis did not decrease the incidence of BRD nor did it have an impact on weight gain. However, metaphylaxis with two injections of tildipirosin at enrollment and 17 days later tended to reduce mortality and improved the systemic inflammatory status of calves during the preweaning period.

Keywords: dairy calves, metaphylaxis, BRD, tildipirosin, transportation

INTRODUCTION

Bovine respiratory disease (**BRD**) is a highly prevalent and multifactorial illness responsible for production losses in pre-weaned dairy calves. Clinical signs associated with BRD are nasal and ocular discharge, cough, fever, and droopy ears (1, 2). According to the United States Department of Agriculture (**USDA**) National Animal Health Monitoring Survey conducted in 2014, BRD affected 27% of pre-weaned calves and caused 14.1% of deaths (3). Recently, a study performed in California dairies between 2015 and 2016, reported 22.8% BRD incidence in pre-weaned dairy calves totaling 19.3% case fatality rate (4). The short-term economic impact of BRD on farm operations are due to labor (i.e., for disease detection and care of sick animals), medications, veterinary fees, and replacement of dead animals (5, 6). Dubrovsky et al. (4), calculated short-term cost of treating recurrent cases of BRD in dairy calves was $42.15 per calf. Additionally, the long-term costs of BRD are complex and involve impaired performance of animals even when they have received treatment (7, 8). These animals undergo delayed growth during the pre-weaning period (7, 9), decreased reproductive performance (10), increased chance of leaving the herd prior to first calving (7, 8, 11), increased age at first calving (11) and decreased milk production during first lactation (8).

The etiopathogenesis of BRD involves an interaction between host and environmental factors, stressors, pathogens and management practices (12, 13). The disease is usually initiated by a stressful event (i.e., transportation, comingling) followed by a viral or bacterial infection, which predisposes the animals to bacterial infections (14, 15). Viruses such as bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), parainfluenza type 3 virus (PI-3), bovine corona virus (BCV), bovine adeno- virus (BAV) and bovine herpes virus 1 (BoHV-1) have been described as causative agents of the BRD complex (15, 16). The most common bacterial agents associated with BRD cases are *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni,* and *Mycoplasma bovis*, with the most predominant pathogen being *Mannheimia haemolytica* (7, 15). These pathogens can be found in the upper respiratory tract of both healthy and diseased calves (17). Thus, the onset of the disease will be dependent on bacterial load and risk factors, such as season of birth, failure of transfer of passive immunity, and occurrence of other diseases within the first 14 days of life (18, 19). Also, commingling of animals and long-distance transportation can increase the risk for BRD (7, 20, 21). In the modern U.S. dairy industry, 10% calves are raised in specialized facilities, where calves are acquired from different sources and are transported for long periods of time, which are known stressors leading to increased BRD risk (22).

To minimize deleterious impacts of BRD, metaphylactic antimicrobial administration before the main peak of BRD incidence is a common management practice to reduce pathogen load in a high-risk population (12). The anti-infective tildipirosin (Zuprevo^{TM}, Merck Animal Health) is a long-acting macrolide that is indicated for the treatment and control of BRD in high risk cattle. The pharmacokinetics properties of tildipirosin include rapid distribution to lung tissue and bronchial fluid with a long half-life, which leads to a sustained concentration of the macrolide in the lower respiratory tract (23). Reports regarding the effectiveness of the metaphylactic use of tildipirosin to control BRD in high-risk calves have been inconsistent. Metaphylactic use of tildipirosin at arrival did not reduce the number of BRD treated cases in veal calves (13). However, others have shown that metaphylactic injections of tildipirosin reduces the incidence of pneumonia and otitis during the pre-weaning period of dairy calves housed in group pens (9). The efficacy of tildipirosin to control and mitigate the deleterious effects of BRD in dairy calves transported to calf raising facilities are unknown. The objective of this study was to evaluate the effect of two metaphylactic strategies using tildipirosin in the incidence of BRD, growth, and mortality of dairy calves originating from multiple sources and following long transport time within the first week of life (i.e., high risk).

MATERIALS AND METHODS

All activities performed in this study were reviewed and approved by the Texas Tech University Institutional Animal Care and Use Committee (#18081-10).

Animals and Facilities

The study was conducted in a commercial heifer raising facility located in eastern NM, from January 11, 2019 to July 15, 2019. Calves were born in 13 different farms located in Minnesota. General management practices of these farms included immediate separation of calves from their dams and feeding of 4 L of pasteurized (60°C for 60 min) pooled colostrum within the first 6 h of life. Total serum protein was assessed for a subset of calves by farm employees to evaluate and ensure proper colostrum management. Within the first week of life (mean ± SD = 3.78 ± 1.3 days of life), calves were transported from their farm of origin to the calf raising facility located in NM. The approximate transportation distance was 1,715 km. At arrival, calves were individually housed in hutches. Whole milk was fed twice a day (4 L/d), water and calf starter were offered ad libitum during the pre-weaning period. Calves were vaccinated at birth intranasally with BRSV, IBR, and PI3 (Inforce 3, Zoetis, MI), and at 30 days of age and at weaning, with a *Mannheimia haemolytica* bacterin-toxoid bacteria (One Shot, Zoetis, MI).

Treatment Allocation, Data Collection, and Case Definition

Calves were enrolled in the study at 3 days after arrival and were randomly allocated into three different groups: (1) CON: untreated controls, $n = 700$; (2) META1: single SQ injection of tildipirosin at enrollment, $n = 700$; and (3) META2: one SQ injection of tildipirosin at enrollment and a subsequent SQ tildipirosin injection 17 days later, $n = 700$. Tildipirosin treatments followed the label dose of 4 mg/kg of body weight. Calves were included in the study if they did not present clinical signs associated with BRD, such as ocular or nasal discharge, ear droop, cough, or rectal temperature $\geq 39.2^{\circ}$C. All animals were tested for BVD at enrollment. Fresh skin samples (ear notch) were submitted to the Texas A&M Veterinary Medical

Diagnostic Laboratory in Amarillo, TX and tested using the antigen capture ELISA method. Persistently infected animals were excluded from the study. Animals that had been previously treated with antibiotics for BRD or other conditions were not eligible to be enrolled in the study.

Calves were visually inspected by the research team members three times per week (on a M-W-F basis) from enrollment until weaning (60 days of life). The research team used a systematic scoring system developed for the assessment of BRD in pre-weaned dairy calves (2). This validated scoring system assesses six clinical signs (cough, eye discharge, abnormal respiration, nasal discharge, ear droop or head tilt, and rectal temperature $\geq$ 39.2°C: Cough = 2 points, Eye discharge = 2 points, Fever ($\geq$ 39.2°C) = 2 points, Abnormal respiration = 2 points, Nasal discharge = 4 points, Ear droop or head tilt = 5 points. A total score of five points or higher characterizes a BRD case and treatment was warranted. To achieve blinding of research and farm personnel, treatment allocation and administration were performed by a veterinarian from the research team in the mornings, and BRD scoring for diagnosis was performed by another veterinarian from the research team (unaware of treatment assignment) in the afternoons. In addition to the research group monitoring and scoring recording, animals were visually monitored daily by trained farm employees following the same BRD scoring system utilized by the research team. Farm personnel was also blinded to treatments. Animals diagnosed with BRD were treated with 40 mg/kg florfenicol and 2.2 mg/kg flunixin meglumine (Resflor Gold®, Merck Animal Health, NJ). Treated animals had a 4-day post-treatment interval, when they were not eligible to receive subsequent treatment, unless authorized by the herd veterinarian. If clinical signs persisted after 4 days of initial treatment, animals were re-treated with a different drug class (e.g., Enrofloxacin, Baytril® 100, Bayer, NJ).

At enrollment and weaning (60 days of life), ultrasonography of the lungs was assessed for a random subset of 200 calves per treatment. Thoracic ultrasonography was performed by a trained veterinarian using an Ibex-pro device with a 6.2-MHz linear transducer (E.I. Medical Imaging, Loveland, CO). The examination of the lungs was carried out by a dorsal to ventral screening of the thorax. The area from the 1st to the 10th intercostal spaces was screened on the right side of the thorax, and from the 3rd to the 9th on the left side. Consolidation of the lungs was detected based on heterogeneous hypoechoic area in the absence of the pleural surface clear line. Body weight measurements were assessed at enrollment and at the end of the study period (49 days after enrollment) using a digital scale (Calf Cart™, Raytec®, Ephrata, PA). These measurements were used to calculate the average daily gain (ADG) during the study period (final weight–initial weight/days in study).

Data regarding mortality, source (farm of origin), total serum protein, date of birth, dam's parity, dam's gestation length were extracted from the farms' database software (DairyComp 305, Valley Agricultural Software, Tulare, CA).

Blood Sampling and Analysis

Blood was collected for a random subset of 100 calves per treatment group to determine evidence of stress and inflammation. Blood samples were collected at enrollment, 10, 27, and 49 days later by jugular venipuncture using a Vacutainer tube without anticoagulant and a Vacutainer tube with EDTA, and a 20-gauge × 2.54-cm Vacutainer needle (Becton, Dickinson and Company, Franklin Lakes, NJ). After collection, tubes were immediately placed in a cooler containing iced water and transported to Texas Tech University (Lubbock, TX) within 2 h after collection for processing. Blood samples collected without anticoagulant were centrifuged for serum separation, and frozen at −80°C. Samples collected with EDTA were evaluated for complete blood cell (CBC) counts, using a hematology analyzer (IDEXX Procyte DX, Westbrook, ME).

Serum haptoglobin (Hp) concentration was determined using a colorimetric assay via quantification of the haptoglobin/hemoglobin complex by the estimation of differences in peroxidase activity (24). Assays were performed in 16 × 100 borosilicate tubes. Briefly, 5 μL of serum sample or deionized water (blank) were added to 7.5 mL of a solution containing 0.6 g/L of O dianisidine, 13.8 g/L of sodium phosphate monobasic, and 0.5 g/L EDTA (pH = 4.1). Immediately, 25 μL of 0.3 g/L bovine hemoglobin solution was added to each assay, followed by a water bath incubation at 37°C for 45 min. After incubation, 100 μL of freshly prepared 156 mM hydrogen peroxidase solution was added to each assay. Samples were incubated at room temperature for 60 min. Then, 200 μL of each assay was transferred to a 96-well polystyrene flat-bottom microplate. Optical density at 450 nm was measured on the Epoch2 Microplate Spectrophotometer (BioTek, Winoosk, VT). Finally, the final OD of each assay was subtracted by the blank assay OD. Optical density data was converted to a concentration unit (μg/mL) using standard curves generated by serial dilutions of a sample of known concentration determined by a commercially available ELISA kit following the manufacturer's instructions (Life Diagnostics, West Chester, PA) as previously described (25). The intra and inter-assay CV for serum Hp were 6.9 and 7.7%, respectively. Serum amyloid A (SAA) was determined by a commercially available ELISA kit following the manufacturer's instructions (Life Diagnostics, West Chester, PA). The intra and inter-assay CV for serum Hp were 5.2 and 7.3%, respectively. Samples were analyzed for Zinc concentration using a chemistry analyzer (RX Daytona; RANDOX Laboratories, Crumlin, UK) in a single assay, and the intra-assay CV was 1.9%.

A 0.5 mL aliquot of serum was submitted to the Texas A&M Veterinary Medical Diagnostic Laboratory for ruminant chemistry profile (total protein, albumin, albumin to globulin ratio, globulin, glucose, blood urea nitrogen, calcium, phosphorus, creatinine kinase, total bilirubin, aspartate aminotransferase, gamma-glutamyl transferase, magnesium, sodium, potassium, chloride, and glutamate dehydrogenase activity).

Sample Size Calculation

Based on previous year BRD incidence data from the studied herd, baseline incidence of BRD in pre-weaned calves housed in hutches was anticipated to average 15%, with an assumption that tildipirosin metaphylaxis would reduce BRD incidence by at least 5%. To detect this reduction, 686 calves per treatment

group were needed for a study with 80% power and significant differences declared at $\alpha = 0.05$. To account for eventual data loss (i.e., calves excluded due to BVD diagnosis) a total of 700 calves per treatment group (three treatment groups, $n = 2{,}100$ total) was enrolled in the study.

Statistical Analysis

Descriptive statistics were undertaken using the chi-square and ANOVA functions of JMP 14 (SAS Institute Inc., Cary, NC). To evaluate the effect of metaphylaxis on BRD incidence and presence of lung lesions diagnosed by ultrasonography, two multivariable logistic regressions models were fitted to the data using the GLIMMIX procedure of SAS 9.4 (SAS Institute Inc.). Mortality was evaluated using a multivariable Cox's proportional hazard model (PHREG procedure in SAS). Calves were right-censored if they were alive at the end of the data collection period. The effect of metaphylaxis on growth during the pre-weaning period (ADG) was evaluated using the MIXED procedure of SAS. To evaluate the effect of metaphylaxis on the circulating concentration of metabolic and inflammatory markers, multiple mixed general linear models were fitted to the data using the MIXED procedure of SAS. The data comprised a series of repeated measures of each dependent variable throughout the four blood collection days. To account appropriately for within-calf correlation, the error term was modeled by imposing a first-order autoregressive covariance structure for all models. Visual assessment of the distribution plots of the studentized residuals were used to confirm that the residuals were normally distributed.

For all multivariate models described above, independent variables and their respective interactions were kept when $P < 0.10$. Treatment was forced into all statistical models even in the absence of statistical significance. Age in days at enrollment, body weight at enrollment, dam's parity (primiparous or multiparous), season (Winter or Spring), and rectal temperature at enrollment were offered to all models. Origin of source was included under the STRATA statement in the Cox proportional hazard analyses and as a random variable in all other statistical models described above.

RESULTS

Descriptive Statistics

Descriptive statistics on averages for age at enrollment (in days), body weight at enrollment, rectal temperature at enrollment, total serum protein (subset of animals), dam's gestation length, parity of dam, total number of animals enrolled by season,

TABLE 2 | Effect of tildipirosin metaphylaxis on the incidence of bovine respiratory disease (BRD), ultrasonographic lung consolidation (ULC) at weaning, mortality, and average daily gain (ADG).

	CON[1]	META1[2]	META2[3]
BRD			
Incidence (%)	11.4	10.8	9.4
Odds ratio (95% CI)	*baseline*	0.95 (0.68–1.31)	0.80 (0.57–1.13)
P		0.75	0.21
ULC			
Affected calves (%)	21.0	21.0	21.8
Odds ratio (95% CI)	*baseline*	1.00 (0.62–1.60)	1.05 (0.66–1.68)
P		1.00	0.97
Mortality			
Dead calves (%)	1.5	1.2	0.6
Hazard ratio (95% CI)	*Baseline*	0.77 (0.32–1.82)	0.34 (0.11–1.06)
P		0.55	0.06
ADG (g)	517	518	525
95% CI	(508–525)	(509–526)	(516–533)
P		0.84	0.12

[1]*CON: untreated controls.*
[2]*META1: single SQ injection of tildipirosin (4 mg/kg) at enrollment.*
[3]*META2: one SQ injection of tildipirosin at enrollment and a subsequent SQ tildipirosin injection 17 days after the first injection.*

TABLE 1 | Descriptive statistics of treatment groups.

	CON[1]	META1[2]	META2[3]	*P*
Average age (days) at enrollment (± SE)	7.8 (0.05)	7.8 (0.05)	7.8 (0.05)	0.96
Average body weight (kg) at enrollment (± SE)	32.7 (0.16)	33.0 (0.16)	32.8 (0.16)	0.46
Average rectal temperature (°C) at enrollment (± SE)	38.7 (0.01)	38.7 (0.01)	38.7 (0.01)	0.98
Average total serum protein g/dL (± SE)[4]	6.5 (0.03)	6.5 (0.03)	6.5 (0.03)	0.25
Average days of gestation of dam (± SE)	278.3 (0.61)	277.0 (0.61)	277.7 (0.61)	0.30
Average parity of dam (± SE)	2.3 (0.02)	2.3 (0.02)	2.3 (0.02)	0.87
Total enrolled animals during winter (%)	330 (47.1)	330 (47.1)	330 (47.1)	1.00
Total enrolled animals during spring (%)	370 (52.9)	370 (52.9)	370 (52.9)	
Total enrolled animals (%)	700 (33.3)	700 (33.3)	700 (33.3)	
Total excluded animals (%)	3 (0.43)	1 (0.14)	3 (0.43)	0.51

[1]*CON: untreated controls.*
[2]*META1: single SQ injection of tildipirosin (4 mg/kg) at enrollment.*
[3]*META2: one SQ injection of tildipirosin at enrollment and a subsequent SQ tildipirosin injection 17 days after the first injection.*
[4]*Total serum protein was assessed for 310, 327, and 325 calves enrolled in CON, META1, and META2 treatment groups, respectively.*

and total number of excluded animals are presented in **Table 1**. Six animals were excluded from the study because they were diagnosed as BVD-PI. One animal was excluded from the study because a few weeks after enrollment, it was noticed that it was a male calf.

Effect of Metaphylaxis on BRD Incidence and Lung Consolidation Diagnosed Through Thoracic Ultrasonography

Tildipirosin metaphylaxis did not decrease the incidence of BRD during the pre-weaning period of dairy calves (**Table 2**, $P = 0.44$). The BRD incidence was 11.4, 10.8, and 9.4% for calves enrolled in CON, META1, and META 2, respectively. Similarly, tildipirosin metaphylaxis did not decrease the proportion of calves diagnosed with lung lesions through ultrasonography at weaning (**Table 2**, $P = 0.99$). The proportion of calves diagnosed with lung consolidation at the end of the study period was 21.0, 21.0, and 21.8% for CON, META1 and META2 calves, respectively.

Effect of Metaphylaxis on Mortality and Average Daily Gain

Although we did not observe treatment differences in lung health outcomes, tildipirosin metaphylaxis at enrollment and 17 days later tended to decrease mortality (**Table 2**). Hazard of death was 2.94 times higher for CON calves in comparison to META2 calves ($P = 0.06$). However, the hazard of death did not differ between CON and META1 calves ($P = 0.55$). Additionally, tildipirosin metaphylaxis did not influence growth of pre-weaned calves (**Table 2**, $P = 0.25$). The ADG during the study period for CON, META1, and META2 calves were 517, 518, and 525 g, respectively.

Effect of Metaphylaxis on Blood Variables

The effect of tildipirosin metaphylaxis on white blood cell, neutrophil, and lymphocyte counts, and neutrophil to lymphocyte ratio is depicted in **Figure 1**. Metaphylaxis did not influence the white blood cell count of calves during the study ($P = 0.38$). However, neutrophils, and neutrophil to lymphocyte ratio at 27 days after enrollment were greater for META1 calves in comparison to CON calves. Additionally, META 2 calves had greater lymphocyte counts than CON calves at the last day of the study. The effect of metaphylaxis on circulating concentrations of Hp, SAA, and zinc is presented in **Figure 2**. Calves enrolled in META2 had decreased concentrations of the acute phase proteins Hp and serum-amyloid A than CON calves at 27 days after enrollment. Metaphylaxis did not influence the circulating concentrations of zinc throughout the study period.

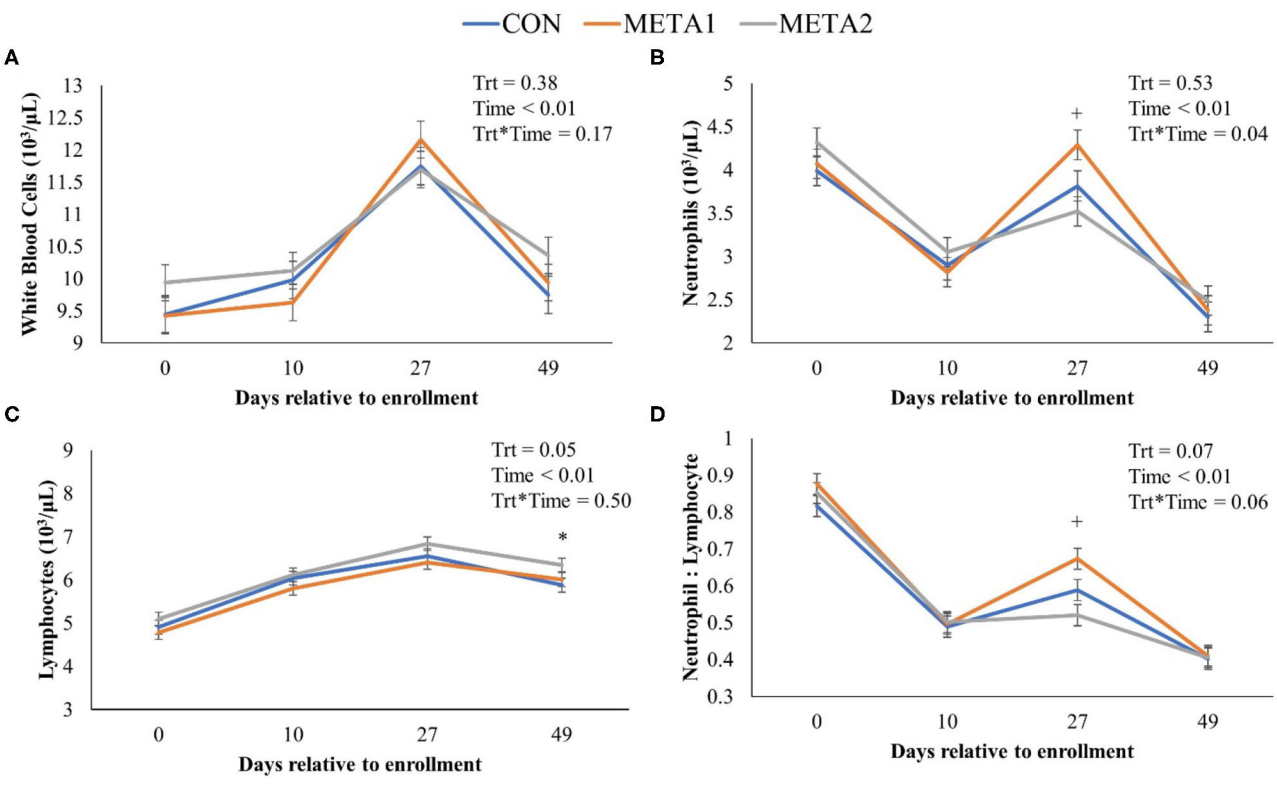

FIGURE 1 | Effect of metaphylaxis on white blood cells **(A)**, neutrophils **(B)**, lymphocytes **(C)**, and neutrophils to lymphocytes ratio **(D)**. A cross (+) or asterisk (*) indicates a $P < 0.05$ when comparing CON with META1 or META2, respectively. Calves enrolled in META1 received single SQ injection of tildipirosin (4 mg/kg) at enrollment, calves enrolled in META2 received one SQ injection of tildipirosin at enrollment and a subsequent SQ tildipirosin injection 17 days after the first injection, and CON calves remained untreated.

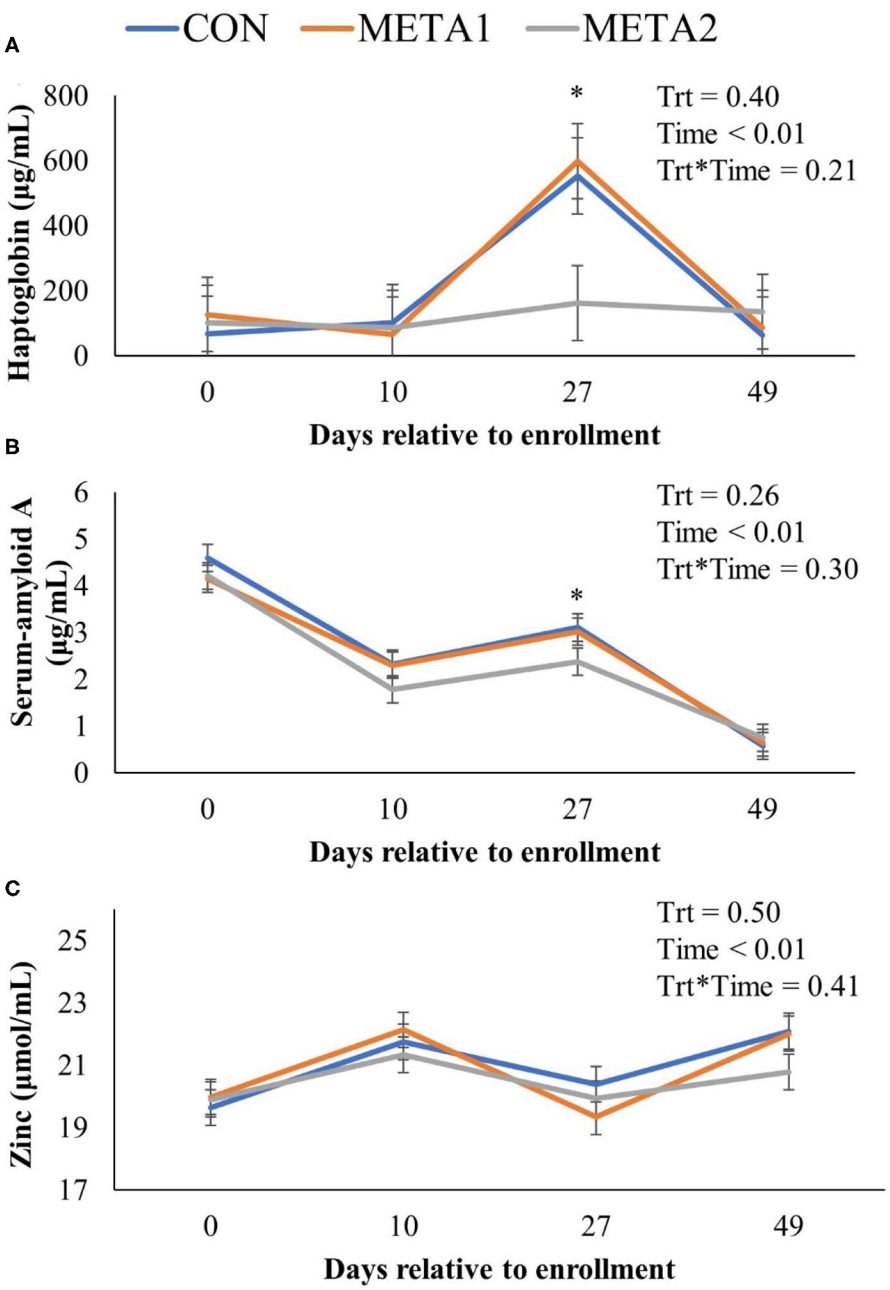

FIGURE 2 | Effect of metaphylaxis on circulating concentrations of haptoglobin **(A)**, serum-amyloid A **(B)**, and zinc **(C)**. An asterisk (*) indicates a $P < 0.05$ when comparing CON with META2. Calves enrolled in META1 received single SQ injection of tildipirosin (4 mg/kg) at enrollment, calves enrolled in META2 received one SQ injection of tildipirosin at enrollment and a subsequent SQ tildipirosin injection 17 days after the first injection, and CON calves remained untreated.

The effect of tildipirosin metaphylaxis on blood chemical panel variables is presented in **Table 3**. Metaphylaxis did not influence the concentration of the blood analytes assessed. However, CON calves tended to have increased circulating concentration of globulins throughout the study period compared to META1 and META 2 calves ($P = 0.07$, **Table 3**). Additionally, the dynamics of the serum concentration of globulin, albumin to globulin ratio, and aspartate aminotransferase by day of sampling are illustrated in **Figure 3**. Calves enrolled in the CON group calves had increased serum concentration of globulins in comparison to META1 calves at enrollment, and META2 calves at 49 days after enrollment (**Figure 3A**; $P < 0.01$ and $P = 0.01$, respectively). Additionally, serum albumin to globulin ratio was only increased for META2 calves in comparison to CON counterparts at 49 days after enrollment (**Figure 3B**; $P < 0.01$). Aspartate

TABLE 3 | Effect of tildipirosin metaphylaxis on ruminant blood chemical panel variables.

Variable	Treatment			*P*		
	CON[1]	META1[2]	META2[3]	TRT	Time	TRT*Time
Total protein (g/dL)	6.19	6.10	6.11	0.12	<0.01	0.70
Albumin (g/dL)	3.27	3.27	3.27	1.00	<0.01	0.85
Calcium (mg/dL)	10.7	10.7	10.6	0.29	<0.01	0.66
Phosphorus (mg/dL)	9.31	9.36	9.27	0.50	<0.01	0.37
Glucose (mg/ dL)	114.9	113.1	113.1	0.46	<0.01	0.27
BUN[4] (mg/dL)	11.5	11.3	11.4	0.74	<0.01	0.83
Creatinine (mg/dL)	0.81	0.82	0.81	0.93	<0.01	0.67
Bilirubin (mg/dL)	0.21	0.22	0.23	0.21	<0.01	0.80
CK[5] (U/L)	121	114	122	0.48	<0.01	0.32
AST[6] (U/L)	46.7	43.7	45.1	0.12	<0.01	0.07
Globulins (g/dL)	2.93	2.84	2.85	0.07	<0.01	0.59
A/G[7]	1.16	1.19	1.22	0.17	<0.01	0.26
GGT[8] (U/L)	157.7	148.4	154.6	0.74	<0.01	0.41
GLDH[9] (U/L)	39.8	34.5	38.7	0.37	<0.01	0.21
Magnesium (mEq/L)	1.93	1.94	1.93	0.36	<0.01	0.45
Sodium (mEq/L)	139.3	139.4	139.3	0.96	<0.01	0.49
Potassium (mEq/L)	5.57	5.53	5.53	0.46	<0.01	0.36
Chloride (mEq/L)	100.5	100.4	100.6	0.78	<0.01	0.48
Na/K[10] (mEq/L)	25.1	25.3	25.3	0.40	<0.01	0.11

[1]*CON: untreated controls.*
[2]*META1: single SQ injection of tildipirosin (4 mg/kg) at enrollment.*
[3]*META2: one SQ injection of tildipirosin at enrollment and a subsequent SQ tildipirosin injection 17 days after the first injection.*
[4]*BUN: blood urea nitrogen.*
[5]*CK: creatine kinase.*
[6]*AST: aspartate aminotransferase.*
[7]*A/G: albumin to globulin ratio.*
[8]*GGT: gamma-glutamyl transferase.*
[9]*GLDH: Glutamate dehydrogenase.*
[10]*Na/K: sodium to potassium ratio.*

aminotransferase serum concentration was greater for CON calves than for META1 and META2 calves at 27 days after enrollment (**Figure 3C**; $P < 0.01$ and $P = 0.01$, respectively).

DISCUSSION

Transportation of dairy calves to an off-site calf raising facility has become a common management strategy for many dairy enterprises (26, 27). Approximately 10% of heifer calves born in the United States are transported for long distances to be raised in specialized facilities and commingled with other calves from different sources (22). Among these calves, the risk for BRD is elevated, and the use of metaphylaxis is often utilized to control BRD. The long-acting macrolide tildipirosin has desirable properties for BRD metaphylaxis in high risk cattle because it has a long half-life leading to a sustained concentration of the macrolide in lung tissue and bronchial fluid (23). Previous studies have evaluated the efficacy of metaphylactic use of tildipirosin to prevent BRD in group housed pre-weaned dairy calves (9), and in calves transported to a veal facility (13). Furthermore, other studies evaluated the development of lung lesions and other measures of health in animals submitted to microbial challenges after metaphylactic administration of tildipirosin (28, 29). To the best of our knowledge, metaphylactic approaches to high-risk dairy calves housed in individual hutches has not been fully investigated.

Metaphylaxis did not decrease the incidence of BRD during the pre-weaning period. We only observed a numerical decrease in BRD incidence for calves that received metaphylaxis. In veal calves, metaphylactic treatment using tildipirosin 12 days after arrival was not associated with the number of BRD treatments (13). However, others have reported that metaphylaxis can improve respiratory tract health of pre-weaned calves. Teixeira et al. (10) showed that tildipirosin metaphylactic injections decreased the likelihood of BRD in pre-weaned calves housed in group pens. Moreover, metaphylactic injection of tildipirosin 5 days prior to *Histophilus somni* inoculation decreased the presence of this bacterium in bronchial secretion samples collected three days after challenge (29). The BRD incidence in our study calves was lower than we expected. For instance, two recent studies showed BRD incidences of ∼22% for calves being diagnosed and treated at least once in the first 3 months of age (4, 19). It is plausible that the lack of impact of metaphylaxis

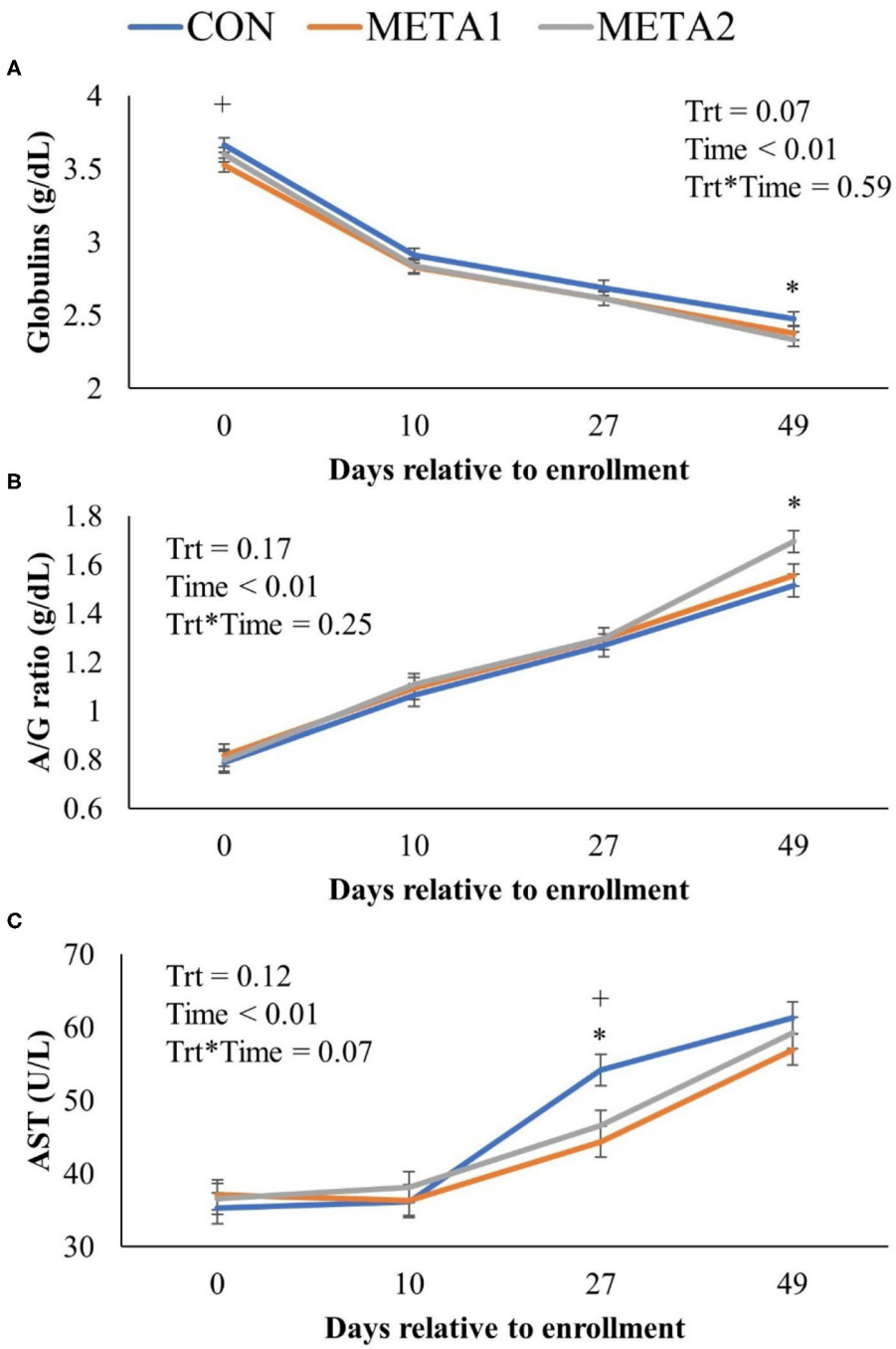

FIGURE 3 | Effect of metaphylaxis on blood concentration of globulins **(A)**, albumin to globulin ratio **(B)**, and aspartate aminotransferase **(C)**. A cross (+) or asterisk (*) indicates a $P < 0.05$ when comparing CON with META1 or META2, respectively. Calves enrolled in META1 received single SQ injection of tildipirosin (4 mg/kg) at enrollment, calves enrolled in META2 received one SQ injection of tildipirosin at enrollment and a subsequent SQ tildipirosin injection 17 days after the first injection, and CON calves remained untreated.

on BRD incidence might be due to the low BRD incidence and consequently low statistical power of our study. Perhaps transportation in our study was not as stressful as initially assumed, and the calves were not in high risk of BRD as we had expected. Because calves were housed in individual hutches after arrival, there was not close contact between them, and it is likely that pathogen transmission between calves was reduced during the pre-weaning period, which resulted in low BRD incidence. Our metaphylactic strategies were designed based on the BRD incidence curve that was built during study design (**Figure S1**),

and injection were administered close to the peaks of BRD incidence during the pre-weaning period. In feedlots, the use of epidemiologic curve plots is helpful to determine a temporal pattern of diseases and may influence management strategies such as metaphylaxis (30). Berman et al. (13) also determined their metaphylactic injection timing (3 weeks after veal calves' arrival) based on the expected BRD incidence peak. Because they also reported a lower than expected BRD incidence, they highlighted the importance of a cohort risk assessment before the development of metaphylactic treatment protocols (13).

Like BRD incidence, lung health assessed by thoracic ultrasonography at weaning was not influenced by metaphylaxis in our study. Thoracic ultrasonography is an accurate and practical diagnostic tool for BRD-related lung lesions in calves (31), and it could represent BRD cases that did not manifest in clinical signs evaluated by the researchers. Berman et al. (13) also did not observe a reduction in lung lesions diagnosed by thoracic ultrasonography in veal calves that received metaphylactic injection of tildipirosin. However, studies involving pathogen-challenges reported that metaphylaxis with tildipirosin improved lung health when assessed through thoracic ultrasonography. Heifers that received a tildipirosin injection 10 days prior to *Mannheimia haemolytica* challenge had decreased lung lesion scores than heifers that received tulathromycin injection or negative saline controls (28). Furthermore, lung lesions were less severe for calves that received tildipirosin injection 5 days prior to *Histophilus somni* inoculation, with a lack of necrosis and only areas of acute bronchopneumonia surrounded by normal lung tissue (29).

Additionally, we observed that mortality during the pre-weaning period tended to be reduced in calves enrolled in the META2 treatment group compared to CON calves. In general, the pre-weaning mortality average was 1.2%, which is considerably lower than the mortality rates previously described. For instance, the overall mortality of calves during the pre-weaning period in the United States has been recently reported to be 5.0%, according to the latest USDA National Animal Health Monitoring Survey (3). Others have reported pre-weaning mortality in herds located in New Mexico, California, and Minnesota to be 14% (range from 7.0 to 29.1%), 2.8% (range from 1.7 to 7.2%) and 3.5% (range from 0 to 10%), respectively (4, 19, 32). In contrast to our results, the metaphylactic use of tildipirosin did not impact mortality in group-housed pre-weaned calves (9).

Growth during the pre-weaning period of dairy calves is affected by BRD (9, 27). Hence, strategies to mitigate BRD during the pre-weaning period can potentially result in improved weight gain of calves. Additionally, calves that received tildipirosin metaphylaxis prior to a *Mannheimia haemolytica* respiratory challenge had greater feed consumption during the 3-day observation period after inoculation, suggesting that metaphylaxis could potentially increase growth; however the animals were euthanized for data collection purposes and no conclusions in long-term ADG could be made (28). Because BRD incidence was not reduced by metaphylaxis in our study, it is not surprising that growth was also not influenced. Others have also reported that metaphylactic administration of tildipirosin had no effect on ADG of calves during the pre-weaning period (9, 13).

Some biomarkers of inflammation that have been previously associated with BRD or stress were affected by metaphylaxis. For instance, Hp and SAA are acute phase proteins that are elevated in blood in calves that show clinical signs of BRD (33–35). Even though metaphylaxis did not decrease BRD incidence in our study, the concentration of Hp and serum-amyloid A was decreased in META2 calves in comparison with CON calves at 27 days after enrollment. Furthermore, animals from META2 group had decreased concentrations of AST and decreased neutrophil to lymphocyte ratio in comparison to CON calves. Additionally, CON group animals had increased concentration of globulins and lower albumin to globulin ratio at weaning in comparison to META1 and META2 calves. Neutrophil to lymphocyte ratio has been used as a measurement of ruminant stress (36). Calves diagnosed with BRD are reported to have increased levels of AST (37), increased serum globulin concentrations (38) and decreased albumin in comparison to healthy calves (35). Collectively, blood analysis results suggest that even though the clinical disease was not influenced by metaphylaxis, systemic inflammatory state of calves were improved.

In conclusion, metaphylactic use of tildipirosin did not decrease BRD incidence, prevalence of lung lesions diagnosed by ultrasonography at weaning, nor it had an impact on growth during the pre-weaning period of dairy calves transported to a heifer raising facility. However, mortality tended to be lower in calves enrolled in the META2 treatment groups, and systemic inflammation status of calves were improved by metaphylaxis based on circulating biomarkers of inflammation and stress. Given the concern regarding antimicrobial resistance development and judicious use of antimicrobial drugs, our results do not support the metaphylactic use of tildipirosin in field conditions with already low incidence of BRD morbidity and mortality as described herein. However, even with low incidence of disease, metaphylaxis tended to decrease mortality by 60% (1.5 vs. 0.6), and improved the inflammatory status of calves, one could speculate that it could be an efficacious strategy to control BRD and improve welfare in herds where BRD incidence is greater than reported herein. Hence, we believe that more research is needed to evaluate potential benefits of metaphylaxis in herds where the incidence of BRD and mortality are greater than what was observed in our study.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by all activities performed in this study were reviewed and approved by the Texas Tech University Institutional Animal Care and Use Committee

(#18081-10). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

The study was designed by VM, TB, RN, and MB. Data collection was conducted by MC, LF, PM, DP, TR, and TS. Database compilation was done by MC, and data analysis was done by VM. The manuscript was drafted by MC and VM, which was then reviewed by all authors. The research protocol was developed with input of all authors. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00632/full#supplementary-material

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Effects of the Administration of a Non-specific Immune Stimulant Around Transportation on Health and Performance of Jersey and Jersey-Cross Heifer Calves During the Rearing Period: Randomized Clinical Trial

Bobwealth O. Omontese[1,2], Luciano S. Caixeta[1], Vinicius S. Machado[3], Aaron Rendahl[4], Maria L. K. Celestino[3], Paulo R. Menta[3], Daniela Paiva[1], Angel Garcia-Muñoz[1,5] and Aleksandar Masic[6]*

[1] Department of Veterinary Population Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, MN, United States, [2] Department of Food and Animal Sciences, College of Agricultural, Life and Natural Sciences, Alabama A and M University, Huntsville, AL, United States, [3] Department of Veterinary Sciences, College of Agricultural Sciences and Natural Resources, Texas Tech University, Lubbock, TX, United States, [4] Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, Saint Paul, MN, United States, [5] Faculty of Veterinary Sciences, University Cardenal Herrera CEU, CEU Universities, Valencia, Spain, [6] NovaVive Inc., Napanee, ON, Canada

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***Correspondence:**
Luciano S. Caixeta
lcaixeta@umn.edu

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Our objective was to evaluate the effects of a non-specific immune stimulant (IS) administered around transportation on health scores (HS), average daily gain (ADG), disease treatment and mortality of Jersey and Jersey-cross calves during the rearing period. Newborn calves (4 d ± 1) were randomly allocated to receive either 1 mL of saline (CON; $n = 438$), 1 mL of IS before transport (BTIS; $n = 431$), or 1 mL of IS immediately after transport (ATIS; $n = 436$). Calves were health scored weekly for 3 weeks after transport. The data were analyzed using multivariable linear mixed models and multivariable logistic regression models. Kaplan-Meier survival analysis was performed for time to event analysis. Treatment, birth weight, breed, site of birth, serum total solids, dam parity, season of enrollment, and metaphylaxis were offered to models. Differences in respiratory and fecal HS, and ADG between treatment groups were not statistically significant. A total of 196 (15.0%) calves were treated at least once for any disease and 52 calves were treated multiple times. The proportion of calves treated for respiratory disease and/or diarrhea were 14.4, 14.4, and 16.2% for BTIS, ATIS and CON groups, respectively. Although the differences in the likelihood of treatment for both respiratory disease and/or diarrhea during the first 9 weeks of life was not statistically different between groups, we observed that more calves in the control group received disease treatments around 15 days of age compared with calves that received IS. The likelihood of treatment for respiratory diseases alone during the first 30 days of life was smaller in the calves that received IS before transportation when compared to the control group. Only 18 (1.4%) calves died within the study period. The calf mortality likelihood

was not statistically different between study groups; however, fewer calves in the IS groups died when compared to CON. In conclusion, the use of IS around transportation did not influence weekly HS, ADG, and the number of disease treatments during the rearing period, but administering IS before transportation resulted in fewer treatments of respiratory diseases during the first 30 days post-transport and marginally lower mortality rates during the rearing period.

Keywords: Jersey calves, mortality, immune stimulant, disease treatment, average daily gain (ADG)

INTRODUCTION

The occurrence of diseases during the rearing period in dairy heifers is associated with impaired productivity of dairy cows during first lactation (1–4). Thus, calf-hood well-being is important to the economic success of dairy operations. Among the morbidities affecting dairy calves during the rearing period, respiratory diseases (i.e., pneumonia) and diarrhea have been reported as the most prevalent and economically important (3, 5–7). In the United States, ∼11 and 19% of calves show signs of pneumonia and diarrhea at least once, respectively, and 5% die before weaning (7). One of the reasons for the high morbidity among pre-weaned calves is the fact that dairy calves are born nearly agammaglobulinemic and are extremely dependent on acquisition of maternal immune protection through proper ingestion of colostrum immediately after birth (8, 9). Successful passive transfer of maternal immunoglobulins is important to assist with protection against infectious agents by providing specific antibodies and to enhance the cell-mediated immune response in calves (10–13). Although extensive research has demonstrated the importance of adequate passive transfer of immunity to calf health, the quality and quantity of colostrum offered to newborn calves are often inadequate. Consequently, dairy calves are susceptible to infectious diseases early in life and antibiotics are often used to treat pneumonia and diarrhea in commercial dairy farms. According to a nationwide survey in the United States, ∼25% of calves receive an antibiotic for the treatment of illness during the pre-weaning period (7).

Poor housing, inadequate ventilation and transportation are some of the stressful conditions associated with high disease incidence in pre-weaned dairy calves (8). A review by Van Engen and Coetzee (14) described the intricate role of transportation on immune suppression and increased inflammation, predisposing feedlot cattle to pneumonia. Transportation increases disease susceptibility of calves (15) and performing preventive interventions before transportation is associated with enhanced health and performance after transportation (16). However, vaccinations and metaphylaxis are often performed after transportation (17). Treating dairy calves after the disease is diagnosed does not eliminate the negative effects on long-term production and the metaphylactic use of antimicrobials can contribute to the alleged influence of animal agriculture on the selection of antimicrobial resistance genes (18, 19). Thus, there is a need to investigate alternative strategies that can enhance animal health around transportation without the use of antibiotics.

Immune stimulants (IS) offer an alternative method to activate innate immune response of newborn dairy calves and IS have the potential to decrease antibiotic treatments for pneumonia and diarrhea in calf operations during the rearing period (20, 21). Among the products available on the market, a mycobacterium cell wall fraction immune stimulant is approved for the reduction of clinical signs and mortality associated with K99 *Escherichia coli* diarrhea in neonatal calves (Amplimune®, NovaVive Inc., Napanee, ON, Canada). Additionally, mycobacterium cell wall fraction immune stimulants have been shown to modulate innate immune response and stimulate lymphocyte functional activity, *in vivo* and *in vitro*, in other species within hours of administration and last only for a few days (22–25). Considering that immune stimulation can induce early activation of the non-specific innate immune system of newborn dairy calves and provide the first line of defense against microbial pathogens (26, 27), this study was designed to evaluate the effects of this commercially available IS on health and performance of pre-weaned Jersey and Jersey-cross calves following transportation. Our hypothesis was that the use of the IS would improve health and performance due to an improved immune response in newborn calves around transportation, leading to improved health and performance during the pre-weaning period. Our specific objectives were to determine whether this non-specific immune stimulant would improve weekly health score (HS) during the first 3 weeks post-treatment, improve average daily gain (ADG) and decrease disease treatments and mortality of calves transported within their first week of life during the rearing period.

MATERIALS AND METHODS

All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota and Texas Tech University.

Study Design, Calves Management and Data Collection

This randomized controlled clinical trial was conducted in a commercial dairy system from March to December 2018. Calves were born at nine different sites from the same dairy system in Minnesota, immediately separated from their dam after birth, weighed, fed colostrum (4 L within 6 h after birth), and transported to the initial temporary holding facility where they were housed for 3–4 days (depending on day of birth) before transportation. Management and standard operating procedures

were the same in the nine different origin sites. In the study facility, calves were placed in individual hutches bedded with straw inside a large cross-ventilated barn.

Newborn Jersey and Jersey-cross heifer calves were enrolled between 3 and 5 days of life. Only multiparous dairy cows were present in the Minnesota sites of this particular dairy system, therefore no calves born from primiparous animals were enrolled in this study. A day prior to enrollment, the list of eligible calves were allocated randomly to treatments using the Microsoft Excel 2016 randomization generator (Microsoft Corporation, Redmond, WA) by the corresponding author. At the temporary holding facility, calves received 1.8 L per feeding of a reconstitute milk replacer (27% crude protein, 25% crude fat, DM basis) two times a day, *ad libitum* water in individual feeding bottles, and were checked for general health. Briefly, sick calves were identified based on whether they consumed the entire milk replacer volume offered, signs of weakness (i.e., unable to rise), diarrhea or any other visible abnormalities. Sick calves were treated by the on-farm veterinarian according to farm protocols and transportation to the heifer growing facility in New Mexico was withheld until the illness resolved. For this reasons, visibly sick calves were not enrolled in the study. Calves were randomly allocated to receive one of three treatments:(1) 1 mL of sterilized saline (CON); (2) 1 mL of IS before transport to grower facility (BTIS); or (3) 1 mL of IS on arrival (after transportation) at the grower facility (ATIS). All treatments were administered subcutaneously on the neck and within 2 h before (CON and BTIS) or after (ATIS) transportation by the University of Minnesota (BTIS) and Texas Tech University (ATIS) research teams, respectively. All calves were safely loaded into a truck and transported to the calf-rearing facility (∼18 h of transport). In order to facilitate identification of study calves requiring treatment at arrival at the growing facility, calves were fitted with removable plastic ear clips. The ear clips were removed immediately after administration of IS to the calves in the ATIS group to ensure that the assigned treatment stayed masked for the research personnel performing health scoring of animals during the first 3 weeks post-transport at the grower facility, and that farm personnel were also masked when identifying and treating sick animals.

At enrollment, blood samples were collected by jugular venipuncture using Vacutainer tubes (10 mL BD Vacutainer glass serum tubes; Becton Dickinson, Franklin Lakes, NJ) from all calves for the determination of serum total solids concentration. Samples were placed immediately in ice and later centrifuged at 2,000 × *g* for 15 min at 4°C for serum separation. Serum total solids were measured using a digital refractometer (MISCO; Palm Abbe PA203X, Whitewater, WI) to evaluate colostrum management of the farm and failure of passive transfer was defined as serum total solids <5.5 g/dL (28).

At the calf-rearing facility, heifer calves were housed in individual hutches bedded with straw, received on average 1.8 L per feeding of a reconstituted milk replacer (27% crude protein, 22% crude fat, DM basis) two times a day, and had *ad-libitum* access to water and calf starter throughout the rearing period. Calf health was evaluated weekly during the first 3 weeks post-transport using a modified calf health scoring system adapted from McGuirk and Peek (29). Briefly, individual health score measures rectal temperature, cough, nasal discharge, ocular discharge (eye score), ear position (ear score), and fecal consistency were scored from 0 to 3. For all categories, lower scores for individual health measures indicated apparently healthier animals. Health score was assessed by one trained observer from the Texas Tech University research team during a weekly visit to the heifer raising facility during the 3 weeks following transportation. For the purpose of this study, a veterinary attention score was created based on respiratory and fecal scores for each week post-transport separately. Calves with respiratory score >4 and calves with fecal score >2 were considered as in need of veterinary attention because of respiratory disease and diarrhea, respectively. Although the research group assessed HS in study animals on a weekly basis during the first 3 weeks post-transport, HS results and veterinarian attention recommendation was not made available for farm personnel in order to avoid deviations from farm standard operating procedures. The HS data was collected and used as an objective measurement of health status post-transportation, however, only animals that received treatments by farm personnel were considered sick for disease treatment analysis.

According to farm protocols, calves were considered sick when clinical signs including weakness, depression, rectal temperatures of over 40°C (>104°F), difficult, shallow or rapid breathing, dehydration, nasal discharge, diminished appetite, coughing, or watery stools were observed. Treatments followed farm protocols and standard operating procedures and were developed by the on-farm veterinarian. Treatment information including treatment number, date, and farm diagnosis was recorded on on-farm management software (Dairy Comp 305; Valley Ag Software, Tulare, CA). Disease treatment records for the first 9 weeks of life (63 days of age) were used for statistical analysis. Additionally, beginning in September 2018 farm management implemented a metaphylactic treatment (Zuprevo, Tildipirosin, 4 mg/kg of body weight; Merck Animal Health, Summit, NJ) to all calves ($n = 457$) at the facility at 35 days of life. The implementation of the metaphylactic treatment was unrelated to our study and had the goal to decrease a perceived higher occurrence of respiratory cases around 40 days of life. This perceived higher occurrence of respiratory cases was not a disease outbreak. The metaphylactic treatment did not fulfill farm management expectations and was halted few months after the end of our study. Animals in all treatment groups received the metaphylactic treatment and hence a metaphylactic treatment variable was added to the statistical models. All heifer calves were weighed using a portable digital scale (Raytec® 42′ Calf Cart™, Raytec LLC, Ephrata, PA) at 9 weeks of age (∼ 63 days of life).

Statistical Analyses

Sample size was calculated using JMP 14 (SAS Inst., Cary, NC). Sample size calculation was performed based on previous reports of the disease incidence (i.e., pneumonia and diarrhea) in the United States dairy calves and the farm's historical data within the rearing period. We were expecting to see a reduction in

disease treatment from 40 to 30% following treatment with IS. Therefore, a minimum of 294 calves per treatment was required to detect a reduction of 10 percentage points in the incidence of calf-hood diseases treatments between control and IS treatment groups, with 80% power at a 5% significance level. Prior to the beginning of the study, we inflated our sample size by 20% to account to loss of follow up (∼60 animals per group). After preliminary descriptive statistical analysis when the expected number of animals completed the trial and the lower disease treatment rates were observed, the research team decided to enroll animals for another 3 weeks (maximum allowed based on budgetary constraints) as an attempt to achieve sufficient numbers to capture the expected differences.

Incidence of calf diseases and mortality are expressed in percentages. The effect of IS treatment on weekly HS were analyzed for each week post-transport individually. Respiratory and fecal scores were analyzed separately as a continuous variable using a generalized linear model and as a dichotomous outcome based on the calculated veterinary attention score by separate chi-squared test. Average daily gain was calculated by dividing the change in weight by the number of days between birth and weaning and was evaluated using multivariable linear regression. Statistical analyses for disease treatment during the rearing period (9 weeks), treatment of respiratory diseases during the first 30 days, mortality, and re-treatment of calves for respiratory disease or diarrhea were carried out using multivariable logistic regression. In addition to treatment, the following independent variables were included in the models to account for their association with each given outcome: season of enrollment (season 1 = March and April, season 2 = May and June, Season 3 = August and September, season 4 = October and November), breed (Jersey or Jersey-cross), birth weight, site of birth, serum total solids, dam parity (lactation = 2; lactation > 3). Metaphylaxis was included as a covariate to the ADG model and to all disease treatment and mortality models that accounted for the entire rearing period. Homoscedasticity and independence of error assumptions was assessed by visual observation of models' residual plots and the Hosmer-Lemeshow test was used to test goodness-of-fit of logistic models.

Kaplan-Meier survival analysis was performed to show the survival of calves from disease treatment or mortality during the rearing period, and the time to respiratory disease treatment during the first 30 days of life. For the time to first disease treatment analysis, calves were right-censored if dead before receiving treatment for any disease or if they did not receive any treatment until the last day of the follow up period when final weights were measured. For the time to respiratory disease treatment event before 30 days, similar strategy for censoring data was applied but follow up period was arbitrarily set to end at 30 days of age. For the time to death analysis, calves were right-censored if they were alive at the end of the data collection period when final weight data was collected (9 weeks of life). Backward stepwise elimination process was used to create the most parsimonious statistical models. Treatment and metaphylaxis (when present) were forced into all statistical models while all other covariates were excluded if $P > 0.20$. Differences with $P < 0.05$ were considered statistically significant. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC) and Kaplan-Meier curves were created in R 3.6.0 (30).

RESULTS

Descriptive Analysis

A total of 1,332 heifer calves were enrolled in the study; however, 27 calves were excluded from statistical analysis. Reasons for exclusion were lost of follow up ($n = 14$) and development of morbidities ($n = 13$; four calves from CON, four calves from BTIS, and six calves from ATIS) that were not defined prior to the beginning of the study (i.e., arthritis, navel infection, or pink eye). Therefore, 1,305 calves including Jersey ($n = 568$) and Jersey-cross ($n = 737$) completed the study. Information on the number of animals from each breed, dam parity, age at enrollment, birth weight, serum total solids at enrollment, age at weaning, final weight (weaning weight), and number of animals that received metaphylactic treatment are presented in **Table 1**. No numerical differences were observed between the three treatment groups at enrollment. Furthermore, no adverse reaction after the administration of the IS subcutaneously was observed during study.

Weekly Health Scores

Weekly health scores are presented in **Table 2**. Overall, there were no statistical differences in respiratory score when comparing BTIS and ATIS with CON during the 3 weeks when HS was assessed. A numerical difference was observed for fecal

TABLE 1 | Descriptive characteristics (mean ± SD) of Jersey and Jersey-cross calves enrolled in a study to evaluate the effects of a non-specific immune stimulant on calf health and performance during the rearing period.

Variable	Treatment		
	Control ($n = 438$)	BTIS[a] ($n = 431$)	ATIS[b] ($n = 436$)
Breed			
Jersey	200	189	179
Jersey-cross[c]	238	242	257
Dam parity	2.64 ± 0.82	2.60 ± 0.83	2.65 ± 0.88
Age at enrollment, *d*	4.86 ± 0.34	4.83 ± 0.39	4.83 ± 0.39
Birth weight, kg	31.7 ± 4.3	32.0 ± 4.4	31.9 ± 4.5
Serum total solid, g/dL	6.60 ± 0.62	6.66 ± 0.61	6.59 ± 0.62
Age at weaning, d	61.3 ± 1.5	61.2 ± 1.5	61.2 ± 1.4
Weaning weight, kg	59.9 ± 7.9	60.6 ± 8.1	60.3 ± 8.0
Metaphylaxis[d], *n* (%)	153 (35)	151 (35)	143 (33)

[a] *BTIS = before transport immune stimulant.*
[b] *ATIS = after transport immune stimulant.*
[c] *Jersey and Holstein cross heifer calves.*
[d] *Subcutaneous administration of Zuprevo (Tildipirosin, 4 mg/kg of body weight; Merck Animal Health, Summit, NJ) at 35 days of age.*

TABLE 2 | Proportion of Jersey and Jersey-cross calves with recommended veterinary attention based on health scores[a] during the first 3 weeks after transportation.

Week after transport	Treatment			
	Control ($n = 438$)	BTIS[b] ($n = 431$)	ATIS[c] ($n = 436$)	*P*-value
Week 1				
Respiratory score[d], mean ± SD	1.42 ± 0.99	1.37 ± 0.92	1.49 ± 0.99	0.21
Fecal score[e], mean ± SD	1.12 ± 1.21	1.14 ± 1.21	1.30 ± 1.24	0.06
Respiratory score–Attention[f], *n* (%)	13 (3%)	10 (2%)	13 (3%)	0.79
Fecal score–Attention[g], *n* (%)	166 (38%)	162 (38%)	186 (43%)	0.23
Week 2				
Respiratory score	1.37 ± 1.02	1.36 ± 1.01	1.39 ± 1.03	0.73
Fecal Score	0.90 ± 1.12	0.93 ± 1.15	0.96 ± 1.15	0.58
Respiratory score–Attention	18 (4%)	18 (4%)	12 (3%)	0.45
Fecal score–Attention	91 (21%)	95 (22%)	76 (17%)	0.21
Week 3				
Respiratory score	1.22 ± 1.04	1.16 ± 0.91	1.20 ± 0.99	0.63
Fecal Score	0.47 ± 0.92	0.48 ± 0.90	0.43 ± 0.85	0.71
Respiratory score–Attention	9 (2%)	6 (1%)	11 (2%)	0.48
Fecal score–Attention	61 (14%)	63 (15%)	48 (11%)	0.25

[a] *Weekly health score was evaluated for all the calves during the first 3 weeks of life using a calf health scoring systems adapted from McGuirk, University of Wisconsin.*
[b] *BTIS = before transport immune stimulant.*
[c] *ATIS = after transport immune stimulant.*
[d] *Respiratory score = Mean respiratory score per treatment group.*
[e] *Fecal score = Mean fecal score per treatment group.*
[f] *Respiratory score–Attention = Veterinary attention because of respiratory diseases was defined as positive when total respiratory score was equal or >4 based on the health scoring systems adapted from McGuirk, University of Wisconsin (dichotomous outcome).*
[g] *Fecal score–Attention = Veterinary attention because of diarrhea was defined as positive when total fecal score was equal or >2 based on the health scoring systems adapted from McGuirk, University of Wisconsin (dichotomous outcome).*

score during week 1 post-transport ($P = 0.06$). We did not observe a difference in the number of calves that required veterinary attention based on total respiratory score (total respiratory score >4) nor fecal score (total fecal score >2) within each week. The percentage of calves that required veterinary attention based on fecal scores decreased throughout the 3-week period post-transport in all treatment groups while a similar percentage of calves were considered to require veterinary attention based on respiratory scores during the same period.

Average Daily Gain

There were no differences in ADG when comparing treatments during the rearing period ($P = 0.58$). Calves in the control group gained an average of 460 g daily (range = 156–699 g/d), while calves in the BTIS group gained an average of 466 g daily (range = 159–796 g/d), and calves in the ATIS group gained 463 g daily (range = −14–729 g/d). Calves born in May and June had a lower ADG ($P < 0.001$) when compared to calves born in March and April, while calves born in October and November had a greater ADG ($P < 0.001$) when compared to the same referent group of calves. ADG gain was greater ($P < 0.001$) in Jersey-cross calves when compared to Jersey calves, greater ($P < 0.001$) in calves that received methaphylactic treatment during the hearing period when compared to calves that did not receive metaphylaxis, and it was associated with birthweight ($P < 0.001$). Lastly, calves born in all but one of the birth sites had similar ADG when compared the referent birth site (**Table 3**). ADG ranged from 338 to 583 g depending on the week of study when calves were enrollment (week 10 and week 18, respectively).

Disease Treatment and Mortality

A total of 196 (15.0%) calves were treated at least once and 18 (1.4%) calves died during their first 9 weeks of life. The proportion of animals treated for pneumonia and/or diarrhea within each group was 14.4, 14.4, and 16.2% for BTIS, ATIS and CON groups, respectively. Treatments for pneumonia alone accounted for 163 (61.3%) of the 266 treatments administered during the study period, treatments for diarrhea accounted for 93 (35.0%), and treatments for both diseases at the same time accounted for 10 (3.7%). Of the total number of calves treated within each group during the study period, 52 calves were treated multiple times, 13 (3.0%) in the BTIS group, 19 (4.4%) in the ATIS group, and 20 (4.6%) from the CON group. One hundred and eighty-nine (71.1%) of all disease treatments occurred within the first 30 days of life. The cumulative incidence of disease treatments and mortality per treatment group during the rearing period by treatment groups is presented in **Table 4**.

Multivariable logistic regression models were developed to determine the odds of receiving a disease treatment during the rearing period. No differences in the odds of receiving a disease treatment during the entire rearing period was observed when comparing all experimental groups (**Table 5**). However, we observed that more calves in the control group received treatment for pneumonia and/or diarrhea around 15 days of age compared with calves that received IS (**Figure 1A**). The observed change in the Kaplan-Meier curve from day 11 to day 20 was 8.5 percentage points for control, compared with 6.3 and 5.5, for BTIS and ATIS, respectively, and the overall estimated proportion receiving treatment by day 30 was 12.2% for CON, compared with 9.1 and 10.5% for BTIS and ATIS, respectively. Season of enrollment was associated ($P = 0.02$) with different likelihood of receiving a disease treatment during the rearing period while birth weight ($P = 0.05$) and breed ($P = 0.08$) were only marginally associated with the odds of receiving a disease treatment during the same period. Calves born in May and June were more likely to have a treatment event during the rearing period than calves born in March and April (OR = 1.05; 95% CI: 0.69–1.60; $P = 0.02$) while calves born in October and November had a lower likelihood of receiving disease treatment during the rearing period (OR = 0.40; 95% CI: 0.22–0.74; $P = 0.003$). The

TABLE 3 | Multivariable linear model evaluating the effects of a non-specific immune stimulant around transportation, season of enrollment, birth weight, breed, site of birth, dam parity, and metaphylaxis on average daily gain of Jersey and Jersey-cross calves during the rearing period.

Variable	Estimate	Standard error	*P*-value
Intercept	0.657	0.05	<0.001
Treatment[a]			
Control	Referent		
BTIS	0.005	0.005	0.32
ATIS	0.004	0.005	0.45
Season[b]			
March–April	Referent		
May–June	−0.053	0.01	<0.001
August–September	0.009	0.01	0.33
October–November	0.048	0.01	<0.001
Birth weight	−0.001	0.00	<0.001
Breed[c]			
Jersey	Referent		
Jersey-bred	0.029	0.005	<0.001
Source site			
Birth site A	Referent		
Birth site B	−0.028	0.02	0.27
Birth site C	0.012	0.01	0.49
Birth site D	−0.001	0.01	0.98
Birth site E	−0.026	0.01	0.05
Birth site F	0.009	0.01	0.58
Birth site G	−0.005	0.01	0.77
Birth site H	−0.006	0.01	0.69
Birth site I	0.026	0.01	0.12
Parity[d]			
Lactation = 2	Referent		
Lactation > 3	−0.008	0.005	0.10
Metaphylaxis[e]			
No	Referent		
Yes	0.09	0.005	<0.001

[a]*Treatment: Calves received subcutaneous administration of 1 mL of a non-specific immune stimulant at 4 ± 1 days of life. CON = calves that receive saline before transport (n = 438); BTIS = calves that received immune stimulant before transport (n = 431) and ATIS = immune stimulant after transport (n = 436).*
[b]*Enrollment season: Period of the study referent to the week when first set of claves was enrolled. Calves were enrolled on a weekly basis from March to November of 2018.*
[c]*Breed: Jersey (n = 568) and Jersey-Holstein cross (n = 737) heifer calves were enrolled in the study.*
[d]*Dam parity: Dam parity was dichotomized (lactation = 2 and lactation > 3) based on the lactation that dams were starting. Only multiparous cows were housed in the different sites where study calves were born.*
[e]*Starting in September 2018 farm management implemented a metaphylactic treatment (Zuprevo, Tildipirosin, 4 mg/kg of body weight; Merck Animal Health, Summit, NJ).*

TABLE 4 | Cumulative incidence of disease treatments and mortality during the rearing period for newborn Jersey and Jersey-cross calves receiving subcutaneous administration of a non-specific immune stimulant around transportation during the rearing period (9 weeks).

Variable	Treatment				
	Control (*n* = 438)	BTIS[a] (*n* = 431)	ATIS[b] (*n* = 436)	*P*-value[c]	Contrast[d]
Disease treatment, *n* (%)					
Pneumonia	55 (12.5)	49 (11.3)	62 (14.2)	0.66	0.60
Diarrhea	38 (8.7)	29 (6.7)	26 (6.0)	0.81	0.68
Pneumonia and diarrhea	4 (0.9)	3 (0.7)	3 (0.7)	0.71	0.49
Mortality, *n* (%)	10 (2.3)	4 (0.9)	4 (0.9)	0.16	0.05

[a]*BTIS = before transport immune stimulant.*
[b]*ATIS = after transport immune stimulant.*
[c]*Overall P-value.*
[d]*P-value when comparing both treatment groups combined vs. the control group.*

odds of receiving a treatment during the rearing period was smaller for Jersey-cross calves when compared to Jersey calves (OR = 0.73; 95% CI: 0.52–1.04; $P = 0.05$), and the likelihood of receiving a treatment for any disease during the rearing period was smaller in calves that were heavier at birth (OR = 0.98; 95% CI: 0.96–0.99; $P = 0.05$). When controlling for all other variables, calves that received metaphylactic treatment at 35 d of age had a 40% lower likelihood of receiving treatment for a disease during the rearing period (OR = 0.60; 95% CI: 0.37–0.97; $P = 0.04$) when compared to calves that did not receive metaphylactic treatment (**Table 5**). When analyzing treatments for respiratory disease alone during the first 30 days of life, we found that BTIS had a significantly lower odds of receiving a treatment for respiratory disease compared with CON (OR = 0.53; 95% CI: 0.29–0.97; $P = 0.03$). No difference was observed when comparing ATIS (OR = 0.92; 95% CI: 0.54–1.55; $P = 0.35$) and CON. Season of enrollment ($P < 0.001$) was also associated with respiratory treatment during the first 30 days of life. Calves born in May and June were more likely to receive a treatment for respiratory diseases during the first 30 days of life than calves born in March and April (OR = 1.50; 95% CI: 0.90–2.50; $P < 0.001$). In the contrary, the odds of receiving treatment for respiratory diseases during the first 30 days of life was smaller for calves born in August and September (OR = 0.16; 95% CI: 0.07–0.33; $P = 0.001$) and in October and November (OR = 0.10; 95% CI: 0.03–0.27; $P < 0.001$) when compared to the referent group (**Table 6**). A Kaplan-Meier curve showing the hazard of being treated for a respiratory disease only during the first 30 days of life is presented in **Figure 1B**.

The effect of the non-specific immune stimulant around transportation and metaphylaxis on the re-treatment of Jersey and Jersey-cross calves during the rearing period is presented in **Table 7**. Compared with CON, there was no difference in the odds of retreatments during the rearing period for BTIS (OR = 1.53, 95% CI: 0.68–3.43; $P = 0.20$) and ATIS (OR = 0.96, 95% CI: 0.43–1.94; $P = 0.38$). Similarly, metaphylactic treatment was not associated ($P = 0.36$) with differences in retreatment during the rearing period.

Mortality rates in the study population were low with 18 (1.4%) calves dying within the study period. The number of animals that died was small, so it is not surprising the differences in survivability of animals in different IS treatment groups were not statistically significant. However, the observed differences were important; the overall mortality rate during the

TABLE 5 | Multivariable logistic regression evaluating the effect of the administration of a non-specific immune stimulant around transportation on disease treatment events during the rearing period (first 9 weeks of life).

Variable	*n*[a]	Disease treatment (%)	Odds ratio	95% C.I.	*P*-value
Treatment[b]					
Control	438	71 (16.2)	Referent		
BTIS	431	63 (14.4)	0.89	0.61–1.30	0.75
ATIS	436	62 (14.4)	0.89	0.61–1.30	0.71
Season[c]					
March–April	286	65 (22.8)	Referent		
May–June	216	51 (23.6)	1.05	0.69–1.60	0.02
August–September	485	59 (12.2)	0.70	0.44–1.10	0.71
October–November	318	21 (6.6)	0.40	0.22–0.74	0.003
Birth weight	1305	196 (15.0)	0.98	0.96–0.99	0.05
Breed[d]					
Jersey	568	115 (20.2)	Referent		
Jersey-cross	737	81 (11.0)	0.73	0.52–1.04	0.08
Metaphylaxis[e]					
No	858	161 (18.8)	Referent		
Yes	457	35 (7.8)	0.60	0.37–0.97	0.04

The variables season of enrollment, birth weight, breed, and metaphylaxis were retained in the model.

[a] *Total number of animals that had disease event (i.e., respiratory disease and/or diarrhea) in each group during the rearing period.*

[b] *Treatment: Animals received subcutaneous administration of 1 mL of a non-specific immune stimulant at 4 ± 1 days of life. CON = calves that receive saline before transport; BTIS = calves that received immune stimulant before transport and ATIS = immune stimulant after transport.*

[c] *Enrollment season: Period of the study referent to the week when first set of calves was enrolled. Calves were enrolled on a weekly basis from March to November of 2018.*

[d] *Breed: Jersey and Jersey-Holstein cross heifer calves were enrolled in the study.*

[e] *Starting in September 2018 farm management implemented a metaphylactic treatment (Zuprevo, Tildipirosin, 4 mg/kg of body weight; Merck Animal Health, Summit, NJ).*

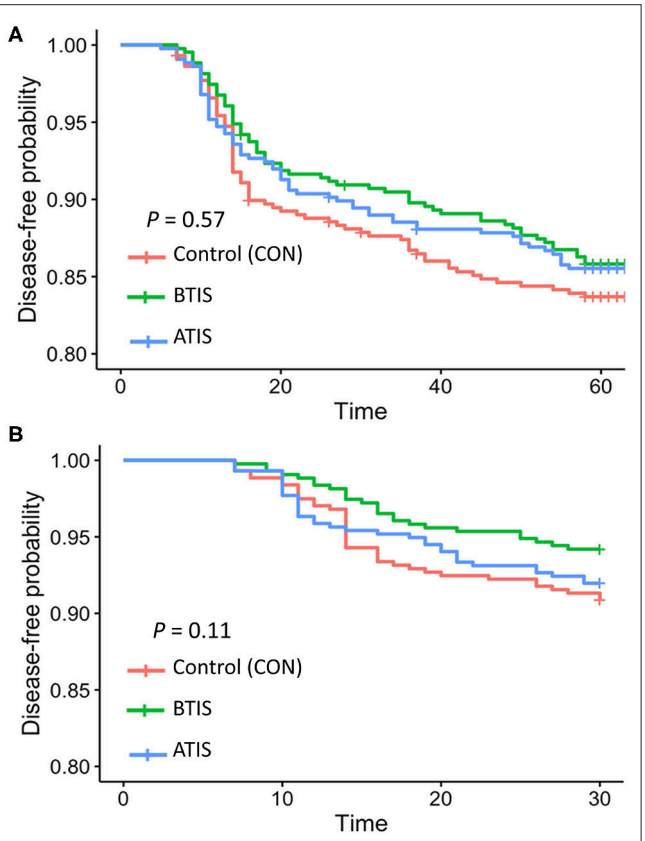

FIGURE 1 | Kaplan-Meier survival curves showing the cumulative proportion of calves that received treatment for any disease during the first 9 weeks of life **(A)** and calves that received treatment for respiratory diseases during the first 30 days of life **(B)**. CON = calves that receive saline before transport ($n = 438$; red line); BTIS = calves that received immune stimulant before transport ($n = 431$; green line) and ATIS = immune stimulant after transport ($n = 436$; blue line).

rearing period was only 0.9% (4 deaths each) in the BTIS and ATIS groups, compared with 2.3% (10 deaths) in the control group. A marginal difference ($P = 0.05$) was observed when contrasting the mortality in both IS groups combined to the CON group (**Table 4**). In the multivariable logistic regression model, no statistical differences were observed even though the odds of death for BTIS and ATIS calves was 60% smaller (BTIS; OR = 0.40; 95% CI = 0.12–1.28; $P = 0.43$; ATIS; OR = 0.40; 95% CI = 0.12–1.27; $P = 0.42$) than CON calves. Similarly, a 60% lower likelihood of mortality (OR = 0.40; 95% CI = 0.16–1.01; $P = 0.05$) was observed when comparing calves from both treatment groups to calves in the CON group. Metaphylactic treatment did not influence the odds of death (OR = 0.96; 95% CI = 0.36–2.60; $P = 0.94$). Full model output is presented in **Table 8** and a Kaplan-Meier curve showing the hazard of dying during the rearing period is presented in **Figure 2**.

DISCUSSION

Raising replacement heifer calves free of disease and that perform well during the rearing period results in a more productive and profitable adult dairy cow (1–4). Unfortunately, the occurrence of calf-hood diseases continues to be a challenge to dairy producers and transportation exacerbates this challenge. Dairy producers use antibiotics to treat and control diseases outbreaks and decrease mortality during the pre-weaning period (7). However, the use of antibiotics in food-producing animals has been associated with the alleged contribution of animal agriculture on the selection of antimicrobial resistance genes (18, 19). For this reason, immune stimulants that can induce early activation of the non-specific innate immune system and provide the first line of defense against microbial pathogens have emerged as an alternative to treat and prevent diseases and mortality in dairy cattle. The administration of mycobacterium cell wall fraction immune stimulant has been demonstrated to be effective on the reduction of severity, duration and mortality of induced bacterial diarrhea in dairy calves (21, 31). However, it was unknown at the start of this study whether it would be effective to improve health and performance of dairy calves transported within days of birth when they are not experimentally challenged to induce bacterial diarrhea but instead experience naturally occurring diseases.

TABLE 6 | Multivariable logistic regression evaluating the effect of the administration of a non-specific immune stimulant around transportation on the treatment of respiratory disease during the first 30 days of life.

Variable	*n*[a]	Disease treatment (%)	Odds ratio	95% C.I.	*P*-value
Treatment[b]					
Control	438	33 (7.5)	Referent		
BTIS	431	19 (4.4)	0.53	0.29–0.97	0.03
ATIS	436	32 (7.3)	0.92	0.54–1.55	0.35
Season[c]					
March–April	286	34 (11.9)	Referent		
May–June	216	36 (16.7)	1.50	0.90–2.50	<0.001
August–September	485	10 (2.1)	0.16	0.07–0.33	0.001
October–November	318	4 (1.3)	0.10	0.03–0.27	<0.001

The variable season of enrollment was retained in the model.
[a] *Total number of animals that had disease event (i.e., respiratory disease and/or diarrhea) in each group during the rearing period.*
[b] *Treatment: Animals received subcutaneous administration of 1 mL of a non-specific immune stimulant at 4 ± 1 days of life. CON = calves that receive saline before transport; BTIS = calves that received immune stimulant before transport and ATIS = immune stimulant after transport.*
[c] *Enrollment season: Period of the study referent to the week when first set of calves was enrolled. Calves were enrolled on a weekly basis from March to November of 2018.*

TABLE 7 | Logistic regression for the effects of a non-specific immune stimulant around transportation and metaphylaxis on the re-treatment of Jersey and Jersey-cross calves during the rearing period (first 9 weeks of life).

Variable	*n*[a]	Second treatment (%)	Odds ratio	95% CI	*P*-value
Treatment[b]					
Control	71	28	Referent		
BTIS	62	21	1.53	0.68–3.43	0.20
ATIS	63	30	0.96	0.43–1.94	0.38
Metaphylaxis[c]					
No	161	25	Referent		
Yes	35	31	0.68	0.30–1.54	0.36

[a] *Total number of animals that had disease event (i.e., respiratory disease and/or diarrhea) in each group during the rearing period.*
[b] *Treatment: Animals received subcutaneous administration of 1 mL of a non-specific immune stimulant at 4 ± 1 days of life. CON = calves that receive saline before transport (n = 438); BTIS = calves that received immune stimulant before transport (n = 431) and ATIS = immune stimulant after transport (n = 436).*
[c] *Starting in September 2018 farm management implemented a metaphylactic treatment (Zuprevo, Tildipirosin, 4 mg/kg of body weight; Merck Animal Health, Summit, NJ).*

TABLE 8 | Multivariable logistic regression evaluating the effect of the administration of a non-specific immune stimulant around transportation on the likelihood of death during the rearing period (first 9 weeks of life).

Variable	*n*[a]	Disease treatment (%)	Odds ratio	95% C.I.	*P*-value
Treatment[b]					
Control	438	10 (2.3)	Referent		
BTIS	431	4 (0.9)	0.40	0.12–1.28	0.43
ATIS	436	4 (0.9)	0.40	0.12–1.27	0.42
Metaphylaxis[c]					
No	858	12 (1.4)	Referent		
Yes	457	6 (1.3)	0.96	0.36–2.60	0.94

The variable metaphylaxis was also retained in the model.
[a] *Total number of animals that had disease event (i.e., respiratory disease and/or diarrhea) in each group during the rearing period.*
[b] *Treatment: Animals received subcutaneous administration of 1 mL of a non-specific immune stimulant at 4 ± 1 days of life. CON = calves that receive saline before transport; BTIS = calves that received immune stimulant before transport and ATIS = immune stimulant after transport.*
[c] *Starting in September 2018 farm management implemented a metaphylactic treatment (Zuprevo, Tildipirosin, 4 mg/kg of body weight; Merck Animal Health, Summit, NJ).*

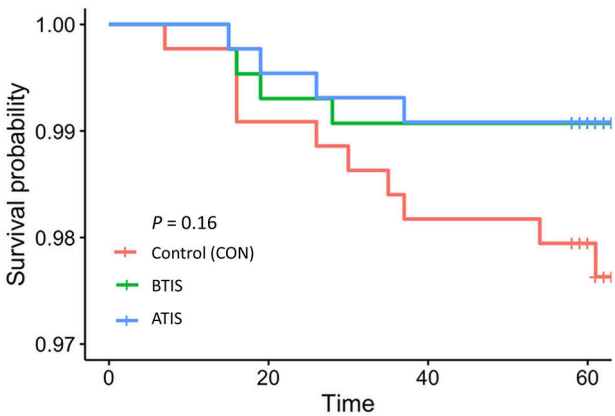

FIGURE 2 | Kaplan-Meier survival curves showing the survival of calves, independently of the cause of death determined by farm personnel, during the first 9 weeks of life (time). CON = calves that receive saline before transport (*n* = 438; red line); BTIS = calves that received immune stimulant before transport (*n* = 431; green line) and ATIS = immune stimulant after transport (*n* = 436; blue line).

In order to address this question, we administered the selected IS subcutaneously to newborn calves around transportation to obtain evidence that it would lead to better health and performance of dairy calves. We decided to use a 1 mL dose subcutaneously based on available information describing this dose as effective to decrease morbidity and improve weight gain in feedlot calves (20).

Considering a rearing period of 9 weeks (63 days of age), this study did not find statistically significant differences in weekly HS during the first 3 weeks, ADG, overall disease treatment rate, or mortality rate, when comparing the overall response of the administration of IS around transportation to newborn dairy calves. However, the likelihood of respiratory disease treatment during the first 30 days of life was lower for calves that received IS before transportation when compared to the calves in the control group and the percentage of calves that died during the rearing period was marginally smaller when comparing calves that received IS to calves in the control group.

The HS of all calves enrolled in our study was lower than expected and very few calves were considered to need extra

attention from farm personnel based on respiratory issues. It is somewhat surprising that HS results were so low, especially following transportation. Transportation is a major cause of stress in calves (32–34), and has been associated with increased prevalence of diseases, especially respiratory diseases (14, 15). However, the differences from our results to previous reports are likely explained by the fact that calves in our study received adequate amounts of good quality colostrum, were housed individually, and were transported at a very young age. To the best of our knowledge, this is the first study to evaluate the effects of IS administered to newborn dairy calves immediately before and after transport and the first to assess HS information following IS administration and transportation. Although we expected to identify subtle biologically relevant differences in health between the different experimental groups by assessing HS during the first 3 weeks post-transport, assessing HS on a weekly basis hindered our ability to capture all the variation in HS for animals enrolled in our study. Assessing HS once per week is unlikely to capture the true incidence of diseases in a herd because the clinical signs used by the HS systems might appear and disappear in the period between two consecutive HS because of treatments given or spontaneous cure of the disease. In future studies, measurement of daily behavior and health assessments by adopting precision technologies such as activity monitoring systems and video cameras is likely to be beneficial compared to a once a week health scoring method. Lastly, caution must be applied when interpreting the respiratory HS results in our study because very few calves were deemed to need extra attention within each treatment group.

Aligned with the HS results, the differences in the ADG for calves in the different treatment groups were also not statistically significant, and were <10 g/day. Previous reports have shown that diseases during the pre-weaning period are associated with decreased growth because of decreased appetite and feed intake, and increased energy demands to support immune response (1, 35, 36). The absence of significant differences in ADG between groups in the current study agrees with other reports showing a lack of effect of immune stimulants on growth and performance of calves (37, 38), even though increased ADG was reported in feedlot calves following the administration of the same IS used in this study (20). The overall good health described in our study population contributed to the similar ADG observed in the three treatment groups. Several factors may have played a role in improving animal health in the current study including individual housing and age of the calves. Differently from other studies, in our study, calves were housed individually during the experimental period and, therefore, were less likely to experience diarrhea and respiratory problems, especially when compared to group housed calves (39). Moreover, in our study, the most stressful events (i.e., enrollment and transport) occurred within the first 4-weeks of life when passive immunity transferred from cows via colostrum provides immunologic protection to calves (40).

In our study, the disease treatment rates were lower than morbidity rates reported in the latest nationwide survey and other epidemiological studies (41, 42) and historical data from the farm where the study was conducted. A larger sample size would have been determined if a more accurate estimate of disease treatment and mortality rates were known. The lack of statistical significance for some of the analysis in our study is likely a consequence of this inadequate sample size leading to imprecise confidence intervals around the point estimates. Thus, results are discussed emphasizing estimates and the uncertainty in them as previously recommended (43).

The reduced disease treatment and mortality incidence in our study are likely related to the reduced prevalence of failure of passive transfer (96.2% of enrolled calves had >5.5 g/dL serum total solids). The transfer of passive immunity via colostrum provides neonates with immunologic protection during early life with a successful colostrum management program having 80% of the calves with serum total solids values of 5.5 g/dL or higher (28). Lastly, it is also important to keep in mind that the disease events and treatments were self-reported by farm personnel, which is a limitation of the study and could have contributed to the lower treatment rates. The authors acknowledge this limitation but we are confident that treatment assignment stayed masked for farm personnel identifying and treating sick animals, thus decreasing the risk of bias when examining and making disease treatment decisions for the study population.

In our study we observed that, in all groups, the number of calves that received treatment for respiratory disease was greater than the number of animals considered to be in need of veterinary attention based on respiratory score. In contrast, very few calves received treatment for diarrhea when compared to the number of animals considered to need veterinary attention based on fecal scores. While in an ideal scenario the disease treatment and morbidity rates would be equivalent, a discrepancy between treatment decisions by farm personnel and HS by observers using clinical score systems to identify sick calves have been described (44). Although lack of employee training on using scoring systems to make treatment decisions and discordance between scoring systems guidelines and criteria used for treatment decisions by farm personnel are probable explanations for this discrepancy (44), infrequent HS assessment and inconsistent disease recording are the likely explanation for the differences observed in our study for respiratory disease and diarrhea, respectively. Health scoring systems rely on observation of abnormal clinical signs to determine the health status of calves. However, dairy calves that exhibited abnormal clinical signs in between subsequent HS were treated by farm personnel and were unlikely to display abnormal clinical signs at the next HS assessment, accounting for the discrepancy in our dataset when comparing respiratory scores and respiratory disease treatments. These findings suggest that weekly HS likely results in under-reporting of sick calves and, therefore, should be used with caution in research studies aimed to describe respiratory disease incidence. For diarrhea treatment, farm personnel only recorded a diarrhea treatment event when administering intravenous fluids. Dairy calves with fecal score >1 received oral electrolytes in their water and our research group could not capture this

treatment information in the farm management software. Thus, many more calves were considered to need veterinary attention in comparison to the number of calves that received a treatment for diarrhea.

Despite the lower disease treatment and mortality rates, important numerical differences were observed when comparing the treatment groups. It is interesting to note that calves receiving IS treatment before transportation had a significant lower likelihood of being treated for respiratory diseases during the first 30 days of life. Additionally, fewer calves that received IS administration died compared to CON. These results further support the idea that the administration of IS can induce innate immune response in calves and, consequently, decrease their susceptibility to infectious diseases (21, 31).

According to several reports, calf morbidity and mortality peaks during the first month of life with bovine respiratory disease and diarrhea as the major culprit (29, 45). The increased calf morbidity and mortality during this period is associated with reduced immunity, hence the opportunity for the use of immune stimulants. In our study, 70% of all disease treatments occurred within the first 30 days of life. The proportion of calves treated for respiratory disease within the first 30 days of life was smaller in the groups receiving IS before transportation, but no differences were observed for diarrhea. The effect of IS reducing the treatments for respiratory diseases in the current study agrees with previous work (31). We speculate that the strength of the immune response immediately after the administration of the IS and the multifactorial nature of the infectious diseases of neonatal calves played a role in this different response during the first 30 days of life. In addition, the implementation of a blanket administration of antibiotics to all animals enrolled in this study at ∼35 days of life could have influenced disease progression and reduced calf morbidity and mortality, especially for the last 457 calves enrolled in the study. However, the majority of the disease treatments in the study occurred before the metaphylactic treatment. Moreover, the variable metaphylaxis was included as a covariate in our statistical models. For this reason, we did not analyze our data considering the periods pre- and post-metaphylaxis implementation separately. Although this particular management strategy introduced a potential confounding variable to the study, it also reflects the challenges inherent to performing clinical trials in commercial dairy farms.

Although the estimated difference in disease treatment and mortality were within the range described by previous studies using immune stimulants (20, 21), the rather low disease treatment and mortality rates encountered in our study may have contributed to the lack of statistically significant differences. Nonetheless, it is also possible that the administration of IS to dairy calves alters the duration of diseases events as well as the time to disease event. The authors considered this hypothesis prior to the beginning of the study, but the logistics for collecting information on disease events duration was challenging and authors decided to analyze "retreatments" as a proxy for unresolved disease cases. Unfortunately, the low disease treatment rate also resulted in a very low recurrence of disease treatments and we were unable to derive conclusions from our results. Further investigation of the effect of IS on disease events duration is warranted.

Previously published studies have shown that calves benefit from adequate transfer of passive immunity leading to fewer diseases and lower mortality, and consequently fewer antibiotic treatments (46). Although, the administration of antibiotics to newborn calves has also been associated with decreased incidence of bovine respiratory diseases and increased calf survivability (46, 47), major concerns about antibiotic resistance, antibiotic-associated diarrhea and calf-rearing costs make their continued use less favorable (48). For this reason, the results of our study provide some support for the conceptual premise that administration of IS can be another tool to improve calf health during the rearing period, especially if administered prior to transportation and periods when naturally occurring disease events are elevated. Additional studies to determine the effect of IS in multiple herds, including herds with treatment and mortality rates higher than the one reported in this manuscript are warranted to confirm the effectiveness of this intervention.

CONCLUSION

The administration of IS did not significantly improve HS, ADG, and the differences in the likelihood of disease treatment within the first 9 weeks of life. However, administration of IS prior to transportation reduced the likelihood of treatment for respiratory diseases during the first 30 days of life and led to a marginal decrease in mortality during the rearing period when compared to calves that did not receive IS.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota and Texas Tech University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

BO was responsible for sample collection and original manuscript draft. LC was responsible for funding acquisition, study conceptualization, and manuscript review and editing. MC, PM, DP, and AG-M were responsible for sample collection and manuscript review. VM was responsible for study conceptualization and manuscript review and editing. AM was responsible for manuscript review. AR was responsible for data analysis and manuscript review. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: NovaVive Inc. employed AM during the period of this study. AM reviewed the manuscript but was not involved with any of the data analyses.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
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Efficacy of a Lactobacillus-Based Teat Spray on Udder Health in Lactating Dairy Cows

John I. Alawneh[1,2]*, *Ameh S. James*[1,2], *Nancy Phillips*[1], *Brandon Fraser*[1], *Karen Jury*[3], *Martin Soust*[3] and *Timothy W. J. Olchowy*[1,4]

[1] School of Veterinary Science, The University of Queensland, Gatton, QLD, Australia, [2] Good Clinical Practice Research Group, The University of Queensland, Gatton, QLD, Australia, [3] Terragen Biotech Pty Ltd., Coolum Beach, QLD, Australia, [4] Faculty of Veterinary Medicine, University of Calgary, Calgary, AB, Canada

Teat disinfection is a common pre- and post-milking mastitis prevention practice that is part of a mastitis control program in dairy herds. Commercially available teat disinfectants are generally chemical-based products. The use of these products has occasionally raised concerns about the risk of chemical residues in milk. An alternative treatment or prevention strategy based on probiotics has the potential to circumvent this risk. Two treatments were compared in a cross-over clinical trial in a single herd: a lactobacillus-based, post-milking teat spray (LACT), and a commercial iodine-based post-milking teat disinfectant product as (positive control, PC). The effect of the two treatments on cow somatic cell counts was quantified using a multivariate mixed-effects linear regression model with cow fitted as a random effect. The odds of teat end scores increasing from a low to a high score tended to be lower (OR = 0.74, 95% CI 0.54–1.01, $P = 0.06$) for cows receiving LACT treatment. On average, there was also a tendency for a lower somatic cell counts in the LACT treated cows (antilog of coefficient = 0.91, 95% CI 0.80–1.03, $P = 0.13$) compared with the PC treated cows. The application of the lactobacillus-based product to teats could reduce the rate of teat end scores progression from low to higher scores, and potentially improve teat end sphincter functions and udder health. Further, larger scale validation work is required to support the findings of the current study.

Keywords: teat end scores, lactobacillus-based, dairy cattle, mastitis, somatic cell counts, udder health

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***Correspondence:**
John I. Alawneh
j.alawneh@uq.edu.au

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INTRODUCTION

Mastitis is the most prevalent production problem of animal welfare, production, and economic loss facing the dairy industry worldwide (1). The prevalence risk of mastitis is high (2, 3) and is influenced by animal (e.g., parity, stage of lactation), farm (e.g., herd-size, geographical location), and nutritional factors in the herd (1, 4). Teat canal and the integrity of teat-end tissue play a pivotal role against the introduction of mastitis-associated pathogens into the udder. Teat-end hyperkeratosis is the teat canal response to the forces imposed by milking. Milking machine and animal level factors can lead to severe teat-end hyperkeratosis and increase the roughness of the teat end (5, 6), and increase the risk of intra-mammary infections (IMI) by mastitis-causing pathogens in the herd (1, 6). Somatic cell counts (SCC) concentration in the milk is considered a biomarker of mammary gland inflammation and used as a proxy for IMIs (7, 8). A relative reduction in SCC while holding constant all other animal and herd-level risk factors, reflects a lower risk of exposure to IMIs (9). Reduced milk SCC is a reasonable indicator of effective mastitis management practices in the herd (10, 11).

Teat disinfection is a common mastitis prevention practice that has proven to be an excellent tool in the control of mastitis (12–14). This practice has been associated with a lower incidence of new IMI, a reduction in bulk milk SCC and fewer teat skin abnormalities (9). Ideally teat products have disinfectant properties and do not cause any harmful changes to the health of the teat skin [National Mastitis Council (NMC), www.nmconline.org]. Teat disinfectant formulations often include skin conditioning agents: emollients (lanolin) or humectants (glycerine, propylene glycol, or polyvinylpyrridone) (15). Some formulations contain aloe and allantoin which have been shown to have skin-healing properties (16). Teat disinfection that effectively and safely reduces bacterial load on teat skin reduces the risk of mastitis in the herd (17–19), improves teat skin condition (9), and reduces the risk of milk contamination (20). The observed efficacy of a teat disinfectant will vary depending on the production system, season, and the particular mastitis causing pathogens affecting the herd (18, 21, 22).

Gleeson *et al.* (17) conducted a study on two dairy farms in Ireland to explore udder health benefits of pre-milking teat disinfectant practice. In that study, bacterial numbers on teat skin were reduced and the practice was effective against environmental bacteria (*Escherichia coli* and *Streptococcus uberis*). Teat disinfection can also be a safe and effective method to reduce the incidence risk of mastitis caused by contagious pathogens such as *Staphylococcus aureus* (23, 24). However, it is less effective against environmental pathogens (15). A 2018 Australian study failed to demonstrate a benefit of iodine-based pre-milking teat disinfection (25). Treated multiparous animals had higher odds of clinical mastitis associated with environmental pathogens. Teat disinfection of primiparous animals did not reduce the odds of developing clinical mastitis compared to the untreated animals (Odd ratio [OR] = 1.31, 95% CI = 0.52–3.29). A combined pre- and post-milking teat disinfection program neither reduced the incidence of new IMI nor did it result in a reduction in SCC in a New Zealand dairy cattle study (9).

Commercially available teat disinfectants are generally chemical-based (iodophor, chlorhexidine) products (23). The use of these products has occasionally raised concerns about the risk of chemical residues in milk (26–28). Lactic acid bacteria (LAB) are part of the healthy alimentary microbiota (29), and have been proposed as a potential alternative therapy for the control of bovine mastitis (30, 31). A liquid product containing a mixture of *Lactobacillus* organisms (LACT) was developed as a post-milking teat spray. Therefore, the objective of the present study was to evaluate the short-term effect of LACT on mammary health as defined by SCC and teat end score (TES). It was hypothesized that LACT would be at least as effective as a commercially available iodine-based post-milking teat disinfectant in improving udder health.

MATERIALS AND METHODS

Study Design

This was a positive-controlled, randomized 2 × 3 cross-over study involving two experimental groups [LACT treated, positive control (PC) treated] and three treatment periods (**Figure 1A**). The study was conducted between 01 June and 26 July 2018 using the year-round calving University of Queensland-Gatton 230 milking cow research dairy herd. The herd is managed as two groups of milking cows typical of Queensland dairies: a combined fresh and early lactation group [up to 100 days in milk (DIM); fed a total mixed ration; $n = 90$]; and a second group comprising mid- and late lactation cows (>100 DIM; fed pasture and a mixed ration; $n = 140$). Grazing pasture consisted of a mixture of temperate and tropical plant species. Pasture supplemented with a silage-based mixed ration was sufficient to meet the maintenance and production requirements of a cow producing 25 L of milk per day. The experimental procedures were approved by the University of Queensland Animal Ethics Committee prior to the start of the study (Approval number: SVS/043/18/TERRAGEN).

The sample size required to evaluate changes in udder health (i.e., SCC), 13 cows, was based on the *a priori* assumptions for SCC of an alpha of 95%, a power of 90%, a common standard deviation of 20,000 cells/mL, a difference of 40,000 cells/mL and a correlation between group means of 0.1. For TES comparison, it was assumed that TES improvements (lower scores) would be associated with a decreased risk of mastitis in this herd. Sample size calculations determined that fifty cows would allow detection (with 95% confidence, power of 90%, pooled variance one teat end score, 1:1 ratio treatment to control sample size) of a difference of one teat end score (one to five scoring scale, see below) between the mean TES before and after LACT teat spray treatment. Therefore, the study sample size was 50 cows divided equally into the two experimental groups.

Animal Management

Cows were milked twice daily at 04:00 and 15:00 h in a double 14 rapid exit high line parallel parlor (GEA Westfalia™, Victoria, Australia). The automatic cup removal (ACR) system was set to detach milking clusters when milk flow decreased to 0.2 L/min. Milk-line vacuum pressure was checked during the milking of each turn of cows in the parlor. The vacuum pressure was maintained between 45 and 48 kPa by a variable frequency vacuum pump. To ensure a consistent milking routine, all milking staff received ongoing training in milking machine operation and milking protocols. Based on study farm animal health records, the estimated incidence risk of clinical mastitis in the source herd was, on average, 22% [95% confidence interval (CI) = 17–33%].

Teat End Scoring

The TES for each cow were determined by a single individual during a single milking session using the one to five TES scale adapted from Mein et al. (5): score 1 = normal with no apparent ring present at the teat end; score 2 = smooth and slightly rough ring; score 3 = rough ring; score 4 = very rough ring; score 5 = open lesions or scabs. The scorer was blinded to the treatment allocations to study animals. The TES were averaged (median) at the cow level. The median TES were used in subsequent analyses.

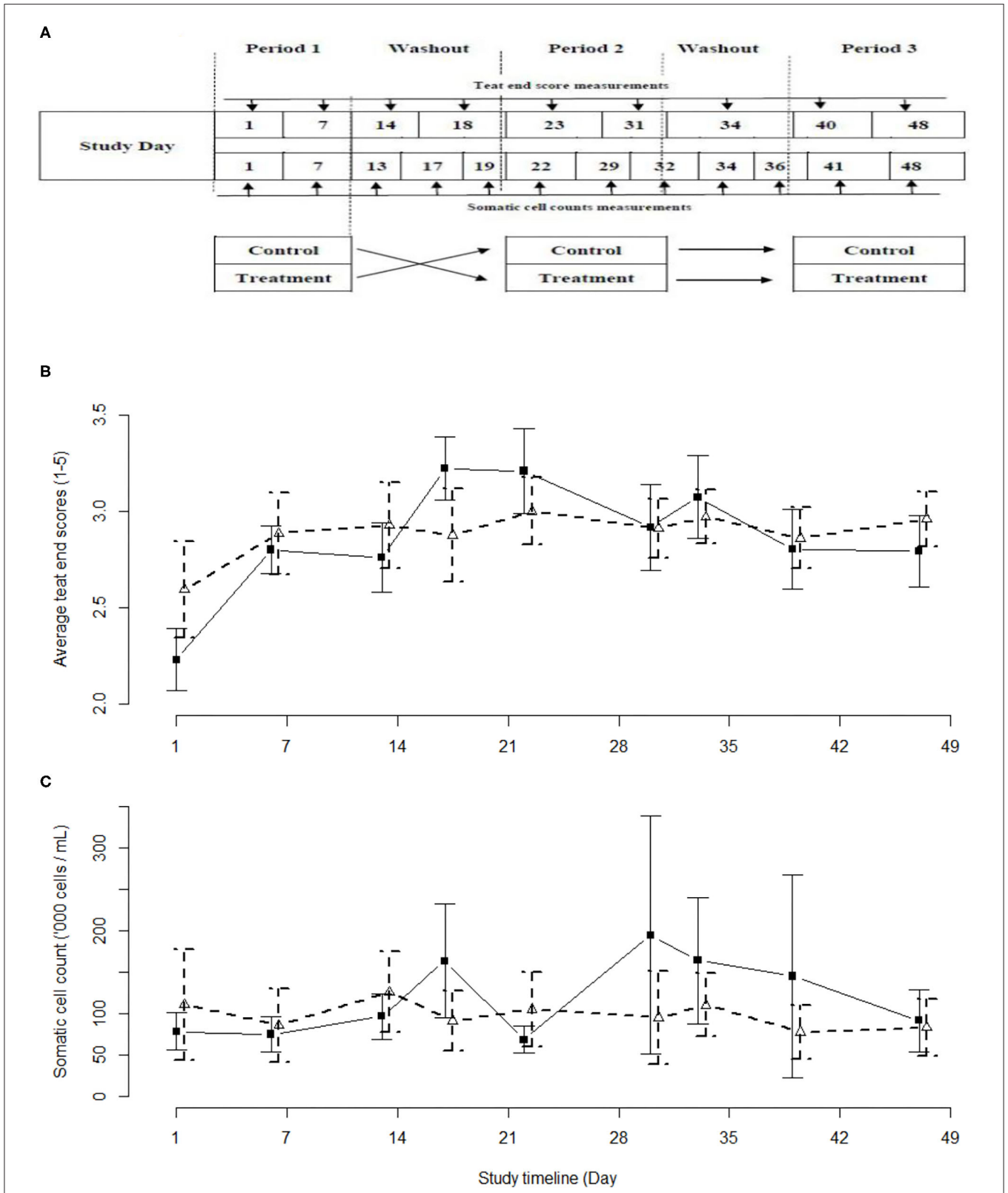

FIGURE 1 | Schemata of the study design **(A)**, and the observed average teat end scores **(B)** and average somatic cell counts **(C)** with 95% confidence intervals as observed in the current study. Iodine-based (PC) group - black solid line and solid squares. Lactobacillus-based (LACT) group – black dashed line, white triangles. Treatment periods were between Study Days 1–7, 23–31, and 40–48. Washout periods were between study days 14–18, and 31–34.

Selection of Study Animals

Fifty apparently healthy lactating dairy cows of mixed age, stage of lactation, and breed, were randomly selected (simple random sampling without replacement) from a pool of 60 eligible cows (median TES of 2 or less, apparently normal quarter milk, SCC <300,000 for at least the past 8 weeks, no history of systemic disease or clinical mastitis in the 8 weeks preceding the study start date) (**Supplementary Table 1**). Enrolled cows were managed, fed, and milked as per the routine farm practices with the exception that the study animals were maintained as separate groups from the main herd.

Experimental Design

The Lactobacillus based teat spray being evaluated was a proprietary liquid formulation (LACT; Lactolin™, Terragen Biotechnology Pty Ltd, Queensland, Australia) suitable for application as a post-milking teat spray. The preparation consisted of a mixture of three *Lactobacillus* spp. (*Lactobacillus paracasei*, *L. buchneri*, *L. casei*; minimum 10^6 cfu/mL of each strain) in saline (0.9%NaCl). This preparation was stored at 4°C until applied to the teats (without an emollient) using a hip mounted, hand operated mobile teat sprayer (HipSpray™, Ambic Equipment Ltd., Davies Way, Brisbane, Australia) set to deliver 10 mL per teat as per manufacturer instructions. The positive control treatment (PC) was a commercial, iodine-based, post-milking teat disinfectant (Dairy Power Mastidyne™ Iodophor 20 g/L available iodine, 2% free iodine, ECOLAB, Sydney, Australia) supplied as a 2-part concentrate and automatically mixed with potable water on an as needed basis for application to the teats using a spray wand. The product as applied consisted of three parts Dairy Power Mastidyne™ Iodophor teat sanitiser, eight parts cool potable water, and one-part Dairy Power Glysoft™ udder emollient 10% solution (ECOLAB, Sydney, Australia) as per the manufacturer instructions. The inline sprayer gun (AMBIC™, Davisway/DASCO, Victoria, Australia) was set to pump a volume of 10 mL per teat covering the entire teat as per manufacturer instructions.

Cows were assigned to groups using simple random sampling without replacement. Simple random sampling was also used to assign a specific teat treatment (either LACT or PC) to one of the two 25-cow study groups for the first and second 2-week long experimental periods. The third 2-week long experimental period was a replica of the second period (**Figure 1A**). The two groups of animals were milked separately with an abbreviated milking machine cleaning cycle run before and between the milkings. To minimize the risk of residual effect of the two treatments, each treatment period was separated by a minimum washout period of 48 h during which there were no applications of either treatments. This minimum washout period was based on an absence of detectable (qPCR) biological residues of the LACT organisms at 36 h post-treatment.

Composite milk samples (50 mL) were collected bi-weekly from each cow at the Monday and Friday morning milkings, preserved with Acticide L-Bronopol, and SCC determined by the Australian Herd Recording Services (Kenilworth, Queensland, Australia) using an automated cell counter (Fossomatic 5000, Foss Electric). Teat ends were evaluated and scored weekly.

Statistical Analysis

Summary statistics generated for continuous or categorical variables (as appropriate) included: mean, median, standard deviation (SD), first and third quartiles (1st and 3rd Q), minimum, maximum, counts and percentage, as appropriate. *Chi-squared* test was used to assess the homogeneity of TES count distribution between the treatment groups. The statistical analysis was conducted in R (43).

The association between the LACT and PC groups and TES was assessed using a multivariate, mixed effects, ordered logistic regression model. The analysis was performed at the quarter-level to allow the model to account for the repeated measurements in TES and clustering of teats within cows (the sampling unit, i.e., the udder quarters nested within the cow, i.e., the experimental unit). Square root transformation was applied to the study day to maintain the linearity of the modeled log odds across the study days and reduce the risk of violating the proportional odds assumption (see below). Statistical significance was declared at an alpha of 0.05 or less. The association between LACT and PC and individual cow SCC was quantified using a multivariate mixed-effects linear regression model with cow fitted as a random effect. Model building followed forward selection procedure. First order interaction terms were tested and were retained if interaction terms was significant at a likelihood ratio test P value of 0.05 or less. Models specification followed that described by St-Pierre (44) and took the following generic forms:

Somatic cell counts – animal level data [1]

$$y_{ijk} = Intercept + Treatment_i + Period_j + \beta_1 Time + S_{ik} + \epsilon_{ijk}$$

Teat end scores – quarter level data [2]

$$y_{ijmk} = Intercept + Tretament_i + Period_j + \beta_1 Time + Quarter_m + S_{mik} + \epsilon_{ijmk}$$

Where y_{ijk} and y_{ijmk} denote the response observed at the cow and quarter levels, respectively for model [1] and [2], in period_j of treatment_i, and Si_k and S_{imk} are the random error term for the k^{th} cow (or $Quarter_m$ nested within cow, respectively) in the i^{th} treatment group. The outcome variable (SCC) was log transformed to stabilize the SCC variance and restore normality of the data. Study day was modeled as a polynomial (of the 4th order) variable with the order of the polynomial determined using the Akaike Information Criterion (AIC). The residuals (ε_{ijk} *and* ε_{ijmk}) of the random effect term were assumed to be normally distributed with a mean of zero, a variance of σ^2, and an autoregressive correlation structure of the first order. Overall model fit was based on AIC, Bayesian information criterion (BIC) and visual assessment of *Pearson's* residuals against fitted values, *Q-Q* standardized residuals against standardized normal quantiles violated the normality assumption (32). The proportional odds assumption was checked visually by examining the vertical consistency of distances between any two of the orders TES scores (at the logit scale) within explanatory

variable in the model. The overall effect of LACT and PC over the three treatment periods was further explored using least-squares means (LSM) prediction from the final mixed-effects linear model. (LSM means predictions were averaged predictions across all covariates in the model fixed at the reference levels). Statistical significance was declared at an alpha of 0.05 or less. Because SCC were log transformed, interpretation of the coefficient represents a unit change in log SCC. The antilog of each coefficient is interpreted as follows: for a continuous explanatory variable, the antilog of the coefficient represent a change in average (geometric mean) SCC for each unit change in the continuous variable. For a categorical variable, the antilog of the coefficient is the ratio of the means for each level of the categorical variable compared with the reference category. All analysis using ordinal (33), emmenas (34), visreg (35), nlme and lme4 (32, 36) statistical packages in R.

RESULTS

The teat end scores of the cows in the LACT and PC groups followed a similar curvilinear trend (**Figure 1B**). The TES values were highest during the second of the three 2-week treatment periods. Overall, the LACT group was associated with fewer TES 4 (13%) and 5 (1%) and more TES 1 (7%), ($\chi^2_{29.042,\ \mathrm{df=4}}$, $P < 0.01$) compared to the PC group (15, 2, and 2%, respectively, **Figure 1B** and **Supplementary Table 2**). In parallel with the TES results, the SCC associated with both treatments followed a similar trend (**Figure 1C**).

The results from the multivariable model for TES are shown in **Table 1**. Holding the covariates at their reference, on average, the odds of a shift from low to high TES tended to be lower for cows in the LACT group compared to TES for cows in the PC group (OR = 0.74, 95% CI 0.54–1.01, $P = 0.06$; **Table 1**). The TES value of individual cows at baseline influenced the odds of observing a high TES during the study. Irrespective of treatment assignment, for each unit TES score above score 1 at baseline, the odds of TES changing from a low to a high score during the study increased ∼3-fold (OR = 3.46, 95% 2.07–5.78, $P < 0.01$; **Table 1**).

The results from the multivariable model for SCC are shown in **Table 2**. After controlling for the effect of TES at baseline, milk production, and holding the remaining covariates at their reference, on average, there was a tendency for SCC in the LACT group to be 9% lower (antilog of coefficient = 0.91, 95% CI 0.80–1.03, $P = 0.13$) compared to the PC group (**Table 2**). Across all three treatment periods, the average SCC for cows in the PC group was 14% higher (1.14, 95% CI = 0.97–1.33, $P = 0.07$; (**Table 3** and **Supplementary Figure 1**) compared to the LACT treated cows.

TABLE 1 | Coefficient (standard errors) and odd ratios (95% confidence interval) from final multivariate mixed-effects ordered logistic regression model fitted on cows teat end scores (TES†) for the study animals.

Variable	Coefficient (SE)	Odd ratio (95% CI)	*P*-value
Teat end scores at baseline	1.24 (0.26)	3.46 (2.07–5.78)	<0.01
Daily milk production	−0.01 (0.02)	0.99 (0.95–1.03)	0.59
(L; centered‡)			
Time (Day; square root)	−0.01 (0.01)	0.99 (0.98–1.01)	0.15
Treatment group			
PC	**Reference**	1	
LACT	−0.30 (0.16)	0.74 (0.54–1.01)	0.06
Treatment period			
Period 1	**Reference**	1	
Period 2	2.03 (0.18)	7.61 (5.36–10.80)	<0.01
Period 3	1.95 (0.29)	7.01 (3.98–12.39)	<0.01
Udder quarter			
Fore quarters	**Reference**	1	
Hind quarters	−1.32 (0.19)	0.27 (0.18–0.39)	<0.01
Random effect	Variance (SE)	95% Confidence Interval	
Cow	0.33 (0.18)	0.11–0.94	
Quarter	0.60 (0.20)	0.31–1.15	

† *Teat end scores (scale 1–5; one is a normal teat end with no ring apparent; 5 is a severely abnormal teat end, rough, raised, and obvious ring at teat end).*

‡ *Centered on the mean.*

SE, Standard Error; CI, Confidence Interval; L, liters; PC, iodine-based positive control; LACT, lactobacillus based product. Model fitted using mixed-effect linear regression procedure in R. Model fitted with quarter nested with cow and animal fitted as random effect. Robust standard error estimation was used to adjust for clustering within cow. Final model AIC = 2700.561, Wald Chi-squared = 237.20 P < 0.001, Log psuedolikelihood = −1337.2804.

DISCUSSION

Our study evaluated the effect of a probiotic-based, post-milking teat skin spray on the health of the mammary glands using the proxy parameters of SCC and TES. This short-term crossover designed pilot study does support the hypothesis that the probiotic product was at least as effective as a commercial iodine-based post-milking teat disinfectant product. There were fewer abnormal TES in the LACT group. The odds of a TES shifting (throughout the study) from a lower to a higher score was lower for cows in the LACT group. Even though there was no statistically significant differences in the SCC values in response to the two treatments, when the effect of the explanatory variables was controlled, there was a trend to a lower mean SCC in the probiotic group.

The study did not identify any significant difference in TES between LACT and PC groups. Teat end scores for cows receiving either treatment followed a similar curvilinear trend. The odds of a reduction in the average TES did not differ between groups. There was a relatively increased number of chapped teat ends in the control group cows. This occurred despite the skin conditioning properties of this commercial product relative to the product under study. The conditioner and sanitizer components were mixed and used throughout the study as per manufacturer recommendations. The mixing system for the commercial product was serviced regularly by a qualified milking machine technician to ensure correct operation. However, no iodine analyses were performed on the final product as delivered to the cows. In contrast to treatment with the commercial product, cows receiving the probiotic product showed a strong propensity toward a lower risk of an increase in TES. This suggests the *Lactobacillus*-based product has a protective effect

TABLE 2 | Coefficient (standard errors) and antilog of the estimated coefficients (95% confidence interval) from final multivariate mixed-effects linear regression model fitted on individual cows somatic cell counts (SCC; 000's cells/mL) for the study animals.

Variable	Coefficient (SE)	Antilog of coefficient (95% CI)	*P*-value
Intercept	4.38 (0.18)	82 (56.03–119.99)	<0.01
Log SCC at baseline (centered†)	0.81 (0.08)	2.24 (1.91–2.61)	<0.01
Study day (4th order polynomial‡)			
1st order	4.38 (2.19)	79.55 (1.07–5922.20)	0.04
2nd order	−1.13 (0.95)	0.32 (0.05–2.05)	0.23
3rd order	−1.31 (0.75)	0.27 (0.06–1.17)	0.08
4th order	1.56 (0.74)	4.77 (1.12–20.40)	0.04
Treatment group			
PC	**Reference**	–	
LACT	−0.09 (0.06)	0.91 (0.80–1.03)	0.13
Treatment period			
Period 1	**Reference**	–	
Period 2	−0.37 (0.21)	0.69 (0.45–1.04)	0.07
Period 3	−0.53 (0.30)	0.59 (0.32–1.05)	0.07
Random effect	Variance (SE)	95% Confidence Interval	
Cow	0.52 (0.02)	0.48–0.56	

†*Centered around the mean.*
‡*The order of the fitted polynomial was assessed using model AIC.*
Se, Standard Error; CI, Confidence Interval; PC, iodine-based positive control; LACT, lactobacillus-based product. Model fitted using mixed-effect linear regression procedure in R. Model fitted with cow fitted as random effects. Final model AIC = 628.929, Loglikelihood = −303.4645.

TABLE 3 | Least-square means predictions (marginal means; at the log and antilog scales) and mean ratios obtained over the grid of predictors settings from linear mixed-effects model shown in **Table 2**.

Experimental group	Predicted marginal effect				
	Log scale		Antilog scale		
	Mean (SE)	95% CI	Mean (SE)†	95% CI†	*P*-value‡
PC (Iodine-based Positive control)	4.62 (0.13)	4.35–4.89	102 (14)	75–138	
LACT (Lactobacillus-based)	4.50 (0.13)	4.23–4.76	90 (12)	66–121	
PC / LACT means ratio	0.13 (0.07)	0.08–0.94	1.14 (0.08)	0.97–1.33	0.07

†*Values are in 000's cells/mL'.*
‡*Bonferroni adjusted for multiple comparisons.*
SE, Standard Error; CI, Confidence Interval.

on teat ends. The pattern of change in SCC (tendency for reduced SCC in the LACT group) in this study is consistent with the reduction in subclinical mastitis in association with the use of a *Lactobacilli* spp. based teat treatment observed by Yu et al. (20). They suggested that the effect of the biologic-based treatment was to decrease the exposure of the teat to mastitis-associated bacteria by improving the microbial environment of the cow teat. The current study focused on the effect on milk SCC and did not investigate the potential effect on the microbiota of the teat.

The specific mechanisms responsible for the observed beneficial trends associated with the *Lactobacillus*-based product were not investigated in the present study, but several possibilities exist. If the organisms in the LACT group grow faster than mastitis causing pathogens, there may be fewer sites on the teat skin for pathogens to adhere or colonize thereby reducing the exposure risk to the udder (19, 37). The development of bovine mastitis has been associated with dysbiosis, an imbalance between the healthy microbiota of the mammary gland and mastitis causing pathogens (37). It is possible that the presence of the LACT organisms inhibited the development of any teat skin or mammary gland dysbiosis. The use of probiotics to minimize the risk of (or correct existing) dysbiosis has been proposed as a method to both reduce mastitis risk and the need for antimicrobial use (29). An additional potential protective effect may be the result of barrier-like biofilm properties of the organisms (20). *Lactobacillus* spp. have some characteristics needed for biofilm formation. They do colonize and are retained for long periods, a critical factor in preventing colonization by pathogenic bacteria (38). The role of the established resident microbiota of the teat skin and mammary gland and the potential changes to the microbiota in response to treatment were not investigated. Changes in TES and SCC in response to potential pathogen exposure were not investigated by culture or PCR based examination of epithelial surfaces or milk samples. This is a limitation of the current study and should be addressed in future and larger scale study. Therefore, the results presented in the current study should be interpreted with caution.

The product tested in this study contained live *Lactobacilli* organisms. This type of product offers advantages over those which do not contain living organisms (i.e., lactic acid, iodine, and other chemical products) as several mechanisms are in force: bacterial competition and/or displacement from an ecological niche(s), and production of anti-bacterial substances [bacteriocins such as lacticin (39)]. This product's characteristics may result in an ongoing effect in contrast to the one-time high dose exposure associated with commercial chemical based teat disinfectants; a high initial dose which may taper off below the threshold of efficacy. In addition, this type of product would benefit from the lack of harmful residues, a characteristic associated with GRAS ("generally regarded as safe") organisms. The future role of lactobacillus-based udder health products is most likely as externally acting formulations. Intra-mammary infusion of these GRAS organisms has been associated with increases in SCC of the infused quarter, especially if the initial SCC of the quarter was quite low (40). Similar outcomes have been observed by others (15, 41). This effect was not observed in quarters with a pre-existing high SCC (IMI affected quarters). Cure rate of infected high SCC quarters following administration of the probiotic product (Lacto-bac; *Lactobacillus acidophilus*, *Lactobacillus casei*) was inferior to the antimicrobial treated group of cows. The authors did not provide any *in vitro*

antibacterial test results so it is possible that these organisms were either not producing, or were producing insufficient amounts of bacteriocins.

One of the challenges of field studies is the inability to control factors that may have significant impact on the study outcomes. This study was no exception with the deterioration of weather conditions (dry, cold); and the lack of treatment of application in the washout phase, during the second treatment period that may have influenced the observed outcomes. With a few exceptions, dairy cows in Australia and New Zealand are not housed in barns. Outside of the time spent in a milking parlor (more often than not with open side walls), under shade structures (purpose-built and/or trees), or sheltering behind wind breaks, the cows are subjected to the weather conditions of the pasture/paddock. The cows in this study had access to partial protection offered by fixed sun shade structures and a modest wind break created by the side of a building. Dry cold weather conditions during the second treatment period were associated with higher SCC values and TES in both groups. The effect was greater in the PC group, but not statistically significant (**Figure 1**). Martins et al. (42) reported a similar increase in SCC and TES and described an increased growth rate of mastitis pathogens during the winter season. It is possible this lack of significant group differences in response to adverse weather was influenced by the sample size of the present study. In cold temperate climates, it is not uncommon for producers to use a "winter formulation" teat disinfectant to reduce the "teat end chapping effect" of the weather and reduce the risk of intra-mammary infection. Further comparative formulation studies would be required to test this potential effect and evaluate the value of a "winter formulation" under Australian conditions.

By their nature, preliminary, pilot or proof-of-concept type studies have limitations. This study is no exception with limitations being expected. The sample size may have been too small to detect significant differences in treatment effects. The short duration and relatively restricted exposure of cows to seasonal variations may have hidden potential long-term beneficial effects of the treatments. The purpose of the study was to test a hypothesis and determine if any potential benefit existed to support the conduct of a long term multiple season (hot and humid, cool, and dry) study. As the observational data from this pilot study was limited, it was not possible to determine if beneficial effects may develop over a longer term, such as a complete lactation, or when cows in both experimental groups are allowed to comingle (e.g., shared risk of exposure mastitis causing pathogens, homogenous animal management within groups). No cows developed any adverse reactions or illnesses, either local (mastitis) or systemic, during the course of the study supporting the general acceptance that this probiotic product is indeed appropriately categorized as GRAS when used in this manner. Finally, determination of the mechanisms underlying any beneficial effects require further laboratory and field studies. Enough evidence was acquired to encourage further investigation of the interaction of these organisms with the teat and udder microbiota.

CONCLUSIONS

Somatic cell counts followed a similar trend for cows receiving either lactobacillus based LACT product or iodine-based PC product. Overall, cows in the LACT group had fewer teat end scores of one, four, and five. The odds of an increase in the teat end scores and average somatic cell counts over the three treatment periods tended to be lower for cows treated with lactobacillus based product compared with the iodine-based PC treated cows. The results from this pilot study suggest that lactobacillus-based product treatment could improve teat end sphincter functions and udder health. Further, larger scale validation work is required to support the findings of the current study.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Queensland Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

JA study design, sample collection, sample processing, data analyses, and drafting of the manuscript. AJ data interpretation and drafting of the manuscript. NP and BF data collection. MS and KJ supply of LACT product and LACT product quality control. TO study design, data interpretation, and drafting of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.584436/full#supplementary-material

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Quantitative Analysis of Colostrum Bacteriology on British Dairy Farms

Robert M. Hyde, Martin J. Green, Chris Hudson and Peter M. Down*

School of Veterinary Medicine and Science, University of Nottingham, Leicestershire, United Kingdom

Total bacterial counts (TBC) and coliform counts (CC) were estimated for 328 colostrum samples from 56 British dairy farms. Samples collected directly from cows' teats had lower mean TBC (32,079) and CC (21) than those collected from both colostrum collection buckets (TBC: 327,879, CC: 13,294) and feeding equipment (TBC: 439,438, CC: 17,859). Mixed effects models were built using an automated backwards stepwise process in conjunction with repeated bootstrap sampling to provide robust estimates of both effect size and 95% bootstrap confidence intervals (BCI) as well as an estimate of the reproducibility of a variable effect within a target population (stability). Colostrum collected using parlor (2.06 log cfu/ml, 95% BCI: 0.35–3.71) or robot (3.38 log cfu/ml, 95% BCI: 1.29–5.80) milking systems, and samples collected from feeding equipment (2.36 log cfu/ml, 95% BCI: 0.77–5.45) were associated with higher TBC than those collected from the teat, suggesting interventions to reduce bacterial contamination should focus on the hygiene of collection and feeding equipment. The use of hot water to clean feeding equipment (−2.54 log cfu/ml, 95% BCI: −3.76 to −1.74) was associated with reductions in TBC, and the use of peracetic acid (−2.04 log cfu/ml, 95% BCI: −3.49 to −0.56) or hypochlorite (−1.60 log cfu/ml, 95% BCI: −3.01 to 0.27) to clean collection equipment was associated with reductions in TBC compared with water. Cleaning collection equipment less frequently than every use (1.75 log cfu/ml, 95% BCI: 1.30–2.49) was associated with increased TBC, the use of pre-milking teat disinfection prior to colostrum collection (−1.85 log cfu/ml, 95% BCI: −3.39 to 2.23) and the pasteurization of colostrum (−3.79 log cfu/ml, 95% BCI: −5.87 to −2.93) were associated with reduced TBC. Colostrum collection protocols should include the cleaning of colostrum collection and feeding equipment after every use with hot water as opposed to cold water, and hypochlorite or peracetic acid as opposed to water or parlor wash. Cows' teats should be prepared with a pre-milking teat disinfectant and wiped with a clean, dry paper towel prior to colostrum collection, and colostrum should be pasteurized where possible.

Keywords: cattle, dairy, colostrum, bacteriology, bootstrap

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Vinicius Machado,
Texas Tech University, United States

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John Z. Alawneh,
The University of Queensland, Australia
Patrick Mester,
University of Veterinary Medicine Vienna, Austria

****Correspondence:***
Robert M. Hyde
robert.hyde1@nottingham.ac.uk

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INTRODUCTION

Bovine neonates are born agammaglobulinemic (1) and consequently must acquire immunity via the ingestion of appropriate quantities of high quality colostrum within the first few hours of life (2). A failure of passive immunity transfer (FPT) in dairy calves has been associated with an increased risk of preweaning morbidity and mortality (3, 4), as well as longer term effects such as increased age at first calving and reduced milk production (5). A recent meta-analysis concluded that calves

experiencing FPT are 1.5 times more likely to be treated for diarrhea, 1.8 times as likely to be treated for respiratory disease and two times as likely to die (6). Despite the clear negative implications, recent research suggests FPT is common in UK dairy calves, with around 26% experiencing FPT as measured by total protein (TP) < 5.6 g/dl (7).

Failure of passive immunity transfer has been associated with suboptimal colostrum feeding volume, timing, quality and also microbiological hygiene (2). Microbiological contamination of colostrum can not only represent a significant risk for FPT through reduced efficiency of immunoglobulin absorption (8), but also act as a vehicle for the transmission of pathogenic organisms to the neonatal calf (9). Higher bacterial levels have been hypothesized to reduce immunoglobulin (Ig) absorption by the binding and neutralizing of Ig by bacteria, pathogenic bacteria damaging intestinal epithelial cells and reducing permeability to Ig, and nonspecific pinocytosis of bacteria blocking the absorption of Ig molecules (10). In addition to effects on FPT and disease early in life, colostrum hygiene has also been identified as a critical control point in the prevention of paratuberculosis (11).

Given the importance of colostrum bacterial levels, several researchers have attempted to provide benchmarking estimates of contamination levels at a national level. Previous thresholds for classification of bacterial contamination have been suggested at >100,000 colony-forming units (cfu) per ml and >10,000 cfu/ml for total bacterial counts (TBC) and coliform counts (CC), respectively (12). North American studies have reported 36% of samples exceeding TBC thresholds in Canada (13), and 85% in the US (12), with later studies of 67 farms in 12 states finding 43 and 17% samples exceeding TBC and CC thresholds, respectively (14). Only 18% of 255 samples from 44 Columbian herds were found to fail on TBC (15). Of 268 NZ samples taken of pooled colostrum, 91 and 91% failed for TBC and CC, respectively (16). Of 221 Australian colostrum samples, 42 and 28% of samples failed on TBC and CC, respectively, with only 20% meeting both standards for immunoglobulin (>50 g/L) and microbiological quality (17), reinforcing previous Australian studies which found 42 and 6% of samples exceeded TBC and CC, respectively, with only 23% meeting all standards for TBC, CC and immunoglobulin levels (18). Previous studies examining colostrum bacteriological levels in Irish dairy herds reported 57% of 214 samples exceeding TBC and 33% exceeding CC thresholds, with significant variation between farms (9), however, there have been no published studies on colostrum hygiene levels on GB dairy farms to date. In addition to a paucity of information around current GB colostrum bacterial levels, there is also a lack of knowledge around specific factors that may influence the bacterial contamination of colostrum (18).

The source of bacteria present in colostrum includes the mammary gland itself as well as contamination or proliferation during harvesting, storage or feeding (12, 13, 19). Previous research has shown that colostrum contamination is generally extremely low or zero when collected directly from the gland (mean log TBC: 1.44 cfu/ml), with significant bacterial contamination occurring during the harvest process (mean log TBC: 4.99 cfu/ml), suggesting steps to prevent colostrum contamination should focus largely on collection methods (19). Storage method also has an effect on bacterial levels, with colostrum stored at warmer conditions (22 degrees C) having >42 times more bacteria present and resulting in a serum IgG concentration almost twice as low compared with colostrum either pasteurized, untreated or stored at 4°C for 2 d (20), and bacterial levels have been shown to be significantly reduced when freeze-thawing colostrum (21). Irrespective of the source of colostrum contamination, it has been found that heat treatment is associated with reduced bacterial levels, improved health status and decreased mortality, even when receiving appropriate colostrum volume (22). This reinforces previous findings that calves fed heat-treated colostrum have significantly higher serum IgG concentrations, and a significantly lower risk of diarrhea than those fed fresh colostrum (10), suggesting colostrum pasteurization may be an effective method of reducing colostrum contamination levels. Whilst colostrum pasteurization is likely to be effective at reducing colostrum bacterial levels, it is unknown how many GB farms currently pasteurize colostrum.

Whilst there are many farm level factors associated with colostrum contamination levels, it is likely that protocols aiming to prevent colostrum contamination are likely to vary between farms (9), and it is important that veterinary advisors are able to recommend interventions that are relevant to the majority of farms. The relative importance of management factors is essential for optimal decision making on-farm (23). Bootstrapping allows for the estimation of robust coefficients (24) and estimates of variable stability: an estimate of the reproducibility of a variable effect within a target population. The use of bootstrapping alongside regression techniques has been utilized in studies identifying a relatively small number of variables having a large and consistent effect on animal health outcomes (23), and the identification of these variables can provide a succinct number of practical recommendations for veterinarians.

This study aims to provide a current benchmark of colostrum bacterial levels, investigate factors associated with increased levels of bacterial contamination of colostrum on GB dairy farms and provide practical recommendations for a small number of factors found to have the largest effect on colostrum hygiene on the largest number of farms.

MATERIALS AND METHODS

Farm Selection

Dairy farms were selected at random from a list of suppliers to a large supermarket consisting of dairy farms in England, Scotland and Wales. Randomization was performed using the sample_n() function from the tidyverse package (25) in R statistical software (26). 120 farms were initially selected and were sent an initial information letter, followed up with a telephone call to recruit farms until 60 were recruited as part of a wider calf research project. Recruited farms were visited by one researcher between 17th December 2018 - 14th February 2019 and provided with a colostrum collection kit (Quality Milk Management Services, Wells UK). One farmer on each farm was trained by the researcher on the collection and posting of colostrum samples. To replace farms leaving the wider calf research project an additional

31 farms were enrolled by the same methodology and were visited by the same researcher between 6th December 2019 and 29th January 2020 and were also provided with colostrum collection kits and training on collection protocols.

Colostrum Collection Protocol

Farmers were encouraged to take six colostrum samples from as close to the calf feeding point as possible, for example farms bottle-feeding colostrum to all calves should take samples from the bottle teat. Where colostrum feeding involved multiple sources, farmers were asked to collect samples from all sources, for example farms where calves would suckle colostrum from the dam in most cases and receive supplementary colostrum by esophageal tube feeding when necessary should take some samples from the cows' teat, and some from the esophageal tube. Farmers were asked to freeze samples as soon as possible after collection, with all six samples being collected within 1 month of each other. Sample pots contained glycerol as a cryopreservative. After collecting 6 samples, farmers were asked to place the samples in a pre-packaged insulated box containing ice packs provided to the farmer. These boxes were couriered to the laboratory (Quality Milk Management Services, Wells UK) for analysis.

Microbiological Analysis

Samples were analyzed using standard laboratory methods for milk (27) as previously described (28). Ten microlitres of secretion was inoculated onto sheep blood agar and Edwards agar and 100 μl was inoculated onto MacConkey agar. Total bacterial count and coliform counts were estimated after incubation for 72 h at 30 degrees C and 37 degrees C, respectively.

Colostrum Collection Questionnaire

A submission form was submitted alongside colostrum samples including the cow id, date of sampling and sample collection location. Upon receiving the colostrum samples, farmers were contacted via telephone and asked about colostrum collection and equipment cleaning protocols used to collect the colostrum samples. Where samples were taken directly from the cows' teat, the method used to clean collection buckets or feeding equipment was deemed irrelevant, and a "Not applicable" factor level was created. Similarly, where samples collected from the collection bucket but before using any feeding equipment, the methods used to clean feeding equipment was deemed irrelevant and a "Not applicable" factor level was created. The percentage of calves receiving manually fed colostrum feeds (feeds by bottle or tube as opposed to suckling from the dam) and the volume of first feeds was also recorded.

Descriptive Analysis

All data analysis was conducted in R (26). The percentage of colostrum samples failing in terms of TBC and CC was calculated for each farm, with a "failure" being when >100,000 for TBC and >10,000 for CC as previously suggested by McGuirk and Collins (12).

Statistical Analysis

Both TBC and coliform counts were natural log transformed after the addition of 1 to all counts. Samples with missing data were removed from the dataset. Continuous variables were scaled (divided by one standard deviation) and centered prior to modeling using the preProcess function within the caret package (29). Categorical variables for cleaning frequency were releveled to include as few relevant categories as possible; when cleaning frequency was recorded as "Less than daily" or "Daily" these were recategorised to "Less than each use".

For model building, a bootstrap sample was taken from the dataset (sampling with replacement to create a sample of the same size as the original dataset). A mixed model was created from the bootstrapped data sample using the lmer function from the lmerTest package (30) with log TBC or log CC as model outcomes in respective models. Farm was included as a random effect, and all other colostrum management variables were included as fixed effects as shown in **Table 1**. The following model equation was used for the mixed model:

$$Y_{ij} = \mu + \beta_1 X_{1ij} + \beta_2 X_{2ij} \ldots + U_j + \epsilon$$

Where Y_{ij} is the log TBC or CC of the ith sample on the jth farm. X_{1ij} represents covariates at the sample farm level, with corresponding coefficients represented by β_1, and X_{2ij} representing covariates at the sample farm level, with corresponding coefficients represented by β_2. μ represents the intercept, β represents explanatory variables, U_j as the farm specific random effect for the jth farm, and ϵ as the random error. The assumed distributions of U and ϵ are normal, with mean zero and variance θ U and θ ϵ respectively.

An automated backwards stepwise selection process based on Akaike information criterion was used using the step function from the lmerTest package (30) to create a final mixed effects model for a given bootstrap sample, and variables from the final model were recorded alongside coefficient values. This process was then repeated 1,000 times, recording the presence of variables and their corresponding effect size in each iteration. Residuals were checked to ensure near normal distribution after building an automated backwards stepwise mixed effects model on the full dataset (i.e., without bootstrap sampling). Cross-validation (10-fold, repeated 10 times) was use for the full model and both internal and cross-validated R2 and MAE were assessed to ensure the model was not overfit. Interactions between significant predictors in the full model were checked and were included if $p < 0.05$.

Variable stability was calculated as the percentage of bootstrap models in which a given variable was selected. Mean coefficient values and 95% bootstrap confidence intervals (BCI) were calculated from coefficient values across all bootstrap samples in which a variable was selected. An estimate of significance as a "bootstrap p-value" was calculated as one minus the proportion of coefficient estimates on the majority side of zero (proportion below zero if the mean coefficient was above zero, and proportion above zero if mean coefficient was above zero). Variables with a bootstrap stability >10% and a bootstrap p-value < 0.025

TABLE 1 | Colostrum management variables and factor levels available as fixed effects for model building.

Variable	Levels (% of samples)
Sample collection point	Cows teat (17.8%), Colostrum collection bucket (36.3%), Feeding teat (24.4%), Esophageal tube (21.6%)
Number of days between calving pen clean out	Numeric
Pre-milking teat disinfection used	No (17.4%), Yes (82.6%)
Teat dry wiped prior to colostrum collection	No (17.7%), Yes (82.3%)
Milking system	Parlor (67.0%), Robot (15.2%), Not applicable (17.8%)
Frequency of colostrum collection equipment cleaning	Each use (21.0%), Less than each use (61.2%), Not applicable (17.8%)
Method of colostrum collection equipment cleaning	Water (24.1%), Hypochlorite (16.5%), Parlor wash (28.7%), Peracetic acid (9.5%), Soap (3.7%), Not applicable (17.8%)
Hot water used to clean collection equipment	No (38.1%), Yes (44.2%), Not applicable (17.8%)
Frequency of colostrum feeding equipment cleaning	Each calf (31.1%), Less than each calf (14.9%), Not applicable (54.0%)
Method of colostrum feeding equipment cleaning	Water (10.4%), Hypochlorite (13.7%), Parlor wash (7.3%), Peracetic acid (7.3%), Soap (7.3%), Not applicable (54.0%)
Hot water used to clean feeding equipment	No (14.9%), Yes (31.1%), Not applicable (54.0%)
Colostrum frozen prior to sample collection	No (78.0%), Yes (4.3%), Not applicable (17.8%)
Colostrum pasteuriser used	No (78.9%), Yes (3.4%), Not applicable (17.8%)

were deemed to be both relatively stable and have reasonable effect size.

RESULTS

Descriptive Analysis

A total of 356 samples were returned from 59 farms. Fifteen samples from six farms were either missing or damaged in transit and were removed from the dataset. Thirteen samples from three farms were removed due to missing or incomplete data on sample collection. Thirty-two farms failed to return any samples, with 19 farms failing to return samples from the first round of data collection, and 13 farms failing to return samples during the second round of data collection. The final dataset consisted of 328 samples from 56 farms. One hundred and fifty-one samples were collected from feeding equipment, with 80 (53.0%) being collected through a feeding teat and 71 (47.0%) being collected through an esophageal feeding tube. One hundred and nineteen samples were collected from a collection bucket and 58 samples were collected directly from the cow's teat. Pre-milking teat disinfection was used prior to colostrum collection for 271 (82.6%) samples compared with 57 (17.4%) samples where no pre-milking disinfection was used. Dry wiping of teats prior to colostrum collection was conducted for 270 (82.3%) samples compared with 58 (17.7%) with no dry wiping of teats. The frequency of the cleaning out of calving pens was between 3.5 and 90 d, with a mean and median of 27.9 and 28 d, respectively. Farmers reported manually feeding colostrum (by bottle or tube as opposed to suckling from the dam) to between 0 and 100% of calves, with a mean and median of 79.2 and 100%, respectively. Colostrum volume fed by farmers at first feed ranged from 2 to 5 L at first feed, with a mean and median of 3.1 and 3.0 L, respectively.

Of the 270 samples collected using milking equipment (i.e., excluding the 58 samples collected directly from the teat), 220 (81.5%) were collected through a milking parlor, and 50 (18.5%) through a robotic milking unit. Only 69 samples (25.6%) were submitted from farms where collection equipment (i.e., collection bucket) was cleaned after each use, with 201 (74.4%) being collected from farms where collection equipment was cleaned less frequently than after each use. Seventy-nine samples (29.2%) were collected from farms using water to clean collection equipment, 94 (34.8%) using parlor washings, 54 (20.0%) from farms using hypochlorite, 31 (11.5%) using peracetic acid, and 12 (4.4%) using soap. One hundred and forty-five samples (53.7%) were collected from farms using hot water to clean collection equipment, compared with 125 (46.3%) from farms that did not use hot water. Farms that used a colostrum pasteuriser accounted for 11 samples (4.1%), compared with 259 samples (95.9%) where a pasteuriser was not used. Colostrum was frozen prior to sample collection for 14 samples (5.2%) compared with 256 samples (94.8%) which were collected without prior freezing.

Of the 151 samples collected directly from feeding equipment (i.e., excluding the 58 samples collected directly from the teat and the 119 samples collected from the collection bucket) 102 samples (67.5%) were collected from farms where feeding equipment was cleaned every time it was used, and 49 samples (32.4%) when feeding equipment was cleaned less than each use. Thirty-four samples (22.5%) were collected from farms using water alone to clean feeding equipment, 24 (15.9%) using parlor washings, 45 (29.8%) from farms using hypochlorite, 24 (15.9%) using peracetic acid, and 24 (15.9%) from farms using soap. One hundred and two samples (67.5%) were from farms that used hot water to clean feeding equipment, compared with 49 (32.4%) from farms that did not use hot water.

Mean TBC and CC were 326,931 and 13,034 cfu/ml with median values of 14,800 and 1 cfu/ml, respectively. Ninety-seven (29.6%) samples had TBC results above threshold (>100,000 cfu/ml) and 25 samples (7.6%) had coliform counts above threshold (>10,000 cfu/ml).

Mean and median TBC were lower when samples were collected directly from the cow's teat, at 32,079 and 535, respectively, with only 6.9% of samples being above threshold, compared with collection from a collection bucket (mean 327,879, median 44,000, 35.3% above threshold) or feeding equipment (mean 439,438, median 18,100, 33.8% above threshold). Coliform counts were also lower when samples were collected from the cow's teat (mean 21, median 0, 0.0% above threshold) compared with samples taken from the collection bucket (mean 13,294, median 2, 7.6% above threshold) or feeding

equipment (mean 17,859, median 1, 10.6% above threshold). A higher number of samples collected directly from the cow's teat had zero coliforms present, with 43 samples (74.1%) having zero coliforms when collected directly from the cow's teat compared with 48 samples (40.3%) taken from the collection bucket and 62 samples (41.0%) when collected from feeding equipment. In contrast, only one sample collected directly from a cows' teat, and zero samples collected from collection or feeding equipment had a zero TBC. A lower proportion of samples collected from cows' teats (6.9%) were above threshold for either TBC or CC than those collected from collection buckets (37.0%) or feeding equipment (34.4%).

Statistical Analysis

No interactions were detected between significant predictors in the full (non-bootstrapped) model, and analysis of cross-validated and internal R2 and MAE suggested the model was not overfit. Predictor variables were assessed to evaluate correlations; since all correlations were <0.36 and cross validation provided no indication of over fitting, the full model was deemed to provide safe parameter estimates.

Thirteen variables were available for predicting both colostrum TBC and CC. After model building using automated backwards stepwise regression and bootstrap resampling, final models resulting in eight and seven variables being selected for TBC and CC respectively.

Total Bacterial Counts

The use of a milking machine was associated with an increase in TBC compared with those collected directly from the cows' teat with a stability of 92.5% being associated with a 2.06 log cfu/ml (95% BCI: 0.35–3.71) increase when collected through a parlor, and a 3.38 log cfu/ml (95% BCI: 1.29–5.80) increase when collected through a robot. Sample collection point was also associated with increased TBC, with a stability of 87.3%, being associated with a 2.36 log cfu/ml (95% BCI: 0.77–5.45) increase when collected from feeding equipment compared with samples collected directly from cows' teats.

The use of hot water to clean feeding equipment was associated with reduced TBC with a stability of 85.9%, being associated with a −2.54 log cfu/ml (95% BCI: −3.76 to −1.74) reduction when hot water was used. The method of cleaning colostrum collection buckets was associated with TBC with a stability of 29.1%. Compared with water, cleaning colostrum collection buckets with peracetic acid was associated with a −2.04 log cfu/ml (95% BCI: −3.49 to −0.56) reduction in TBC, and a tendency toward reduced TBC was identified when cleaning with hypochlorite (−1.60 log cfu/ml, 95% BCI: −3.01 to 0.27) and soap (−1.14 log cfu/ml, 95% BCI: −3.01 to 0.27). No difference was detected when cleaning with parlor wash (0.47 log/cfu/ml, 95% BCI: −0.76 to 1.89) compared with water. The frequency of colostrum collection equipment cleaning was associated with TBC with a stability of 22.1%, and a 1.75 log cfu/ml (95% BCI: 1.30–2.49) increase when collection equipment was cleaned less than every time it was used. The wiping of teats prior to colostrum collection was associated with a reduction in TBC with a stability of 23.3% and a −1.97 log cfu/ml (95% BCI: −2.85 to −1.45) reduction in TBC. The use of a colostrum pasteuriser was associated with TBC with a stability of 10.9% and a −3.79 log cfu/ml (95% BCI: −5.87 to −2.93) reduction in TBC.

Variables with >10% stability and <0.025 bootstrap *p*-value are depicted in **Figure 1**, with stability estimates of variables being presented in **Figure 2**. Coefficients, 95% BCI and stability estimates for all model variables are presented in **Table 2**.

Coliform Counts

The use of hot water to clean both feeding equipment and collection equipment was associated with CC, with stabilities of 51.9 and 32%, respectively. The use of hot water to clean feeding equipment and collection equipment was associated with a −2.72 log cfu/ml (95% BCI: −4.01 to −1.82) and −1.72 log cfu/ml (95% BCI: −2.35 to −1.26) reduction in CC, respectively. The sample collection point was associated with CC with stabilities of 45.6 and 45% when samples were collected from feeding equipment or collection equipment compared with directly from the cows' teat. Collection of samples from feeding equipment was associated with a 3.40 log cfu/ml increase in CC (95% BCI: 1.26–5.59), and a tendency for increased CC was identified when samples were collected from collection equipment (1.49 log cfu/ml, 95% BCI: −0.28 to 3.03) compared with samples collected directly from the cows' teat.

The method of colostrum collection equipment cleaning was associated with CC with a stability of 19.9%. Compared with using water, the use of peracetic acid was associated with a −1.66 log cfu/ml (95% BCI: −2.73 to −0.54) reduction in CC and the use of parlor wash was associated with a 1.28 log cfu/ml (95% BCI: 0.05–2.46) increase in CC. Hypochlorite tended to decrease CC (−0.64 log cfu/ml, 95% BCI: −2.29 to 0.75) and no difference was found when soap was used (0.03 log cfu/ml, 95% BCI: −2.41 to 3.06). The cleaning of colostrum collection equipment less frequently than every use was associated with a 1.68 log cfu/ml (95% BCI: 1.19–2.18, stability 17.9%) increase in CC, and the wiping of teats prior to colostrum collection was associated with a −2.33 log cfu/ml (95% BCI: −3.46 to −1.53, stability 11.4%) reduction in CC.

Variables with >10% stability and <0.025 bootstrap *p*-value are depicted in **Figure 3**, with stability estimates of variables being presented in **Figure 4**. Coefficients, 95% BCI and stability estimates for all model variables are presented in **Table 3**.

DISCUSSION

This is the first study to report TBC and CC in colostrum samples from GB dairy farms and provides and initial benchmark of colostrum hygiene. Samples collected directly from cows' teats had relatively low levels of bacterial contamination with only 6.9% of samples being above threshold for either TBC or CC, compared with 37.0 and 34.4% of samples collected from collection and feeding equipment, respectively. This suggests that bacterial contamination is not likely to originate from the cow, and rather from the milking machine, collection buckets and feeding equipment as has been previously suggested (19).

Several variables were identified as having both relatively high stability and having a relatively large effect size in reducing

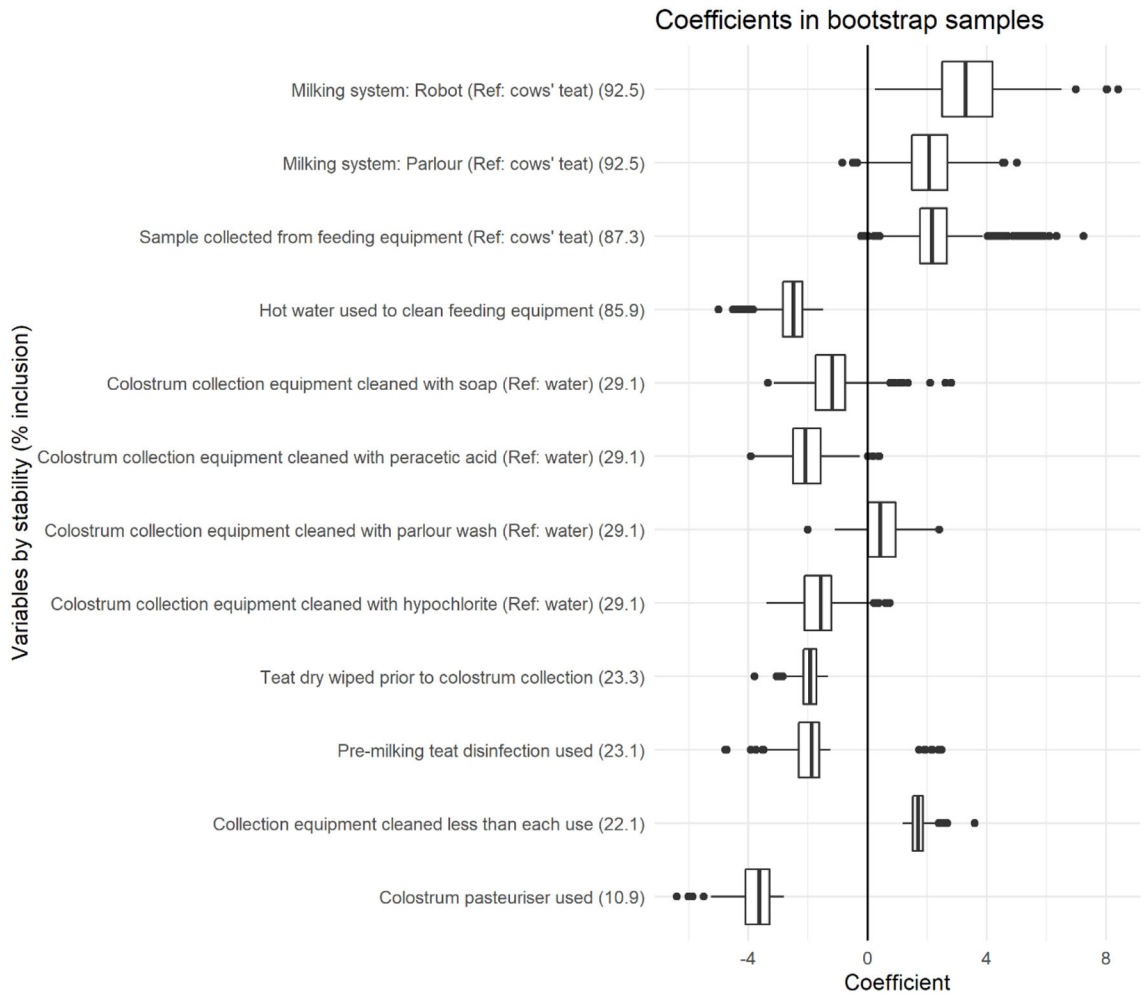

FIGURE 1 | Coefficient distributions and variable stability for variables selected in at least 10% of models across 1,000 bootstrapped samples. Coefficient estimates represent the change in total bacterial count (log cfu/ml), and variable stability is presented within brackets for each variable.

TBC and/or CC in colostrum. The use of a milking machine to harvest colostrum was associated with increased bacterial counts, and samples from both collection and feeding equipment were associated with higher bacterial counts compared with those collected directly from the cows' teat. This large and consistent effect size for both TBC and CC suggests interventions should be targeted primarily at equipment hygiene protocols. The use of hot water was found to have a relatively large effect on bacterial counts, with samples collected from both collection equipment and feeding equipment cleaned with hot water being associated with significantly lower bacterial counts than those where cold water was used. In addition to a large effect size, these were relatively stable variables, suggesting that interventions focused on these variables would have a substantial effect on a large number of farms. As hot water was often not used to clean equipment for collection (46.3% of samples) or feeding (32.4% of samples) equipment, this represents an easy intervention for veterinarians to target on a large number of farms that could have a substantial and immediate impact on colostrum hygiene for GB dairy farms.

Disinfection of collection equipment with either hypochlorite or peracetic acid was found to have a relatively large effect size and high stability for reducing both TBC and CC. Only 31.5% of samples were collected from farms using hypochlorite of peracetic acid to clean colostrum collection equipment, with the remaining farms predominantly using either parlor washings or water. Given the large number of farms following ineffective disinfection protocols this again represents a relatively straightforward intervention to target on the majority of farms. The use of a colostrum pasteuriser was consistently associated with reduced TBC and CC, with a large effect size relative to other variables. The stability however was relatively low, at 10.9 and 7.0% for TBC and CC, respectively, suggesting that whilst colostrum pasteurization is likely to have a large effect size in reducing TBC and CC in a small number of cases, it does not seem to have a significant effect in many of the bootstrap samples taken from the original dataset. This is

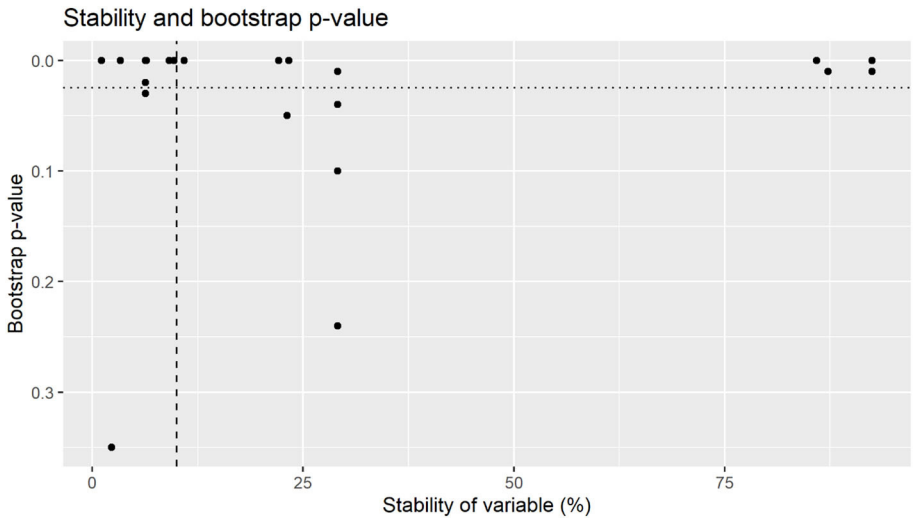

FIGURE 2 | Bootstrap *p*-value by stability of variables for total bacterial counts. Variables were selected for final model were above 10% stability (dashed line) with a bootstrap *p*-value of <0.025 (dotted line).

TABLE 2 | Stability, mean coefficient (log cfu/ml), 95% bootstrap confidence intervals and bootstrap *p*-value for all variables associated with total bacterial count.

Variable	N	Stability (%)	Mean coefficient	95% bootstrap confidence interval	Bootstrap *P*-value
Milking system: parlor (ref: cows' teat)	220	92.5	2.06	(0.35 to 3.71)	0.01
Milking system: robot (ref: cows' teat)	50	92.5	3.38	(1.29 to 5.80)	<0.01
Sample collected from feeding equipment (ref: cows' teat, $n = 58$)	151	87.3	2.36	(0.77 to 5.45)	0.01
Hot water used to clean feeding equipment	102	85.9	−2.54	(−3.76 to −1.74)	<0.01
Colostrum collection equipment cleaned with hypochlorite (ref: water)	54	29.1	−1.60	(−3.01 to 0.27)	0.04
Colostrum collection equipment cleaned with parlor wash (ref: water)	94	29.1	0.47	(−0.76 to 1.89)	0.24
Colostrum collection equipment cleaned with peracetic acid (ref: water)	31	29.1	−2.04	(−3.49 to −0.56)	0.01
Colostrum collection equipment cleaned with soap (Ref: water)	12	29.1	−1.14	(−2.55 to 1.13)	0.10
Teat dry wiped prior to colostrum collection	270	23.3	−1.97	(−2.85 to −1.45)	<0.01
Pre-milking teat disinfection used	271	23.1	−1.85	(−3.39 to 2.23)	0.05
Collection equipment cleaned less than each use	201	22.1	1.75	(1.30 to 2.49)	<0.01
Colostrum pasteuriser used	11	10.9	−3.79	(−5.87 to −2.93)	<0.01
Feeding equipment cleaned less than each calf	49	9.7	−2.13	(−2.98 to −1.63)	<0.01
Hot water used to clean collection equipment	145	9.1	−1.60	(−2.17 to −1.16)	<0.01
Sample collected from collection equipment (ref: cows' teat)	119	6.4	3.51	(2.36 to 4.38)	<0.01
Colostrum feeding equipment cleaned with hypochlorite (ref: water)	45	6.3	3.23	(0.25 to 5.29)	0.03
Colostrum feeding equipment cleaned with parlor wash (ref: water)	24	6.3	2.66	(0.96 to 4.47)	<0.01
Colostrum feeding equipment cleaned with peracetic acid (ref: water)	24	6.3	3.84	(1.34 to 5.75)	0.02
Colostrum feeding equipment cleaned with soap (ref: water)	24	6.3	2.29	(0.09 to 3.83)	0.03
Number of days between calving pen clean out	328	3.3	0.65	(0.54 to 0.84)	<0.01
Colostrum frozen prior to sample collection	14	2.3	0.75	(−2.53 to 3.34)	0.35
Colostrum collection equipment cleaning: not applicable	58	1.1	−4.03	(−4.64 to −3.48)	<0.01

N represents the number of samples where variable was "positive."

likely due to the infrequent use of pasteurization equipment in this sample, with only 4.1% of samples collected from farms using a pasteuriser and therefore many bootstrap samples will not contain any samples collected after pasteurization. Whilst colostrum pasteurization appears to have a relatively large effect size in reducing TBC and CC as described previously (8, 10), the relative scarcity of colostrum pasteurization and the requirement for initial financial investment may make this a more challenging intervention for veterinarians to implement on a large number of farms.

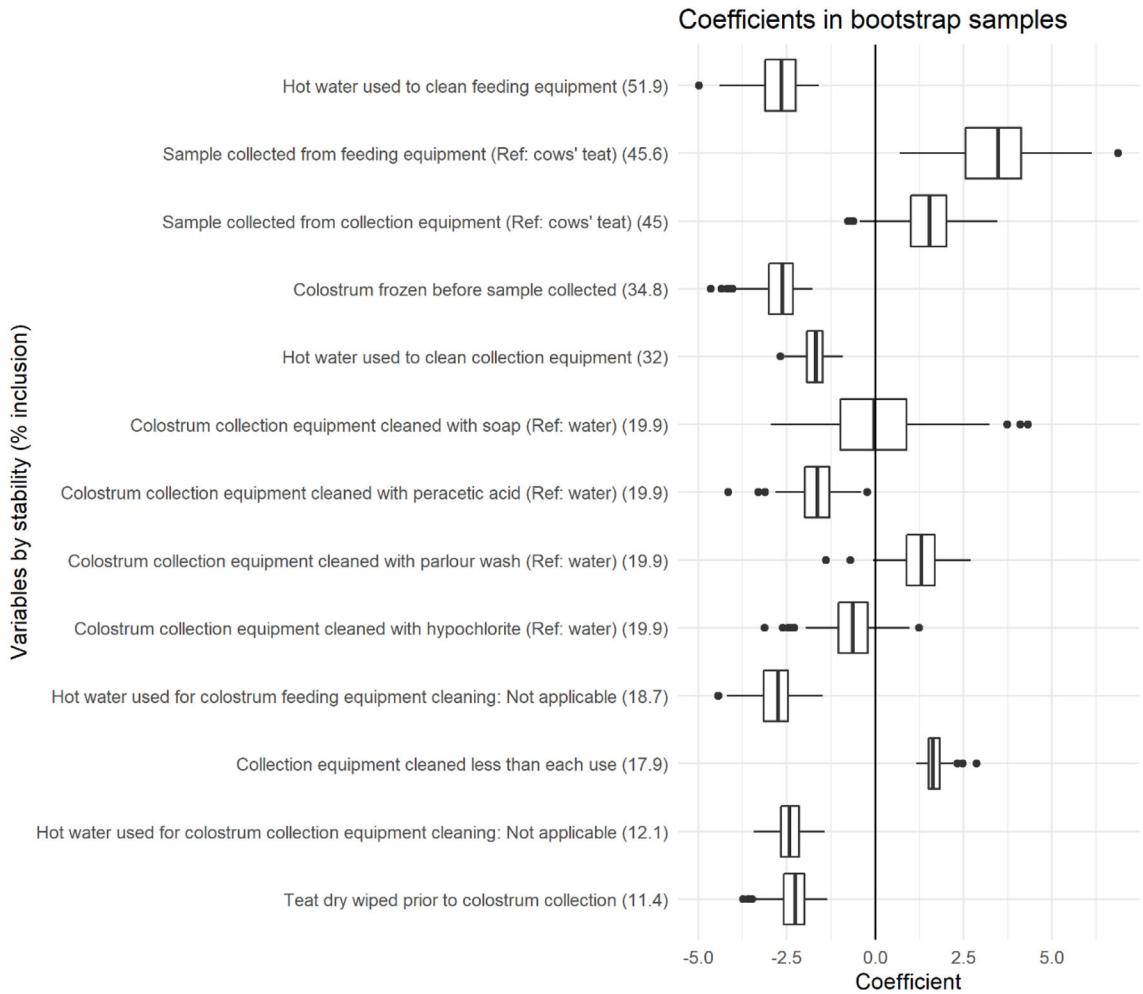

FIGURE 3 | Coefficient distributions and variable stability for variables selected in at least 10% of models across 1,000 bootstrapped samples. Coefficient estimates represent the change in coliform count (log cfu/ml), and variable stability is presented within brackets for each variable.

Based on the results from this trial, practical recommendations for veterinary intervention should focus on the effective cleaning of colostrum collection and feeding equipment after every use with hot water as opposed to cold water, and hypochlorite or peracetic acid as opposed to water or parlor wash. Cows' teats should be prepared with a pre-milking teat disinfectant and wiped with a clean, dry paper towel prior to colostrum collection, and colostrum should be pasteurized where possible. Only 23 samples in the current dataset were collected from farms following the optimal collection practices identified in this study (cleaning both collection and feeding equipment each time they were used with hot hypochlorite or peracetic acid and using a pre-milking teat disinfection and dry wipe prior to collection, but excluding pasteurization). By following these simple recommendations, it is likely that significant reductions in both TBC and CC will be achieved, although these results should be validated in a randomized controlled trial in future research.

Whilst some factors might have a large impact on colostrum hygiene (relatively large effect size), this might only be applicable to a small number of farms (low stability). Similarly, some factors are applicable to a large number of farms (high stability) however only have a small impact on colostrum hygiene [relatively small effect size. The recommendations from this trial are not intended to provide an exhaustive list of all factors affecting colostrum hygiene, rather identify a small number of practically implementable interventions that had the largest effect size on the majority of farms. The stability thresholds applied to select influential variables aimed to identify the most stable variables with the largest effect size (31) as shown in **Figures 2**, **4**. Whilst there are several key variables identified in this research, it is likely that there are other variables that also impact colostrum bacterial levels to some degree Whilst the bootstrapped regression methods utilized in this research have identified several variables with both high stability and relatively large effect sizes, it is possible that variables with an effect on colostrum bacteriology levels may have remained undetected due to sample size constraints. An a priori sample size calculation was not performed, and in the absence of prior literature to

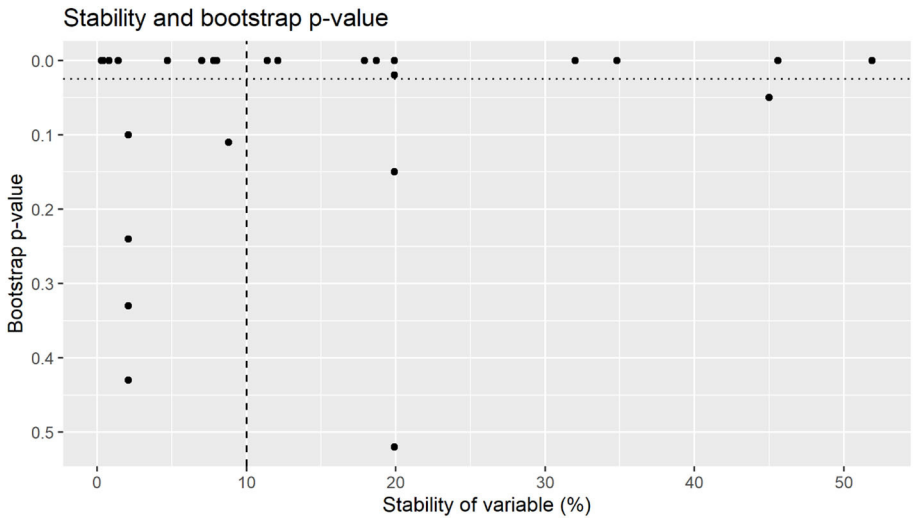

FIGURE 4 | Bootstrap *p*-value by stability of variables for coliform counts. Variables were selected for final model were above 10% stability (dashed line) with a bootstrap *p*-value of <0.025 (dotted line).

TABLE 3 | Stability, mean coefficient (log cfu/ml), 95% bootstrap confidence intervals and bootstrap *p*-value for all variables associated with coliform count.

Variable	*N*	Stability	Mean coefficient	95% bootstrap confidence interval	Bootstrap *P*-value
Hot water used to clean feeding equipment	102	51.9	−2.72	(−4.01 to −1.82)	<0.01
Sample collected from feeding equipment (ref: cows' teat)	151	45.6	3.40	(1.26 to 5.59)	<0.01
Sample collected from collection equipment (ref: cows' teat)	119	45	1.49	(−0.28 to 3.03)	0.05
Colostrum frozen prior to sample collection	14	34.8	−2.70	(−3.93 to −1.92)	<0.01
Hot water used to clean collection equipment	145	32	−1.72	(−2.35 to −1.26)	<0.01
Colostrum collection equipment cleaned with hypochlorite (ref: water)	54	19.9	−0.64	(−2.29 to 0.75)	0.15
Colostrum collection equipment cleaned with parlor wash (ref: water)	94	19.9	1.28	(0.05 to 2.46)	0.02
Colostrum collection equipment cleaned with peracetic acid (ref: water)	31	19.9	−1.66	(−2.73 to −0.54)	<0.01
Colostrum collection equipment cleaned with soap (ref: water)	12	19.9	0.03	(−2.41 to 3.06)	0.52
Hot water used for colostrum feeding equipment cleaning: not applicable	177	18.7	−2.82	(−4.15 to −2.00)	<0.01
Collection equipment cleaned less than each use	201	17.9	1.68	(1.19 to 2.18)	<0.01
Hot water used for colostrum collection equipment cleaning: not applicable	58	12.1	−2.40	(−3.32 to −1.64)	<0.01
Teat dry wiped prior to colostrum collection	270	11.4	−2.33	(−3.46 to −1.53)	<0.01
Colostrum frozen prior to sample collection: not applicable	58	9.6	−0.63	(−1.90 to 0.13)	0.10
Pre-milking teat disinfection used	271	8	1.99	(1.46 to 2.70)	<0.01
Colostrum collection equipment cleaning: not applicable	58	7.8	−1.89	(−2.82 to −0.84)	<0.01
Colostrum pasteuriser used	11	7	−3.89	(−5.36 to −3.13)	<0.01
Collection equipment cleaned less than each calf	201	4.7	−2.13	(−2.57 to −1.73)	<0.01
Colostrum feeding equipment cleaned with hypochlorite (ref: water)	45	2.1	−0.78	(−4.47 to 1.99)	0.33
Colostrum feeding equipment cleaned with parlor wash (ref: water)	24	2.1	−0.17	(−3.96 to 3.49)	0.43
Colostrum feeding equipment cleaned with peracetic acid (ref: water)	24	2.1	−0.76	(−3.85 to 0.57)	0.24
Colostrum feeding equipment cleaned with soap (ref: water)	24	2.1	3.13	(−2.72 to 5.37)	0.10
Milking system: parlor (ref: cows' teat)	220	1.4	1.75	(0.57 to 3.23)	<0.01
Milking system: robot (ref: cows' teat)	50	1.4	2.18	(0.40 to 3.97)	<0.01
Number of days between calving pen clean out	328	0.4	0.68	(0.65 to 0.74)	<0.01

N represents the number of samples where variable was "positive."

base a sampling number six samples from each farm was chosen to establish a representative set of samples for each farm given financial constraints. Whilst the use of bootstrapped mixed effects models means a conventional sample size calculation is unlikely to be appropriate, the standard deviation for TBC from the current research (log 3.3 cfu/ml) and 328 samples, a conventional sample size calculation indicate an 80% chance of detecting a log 1.0 cfu/ml change in TBC. Whilst this method of sample size calculation would not be appropriate when using bootstrapped mixed effects models, it suggests that variables with relatively small effect sizes might only be detected if a larger sample size was available.

Whilst TBC and CC are highly correlated, it has been suggested that CC might be a better predictor of disease. Whilst a threshold of CC < 10,000 cfu/ml is a reasonable threshold to aim for, the negative linear relationship between CC and serum IgG suggests there is no optimal threshold, and it may be better to aim for as low as possible (10). A CC target of as low as possible would be supported by the current trial, with the 41% of samples from feeding equipment having zero CC suggesting that 0 cfu/ml is an achievable target for coliforms in colostrum from GB dairy producers.

Whilst all milking machine use was associated with higher levels of bacterial contamination, robot milking machines were associated with a particularly high level of contamination. This may be due to default settings for the collection of colostrum by the robot rather than any inherent issue with robot systems themselves. Although the default setting for robots participating in this trial for routine milk collection was generally to perform a full machine wash and perform pre-milking teat cleaning prior to milking, these were often not performed prior to the collection of first colostrum. Veterinarians on robotically milked dairy farms should investigate default colostrum collection settings and ensure that settings are configured for optimal colostrum hygiene, and future research should aim to validate how the hygiene of robotically collected colostrum might be optimized.

Farmer collection of colostrum samples represents a potential limitation of this study, as there is likely to be a degree of inconsistency in sampling technique. Variability in sample collection technique between farmers is likely to be random and is unlikely to introduce bias into models. It is possible that by being enrolled in a trial, an element of bias may have been introduced with farmers being keen to process colostrum in a relatively hygienic manner. The simple act of benchmarking has been reported to decrease levels of FPT from 21 to 11% after a benchmark report and change in management (32), although every effort was made to encourage farmers to collect samples as normal. This bias was limited by the design of the study to some degree, as farmers were only asked about collection protocols used for the samples after the samples were received. As enrolment in the trial was voluntary it is possible that farmers on this trial represent a more progressive population, and therefore estimates of bacterial contamination levels are likely to be conservative. Concurrent research was also being undertaken on the study farms which may have introduced a level of bias, particularly an intervention trial aimed at increasing growth rates in preweaned calves. One component of this was encouraging farmers to use hypochlorite or peracetic acid when collecting colostrum, however, on further analysis, only 28 samples (8.5%) were from intervention farms where farmers were now using a recommended cleaning product where they were not previously, compared with 15 samples (4.6%) from control farms. Differences between control and intervention groups were not significant after performing a chi-squared test ($p = 0.16$), and the authors feel that whilst this may have had a small effect on the numbers of farms using hypochlorite or peracetic acid overall, this is unlikely to have a significant effect on bacterial estimations and have no effect on model performance or recommendations from this trial.

Stability of variables in predicting coliform counts were far lower than TBC. Log transforming total bacterial counts resulted in a gaussian distribution for TBC. Due to a large number of zero counts, however, CC did not fit a gaussian distribution after log transformation. The distribution of residuals for CC models were carefully checked however and were deemed to provide satisfactory evidence of model fit. Furthermore, any prediction errors at extreme values are likely to be ameliorated by the bootstrapping process. The use of regularized regression models was also considered due to their effective performance in robust variable selection (33), however, due to the presence of multiple samples from each farm and relatively few explanatory variables, mixed models were better suited to the dataset. The recommendations from this research are likely to be applicable to dairy farms in GB, however caution should be taken when extrapolating the results to dairy farms in other countries.

CONCLUSION

Colostrum sampled from collection or feeding equipment had higher levels of TBC and CC than those taken directly from cows' teats suggesting microbiological contamination is likely to occur from improperly cleaned equipment rather than the cow. Whilst extremely low bacterial counts were achievable, this study indicates over one third of samples collected from either collection buckets or feeding equipment were over conventional thresholds for either TBC or CC, and would, therefore, represent a significant risk for both the ingestion of pathogens and the failure of passive transfer of immunity on GB dairy farms.

Routine testing of colostrum bacteriology is relatively cheap and straightforward and is likely to be currently underutilized in the UK. Veterinarians should consider routine colostrum hygiene testing as part of a preventative calf health approach, and this trial has identified a small number of variables that are likely to have a substantial impact on colostrum hygiene for a large proportion of farms. Key recommendations based on this research to reduce bacterial levels in colostrum suggest protocols should include the cleaning of colostrum collection and feeding equipment after every use with hot water as opposed to cold water, and hypochlorite or peracetic acid as opposed to water or parlor wash. Cows' teats should be prepared with a pre-milking teat disinfectant and wiped with a clean, dry paper towel prior to colostrum collection, and colostrum should be pasteurized where possible.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by University of Nottingham. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

RH conducted farm visits and farmer training. RH, MG, CH, and PD wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Factors Associated With Lameness in Tie Stall Housed Dairy Cows in South Germany

Andreas W. Oehm[1], Katharina Charlotte Jensen[2], Annegret Tautenhahn[3], Kerstin-Elisabeth Mueller[3], Melanie Feist[1] and Roswitha Merle[4]*

[1] Clinic for Ruminants With Ambulatory and Herd Health Services, Ludwig-Maximilians Universität Munich, Munich, Germany, [2] Clinic for Cattle, Foundation, University of Veterinary Medicine, Hanover, Germany, [3] Clinic for Ruminants and Swine, Faculty of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany, [4] Institute for Veterinary Epidemiology and Biostatistics, Freie Universität Berlin, Berlin, Germany

Lameness remains a major concern for animal welfare and productivity in modern dairy production. Even though a trend toward loose housing systems exists and the public expects livestock to be kept under conditions where freedom of movement and the expression of natural behavior are ensured, restrictive housing systems continue to be the predominant type of housing in some regions. Factors associated with lameness were evaluated by application of multiple logistic regression modeling on data of 1,006 dairy cows from 56 tie stall farms in Bavaria, South Germany. In this population, approximately every fourth cow was lame (24.44% of scored animals). The mean farm level prevalence of lameness was 23.28%. In total, 22 factors were analyzed regarding their association with lameness. A low Body Condition Score (BCS) (OR 1.54 [95%-CI 1.05–2.25]) as well as increasing parity (OR 1.41 [95%-CI 1.29–1.54]) entailed greater odds of lameness. Moreover, higher milk yield (OR 0.98 [95%-CI 0.96–1.00]) and organic farming (OR 0.48 [95%-0.25–0.92]) appeared to be protectively associated with lameness. Cows with hock injuries (OR 2.57 [95%-CI 1.41–4.67]) or with swellings of the ribs (OR 2.55 [95%-CI 1.53–4.23]) had higher odds of lameness. A similar association was observed for the contamination of the lower legs with distinct plaques of manure (OR 1.88 [95%-CI 1.14–3.10]). As a central aspect of tie stall housing, the length of the stalls was associated with lameness; with stalls of medium [(>158–171 cm) (OR 2.15 [95%-CI 1.29–3.58]) and short (≤158 cm) length (OR 4.07 [95%-CI 2.35–7.05]) increasing the odds compared with long stalls (>171 cm). These results can help both gaining knowledge on relevant factors associated with lameness as well as approaching the problem of dairy cow lameness in tie stall operations.

Keywords: locomotion, cattle, risk factor analysis, housing conditions, tie stall, lameness

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****Correspondence:***
Andreas W. Oehm
andreas.oehm@Outlook.com

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INTRODUCTION

Lameness, defined as impaired locomotion regardless of the underlying cause (1–3), is the most important matter for economic and animal welfare concerns in modern dairy production (4–8). It has considerable adverse effects on longevity, milk yield, reproductive performance, and general well-being (9–12). Although muscle damage and nerve paralysis contribute to lameness, by far

the most cases originate from claw disorders (13). While the source of pain in the initial phase of a claw disorder is the lesion itself, hyperalgesia is present in chronic cases, which does not need to be related to the severity of the lesion (14–16). Painful disorders impair the natural behavior of affected animals (16–19). Lameness is multifactorial by origin with housing conditions, on-farm management practices, and the individual animal having the greatest impact (20, 21).

Even though modern dairy husbandry has been experiencing a shift toward loose housing systems, keeping dairy cows in tie stall facilities is still a common husbandry method worldwide (8, 22, 23). This practice has yet been criticized due to increasing concerns of consumers about the well-being and quality of life of livestock (24, 25). Even though tie stall housing often incorporates pasture access, animals are mostly restrained in their individual stalls throughout their productive life, they are unable to move freely or express natural behavioral patterns. Concerning lameness however, lower prevalence has been reported for tie stall facilities compared with free stall barns (26).

The aim of the present study was to assess the prevalence of lameness in tie stall housed dairy cows in South Germany and to evaluate the association of lameness with potential risk factors.

MATERIALS AND METHODS

Farm Recruitment

This study was conducted as part of a large cross-sectional study on health, biosecurity, and housing environment on dairy farms in Germany. The project was initiated and funded by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE) grant number 2814HS008. A total amount of 265 dairy farms in the German federal state of Bavaria were visited. Farms were randomly selected stratified by administrative district and farm size within their federal states. Sampling was based on the national animal information data base (HIT) and on the farm data from the Milchprüfring Bayern e.V. Selected farms received a letter including information on the study and an invitation to participate. Interested farmers contacted the study team voluntarily and gave their written consent to participate in the study. Farms were visited once between December 2016 and March 2019. In the present study, farms housing their cows in tie stall facilities were included.

On-Farm Data Collection

Inter-observer reliability between all of the seven researchers collecting the data was assessed three times during the study period. Each of these assessments took place in the form of a 2 day practical course. During the first assessment, 44 cows were scored, 59 cows were scored during the second assessment, and 73 cows were scored at the third assessment date. Furthermore, video as well as photo material was evaluated in group discussions conducted after each of the meetings. Based on these assessments, a weak/moderate, substantial, and fair agreement was present between the observers (overall weak to moderate, kappa values of 0.57, 0.63, and 0.44, respectively) (27, 28).

On each farm, all cows were assessed. The individual ear tag number of the animals (last five digits) was documented. All lactating and dry cows that were tied at the day of the farm visit individually underwent scoring for lameness, body condition, rib swellings, cleanliness of the lower legs and udder, and the presence of observable abnormalities of the hock, neck, back, and tail.

Lameness was assessed using the Stall Lameness Score (SLS) introduced by Leach et al. (29). Four criteria were observed during a 90 s observation period: weight shifting between feet, sparing a foot while standing, unequal weight bearing when stepping from side to side, and standing on the edge of the kerb (29). A cow displaying two out of the four criteria patterns was classified as lame. Body condition was scored according to the Body Condition Score (BCS) established by Edmonson et al. (30), later modified by Metzner et al. (31). As body condition changes during lactation, breed-specific categories exist in regard to days in milk. Therefore, cows were assigned to one of the three body condition categories "under," "opt," and "over" in relation to breed and stage of lactation (32–34), which can be seen in **Table 1**.

The presence of rib swellings was visually assessed in the lateral thoracic region between the 7 and the 9th rib at the transition from the bony part to the cartilaginous part of the rib (35).

A modified scoring approach was implemented to record skin changes of the hock (36, 37). Accordingly, hocks were assessed from a caudolateral perspective as follows: 1 = no skin change, 2 = hairless patch, 3 = swelling (no wound), 4 = wound (no swelling), 5 = wound and swelling, 6 = no assessment possible due to solid plaque of manure. The most severe of the present abnormalities on both sides was recorded. Skin changes of the neck were documented if present in the region between the first cervical and the first thoracic vertebra. A modified score according to Kielland et al. (38) was implemented: 1 = no observable skin change, 2 = hairless patch, 3 = wound or swelling. To assess the back, the region between the first cervical and the first caudal vertebra in an area of 10 cm on both sides of the median line of the back was examined. As for the tail, only visible abnormalities were documented: 1 = no abnormalities, 2 = swelling or deviation of the tail, 3 = amputated tail. Cleanliness of the udder and the lower legs was appraised according to Cook and Reinemann (39): 1 = little or no manure, 2 = minor splashing, 3 = distinct plaques of manure, 4 = solid plaque of manure.

The type of tying system, type of stall base, use of bedding material, and gutter design were assessed by visual inspection. An a priori determined number of stanchions per farm was measured for length and width: up to 30 stanchions with cows: 10 stanchions were measured; 30–49 stanchions: 15 stanchions were measured; 50–99 stanchions: 17 stanchions were measured. This number had been calculated prior to farm visits in accordance with farm size (i.e., the number of stalls present on farm in this context). For example, if 30 stanchions were present on farm and 10 had to be measured according to the pre-defined plan, every 3rd stall was assessed. The median value per farm was calculated and used for further statistical analysis.

TABLE 1 | BCS categories in accordance with stage of lactation and breed (32–34).

Days in milk	Breed								
	Holstein			Brown swiss			Simmental/other		
	Under	Optimal	Over	Under	Optimal	Over	Under	Optimal	Over
0–29	≤ 2.75	3.0–3.75	> 3.75	≤ 2.75	3.0–3.75	> 3.75	≤ 3.25	3.5–4.25	> 4.25
30–99	≤ 2.5	2.75–3.25	> 3.25	≤ 2.5	2.75–3.25	> 3.25	≤ 3.0	3.25–4.0	> 4.0
100–199	≤ 2.5	2.75–3.25	> 3.25	≤ 2.5	2.75–3.25	> 3.25	≤ 3.0	3.25–4.0	> 4.0
200–299	≤ 2.75	3.0–3.75	> 3.75	≤ 2.75	3.0–3.75	> 3.75	≤ 3.25	3.5–4.25	> 4.25
> 300	< 3.25	3.25–3.75	> 3.75	< 3.25	3.25–3.75	> 3.75	< 3.75	3.75–4.25	> 4.25

Farmers were interviewed during the farm visit in order to collect information on the operational type of the farm (main source of income, organic farming) and if cows were provided with access to pasture or an outdoor exercise area at any given time during the year. Data on milk yield, parity, age, breed, and days in milk were retrieved from the national animal information data base HIT and from the national milk recording system (DHI). Farm records for milk yield were available for each cow up to 12 months prior to the farm visit. Test day milk yield is assessed once a month. In the present study, the most recent test day milk yield was used.

Data Handling and Statistical Analysis

All data were collected using questionnaires and data entry forms. After the farm visit, data were manually entered into a central SQL-data base. From there Microsoft Excel (40) datasheets were extracted and imported into R.

Statistical analyses were conducted with the statistical software R version 1.2.1335 (41). We used the following five packages: `tidyverse` (42), `ggstatsplot` (43), `sjPlot` (44), `effects` (45), and `caret` (46).

Descriptive statistics were carried out to investigate the distribution of predictors with the Stall Lameness Score. Abnormalities of the back and the tail were dichotomized. As for hocks, all cows that scored 6 were excluded from further analyses. Moreover, observable skin changes of the hocks were further categorized to 1 (no observable skin changes present), 2 (hairless patches), and 3 (swelling and/or wound). The continuous variables *stall length* and *stall width* were transformed into categorical variables depending on their distribution and the values of their quartiles. Three categories were created: short (≤ 158 cm), medium (>158–171 cm), and long (>171 cm). Farm size was grouped into three categories: small (<24 cows), medium (24–30 cows), and large (>30 cows). Subsequently, univariable analyses were performed on cow level for each variable in regard to the targeted variable *lame* (1/0) using logistic regression. A $p \leq 0.05$ was regarded as statistically significant.

Multiple mixed logistic regression models were built on cow level in a manual stepwise forward selection process adding one predictor at a time to the model; *farm* was included as random factor. *Year* and *farm size* (categorized) were included as fixed effects. After every newly included variable, the model was assessed using the Akaike's Information (AIC) and Conditional R^2. The lower the AIC the better the quality of the model (47). If a significant improvement of the AIC was perceived, a variable was kept within the model. Furthermore, after each step, the R function `car::vif()` was implemented for variable inflation in order to detect potential (multi-)collinearity among predictors.

RESULTS

A total number of 1,170 dairy cows on 56 farms in the south German state of Bavaria were included in the data set of this study based on the housing system of their cows. If cows were housed in tie stalls at farm visit, these farms were included in the present analysis which led to the inclusion of 56 farms out of the initial 265 farms. The mean farm size was 25.60 cows (range 4–61 animals). Of the 56 farms, 47 were run conventionally whereas 9 farms were managed according to principles of organic farming. The predominant breed was German Simmental (84.53%), followed by Brown Swiss (10.77%), German Holstein (2.65%) and others (2.05%), i.e., crossbreds of the aforementioned. On 33 farms, dairy farming was the main source of income, whereas dairy farming provided subsidiary income on 23 farms. Among the 1,170 cows, 286 were classified as *lame* which equals a lameness prevalence of 24.44%. On farm level, the mean lameness prevalence was 23.28% (5.26–51.58%). Descriptive statistics of all categorical variables within the data set are presented in **Table 2**. Descriptive statistics of numerical variables within the data set can be seen in **Table 3**.

Table 4 summarizes the results of the univariable analyses. All predictors were analyzed in relation to the outcome *lame*.

The multiple logistic regression approach required a complete cases data set. Accordingly, missing observations were removed which resulted in a total number of 1,006 cows on 56 farms. The final model maintained 8 out of the 22 predictors as well as the fixed effects *year* and *farm size* (categorized). **Table 5** displays an overview of the results from the final multiple mixed logistic regression model. Low BCS was associated with greater odds of lameness. Compared with optimally conditioned cows, underconditioned animals experience higher odds of being lame (OR 1.59 [CI 1.10–2.30], $p = 0.014$). Higher odds of lameness were observed in animals of parities 3 or higher compared with animals in their first lactation (OR 2.71 [CI 1.83–4.01], $p < 0.001$). Furthermore, increasing milk yield was associated with lameness (OR 0.98 [CI 0.96–1.00], $p = 0.05$). With increasing

TABLE 2 | Distribution of categorical variables within the data set.

Predictor	Categories	n cows (%)
Breed	German Simmental	989 (84.53)
	other	181 (15.47)
Udder hygiene	1 (little or no manure)	344 (29.40)
	2 (minor splashing)	405 (34.62)
	3 (distinct plaques of manure)	246 (21.03)
	4 (solid plaque of manure)	175 (14.96)
Cleanliness of lower legs	1 (little or no manure)	357 (30.51)
	2 (minor splashing)	519 (44.36)
	3 (distinct plaques of manure)	199 (17.01)
	4 (solid plaque of manure)	95 (8.12)
Hock	1 (no observable skin change)	160 (15.90)
	2 (hairless patch)	604 (60.04)
	3 (swelling and/ or wound)	242 (24.06)
Swelling of the ribs	No	1,072 (91.62)
	Yes	98 (8.38)
Neck	1 (no observable skin change)	603 (51.54)
	2 (hairless patch)	473 (40.43)
	3 (swelling and/ or wound)	94 (8.03)
Back	0 (no observable skin change)	1,133 (96.84)
	1 (skin change present)	37 (3.16)
Tail	0 (no observable skin change)	1,103 (94.43)
	1 (skin change present)	65 (5.57)
Income from dairy farming	Main income	794 (69.22)
	Subsidiary income	353 (30.78)
BCS	Underconditioned	262 (22.39)
	Optimally conditioned	824 (70.43)
	Overconditioned	84 (7.12)
Type of tying system	Grabner tie[a]	713 (62.65)
	Vertical neck frame Collar	150 (13.18)
	and chain	198 (17.40)
	Other	77 (6.77)
Stall base	Concrete	181 (16.20)
	Rubber	936 (83.80)
Use of bedding	No	1,137 (97.26)
	Yes	32 (2.74)
Gutter design	Concrete	205 (18.22)
	Grate	920 (81.78)
Farming type	Conventional farming	1,006 (86.00)
	Organic farming	164 (14.00)
Access to pasture	No	718 (61.37)
	Yes	452 (38.63)
Exercise area present	No	1,035 (88.46)
	Yes	135 (11.54)
Length of stalls (categorized)[b]	1 (short)	291 (24.87)
	2 (medium)	539 (46.07)
	3 (long)	340 (29.6)
Width of stalls (categorized)[c]	1 (narrow)	318 (28.14)
	2 (medium)	519 (45.93)
	3 (broad)	293 (25.93)
Farm size (categorized)[d]	1 (small)	583 (49.83)
	2 (medium)	282 (24.10)
	3 (large)	305 (26.07)
Observer	1	132 (11.28)
	2	331 (28.29)
	3	85 (7.26)
	4	113 (9.66)
	5	274 (23.42)
	6	126 (10.77)
	7	109 (9.32)

(Continued)

n_{cows}: absolute number of cows. [a]chain/belt fixed vertically with attached sliding frame around the cow's neck. [b]length of stalls was categorized according to the distribution of the measured values and the medians calculated from these (≤ 158 cm: 1; > 158–171 cm: 2; > 171 cm: 3).

[c]width of stalls was categorized according to the distribution of the measured values and the medians calculated from these (≤ 98.5 cm: 1; > 98.5–103 cm: 2; > 103 cm: 3).

[d] farm size was categorized (small < 24 cows; medium 24–30 cows; large > 30 cows).

levels of contamination of the lower legs, cows experienced higher odds of lameness. This was noticeable for the presence of distinct plaques of manure (OR 1.61 [CI 1.00–2.61], $p = 0.05$), but not for solid plaques of manure (OR 1.30 [CI 0.66–2.57], $p = 0.443$). Swellings and/or open wounds in the hock region were associated with lameness (OR 2.56 [CI 1.43–4.61], $p = 0.002$) as well as the presence of rib swellings (OR 2.81 [1.70–4.64], $p < 0.001$). Compared with long stalls, cows kept in medium (OR 1.76 [CI 1.07–2.87], $p = 0.025$) or short (OR 3.17 [CI 1.93–5.19], $p < 0.001$) stalls appeared to have greater odds of lameness. Cows living on farms with more than 30 cows have higher odds for lameness compared with cows on small farms (< 24 cows) (OR 1.72 [CI 1.15–2.58], $p = 0.008$). As animals on different farms are not subjected to the same housing and management conditions, a farm-specific random effect was introduced in the modeling procedure in order to account for the presence of random variability in the data due to actual differences in on-farm housing- and management practices. The random effect considered that effects may differ as a consequence of differences across farms and incorporates farm-to-farm-variability within the analysis. In the current study, the percentage of heterogeneity, i.e., the value of $\tau_{00\ farm}$ as the variance between farms, in the final model was 0.20. Hence, 20% of the variance were explained by the variance between farms, e.g., as a consequence of different settings, varying housing conditions, management elements or of a different mindset.

DISCUSSION

As public interest in the welfare and physical integrity of agricultural livestock in modern production systems grows, husbandry conditions are likely to come under close scrutiny which necessitates a critical evaluation in order to both meet animal welfare standards and economic viability (48). This growing public focus on farm animal welfare requires further investigation in current practices and to broaden our knowledge concerning housing conditions of livestock. This is particularly important with regard to lameness prevalence which is often addressed in the context of welfare assessment (49, 50). Against this background, the aim of this study was to determine the prevalence of lameness in tie stall housed dairy cows in Bavaria and to evaluate factors associated with the condition. By including a large number of animals and farms, we are

TABLE 3 | Distribution of continuous variables within the data set.

Predictor	Mean	Range	1st quartile	Median	3rd quartile	*n*
Parity	2.71	1–11	1	2	4	1,170
Days in milk	200.35	0–1,060	92	192	284	1,170
Milk yield (in kg)*	22.91	4.80–51.80	17.12	22.30	28.10	1,170
Farm size	25.60	4.00–61.00	19.00	24.00	30.00	1,170

**Variable on cow level; values obtained from the most recent sampling record.*

convinced to have attained a high level of standardization even though some limitations exist. The mean farm level prevalence of lameness was 23.28 and 24.44% on animal level which is similar to other studies. In a Canadian study, Bouffard et al. (51) also implemented the SLS to determine lameness prevalence and found 25% of the cows assessed to be lame. In general, lameness prevalences are higher in free stall facilities than in tie stall operations and other housing types (6, 52, 53). Regarding the lameness prevalence determined in the current study, it is important to acknowledge, that Leach et al. (29) only observed a moderate sensitivity (0.54–0.77) of the SLS in direct comparison with locomotion scoring according to Sprecher et al. (54). This means that lameness might be underestimated when detected by SLS. The prevalence of lameness was underestimated on average by 27% (11–37%) in the study by Leach et al. (29). Moreover, as farmers had to get in contact with the study team on their own initiative, one might infer that mainly proactive farmers or well-conditioned operations have been enrolled and visited. This circumstance raises the hypothesis that the true lameness prevalence could be even higher in the dairy cow population housed in tie stall facilities. On the other hand, it appears plausible to assume that voluntary participation may have motivated specifically those farms with a lameness problem to participate. In this case, the true lameness prevalence in the current study would be overestimated.

Cows with a BCS lower than recommended (32–34) had higher odds of lameness compared with cows with a higher BCS according to breed and stage of lactation. This association is in accordance with others (20, 55, 56). As loss in body condition is not exclusively related to subcutaneous body fat but also affects the digital cushion, its shock absorbing properties during weight-bearing are impaired exposing the sensitive structures of the claw, i.e., the distal phalanx and the corium to undissipated mechanical forces that subsequently result in the formation of traumatic claw lesions (56–58). On the other hand, lameness itself often entails a loss of body condition as animals show alterations in their feeding behavior (59–61). Regarding the BCS limits in the present study, Holstein cows where considered as optimally conditioned with a BCS of up to 3.75 at the start of lactation as well as in the later stages of lactation and during the dry period. These cut offs where selected in accordance with the above cited literature. It is yet important to be aware that Drackley (62) recommended that BCS may not exceed 3.0 in North American Holstein cows at the beginning of lactation. As Holstein cows represented only a minor part of the study population in the present study and since difference might be present between Holstein cows of the North American type and the European or German type, respectively, we decided to implement the values presented in European publications that also provided cut off values for other breeds of the study population. As outlined previously, the results regarding the association between BCS and lameness are well in accordance with previous work. Using the stricter cut off values for Holstein cows suggested by Drackley (62), the result may have become even more distinct.

Higher parity increased a cow's odds for lameness in the current study and in previous work (63, 64). Prolonged exposition to potentially harmful elements of housing and management environments may increase the odds for cows higher in parity to suffer from recurrent episodes of claw disorders, finally resulting in chronic lameness (63–66). Older animals may also be less able to cope with deficient housing conditions. Furthermore, the tensile strength of the suspensory apparatus progressively wears out with increasing parity which causes the third phalanx to remain in a state of sinking (65, 67, 68). In combination with delivery associated remodeling processes of both the suspensory apparatus of the claw and the digital cushion, the deeper, more sensible structures of the claw may experience impaired shock-absorbing capacity and hence a massive increase of pressure (57, 58, 65, 69, 70). This subsequently fosters the development of traumatic claw lesions and lameness. On the other hand, dairy cows in their first lactation may encounter the most pronounced problems with housing associated changes when they are transferred from a heifer group to the group of lactating animals. The transition from free housing as heifer to tied housing as a lactating cow may create challenges for these animals and they may hence be removed from the herd prematurely which is supported by the fact that dairy cows in Germany survive to an average age of 5.4 years (71–73). This in sharp contrast to the aspiration of keeping dairy cows for a long productive life and highlights the fact that the current housing systems ought to be re-considered in order to be adequate to keep the animals sound and physically intact on the long run. It furthermore emphasizes that with increasing parity cows need to be provided with special care.

The association between high milk yield and the occurrence of disease, e.g., lameness, cows has been subject to ongoing discussions (74–76) with high yielding animals being particularly at risk for metabolic disorders, reproductive deficiencies, and lameness (77, 78). In tie stalls in southern Germany, cows are mostly fed with single components instead of mixed rations provided in free stall barns. Therefore, it is difficult to meet the nutritional requirements of high-yielding cows.

TABLE 4 | Results of the univariable analyses of all factors with the target variable *lame*.

Predictor	Parameter estimate	Standard error	Odds ratio	Confidence interval (95%)	*P*-value
Breed					
Other	Reference	–	–	–	–
German Simmental	0.08	0.19	1.08	0.75–1.59	0.673
Parity					
Increasing parity	0.26	0.04	1.30	1.21–1.39	< 0.001
Days in Milk	0.00045	0.00049	1.00	1.00–1.00	0.353
Milk yield	−0.00062	0.0087	1.00	0.98–1.02	0.943
Udder hygiene					
Little/no manure	Reference	–	–	–	–
Minor splashing	0.12	0.17	1.13	0.80–1.59	0.497
Distinct plaques of manure	0.08	0.19	1.09	0.74–1.60	0.671
Solid plaque of manure	0.45	0.21	1.57	1.04–2.37	0.030
Cleanliness of lower leg					
Little/no manure	Reference	–	–	–	– 0.231
Minor splashing	0.20	0.17	1.22	0.88–1.70	0.011
Distinct plaques of manure	0.52	0.20	1.68	1.13–2.50	0.038
Solid plaque of manure	0.54	0.26	1.71	1.02–2.82	
Hocks					
No observable skin change	Reference	–	–	–	–
Hairless patch	0.50	0.24	1.65	1.04–2.70	0.039
Swelling and/or wound	1.31	0.26	3.73	2.28–6.28	< 0.001
Swelling of the rib					
No	Reference	–	–	–	–
Yes	1.12	0.22	3.07	2.01–4.68	< 0.001
Neck					
No observable skin change	Reference	–	–	–	–
Hairless patch	0.51	0.14	1.66	1.25–2.19	< 0.001
swelling or wound	0.18	0.26	0.70	1.97	0.505
Back					
No observable skin chance	Reference	–	–	–	–
Skin change present	1.00	0.34	2.73	1.39–5.29	0.003
Tail					
No observable abnormality	Reference	–	–	–	–
Deviation and/or swelling, amputated tail	−0.27	0.32	0.76	0.39–1.38	0.397
Income from dairy farming					
Main income	Reference	–	–	–	–
Subsidiary income	−0.05	0.15	0.95	0.71–1.28	0.756
BCS					
Underconditioned	0.58	0.16	1.79	1.31–2.42	< 0.001
Optimally conditioned	Reference	–	–	–	–
Overconditioned	0.32	0.26	1.38	0.82–2.26	0.215
Type of tying system					
Grabner tie[a]	Reference	–	–	–	–
Vertical neck frame	−0.47	0.22	0.62	0.40–0.95	0.032
Collar and chain	−0.59	0.20	0.55	0.37–0.82	0.004
Other	−1.24	0.38	0.29	0.13–0.58	0.001
Stall base					
Concrete	Reference	–	–	–	–
Rubber	- 0.05	0.19	0.95	0.66–1.39	0.791
Use of bedding					
No bedding	Reference	–	–	–	–
Bedding present	0.03	0.41	1.03	0.43–2.22	0.943

(Continued)

TABLE 4 | Continued

Predictor	Parameter estimate	Standard error	Odds ratio	Confidence interval (95%)	*P*-value
Gutter design					
Concrete or gutter without grate	Reference	–	–	–	–
Gutter with grate	0.52	0.20	1.69	1.15–2.52	0.008
Farming type					
Conventional farming	Reference	–	–	–	–
Organic farming	−0.77	0.24	0.46	0.28–0.72	0.001
Access to pasture					
No	Reference	–	–	–	–
Yes	−0.58	0.15	0.56	0.42–0.74	< 0.001
Exercise area present					
No	Reference	–	–	–	–
Yes	−0.56	0.24	0.57	0.34–0.90	0.021
Length of stalls (categorized)[b]					
1 (short)	1.24	0.21	3.45	2.31–5.24	< 0.001
2 (medium)	0.77	0.20	2.16	1.47–3.24	< 0.001
3 (long)	Reference	–	–	–	–
Width of stalls (categorized)[c]					
1 (narrow)	Reference	–	–	–	–
2 (medium)	0.005	0.16	1.01	0.73–1.38	0.975
3 (broad)	−0.49	0.20	0.61	0.41–0.90	0.013
Farm size (categorized)[d]					
1 (small)	Reference	–	–	–	–
2 (medium)	0.15	0.18	1.16	0.82–1.63	0.407
3 (large)	0.71	0.16	2.04	1.48–2.78	< 0.001
Year					
2016	Reference	–	–	–	–
2017	−0.59	0.28	0.55	0.32–0.96	0.034
2018	−1.01	0.29	0.37	0.21–0.64	< 0.001
2019	−1.21	0.35	0.30	0.15–0.59	0.001
Observer					
1	Reference	–	–	–	–
2	0.11	0.24	1.12	0.71–1.80	0.630
3	−0.53	0.35	0.59	0.29–1.17	0.139
4	−0.06	0.30	0.94	0.52–1.69	0.841
5	−0.22	0.25	0.81	0.50–1.32	0.386
6	−0.35	0.30	0.71	0.39–1.27	0.2501
7	0.55	0.28	1.74	1.00–3.04	0.053

[a] *Chain/belt fixed vertically with attached sliding frame around the cow's neck.*
[b] *Length of stalls was categorized according to the distribution of the measured values and the medians calculated from these (≤ 158 cm: 1; > 158–171 cm: 2; > 171 cm: 3).*
[c] *Width of stalls was categorized according to the distribution of the measured values and the medians calculated from these (≤ 98.5 cm: 1; > 98.5–103 cm: 2; > 103 cm: 3).*
[d] *Farm size was categorized (small < 24 cows; medium 24–30 cows; large > 30 cows).*

Counterintuitively, high milk yield appeared to reduce the odds of lameness in the current study (OR 0.98 [0.95–1.00]) which is also confirmed by investigations made by Wangler et al. (73). This may be explained by the fact that cows with a high milk yield may be exposed to improved management and housing procedures which keep animals in a healthy condition (and consequently being less lame) and enable them to meet their productive potential. Another reason might be that lame cows cannot reach their full potential due to changed feeding behavior and inflammation processes (9, 79). It is importance to note that according to Green et al. (79–81) a decrease in milk yield can be observed already 6 weeks before the clinically visible presentation of a lameness case. Hence in regard to milk yield, these cows are not standing out on average. This means that only a continuous assessment of the animals for lameness, for instance every fortnight, in conjunction with an evaluation of their performance immediately after calving would have produced the possibility to make a final assumption that high milk yield or high performance in the initial stage of lactation, respectively, entails a higher risk for lameness.

If cleanliness of the lower legs was compromised to the extent that distinct plaques of manure were present, the odds for lameness were increased. As lame cows spend a greater daily amount of time lying with shorter lying bouts (11, 82), this contamination of the lower legs may arise from increased exposure to excrements so that it would be rather

TABLE 5 | Final multiple logistic regression model for factors associated with lameness.

			Lame		
Predictor	**Category**	**Parameter estimate**	**Odds ratio**	**Confidence interval (95%)**	***P*-value**
Intercept		−1.82	0.16***	0.06–0.43	< 0.001
BCS	Optimal	Reference	–	–	–
	Overconditioned	−0.14	0.87	0.48–1.59	0.656
	Underconditioned	0.46	1.59*	1.10–2.30	0.014
Parity	1	Reference	–	–	–
	2	0.0021	1.00	0.62–1.61	0.993
	≥ 3	1.00	2.71***	1.83–4.01	< 0.001
Milk yield	Continuous[a]	−0.02	0.98*	0.96–1.00	0.05
Leg cleanliness	Little/no manure	Reference	–	–	–
	Minor splashing	0.03	1.03	0.71–1.50	0868
	Distinct plaques of manure	0.47	1.61*	1.00–2.61	0.05
	Solid plaque of manure	0.94	1.30	0.66–2.57	0.443
Hocks	No observable skin change	Reference	–	–	–
	Hairless patch	0.26	1.30	0.76–2.20	0.338
	Swelling and/or wound	0.94	2.56**	1.43–4.61	0.002
Rib swelling	No	Reference	–	–	–
	yes	1.03	2.81***	1.70–4.64	< 0.001
Farming type	Conventional farming	Reference	–	–	–
	Organic farming	−0.46	0.63	0.35–1.14	0.125
Length of stalls	Long	Reference	–	–	–
	Medium	0.56	1.76*	1.07–2.87	0.025
	Short	1.15	3.17***	1.93–5.19	< 0.001
Farm size	Small	Reference	–	–	–
	Medium	0.29	1.34	0.87–2.08	0.189
	Large	0.55	1.72	1.15–2.58	0.008
Year	2016	Reference	–	–	–
	2017	−0.89	0.41	0.20–0.82	0.012
	2018	−0.85	0.43	0.22–0.84	0.014
	2019	−0.84	0.43	0.19–0.96	0.040

Out of the initial 22 predictors, 10 factors associated with housing conditions and the individual animal were maintained within the final model. The model incorporated data from 1,006 dairy cows on 56 farms.

[a] *1 unit increase.*

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

a consequence of lameness. Also, an alternated lying behavior or an unphysiological lying position may further promote the contamination of the legs. As animals in tie stall facilities are constantly fixed in the same stall, they do not have the possibility to evade these conditions. On the other hand, contaminated legs may favor the development of lameness as the lower legs are exposed to increased bacterial contamination (9, 83–85). Urine and feces chemically impinge upon the integrity of the skin that may trigger the development of infectious claw pathologies. Interestingly, solid plaques of manure did not appear to be significantly associated with lameness in the final model. This might be the result of other protective factors attributable to deficient management that cover the influence of heavily contaminated legs. Hence, heavily contaminated legs (solid plaques of manure on the lower legs) may not have been necessary to increase the explanatory power of the model.

The presence of skin changes on the hock was associated with increased odds of lameness in accordance with previous studies and can be mainly traced back to three circumstances (37, 86, 87). Firstly, hock lesions can themselves be painful and hence cause lameness (88). However, this might apply to a minor percentage of cows as most cases of lameness can be traced back to pathologies of the claws (13, 22). Secondly, hock lesions may be a result of lameness. As lame cows are impaired in their ability to lie down and rise physiologically, they may collide with stall control elements which eventually gives rise to the development of lesions on the hock (87, 89). Furthermore, the quality of bedding and the amount of bedding material are other important

factors in the context of lameness and hock lesions that may aggravate the situation. Also, as lame cows spent a greater amount of time lying (11, 82), their risk of developing hock lesions may increase due to abrasive properties of stall surface, low amount of bedding material or soiled bedding material (87, 89, 90). Finally, hock lesions and lameness are associated with similar factors that foster their occurrence (86, 91) which may be an important point when regarding their association.

Knowledge on the occurrence and importance of rib swellings has been scarce. They often rather represent an additional finding and may point to previous rib fractures. They typically occur between the 7 and the 9th rib at the transition from the boney part of the rib to the cartilaginous part (35, 92, 93). In the current study, they were highly associated with lameness. This association is plausible given the fact that lame animals have difficulties in rising and lying down as discussed previously. They hence may frequently slip or fall down harshly with the consequence of lesions of the ribs (35). Another hypothesis on the pathogenesis of rib swellings may be that lame animals tend to lean against dividers of their stalls and when they slip or try to lie down, their thorax collides with these elements (94). The association between lameness and rib swellings has previously been discovered but may need more research to discover the etiologic mechanisms. As rib fractures are likely to be very painful, their relevance to animal welfare is obvious.

The length of the stalls appeared as a factor associated with lameness in the final model. Both medium ($>$ 158–171 cm) and short ($\leq$ 158 cm) stalls increased the odds of lameness compared with long ($>$ 171 cm) stalls. For a physiological lying and rising process, an adequately sized stall, which is the place of permanent inhabitation of a cow in tie stall housing, is of the utmost importance. Short stalls result in cows often lying down with parts of their body in the gutter area which frequently is either covered in manure or built as a grate. This is likely to have adverse effects of microbiological and physical integrity of the claws and facilitate the emergence of infectious and traumatic claw lesions. Short stalls also interfere with the cows' desire to lie down in a comfortable, well-bedded stall and hence significantly compromise the animals' well-being (95–97).

The currently available literature has presented equivocal opinions on the association between farm size and lameness. Whereas, evidence from a recent meta-analysis (20) as well as results from previous work (86, 98) suggest an association between increasing herd size and lameness as a result of less intensive surveillance of the individual animal, decreased availability of qualified staff or overstocking rather than a larger herd *per se*, other studies have yet observed lower lameness prevalences in larger herds (53, 77, 78). The latter studies suggest an increased level of professionalism, more personnel specifically trained for identifying lame cows and automated management elements. The current study suggests that a herd size of $>$ 30 cows entails greater odds for the individual animal to be lame. We yet think this finding is ought to be interpreted cautiously as the general farm size was very low (range 4–61 cows). Nevertheless, this may be a perspective for future research to identify the role of herd size in dairy cow lameness especially in tie stall operations where lameness detection itself might be more challenging as outlined previously.

CONCLUSIONS

The present study determined the prevalence of lameness in tie stall housed dairy cows and identified factors associated with lameness in this housing system. Housing conditions and elements of stall design are paramount in tie stall systems and in regard to lameness, they may possess an even more pivotal role in restrictive housing systems. Moreover, some aspects of housing and management are elements that allow for modification and improvement already in the short or the medium term. Following recommendations for stall design and management in these husbandry systems may be beneficial for both animal welfare and the prevalence of lameness. Furthermore, animal-level factors such as low body condition, higher parity, the presence of hock lesions and of rib swelling are important aspects in the context of dairy cow lameness which ought to be understood in order to tackle lameness problems and to improve animal welfare. Some of these factors may also require future investigations to better understand their inter-relationships especially in tie stall facilities.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the study was initiated by a Federal Ministry. Even though data are anonymized, they are not allowed to be made available to subjects not involved in the initial study. Requests to access the datasets should be directed to Roswitha Merle, roswitha.merle@fu-berlin.de.

AUTHOR CONTRIBUTIONS

AO and RM initiated, conducted, supervised the study, and performed statistical analyses. AO drafted the manuscript with support from KJ, AT, and K-EM. AO, KJ, AT, and RM were involved in data cleaning, handling of the variables, and descriptive analyses. MF and K-EM contributed their professional expertise in the field and critically revised the manuscript. All authors have read and approved of the final manuscript.

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Prudent Use of Antibiotics in Dairy Cows: The Nordic Approach to Udder Health

Päivi Rajala-Schultz[1]*, *Ane Nødtvedt*[2], *Tariq Halasa*[3] and *Karin Persson Waller*[4]

[1] Department of Production Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland, [2] Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Ås, Norway, [3] Section of Welfare and Disease Control, Faculty of Medical and Health Sciences, Institute of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark, [4] Department of Animal Health and Antimicrobial Strategies, National Veterinary Institute (SVA), Uppsala, Sweden

Global concerns regarding bacterial antibiotic resistance demand prudent use of antibiotics in livestock production. Dairy production in the Nordic countries has a low consumption of antibiotics, while animal health, productivity and milk quality are at high levels. Here, we describe the basis of Nordic mastitis control and treatment strategies, as a model for production of high-quality milk with prudent use of antibiotics. We hope this will be beneficial for dairy producers and advisors in other countries and regions that consider limiting antibiotic use in cattle herds. In this perspectives paper we describe the dairy sector in the Nordic countries, and present regulatory aspects of antibiotic use, diagnostics and current guidelines for treatment of clinical mastitis as well as dry cow therapy. We also show summary statistics of udder health indicators in Denmark, Finland, Norway and Sweden, to illustrate the effects of the implemented udder health management practices.

Keywords: mastitis control, bovine, antibiotic use, therapy, bacteriologic diagnosis

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***Correspondence:**
Päivi Rajala-Schultz
paivi.rajala-schultz@helsinki.fi

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INTRODUCTION

Antibiotic resistance is a global concern because of its fast spread not only in human, but also in animal populations (1–3). A common feature for livestock production in the Nordic countries is the constant focus on prudent use of antibiotics. The overall consumption of antibiotics in animal populations in Denmark, Finland, Iceland, Norway and Sweden is the lowest among European countries, as measured by mg of active ingredients per kg of estimated biomass of animal populations (4). Reports on sales of veterinary antimicrobial agents in European countries are published regularly by the European Medicines Agency (EMA) and comparative sales figures can be found on these reports. Moreover, the tradition of prudent antibiotic use in production animals goes back several decades and has contributed to low levels of antibiotic resistance in bacteria isolated from this sector across the Nordic countries (5, 6). In general, antibiotic resistance is quite uncommon in dairy cattle in the region, compared to that in other species and regions.

Dairy production has long traditions in the region and stakeholders across the country borders collaborate actively on various issues. As an example, joint Nordic guidelines for treatment of mastitis in dairy cows were agreed upon in a consensus meeting initiated by the Nordic collaborative group of dairy processors and published in 2009 (7). Generally, initiation of antibiotic treatment for intramammary infections is expected to be based on microbiologic diagnosis and benzylpenicillin is the drug of choice in most cases. Prophylactic use of antibiotics is discouraged in all production animals in the region.

The aim of this paper is to describe the basis of Nordic mastitis control and treatment strategies, as a model for high-quality milk production with prudent use of antibiotics. The focus will be on data from Denmark, Finland, Norway and Sweden. We describe the dairy sector in the region, regulatory aspects of antibiotic use, diagnostics and current guidelines for treatment of clinical mastitis as well as for dry cow therapy. Further, we will present summary statistics regarding udder health indicators to demonstrate some outcomes of the existing control and treatment strategies.

STRUCTURE OF NORDIC DAIRY PRODUCTION

Dairy farming in the Nordic countries is characterized by small, family-run farms. National dairy herd improvement organizations collect data on milk production, milk components and milk somatic cell counts (SCC) and provide support also on nutrition and farm economics. The dairy herd milk recording schemes have a high coverage, which ranges from approximately 80 to over 90% of the cows within each country. Additionally, information on veterinary-diagnosed and -treated diseases is recorded and collected from all farms to a centralized national database in each Nordic country. Data collection and recording is at present largely electronic. In the Nordic countries, producers do not have access to veterinary drugs without a prescription from a veterinarian and thus most treatments are initiated by veterinarians who are also expected to record them. While the disease recording is not 100% complete (8, 9), the system has quite high accuracy and is unique on a global scale. Additionally, a coding scheme for the veterinary diagnoses has been jointly agreed upon and as a result, health and production are well-monitored in the Nordic region and at the national levels. The national dairy organizations are mostly farmer-owned co-operatives, and this contributes to a good climate for collaboration where farmers, veterinarians, researchers and legislators strive together for good milk quality and animal health. There is high trust and confidence among the different stakeholders, who work closely together when developing new recommendations and regulations on issues such as antibiotic use. The goal is to ensure that: (1) the recommendations and regulations are evidence-based and (2) they have high level of acceptability and implementation.

As can be seen in **Table 1**, the average herd size varies across the region with Danish herds being the largest and Norwegian the smallest. However, herd sizes are increasing and number of herds decreasing every year in all four countries, in keeping with the global trend. Automatic milking systems (AMS) have become common in all the Nordic countries since the introduction in 1989, and 30% of all milk produced in the region is estimated to come from cows milked in AMS herds (10). Small tie-stall barns are still common, but their numbers are constantly decreasing and new facilities in all four countries are free-stall barns, often with AMS. The average milk yield per cow in the region is highest in Denmark and lowest in Norway. This is partially explained by genetics, because Danish Holstein is the dominating breed in Denmark, while the combined milk- and meat-producing Norwegian Red dominates its homeland. In addition to focusing on productivity, breeding programs in the Nordic countries have also long focused on health, including resistance to mastitis (11).

REGULATIONS AND RECOMMENDATIONS ON ANTIBIOTIC USE

Legislation in the Nordic countries allows the purchase of antibiotics for use in animals only based on a prescription from a veterinarian. In addition, antibiotics should not be used for prophylactic purposes or as growth promoters. Administration of antibiotics may, however, be carried out by a farmer as instructed by a herd veterinarian, depending on the level of the advisory agreement between them. The sale of antibiotics is generally only through pharmacies or directly from veterinarians who are not allowed to make any profit from these sales. Veterinary use of critically important antibiotics is strongly discouraged across the region. Veterinary and farm records of antibiotic sales and usage can be randomly checked by the authorities for compliance. For example, Nordic countries do not allow routine prophylactic antibiotic use at dry-off and in some countries farmers can be fined, if found to be breaking the law. Similarly, veterinarians could lose their licenses, temporarily or permanently, if their antibiotic prescribing practices are repeatedly found in violation of the regulations, but this happens rarely.

Udder health experts in each country have developed guidelines on treatment of mastitis of dairy cows. These guidelines complement the legal regulations regarding dispensing of antibiotics and provide practical guidance regarding the use of antibiotics in mastitis therapy. They vary to some degree between the Nordic countries due to differences in legislation, availability of drugs, distribution of mastitis-causing pathogens and their susceptibility to antibiotics, national studies, tradition and policies.

DIAGNOSTICS OF BACTERIOLOGY AND ANTIBIOTIC RESISTANCE

Bacteriologic diagnosis is an important part of the Nordic guidelines for mastitis therapy (7). Knowledge regarding aetiologic agents, patterns of udder infections and antibiotic resistance in a herd is essential when choosing the best treatment and control measures. This approach is a part of the basic veterinary training in the Nordic countries and the same message of prudent antibiotic usage is also conveyed to dairy farmers. To obtain this information, milk samples should be taken before treatment decisions, and sent to a laboratory for microbiologic analysis. Culturing of milk samples at veterinary clinics using selective agar plates can also be of value to quickly confirm the drug of choice in acute cases of clinical mastitis. Bacteriologic follow-up after treatment might also be of interest in some cases.

A veterinarian or a farmer typically collects milk samples, which are transported to the laboratory via postal mail or in a milk truck of a dairy co-operative, as some dairy co-operatives have their own diagnostic mastitis laboratories. In acute clinical

TABLE 1 | Descriptive statistics of milk production of dairy cows in four Nordic countries.

	Denmark[a]	Finland[b]	Norway[c]	Sweden[d]
Dairy herds, number	2 715	5 783	7 831	3 253
Average herd size (milking cows)	213	48	28	94
Proportion (%) herds with automatic milking systems[a]	25	20	23	28
Average annual milk yield, kg ECM[e]/cow	11 037	10 534	8 602	10 417

[a]*Data obtained from January 2020 statistics (SEGES (an agricultural knowledge & innovation center), Denmark, www.seges.dk).*
[b]*Number of herds and herd size in December 2019, yield data for herds in milk recording scheme during 2019.*
[c]*Number of herds and herd size 2019 (Norwegian Agriculture Agency; www.landbruksdirektoratet.no), yield data for herds in dairy herd recording system.*
[d]*Number of herds and herd size 2019 (National Board of Agriculture; www.jordbruksverket.se), yield data for herds in milk recording scheme during 2019.*
[e]*ECM = energy-corrected milk, accounts for the variability in milk fat and protein contents.*

cases of mastitis, treatment is often given immediately and adjusted as needed when bacteriologic results become available. In most mastitis cases, whether clinical or subclinical, producers take milk samples for testing, partly hoping that use of antibiotics and consequently discarding of milk could be avoided based on the causal agent, or lack thereof. To date, bacteriologic culturing is the most common method in all countries except in Finland where most milk samples are analyzed using polymerase chain reaction (PCR) technology. Presence of beta-lactamase production in staphylococci is routinely investigated. Evaluation of resistance against other antibiotics or among other bacteria can be performed if needed.

Good knowledge on and monitoring national trends in occurrence of mastitis-causing pathogens and their antibiotic resistance is important when forming recommendations for treatment and control of mastitis. At present, no common Nordic scheme exists for such monitoring, but several studies on clinical or subclinical mastitis have been performed at national levels (12–19). Compiled annual laboratory data are also available (20, 21). Overall, the most common micro-organisms found in association with bovine mastitis in the Nordic countries are staphylococci and streptococci. Streptococci are mostly sensitive to penicillin, as are the majority of the staphylococci although this varies somewhat between staphylococcal species and countries. Contrary to many other countries (22, 23), Gram-negative bacteria, such as *Escherichia coli*, are of lesser importance.

TREATMENT GUIDELINES

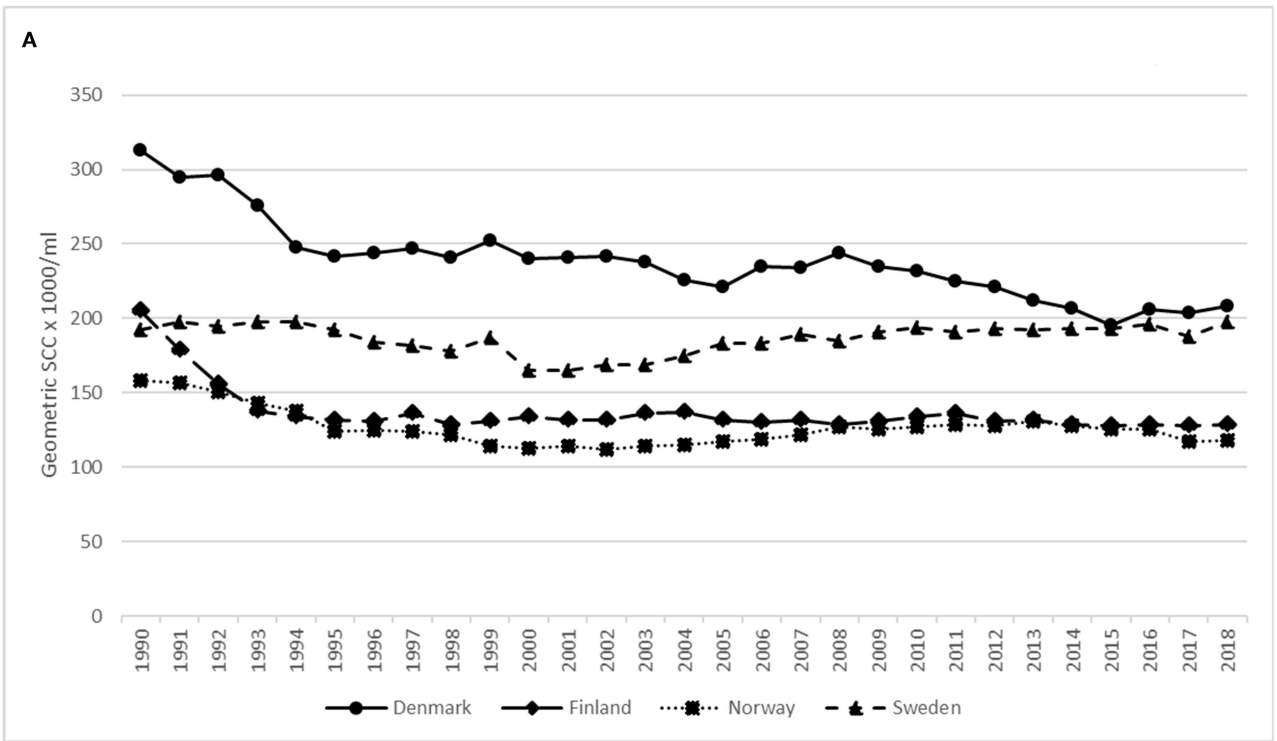
According to the Nordic guidelines for mastitis therapy, decisions on antibiotic treatment should be based on evaluation of prognosis and bacteriologic diagnosis (7). Moreover, the use of antibiotics during lactation should primarily be considered for cases of acute clinical mastitis. Antibiotic treatment of subclinical mastitis should mainly be done at dry-off.

Clinical Mastitis

In clinical mastitis, benzylpenicillin is the drug of choice, unless the causative pathogen is a Gram-negative bacterium or known to be resistant to penicillin. In such cases, mostly supportive therapy is recommended. According to the guidelines, treatment length when using benzylpenicillin varies between 3 and 5 days depending on the pathogen, e.g., for S*taphylococcus aureus* and *Streptococcus uberis* IMI a 5-d treatment is recommended, but for non-aureus staphylococcal (NAS) IMI a shorter therapy is often considered adequate. Recommendations on the route of administration, however, are not given at the Nordic level. Various combinations of local and/or systemic treatments are applied in each country. In all cases, supportive measures (e.g., sick pen, optimal cow comfort) and supportive therapy (e.g., NSAID, fluid therapy) as well as biosecurity actions (e.g., segregation) should always be considered. In a survey, two-thirds of Swedish veterinarians reported using NSAIDs always or almost always when treating clinical mastitis (24).

Dry Cow Therapy

As mentioned above, the Nordic guidelines for mastitis therapy mention the possibility to treat subclinical mastitis with antibiotics at dry-off, but they provide no further details or advice on the practice. The use of dry cow therapy (DCT) has been an important part of most mastitis control programs, but its implementation differs among regions of the world. In many countries, blanket DCT, i.e., treating all quarters of all cows with antibiotics at dry-off, regardless of their infection status, has long been recommended and used (25–28). Currently, however, due to the growing concerns about antibiotic resistance, selective DCT is being studied and considered worldwide in herds with low levels of contagious mastitis problems (29–32). In the Nordic countries, the recommendation has always been to implement selective DCT, i.e., to treat only infected cows (33–36). Selection of cows that are candidates for DCT is mostly done based on SCC data from 1 to 3 milk recordings before drying-off, data from the AMS system, mastitis history and possibly California Mastitis Test (CMT) scores. In all Nordic countries, having a bacteriologic diagnosis is encouraged before antibiotic therapy at dry-off is initiated, or at least knowing the pathogen profile and susceptibility of mastitis-causing pathogens in the herd. For example, if a causal agent of a subclinical IMI was detected earlier during the lactation, but treatment was postponed until dry-off, a new sampling of the cow might not be performed prior to DCT. The focus is to mainly treat penicillin-susceptible intramammary infections using long-acting benzylpenicillin. Penicillin-resistant NAS infections may be treated with cloxacillin-containing products and with older, chronically infected cows, culling is often recommended in the short- to medium-term.



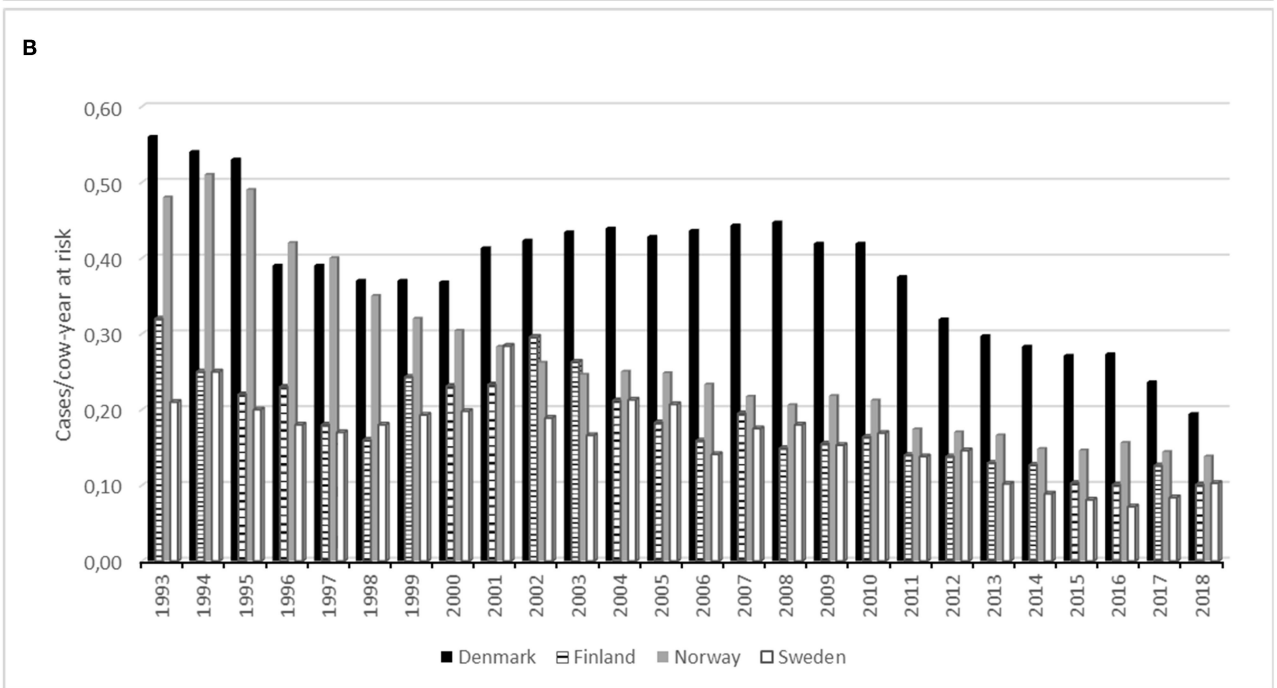


FIGURE 1 | Geometric mean of bulk tank milk somatic cell count (SCC) (cells × 1,000/mL) from 1990 to 2018 **(A)** and incidence rate of clinical mastitis* (cases/cow year at risk) from 1993 to 2018 **(B)** in four Nordic countries. *All treatments are administered or initiated by a veterinarian.

TRENDS IN UDDER HEALTH INDICATORS

Data on udder health indicators compiled by the collaborative group of Nordic dairy processors are presented in **Figure 1** (modified from (37)). The figures show a decreasing trend both in the incidence of clinical mastitis and in SCC during the period from around early 1990-ies until today. Geometric means of bulk tank milk SCC are currently approximately 200,000 cells/ml in

Denmark and Sweden and around 125,000 cells/mL in Finland and Norway. The incidence rates for treatment of clinical mastitis in 2018 were 0.19 and 0.10 per cow year in Denmark and Sweden, respectively. The trend is toward fewer treatments of clinical mastitis across the region during this time-period. As an example, the treatment frequency for clinical mastitis in Norway decreased by 4.2% from 2017 to 2018, while the reduction from 1994 to 2018 was 73% (20) (**Figure 1**). In other European countries with major dairy production, such as The Netherlands, bulk tank SCC is relatively similar to that in the Nordic countries; a mean value of 171,000 cells/mL. However, incidence of clinical mastitis (28.6 cases per 100 cows-year) and frequency of antibiotic treatments were higher than those in the Nordic countries (38).

Overall, the use of antibiotic DCT is low in the Nordic countries, with an estimated one-third or less of cows receiving such treatment at the end of lactation. According to a recent survey of DCT routines, 78% of Finnish producers report using selective DCT and 9% no DCT at all. The remaining proportion of herds reported treating all cows at dry-off and these herds were typically larger and more frequently had an automatic milking system compared to the other groups. In the majority (71%) of the selective DCT herds, less than one-fourth of the cows receive DCT at dry-off (39). In a similar study in Sweden, 96% of the farmers said they use selective DCT and most of those treat 25% or less of the cows (Persson Waller et al., unpublished). Information from Norway indicates that the treatment frequency for DCT there is even lower (O. Østerås, personal communication).

DISCUSSION

In a time when antibiotic resistance is recognized as a threat to animal and human health (3), food animal producers and veterinarians must continue to strive for prudent antibiotic use and sustainable production. This is an obvious One Health challenge and all stakeholders, industry in the front row, must actively participate. If consumers do not find animal-derived food sustainably and ethically produced, demand and markets for these products will likely shrink. In fact, market demands and consumer concerns e.g., on animal welfare and antibiotic resistance can be driving forces for changes in routines and procedures used in animal production (40, 41).

In the Nordic region, strong trust among farmers, consumers, educators, researchers and governmental agencies has enabled introduction and implementation of both strict legislation and recommendations on antibiotic use in animal production. This approach is widely embraced in the society and the recommendations rely on the willingness of all stakeholders to cooperate. This, as well as industry initiatives, have resulted in a marked decrease in antibiotic use across the Nordic countries during the past decades. Simultaneously, milk SCC levels and occurrence of clinical mastitis have decreased in most countries or remained stable. Production and health parameters have been recorded at cow level in the Nordic countries for decades, mainly for breeding reasons, but also to monitor health. These data have played a central role in establishing control and/or eradication programs for different diseases in the region, including mastitis. High quality milk production is also a result of this tradition.

It should be noted that dairy herd sizes in the Nordic countries are small when compared to other regions with major milk production. This has likely contributed to the current situation with low antibiotic usage and good animal health. It is also important to note that while veterinarians and producers in many other countries and regions are now adjusting to a new situation where antibiotic usage is becoming more regulated, the Nordic counterparts have always lived in that situation and consider it the norm. However, herd sizes are currently increasing everywhere, also in the Nordic countries. Optimal management and continued monitoring of animal health, milk quality and antibiotic consumption will be key elements in maintaining our favorable situation even as herds grow larger. Up-to-date knowledge of causal agents of intramammary infections, especially in free-stall and AMS farms will be crucially important in larger herds. This will assist in preventing and rapidly controlling potential spread of contagious pathogens, such as *Streptococcus agalactiae*. This pathogen had been eradicated decades ago from all Nordic countries, but it has recently re-emerged in some Nordic dairy herds, and interestingly, it is displaying also a potential feco-oral transmission routes (42).

We hope that the Nordic approach to dairy production might serve as an inspiration so that bacteriologic diagnosis of mastitic milk samples before initiation of antibiotic treatments, use of narrow-spectrum antibiotics and selective DCT can become the norm also in other regions. The Nordic experience provides evidence that prudent use of antibiotics is possible, without sacrificing animal health or milk quality. Continuous education of veterinarians and producers is pivotal to maintain the favorable situation in the region. In addition to veterinarians, farmers and legislators, it is also important that the pharmaceutical industry understands this strategy and ensures availability of suitable, narrow-spectrum antibiotics.

In conclusion, the Nordic experience shows that it is possible to maintain low incidence of clinical mastitis and acceptable SCC levels with prudent use of antibiotics and selective DCT based on bacteriologic diagnosis of intramammary infections in a region with high milk production.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

All authors have equally contributed to the planning, writing and editing of the manuscript.

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Parenteral Antimicrobial Treatment Diminishes Fecal *Bifidobacterium* Quantity but Has No Impact on Health in Neonatal Dairy Calves: Data From a Field Trial

*Olivia C. O'Keefe, Dale A. Moore, Craig S. McConnel and William M. Sischo**

Field Disease Investigation Unit, Department of Veterinary Clinical Sciences, Washington State University, Pullman, WA, United States

There is evidence that neonatal calves are over treated with antimicrobials that may disrupt colonization of their gastrointestinal tract (GIT) microbiota. The study objectives were to assess the decision-making process of antimicrobial use on a commercial dairy and impacts of parenteral antibiotics on dairy calves' GIT *Bifidobacterium* and calf health. Unhealthy pre-weaned dairy calves were enrolled based on farm personnel identification with age-matched healthy calves. Half the calves in each group were treated with a 3-day course of IM ampicillin and half were given supportive therapy as needed. Health scores (appetite, fecal consistency, attitude, and temperature) were recorded twice daily throughout the study. Because of inconsistency in employee health decisions, the 121 enrolled calves were reassessed using objective clinical observations plus fecal dry matter and placed into 1 of 3 health categories: healthy, uncomplicated diarrhea (bright attitude and good appetite but with diarrhea), and sick. Accounting for treatment group allocation, this resulted in six post-enrollment health and treatment categories. Calves were followed daily for 14 days post-enrollment and fecal samples collected at 6 time points and *Bifidobacterium* was quantified from these samples using quantitative PCR. The objective criteria for disease definition reclassified many "unhealthy" calves as uncomplicated diarrhea. Including all calves, on average, the quantity of *Bifidobacterium* decreased from the day of enrollment (median 8 days of age) across time to 14 days post-enrollment. Calves given an antibiotic the day of enrollment had a greater decrease in *Bifidobacterium* 4 and 9 days later relative to enrollment *Bifidobacterium* compared to untreated calves. At enrollment, sick calves and those categorized as uncomplicated diarrhea were more likely to have low *Bifidobacterium* counts and less likely to be categorized as healthy following antimicrobial treatment. Our results indicate that relying on farm personnel to identify morbidity may lead to some clinical misclassification. There was no indication that antimicrobials affected subsequent health outcomes, but antimicrobials did impact *Bifidobacterium* dynamics. These results highlight the importance and difficulty in assigning appropriate illness classification on farms and point to a need to develop better point of care diagnostics that improve calf husbandry and stewardship of antimicrobials.

Keywords: *Bifidobacterium*, dairy, calf, antimicrobial, ampicillin, diarrhea

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****Correspondence:***
William M. Sischo
wmsischo@wsu.edu

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INTRODUCTION

Antimicrobials are a common tool used to manage calf health and treat pre-weaning calf diseases. From a U.S, national survey of heifer rearing nearly all calves with respiratory signs and 75% of calves observed with diarrhea were treated with an antimicrobial (1). In a study involving calf raising facilities, 82% of calves observed with respiratory signs and 73% of calves observed with diarrhea were treated with an antimicrobial (2). This has led to discussions about the appropriate use of antimicrobials to treat disease in pre-weaned calves and whether diarrhea (a common reason that an antimicrobial is administered to a calf) is a symptom in a disease spectrum rather than a disease (3). One part of the discussion is that severe diarrhea should receive an antimicrobial to prevent septicemia and reduce mortality (4). A second part of the discussion is that GI disease identified only by observed diarrhea is over diagnosed and consequently antimicrobials are overused. In these cases, supportive therapy should be the first line treatment rather than antimicrobials (5, 6). While investigators have collected data regarding the frequency and type of antibiotics used to treat calves, little is known about the on-farm decision making process regarding calf health, the decision-making process for using antimicrobials, or the consequences of overuse. While there is evidence that overuse of antimicrobials is associated with diarrhea (6), little is known about overuse of antimicrobials on animal health over time, productivity, or gut microbiome subsequent or concomitant to treatment.

After birth, the neonatal GIT is rapidly colonized with a wide array of microorganisms and transitions as the animal matures. The transition to a stable GIT microbiota occurs early on in life; the exact age it occurs depends on the species. In dairy calves, there is an increase in the diversity and stability of the GIT microbiota over the first few months of age (7). This colonization is critical as interaction between the microbiota and the animal plays a key role in the development of the mucosal immune system (8) and is linked to resistance or susceptibility to diseases later in life. This suggests that the relationship between the GIT and immune system is most impacted in early life when the microbiome colonization of the GIT is variable (9). For cattle, if the normal developmental process of the intestinal microbiota in early life is disturbed, there may be long lasting health effects to the host (8) and downstream production performance (10).

There are data suggesting that antimicrobials impact the GIT and have negative health outcomes. Children treated with antimicrobials within the first 6 months of life are associated with an increased susceptibility to allergies, asthma, wheezing, eczema, inflammatory bowel disease, obesity, and type 2 diabetes mellitus later in life (9) It has been reported that intrapartum antibiotics resulted in altered microbiome in infants in the first weeks of life (11). When multiple antibiotics were given intrapartum, infants had lower GIT diversity as well as different bacterial communities at 6 weeks (12). There is evidence demonstrating the detrimental effects of antibiotic exposure in early life on the developing GIT as well as gastrointestinal microbiota composition in the adult (8, 11, 12). In calves, a study evaluating feeding low concentrations of antibiotics suggested they impacted relative abundance of genes coding for microbial cell functions and increased relative abundance of antibiotic resistance genes (13). Another study found no effect of oral antibiotics on ruminal microbiome (14). A study evaluating therapeutic and subtherapeutic oral oxytetracycline found a transient effect on the microbiome in the therapeutic group but observed no impact of subtherapeutic oxytetracycline on the microbiome. Differences between calves was mainly attributed to temporal changes across sampling times likely reflecting normal maturation (15).

Bifidobacterium species have been identified in the human health literature as critical members of the GIT microbiota with important functions within the colon that are associated with host health (16). Decreased abundance of these bacteria has been associated with diarrhea, obesity, and allergies. They also appear to support maturation of the immune system, support gut barrier functions, and protect against pathogens. Because it is presumed that *Bifidobacterium* plays a similar role in calves there are studies investigating probiotic feeding and its impact on GIT *Bifidobacterium* levels and health. Calves fed a supplemental bifidobacterial probiotic in an extensive housing system with their dams and that received mainly whole milk showed persistent, high levels of *Bifidobacterium* compared to calves also supplemented but reared in an intensive system without their dam and received a milk plus a supplemented concentrate diet (17). Although the study was confounded using different calf breeds in the two systems, the authors suggested that a pre-dominant milk diet influenced the persistence of fed supplemental bifidobacterial probiotics. As indirect evidence for probiotic impact on the microbiome, calves fed a multispecies probiotic of bacteria including *Bifdobacterium* spp. at the onset of diarrhea had faster resolution of diarrhea, but there was no difference in average daily weight gain (ADG) compared to placebo-treated control calves, suggesting only a short term effect of supplementation (18). Another study supporting the previous finding showed that probiotics had little to no impact on ADG or feeding behavior (19). Studies have also shown that probiotic supplementation resulted in a transient increase in abundance of *Bifidobacterium* spp. and was associated with fewer *E. coli* in calves' GIT and overall good health (20, 21). Another study showed that colostrum changed the GIT microbial community and enhanced the abundance of *Bifidobacterium* (22). While it appears that management to support and enhance *Bifidobacterium* exists there is a research gap in how other management interventions, particularly parenteral antimicrobials might impact GIT microbiota in calves and specifically *Bifidobacterium* spp.

The objectives of this study were to investigate the effect of parenteral treatment of healthy and unhealthy pre-weaned calves with antimicrobials on objective measures of pre-weaned calf health, growth, subsequent reproduction and the dynamics of fecal *Bifidobacterium* spp. The hypotheses were that parenteral antimicrobial treatment would negatively impact calf health, growth, and reproduction as well as dampen the normal dynamics of fecal *Bifidobacterium*, though these effects may be conditional on the health status of treated calves. In addition, we investigated the relationship between objective measures of

pre-weaned calf health with decisions made by on-farm calf caretakers and their assessment of calf health.

MATERIALS AND METHODS

Ethics Statement

The research protocol was reviewed and approved by the Institutional Animal Care and use Committee of Washington State University (ASAF 04925). All protocols involving calves housed on the commercial dairy farm were authorized by the farm owner, who was aware of all procedures.

Study Design and Calf Enrollment

The study was conducted on a commercial dairy farm in the Pacific Northwest, USA. The farm milked 3,000 Holstein and Holstein-Jersey mix cows and raised all their replacement heifers. Study personnel worked with on-farm staff to identify animals for enrollment and conduct the study. All heifer calves born on the farm were fed previously collected and frozen, single source colostrum (3.8 L) within 2 h of calving and transferred within 24–48 h to the calf rearing facility that was separate but part of the dairy property. All calves entering the calf housing area were eligible to participate in the study unless they were involved in a dystocia, twin birth, or limb abnormality. Calf body weight was recorded at 24–48 h of age (median age = 24 h). At the same time, blood samples were obtained via jugular venipuncture to assess passive transfer of immunity by measuring total serum protein (TSP). From these blood samples, serum was obtained, and TSP values measured using a calibrated, clinical refractometer. Calves with TSP concentrations <5.2 g/dL indicated failure of transfer of passive immunity and were excluded from the study (23). For the study, TSP was summarized using quartiles and two categories created, low (as below the 25th percentile) and adequate (above the 25th percentile).

On-farm personnel were responsible for all the primary care of calves including feeding, cleaning, watering, bedding maintenance, and health assessments. This work involved three employees and the two employees tasked with the full-time care of calves had worked with calves on this farm for more than 5 years. One person was responsible for health decisions and feeding and cleaning protocols 6-days per week, one person supported feeding and cleaning 5-days per week and was responsible for health decisions 1-day per week, and one person supported feeding and cleaning 2-days per week (filling in for the regular team on their days off). Calves were housed in individual hutches with straw bedding that was renewed weekly and fed ∼2.8 liters of whole unpasteurized milk from the farm's bulk tank milk dispensed into a bucket twice daily. Calves had ad-libitum access to grass hay and a grain-based starter feed mixture beginning at 4 days after birth. The starter feed was a farm-made ration that was 10% forage (generally grass hay) and 90% concentrate consisting of ground corn ears, corn dried distillers' grains, canola and soybean meal plus molasses to achieve a crude protein level of 25%. Water was available between milk feedings.

The study was designed to enroll eligible Holstein or Holstein-cross heifers at the first sign of disease (unhealthy). Simultaneously, an age-matched heifer with no clinical signs (healthy) was also enrolled. These initial health status decisions were made by farm personnel following the morning milk feeding. Although calf caretakers received *ad-hoc*, on the job training and a veterinarian was available to answer questions, the farm did not have specific protocols for assessing calf health and calves were identified as either unhealthy or healthy primarily based on workers' experience and supporting visual health observations such as attitude, appetite, posture, stool consistency, and risk age. Study personnel randomly allocated (using a pre-generated list) calves in each worker-identified group (healthy and unhealthy) to be treated by calf caretakers with either 3 mg/kg ampicillin trihydrate (Polyflex, Boehringer Ingelheim Vetmedica, Inc.) by intramuscular (IM) injection and 2.8 L oral bottle-fed electrolytes (Calva Lyte™, Calva Products LLC, Acampo CA) or given oral electrolytes alone. Based on employee discretion, calves could receive an ancillary therapy of bismuth subsalicylate. This resulted in an initial 4 study groups: (1) unhealthy-treated with an antimicrobial, (2) healthy-with no antimicrobial treatments, (3) healthy-treated with an antimicrobial, and (4) unhealthy-with no antimicrobial treatment. Based on farm protocols, calves enrolled in the antibiotic treatment groups were treated at enrollment and at 24-h intervals for a total of 3 treatment days. The choice of antimicrobial and protocol for administering it were a farm decision. If at any point in the study a calf demonstrated declining health indicated by an elevated body temperature (≥39.4°C) with decreased appetite and dull or depressed attitude, the calf was dropped from the study and was medically treated by farm personnel. On-farm personnel were not blinded to calf treatment group assignments.

Data Collection

Fecal Samples

Because enrollment in the study (E1) did not begin until calves were identified as unhealthy and to ensure that we had a fecal sample from the day prior to enrollment (E0), commencing at 24–48 h post-parturition (P2) and daily thereafter, fecal samples from all calves eligible to be enrolled into the study were collected by digital rectal stimulation into sterile sampling bags (Thermo Fisher Scientific, USA). These samples were frozen on dry ice on the farm and subsequently transferred to the laboratory and stored at −80°C. As calves were enrolled into the trial as unhealthy or healthy controls, fecal samples were collected on enrollment day (E1), 4 days post-enrollment and the day following the final day of antimicrobial treatment (E4), 9 days post-enrollment (E9), and 14 days post-enrollment (E14). In addition to those samples, fecal samples analyzed in the study included the P2 and E0 samples.

Health Assessment

Twice daily, prior to feeding, study personnel blinded to calf group assignment independently observed all eligible and enrolled calves and recorded a series of assessments including: attitude (A = alert; AS = alert and sternal; D = dull/depressed; NA = non-responsive), a visual assessment of fecal consistency as observed from outside the hutch (0 = well-formed fecal samples; 1 = semi-formed fecal samples; 2 = loose fecal samples;

3 = watery fecal samples), and respiratory signs (normal, eye discharge, nasal discharge, and spontaneous cough). After the AM and PM feedings, appetite was scored (Y = good appetite, finished milk; N = did not finish milk; S = slow to finish milk; T = tube fed). Assessments began at P2 through E14. Rectal temperature was recorded for all calves at E1 and subsequently on sampling days E4, E9, and E14. Using on-farm records (Dairy Comp 305, VAS, Tulare CA), study calves were followed through to their first calving.

Fecal Dry Matter

Fecal samples collected at P2, E0, E1, E4, E9, and E14 were assessed for total dry matter by weighing out 2.5 grams of raw sample and drying the sample in an incubator at 25°C for 24 h. Percent dry matter was calculated as the difference between dry weight and wet weight divided by wet weight and multiplied by 100.

Average Daily Gain

All eligible calves were weighed (in pounds and converted to kg) at P2 using a balance calf scale (Paul Scale, Livestock Systems, Duncan OK, USA) that was calibrated with free weights before each use. At weaning (average = 57 days old), all enrolled calves were weighed again, and weaning age noted. Average daily gain was the difference between weaning weight and P2 weight relative to the weaning age (days). For analyses using P2 calf weight, it was summarized using quantiles and three categories created as below the 25th percentile, within the interquartile range (IQR), and above the 75th percentile.

Bacterial DNA Extraction From Fecal Samples and qPCR to Quantify Bifidobacterium

Bacterial DNA was extracted from fecal samples using the MagMAX™ Total Nucleic Acid Isolation kit (Thermo Fisher Scientific, USA). Briefly, frozen fecal samples were thawed at room temperature, manually mixed and 300 mg of this sample was removed and suspended in 1 ml of PBS. This suspension was centrifuged at 100 RPM for 1 min to pellet gross solids. After centrifugation, 175 μl of the supernatant was removed and added to 235 μl lysis buffer provided by the kit manufacturer using a bead tube. This mixture was homogenized using a bead mill (Bead Mill, Fisher Scientific). The homogenized sample was centrifuged at 16,000 g for 10 min and 300 μl of the supernatant was removed and centrifuged at 16,000 g for 10 min to clarify. Following this centrifugation step, 115 μl was removed and transferred to the MagMax DNA extraction plate. Isolation was completed following manufacturer's directions in conjunction with the MagMax Express automated system (Applied BioSystems, USA). The final volume of extracted DNA was 90 μL.

The heat shock proteins in bacteria are highly conserved proteins and specific to bacterial genus and species, including the *gro*ES gene. Identification and quantification of *Bifidobacterium* spp. was carried out using qPCR targeting the bifidobacterial specific *gro*ES gene. The following oligonucleotide sequences were used to detect *gro*ES: gro-1 (5-CTCACACCGTTGGAAG-3) (forward) and gro-2 (5-GN(CA)GGAGACGATGAGGTA-3) (reverse) (24). A single qPCR reaction was performed containing 10 μL SsoAdvanced Universal SYBER Green Supermix (BioRad, USA), 1 μL forward and reverse primers (5 μM stock solution), 6 μL PCR nuclease-free water (Thermo Fisher Scientific, USA), and 2 μL fecal DNA template.

Quantification of PCR product was estimated from a standard curve developed from a sequence confirmed *Bifidobacterium longum* (Q349). Briefly, DNA was extracted from Q349 using a 5% chelex resin following a boil cell lysate procedure and the *gro*ES gene was amplified using PCR to obtain amplicons for cloning. PCR products were purified using QuiQuick PCR Purification Kit (Qiagen, MD). Cloning of our target sequence was done using a TOPO TA cloning kit dual promoter pCRII-TOPT vector (Invitrogen-ThermoFischer Scientific, MA, USA) per manufacturer's instructions using a One Shot™ TOP10 chemically competent *E. coli* (Invitrogen) as the host. Transformed cells were plated to LB agar containing 50 μg/ml of ampicillin (imMedia AMP Agar Invitrogen Q60120) and 40 mg/ml of X-Gal. Plates were incubated overnight at 37°C. Following incubation, 2–6 white or light blue colonies were selected and transferred to LB medium containing 50 μg/ml of ampicillin. Plasmid prep on culture was performed using Qiagen Plasmid Max prep kit (Qiagen 12163, Qiagen, USA). Transformation was confirmed by PCR. The number of copies were calculated using a portion of the transformed cells stock solution to create a standard curve (10^2-10^8, 7-points). Copies were calculated using the formula in the TOPO TA cloning kit protocol (Invitrogen, USA). Stock solution of transformed cells were stored in a 20% glycerol solution and kept frozen at −80C.

Amplification reactions were performed on an ABI StepOne Plus real time instrument (Applied Biosystems, USA). Amplification was carried out at 95°C for 1 min followed by 95°C for 30 s and 60°C for 30 s for a total of 40 cycles. Quantification estimates were generated based on the values generated from the standard curve and using the StepOne Plus 2.3v software (Applied BioSystems, USA). These estimates were adjusted to reflect copy number/gram of feces (copies/gm). Samples with a melt temperature between 87°C and 90.9°C but no amplification by 40 cycles were deemed to reflect a positive sample and used in analyses by randomly assigning a value between 0 and level of detection for the assay (10^2 target copies). All samples, including external standards and non-template control, were run in duplicate.

Data Analysis

Sample Size

Sample size was based on detecting differences in the temporal pattern of *Bifidobacterium* in the pre-weaning period. The assumptions for sample size determination were observing at least a 2 $\log_{10}$ difference in change of *Bifidobacterium* between time points and conditional on calf antimicrobial treatment status at E1 with an α and β error of 0.1. Based on experience, we assumed a variance of 3.6 $\log_{10}$. Sample size was calculated using R (R Project for Statistical Computing Version 4.0.2) package pwr. Estimated sample size was 56 calves per group and assuming a 10% loss to follow-up we determined a total sample of at least 61 calves per treatment group.

Disease Categories

The original enrollment criteria for the study as "unhealthy" and "healthy" were based on decisions made by farm personnel and these enrolled calves were then randomly assigned to an antibiotic treatment or supportive care only category. In parallel, all enrolled calves were independently assessed for appetite and pre-meal attitude by study personnel and a fecal sample collected and dry matter determined. Rectal temperature was measured on all calves identified as "unhealthy" by farm personnel. In addition, calves were evaluated for respiratory signs and few calves were identified with either ocular or nasal discharge and none were observed with otitis or voluntary cough. These data (excluding observations of respiratory signs) were used to create three post-enrollment health categories (**Table 1**). These categories were then used to classify calf health at all the sampling time points (P2, E0, E1, E4, E9, and E14) in all data analyses.

Summarizing and Modeling Changes in fecal Bifidobacterium Across Study Follow-Up

From qPCR findings, results were standardized to copy number per gram of fecal material (copies/gm) and $\log_{10}$ transformed. Means, minimum, maximum, medians, interquartile ranges, and contingency tables were determined to assess data distributions and make simple comparisons. Log_{10} *Bifidobacterium* (copies/gm) were summarized at each sampling time and compared using R and packages lme4 and emmean to calculate estimated marginal means. Temporal changes in $\log_{10}$ *Bifidobacterium* qPCR quantity between sampling times (E4, E9, and E14) and enrollment (E1) were determined and these differences summarized using quartiles and difference categories at each time point were developed based on below the 25th percentile, IQR, and above the 75th percentile and used as outcomes in multinomial logistic regression (R package, nnet). Multinomial logistic regression was used to assess relationships between temporal changes in $\log_{10}$ *Bifidobacterium* as the outcome. Initial models included risk factors associated with P2 (TSP categories, breed, and birth weight categories). Additional risk factors included in the initial models included those associated with E1 (antimicrobial exposure and health category), health categories on a sampling date as well as health category at prior sampling times, and interactions between E1 antimicrobial exposure and health categories. The goal for final models was to include risk factors or exposure variables associated with parsimonious models guided by AIC and improving residual deviance. Because antimicrobial exposure at E1 was the main effect evaluated in our study, it was retained in all models. Odds ratios (OR) with their 90% confidence intervals are reported.

TABLE 1 | Post-enrollment health score criteria for categorizing healthy and sick calves at enrollment during pre-weaned period.

Health variable	Health category		
	Healthy	**Uncomplicated diarrhea**	**Sick**[a]
Attitude[b]	Alert or alert-sternal	Alert or alert-sternal	Dull/depressed or non-responsive
Fecal DM[c]	DM >17.0%	DM ≤17.0%	DM ≤17.0%
Appetite[d]	Finished milk meal	Finished milk meal	Did not finish milk meal, or slow to finish milk meal, or milk meal fed via esophageal feeder
Rectal temperature	<39.4°C	<39.4°C	≥39.4°C

[a] *Calf was classified as sick if DM ≤17% and one other abnormal clinical sign or any single or combination of abnormal attitude, appetite, or rectal temperature.*
[b] *Observation of attitude prior to feeding.*
[c] *DM, Dry matter.*
[d] *Appetite at meal prior to enrollment.*

Summarizing and Modeling Health Categories Across Study Follow-Up

Calves were assigned to health categories based on criteria shown in **Table 1** independently for each sampling time point. These categories were used as outcomes in a set of multinomial logistic regression models (R project, nnet) that assessed risk factors for health for sampling points E1, E4, E9, and E14. Initial models included risk factors associated with P2 (TSP category, breed, and birth weight category), those associated with E1 (antimicrobial exposure and health category), and factors associated with the sampling date including $\log_{10}$ *Bifidobacterium* copies/gm categories, bismuth as an ancillary therapy, and health category at prior sampling times. Log_{10} *Bifidobacterium* was summarized for each sampling time using quartiles to create three categories (unique to each sampling time): below the 25th percentile, IQR, and above the 75th percentile. The goal for final models was to include risk factors or exposure variables associated with parsimonious models guided by AIC and improving residual deviance. Because antimicrobial exposure at E1 was the main effect evaluated in our study, it was retained in all models. OR and their 90% confidence intervals are reported.

Modeling Pre-weaning Average Daily Gain

Pre-weaning average daily gain (ADG) was calculated as the difference between weaning weight and P2 weight divided by weaning age (days). Because we were most interested in describing associations with low performers relative to high performers, ADG was summarized using quartiles and three categories created based on ADG below the 25th percentile (low performers), IQR, and above the 75th percentile (high performers). The ADG categories were used as outcomes in a multinomial logistic regression (R package nnet). Initial models included risk factors associated with P2 (TSP, breed, and birth weight category), those associated with E1 (antimicrobial exposure and health category), and factors associated with pre-weaning sampling including appetite (yes, finished milk meals between E1 andE9 or no, did not finish two or more milk meals between E1 andE9), temporal changes in $\log_{10}$ *Bifidobacterium* copies/gm at pre-weaning sampling times, and health categories at sampling times E1, E4, E9, and E14. As described previously, the goal for the final model was to include risk factors or

exposure variables associated with parsimony guided by AIC and improving residual deviance. Because antimicrobial exposure at E1 was the main effect evaluated in our study, it was retained in the final model. OR with their 90% confidence intervals are reported.

Modeling Age to First Calving

A proportional hazards model was used to assess time to first calving. The model building approach was similar to that described for ADG assessment. Risk factors included were those associated with P2, E1, and cumulative events across the follow up period including ADG. The goal for the final model was to include risk factors or exposure variables associated with parsimony guided by a Likelihood-Ratio test. The R package survival was used to create the final model. Hazard ratios were determined with their 90% confidence intervals.

RESULTS

Enrollment Data

A total of 121 heifers were enrolled in this field trial (85 Holstein and 36 Holstein-Jersey cross). No animals were removed from the study because of deteriorating health although five calves died during the pre-weaning period. The median age of enrolled calves was 8 days with an IQR of 2 which ranged between 7 and 9 days. At enrollment (E1), calves were identified by on-farm personnel as needing treatment and a similar calf not needing treatment were randomly allocated to one of the four study groups with 31, 32, 31, and 27 calves assigned to unhealthy and receiving an antimicrobial, healthy and no antimicrobial treatment, healthy and receiving an antimicrobial, and unhealthy and no antimicrobial treatment, respectively. The median value for total serum protein (TSP) for the enrolled calves was 6.2 g/dL with an IQR of 0.9 (range = 5.7–6.6g/dL). At enrollment, the median calf weight was 37 kg with an IQR of 8 kg (range = 34–42 kg).

At E1 a fecal sample was collected, and a portion used to determine dry matter (DM). The fecal DM ranged from 4 to 43% with a median DM value of 26.1%. **Figure 1** shows the box and whisker plots of DM by observed fecal score on day E1. Although there was variability in DM at each of the four fecal scores as well as overlap between scores, the trend was a decreasing median DM with increasing fecal scores. Based on these data, we defined a diarrhea event as a DM ≤17% which was near the 75th percentile for a fecal score of two and the 25th percentile for a fecal score of 1. Using DM ≤17% as a definition of diarrhea, 11/81 calves with fecal scores of 0 or 1 were reclassified as diarrhea and 6/37 calves with fecal scores of 2 or 3 were reclassified as normal.

We compared the decision that a calf was unhealthy made by on-farm personnel to objective criteria noted by study personnel at enrollment (attitude, appetite, and rectal temperature) combined with measured DM and applied the decision tool described in **Table 1**. At enrollment, no calves were observed with respiratory signs suggesting bovine respiratory disease (BRD). Of the 121 calves enrolled in the study, on the day of enrollment, 43 (based on DM ≤17%) were scored with diarrhea, 15 were noted as depressed, 15 did not finish the milk meal prior to enrollment, and seven calves were identified with elevated rectal temperature (≥39.4°C) by study personnel. Based on these data, 66/121 calves (54%) remained in their original enrollment groups based on farm personnel decisions (**Table 2**). Twenty-nine (24%) calves were reclassified as uncomplicated diarrhea; the majority of which were reclassified from the original unhealthy category. Those reclassified calves represented the greatest change in the original risk classification where only 20/58 (34%) remained objectively classified as unhealthy (sick). For all subsequent analyses, calves were allocated to one of six objective disease categories as antibiotic treated or not in the health categories sick, uncomplicated diarrhea, and healthy.

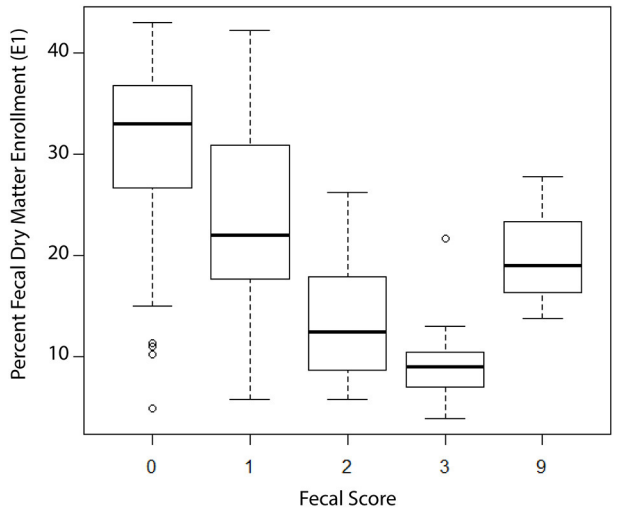

FIGURE 1 | Box and whisker plots of fecal dry matter (DM) stratified by observed fecal score at enrollment of calves into the study, $n = 121$ calves. Fecal Score Definition: 0 = well-formed fecal samples; 1 = semi-formed fecal samples; 2 = loose fecal samples; 3 = watery fecal samples; 9 = not scored.

Across the six treatment and health categories there was variability for median P2 calf weight and TSP determined at P2 (**Table 3**). Median P2 weights tended to be lower for calves at E1 classified as sick relative to healthy calves although there was considerable overlap in the range of weights within the IQR. Median TSP values across the groups ranged from 5.9 to 6.3 g/dl and would be classified as good to excellent based on recent published recommendations (25). We used the farm level TSP distribution to create categories for subsequent analyses. The TSP values equal or below the overall 1st quartile (≤5.7 g/dl) were categorized as low and values above the 1st quartile as adequate. Similarly, P2 body weight was categorized based on "light" being equal or below the 25th percentile (≤33.6 kg) and "heavy" being equal or greater than the 75th percentile (≥41.8 kg). Calves weighing within the IQR were called "medium."

Bifidobacterium spp. Temporal Trends and Effect of Antimicrobial Use and Illness on Those Temporal Trends

The median, IQR, and Estimated Marginal Means (EMM) for calf $\log_{10}$ *Bifidobacterium* spp. copies/gm stratified by study days

TABLE 2 | Comparison of study group allocations based on farm personnel decisions and post-enrollment criteria based on symptoms for 121 pre-weaning dairy calves.

Health category determined by farm personnel and random treatment assignment at enrollment	Post-enrollment health category based on symptoms (Table 1) and treatment assignment at enrollment						
	Sick AM[a]	Sick No AM	Uncomplicated diarrhea AM	Uncomplicated diarrhea No AM	Healthy AM	Healthy No AM	Total
Unhealthy AM	12	0	9	0	10	0	31
Unhealthy No AM	0	8	0	13	0	6	27
Healthy AM	5	0	5	0	21	0	31
Healthy No AM	0	5	0	2	0	25	32
Total	17	13	14	15	31	31	121

[a]*AM = intramuscular antimicrobial administered at enrollment (3 mg/kg ampicillin trihydrate over three consecutive days).*

TABLE 3 | Distribution of calves' total serum protein concentration (TSP), bodyweight the day after birth, and assigned TSP category (adequate >5.7 g/dL, low ≤5.7 g/dL) by *post-hoc* study group at enrollment.

Treatment Category	N	TSP (g/dL)		TSP Category		Day 1 body weight (kg)	
		Median	IQR (range)	Adequate	Low	Median	IQR (range)
Sick AM	17	6.3	0.5 (6.0–6.5)	13	4	35.9	6.3 (33.2–39.5)
Sick No AM	13	6.2	0.7 (5.8–6.5)	11	2	35.9	6.9 (34.5–41.4)
Uncomplicated diarrhea AM	14	6.0	0.6 (5.5–6.1)	8	6	36.8	7.8 (34.0–41.8)
Uncomplicated diarrhea No AM	15	6.0	1.0 (5.6–6.6)	10	5	36.8	5.7 (33.2–38.9)
Healthy AM	31	6.3	0.7 (5.9–6.6)	24	7	38.2	9.3 (33.4–42.7)
Healthy No AM	31	5.9	1.2 (5.5–6.7)	19	12	40.5	8.8 (34.5–42.3)
Overall	121	6.2	0.9 (5.7–6.6)	85	36	37.3	8.2 (33.6–41.8)

are shown (**Table 4**). There was a temporal trend over the course of sampling with $\log_{10}$ *Bifidobacterium* spp. quantity increasing from P2 to E0 with the highest quantities at E0 and E1 and diminishing in subsequent samplings (E4, E9, and E14). This was most notable in the later samplings as *Bifidobacterium* spp. The EMM decreased ∼2 logs between sampling days E1 and E14.

The association of antimicrobial exposure and disease categories with temporal trend of $\log_{10}$ *Bifidobacterium* spp. (copies/gm) was assessed using the outcome measure of difference in the amount of *Bifidobacterium* spp. between sampling times (E4, E9, or E14) and E1. **Figure 2** depicts notched box and whisker plots overlaid with the individual calves' difference values at each of the assessed time points. There was more visible variability in the difference values for antimicrobial treated calves relative to the untreated calves. In addition, while there was discernable overlap for IQR values between treated and untreated calves (less so at E9-E1) and overlap of notches it was clear that treated calves tended to have more relative negative values than those untreated. In addition, there was tendency for some of the distributions to be bimodal. Consequently, for subsequent data analyses at each sampling time point we created three categories as outcome variables to reflect temporal trends.

Difference in Log_{10} Bifidobacterium Quantity Between E4 and E1

Difference in $\log_{10}$ quantity between E4 and E1 ranged in value from −10.4 (decrease) to 10.6 (increase) with a median difference of −0.4. This difference was categorized into three outcome variables based on the quartile distribution of below the 25th percentile (<-1.98), within the IQR (−1.98–1.71), and 75th percentile and above (>1.71).

A multinomial logistic regression using these difference categories in $\log_{10}$ *Bifidobacterium* spp. copies/gm between sampling days E4 and E1 as the outcome (reference group >1.71 $\log_{10}$ change in *Bifidobacterium* copies/gm) and treatment group at E1 (reference group = "did not receive antimicrobials") and objective disease categories at E1 and E4 as risk factors was determined. Calves receiving an antibiotic at E1 for 3

TABLE 4 | Distribution and summary values of $\log_{10}$ *Bifidobacterium* copy number/gram fecal for 121 calves as determined by qPCR, stratified by sampling day (P2 = day 2 of age, E0 = day prior to enrollment, E1= enrollment day and 1st follow-up day, E4 = 4th follow-up day, E9 = 9th follow-up day, and E14 = 14th follow-up day.

Sampling day	Median	IQR	Estimated marginal means (EMM)	90% CI
P2	7.0	3.1 (5.4–8.5)	6.53	6.18–6.88
E0	8.3	2.4 (6.7–9.1)	7.77	7.41–8.12
E1	7.8	2.3 (6.6–8.9)	7.68	7.33–8.02
E4	7.5	2.6 (6.3–8.9)	7.32	6.97–7.67
E9	6.8	2.4 (5.7–8.1)	6.68	6.33–7.03
E14	5.9	1.9 (5.0–6.9)	5.50	5.15–5.85

days were more likely to have a 1.98 $\log_{10}$ or greater decrease in *Bifidobacterium* copies/gm compared to calves receiving no antimicrobial therapy at E1. Calves that were categorized as sick at E1 or uncomplicated diarrhea were less likely to be in either the lowest or IQR *Bifidobacterium* spp. difference categories relative to healthy calves suggesting that sick calves at E1 had lower baseline than healthy calves (**Figure 3**). Breed was not a risk factor in this model or in any of the subsequent models.

Difference in Log_{10} Bifidobacterium Quantity Between E9 and E1

The difference in $\log_{10}$ quantity between E9 and E1 ranged in value from −9.53 to 8.48 with a median difference of −1.11 reflecting the overall trend of decreasing *Bifidobacterium* spp. over the sampling periods. This difference was also categorized into three outcome variables based on the quartile distribution of below the 25th percentile (<-2.54), IQR (−2.54–0.74), and 75th percentile (>0.74–reference group).

A multinomial logistic regression using the E9-E1 *Bifidobacterium* difference categories and risk factors of E1 antimicrobial category and health categories at E1, E4, and E9 found that calves receiving an antibiotic at E1 for 3 days were more likely to experience a 2.5 $\log_{10}$ or greater decrease in *Bifidobacterium* spp. between E1 to E9 compared to calves not receiving an antimicrobial. Calves classified sick at E1 were associated with a decreased likelihood of either a 2.5 $\log_{10}$ or greater decrease in *Bifidobacterium* spp. between E1 to E9 or in the IQR E9-E1 *Bifidobacterium* difference category compared to healthy calves. Calves with uncomplicated diarrhea at E1 were also less likely to experience a 2.5 $\log_{10}$ or greater decrease in *Bifidobacterium* spp. (**Figure 4**). There was no association between disease categories at E4 or E9 on *Bifidobacterium* difference category.

Difference in Log_{10} Bifidobacterium Quantity Between E14 and E1

The difference in $\log_{10}$ *Bifidobacterium* spp. quantity between E14 and E1 ranged in value from −10.8 to 8.5 with a median difference of −1.63 which reflected the overall trend that E14 sampling had the lowest median value for *Bifidobacterium* spp. content. This difference was categorized into three outcome variables based on the quartile distribution of below the 25th percentile (<−3.66), IQR [−3.66–(−0.033)], and above the 75th percentile (>−0.033).

A multinomial logistic regression using the E14-E1 *Bifidobacterium* difference categories found that calves categorized with uncomplicated diarrhea at E4 were less likely to experience a 3.6 $\log_{10}$ or greater decrease in *Bifidobacterium* spp. or in the IQR E14-E1 *Bifidobacterium* difference category compared to healthy calves. Sick calves at E4 were also less likely to be in the IQR *Bifidobacterium category*. In contrast, calves with uncomplicated diarrhea at E9 were more likely to be in the IQR *Bifidobacterium* category compared to healthy calves (**Figure 5**). There was no effect of antimicrobial exposure at E1 on the difference in $\log_{10}$ *Bifidobacterium* spp. between E14 and E1.

Risk Factors Associated With Health Categories at E1, E4, E9, and E14

Risk Factors Associated With Health–E1

The results of a multinomial logistic regression analysis for risks for disease category at enrollment (E1) as the outcome are shown (**Figure 6**). Calves classified as uncomplicated diarrhea were more likely to be in the lowest quantity of three categories for $\log_{10}$ *Bifidobacterium* (<6.6 $\log_{10}$ copies/gm) at E1 relative to healthy calves (quartile distributions shown in **Table 4**). Calves were also more likely to be categorized at enrollment as uncomplicated diarrhea if they were classified uncomplicated diarrhea or sick at E0. Calves categorized as sick compared to healthy on enrollment day were also associated with being in the lowest category for $\log_{10}$ fecal *Bifidobacterium* spp. at E1 or within the IQR for $\log_{10}$ fecal *Bifidobacterium* spp. on E1. If calves were classified as sick at E0 they were likely to be classified as sick at enrollment.

Risk Factors Associated With Health–E4

The results of a multinomial logistic regression for risks for health category at sampling day E4 (4 days following enrollment or 1 day after the last antimicrobial treatment) are shown (**Figure 7**). Calves classified as uncomplicated diarrhea compared to healthy at E4 were associated with being classified as sick at E1. Calves classified as sick compared to healthy at E4 were also associated with being classified as sick at E1 and being in the lowest birth weight classification. Neither antimicrobial treatment at E1

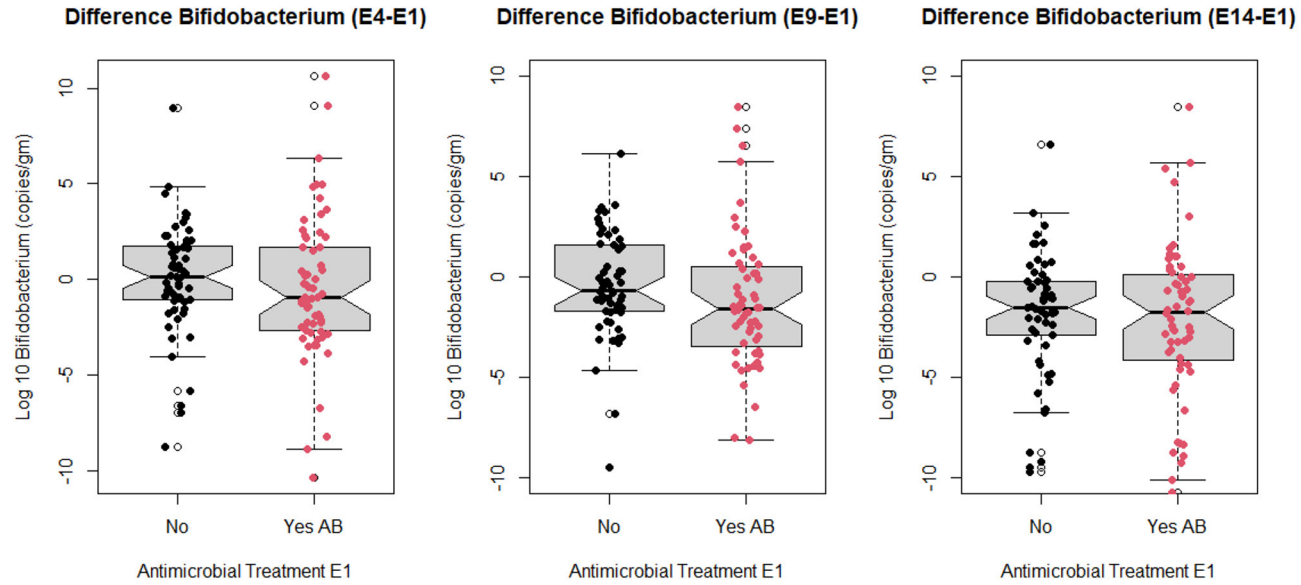

FIGURE 2 | Notched box and whisker plots of change in $\log_{10}$ *Bifidobacterium* copies/gram fecal between sampling day E4, E9, E14, and sampling day E1 stratified by antimicrobial treatment group at E1 (no antimicrobial and yes antimicrobial). Points on graph represent values for study calves at each sampling point (E1 = enrollment day and 1st follow-up day, E4 = 4th follow-up day, E9 = 9th follow-up day, and E14 = 14th follow-up day).

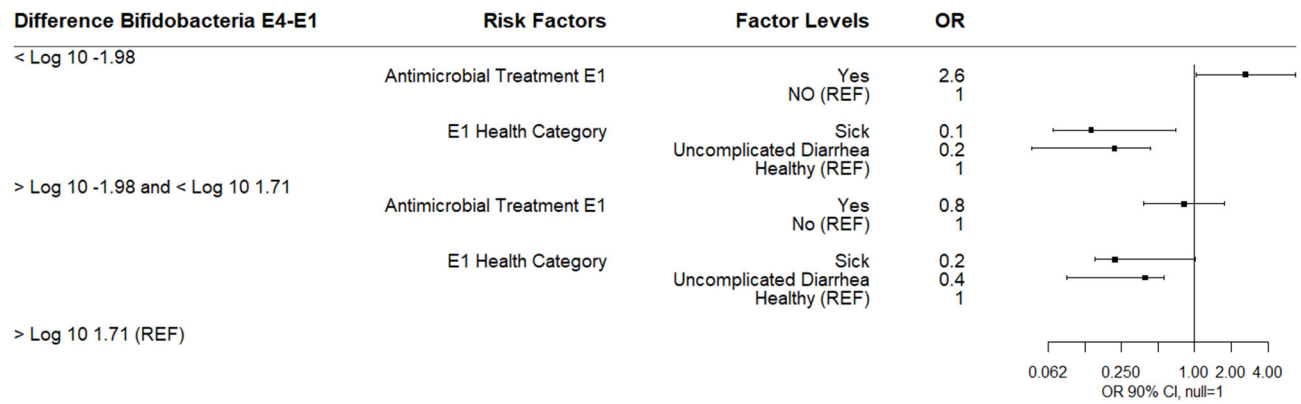

FIGURE 3 | Results of multinomial logistic regression modeling the change in Log_{10} *Bifidobacterium* copies/gram fecal between sampling day E4 and sampling day E1 (enrollment). Odds ratios and 90% confidence intervals are shown. E1 antimicrobial categories were defined as: yes (received 3-day course of intramuscular ampicillin) or no (did not receive an antimicrobial). E1 health categories were defined as: sick (diarrhea with not finishing milk meal or depressed attitude), uncomplicated diarrhea (diarrhea with no additional clinical signs), and healthy.

nor $\log_{10}$ *Bifidobacterium* copies/gm at E4 were associated with health category at E4 (**Figure 6**).

Risk Factors Associated With Health–E9 and E14

The multinomial logistic regression results for risks for health category at sampling day E9 (9 days post-enrollment) are shown (**Figure 8**). Calves categorized as sick at E9 were more likely to be observed as uncomplicated diarrhea or sick at E4 compared to healthy calves. No risk factors for uncomplicated diarrhea were noted. No association between disease category at E9 and antibiotic use at E1 or $\log_{10}$ E9 *Bifidobacterium* was observed.

By E14 (14 days post-enrollment), 74% of study calves were categorized as healthy. The only observed association in a multinomial logistic regression was for calves categorized as sick at E14 were more likely to have been categorized sick at E9. Neither antimicrobial treatment at E1 nor $\log_{10}$ *Bifidobacterium* at E14 were associated with health category at E14 (**Figure 9**).

Impact on Pre-weaning Average Daily Weight Gain

Study calves were weighed at arrival to the calf rearing area (day 1 after birth) and again at weaning. The average pre-weaning period for calves was 61 days (median = 60 days). The average

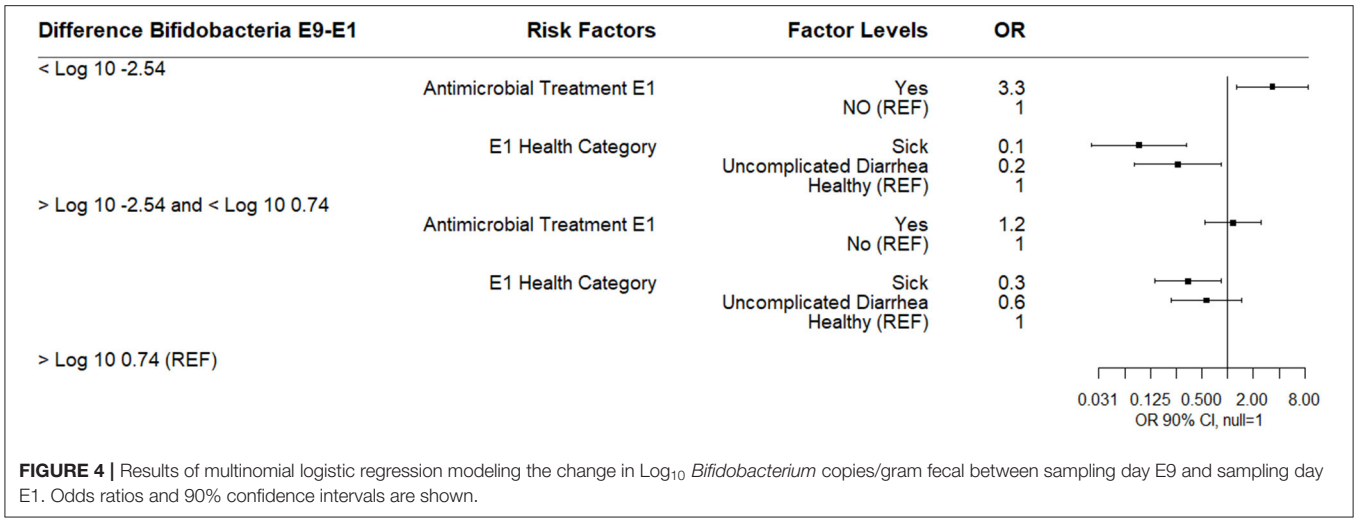

FIGURE 4 | Results of multinomial logistic regression modeling the change in Log_{10} *Bifidobacterium* copies/gram fecal between sampling day E9 and sampling day E1. Odds ratios and 90% confidence intervals are shown.

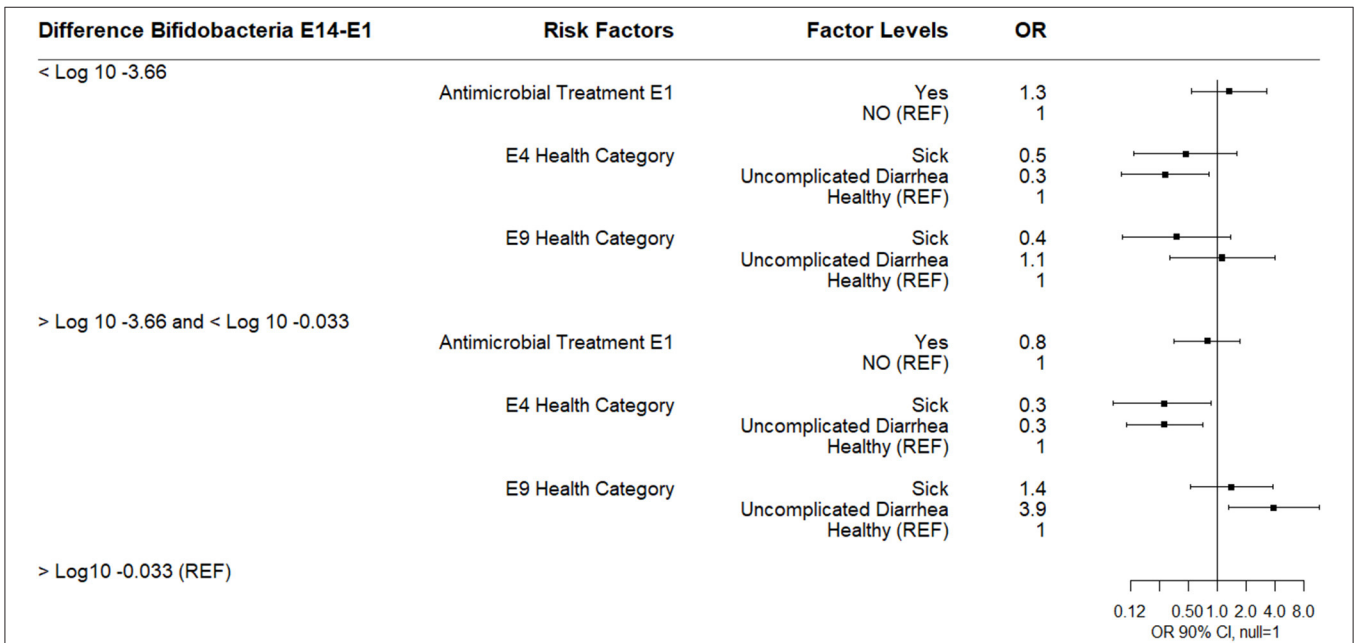

FIGURE 5 | Results of multinomial logistic regression modeling the change in Log_{10} *Bifidobacterium* copies/gram fecal between sampling day E14 and sampling day E1. Odds ratios and 90% confidence intervals are shown. E4 and E9 health categories were defined as: sick (diarrhea with not finishing milk meal and/or depressed attitude), uncomplicated diarrhea (diarrhea with no additional clinical signs), and healthy.

weaning weight was 80.7 kg (median = 80.9 kg) and ADG was 0.7 kg (median = 0.7 kg). For subsequent analysis, ADG was categorized into three levels based on quartile distribution: low <0.6 kg/day (25th percentile), medium ≥0.6 kg/day and <0.8 kg/day (IQR), and high ≥0.8 kg/day (75th percentile).

The results of a multinomial logistic regression for risks for ADG category as the dependent variable (high = reference group) are shown (**Figure 10**). Calves not finishing their milk meal more than two times between sampling times E1-E9, calves categorized as sick or with uncomplicated diarrhea at E9 or sick at E14, and calves categorized in IQR category for the difference in *Bifidobacterium* spp. quantity between E4 and E1, and Holstein Jersey cross were associated with being in the low pre-weaning ADG category. Calves not finishing their milk meal more than two times, calves categorized below the 25th percentile for difference in *Bifidobacterium* spp. quantity between E4 and E1, calves categorized with uncomplicated diarrhea at E9, and Holstein Jersey cross were associated with the medium ADG category. There was no association of E1 antimicrobial treatment with ADG category.

Post-Weaning Events

Using on-farm records, calves were followed post-weaning to assess effect of treatment and pre-weaning events on

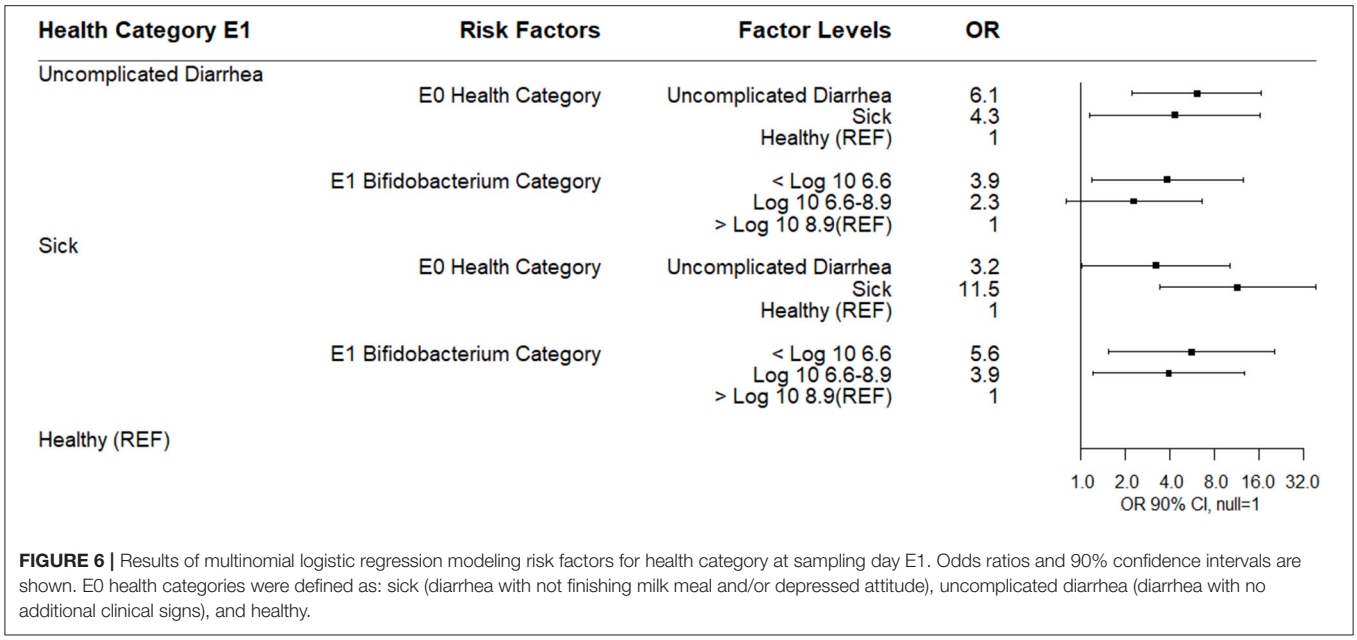

FIGURE 6 | Results of multinomial logistic regression modeling risk factors for health category at sampling day E1. Odds ratios and 90% confidence intervals are shown. E0 health categories were defined as: sick (diarrhea with not finishing milk meal and/or depressed attitude), uncomplicated diarrhea (diarrhea with no additional clinical signs), and healthy.

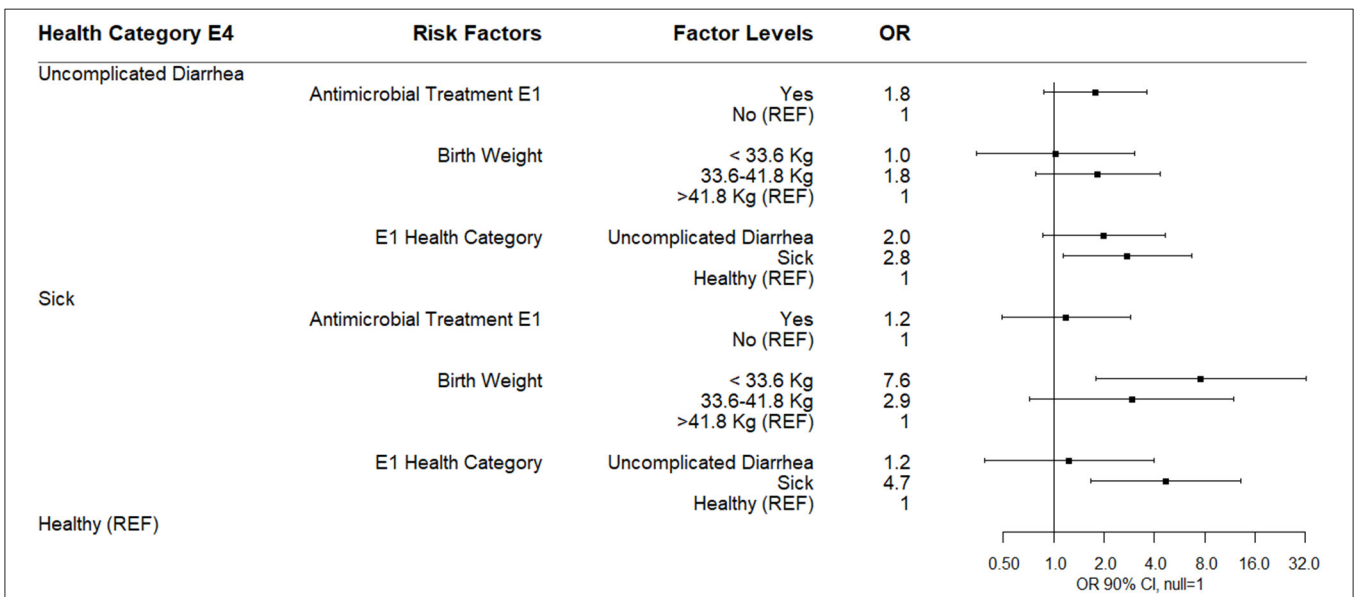

FIGURE 7 | Results of multinomial logistic regression modeling health category at sampling day E4. Odds ratios and 90% confidence intervals are shown.

survival in the herd and time to first calving. Of the 121 calves originally enrolled in the study, 97 entered their first lactation, 14 had died, seven were sold, and two were lost to follow-up post-weaning. Of the 14 that died, five died during the pre-weaning period, two died within 7 days following weaning, five died between 100 and 170 days of age, and one died at 558 days of age. For the 97 study animals that calved, the median and mean age at first calving was 22.5 months with the IQR being ∼30 days. None of the pre-weaning variables (disease status at sampling points, *Bifidobacterium* quantity, E1 antimicrobial treatment, ADG category, breed, or TSP category) were associated with age at first calving.

DISCUSSION

To our knowledge this is the first study of the effects of parenteral antimicrobials given to healthy as well as unhealthy pre-weaning calves on fecal *Bifidobacterium* quantity and health outcomes. On farm detection of calf disease is challenging and in this study was inconsistent in its application. This inconsistency makes it difficult to use farm records to make

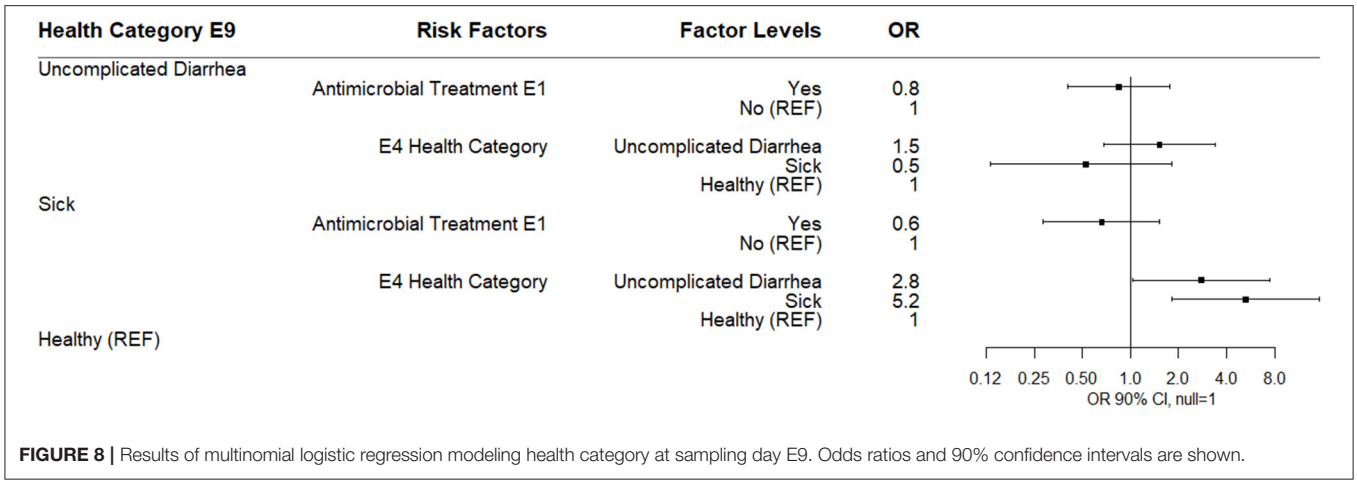

FIGURE 8 | Results of multinomial logistic regression modeling health category at sampling day E9. Odds ratios and 90% confidence intervals are shown.

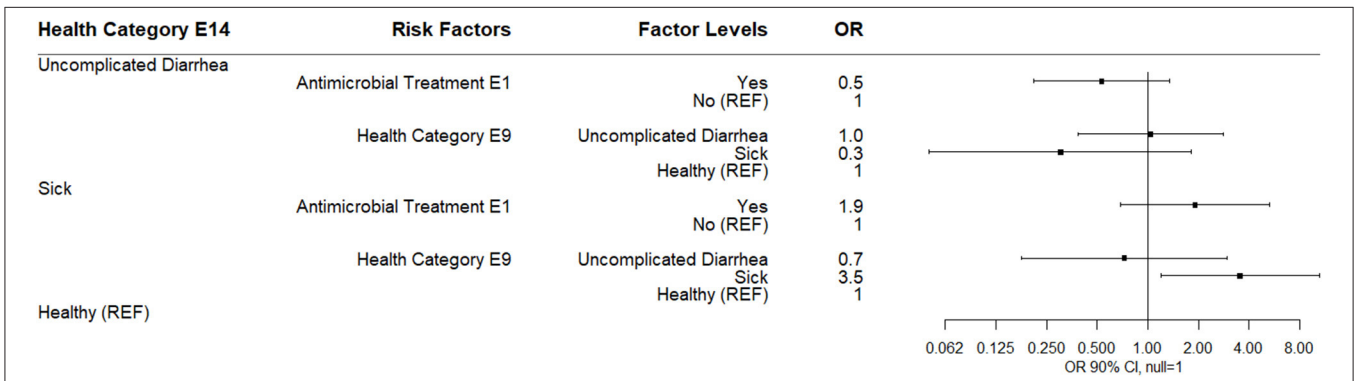

FIGURE 9 | Results of multinomial logistic regression modeling health category at sampling day E14. Odds ratios and 90% confidence intervals are shown.

management decisions on efficacy of treatments. Antimicrobials impacted the temporal pattern of *Bifidobacterium* succession in both healthy and sick calves through 9 days following a 3-day course of parenteral antimicrobials but had no impact on health outcomes or growth after treatment. The temporal pattern of *Bifidobacterium* and health assessments made during the study were closely aligned with calves classified as sick being associated with lower quantities of fecal *Bifidobacterium* and previously being identified as sick.

Identification of the frequent misclassification of illness was an important finding. Based on comparisons with clinical observations, we could not rely on farm personnel decisions for what defined a sick calf. This finding has been observed elsewhere (5) and cautions on farm researchers and dairy advisors to question the utility of farm treatment records to identify or evaluate farm morbidity. The on-farm criteria for determining a sick animal should be as objective as possible and clearly defined to be consistently applied by on-farm personnel charged with health assessments. Even clinical scoring systems have potential for misclassification when compared to more objective measures, such as dry matter content of feces vs. fecal score. The trend we saw with fecal score and fecal DM has been observed by others who used DM to normalize estimates of parasite load (26) and points out the underlying variability of DM associated with a fecal score. In addition, our findings indicate that levels of illness severity should be considered both in classification of a disease as well as to identify treatment options. Diarrhea is a symptom and not a disease and there appear to be gradations of severity that are not obvious through observation. Just as with dairy cow mastitis severity scoring, the outcomes and appropriate therapies for levels of diarrhea severity may differ (27) and this points to the importance of developing quick and easy point of care diagnostics to augment observation and intuition.

Follow-up sampling of neonatal calves revealed a temporal trend over the course of sampling with $\log_{10}$ *Bifidobacterium* quantity increasing from day 2 of life to the day of enrollment (about 8 days of age) with the highest quantities at the day before and the day of enrollment and diminishing in subsequent samplings (4, 9, and 14 days post-enrollment). Others have reported a rise in *Bifidobacterium* spp. fecal bacteria count from the first to the third week of life and a decline in week 4 and 5 (28) and that the relative abundance of *Bifidobacterium* appears to decrease with the age of the calf from day 7–14 (29).

The introduction of new feed is likely to have an influence on the bacterial species presented to the GIT. Despite the fact that the calves at the enrollment age are consuming mostly milk

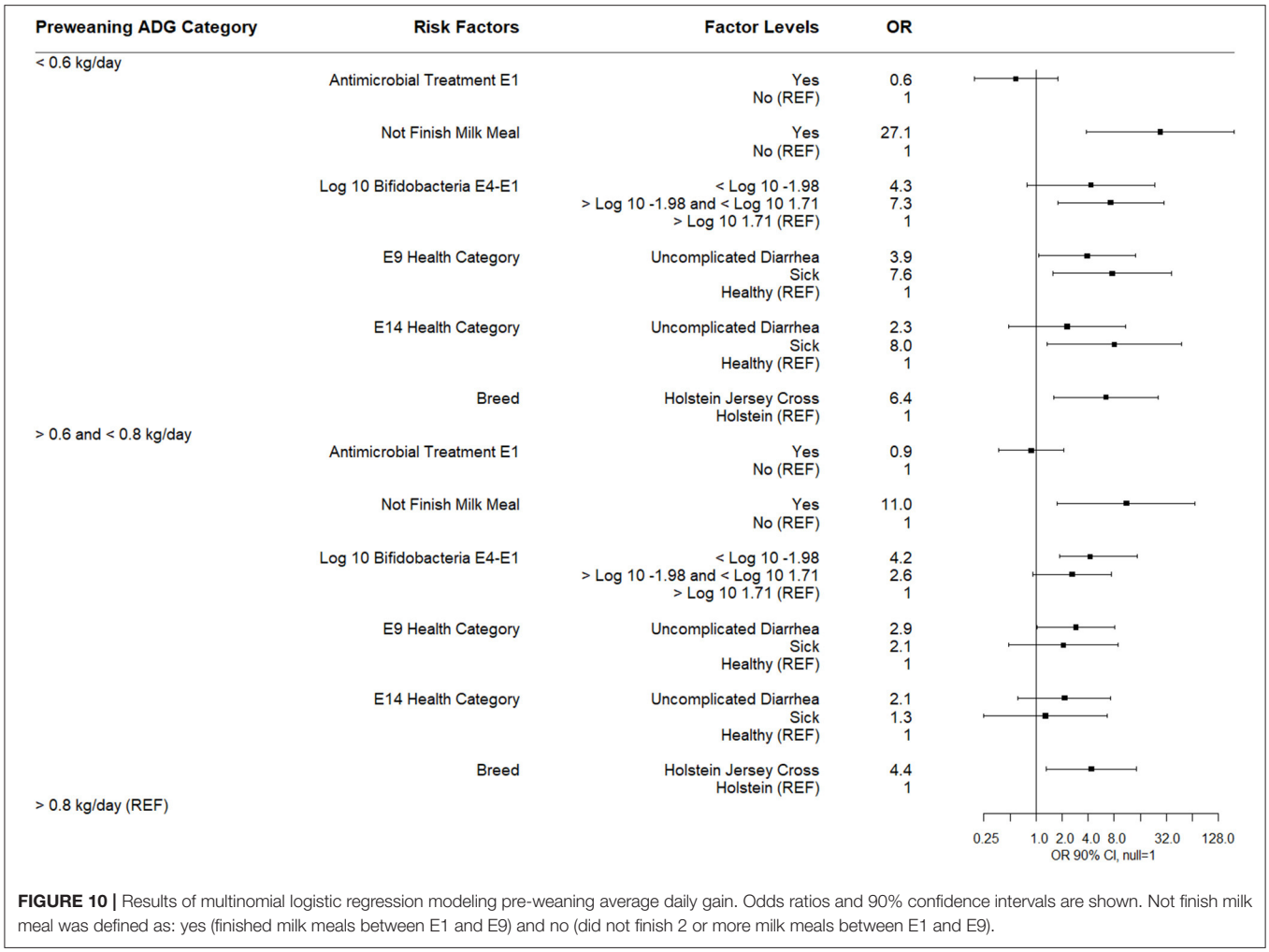

FIGURE 10 | Results of multinomial logistic regression modeling pre-weaning average daily gain. Odds ratios and 90% confidence intervals are shown. Not finish milk meal was defined as: yes (finished milk meals between E1 and E9) and no (did not finish 2 or more milk meals between E1 and E9).

or milk replacer, calves' consumption of starter feed doubles in the first 2 weeks of life and by 3 weeks of age, triples in quantity compared to consumption in the first week of life (30). These diet changes affect both the lower GIT as well as establishment of the rumen bacterial community (31). In addition, fecal *Bifidobacterium* dynamics appears dependent on diet, with higher counts found in all milk diets compared to diets with milk and grains (32).

Regardless of health status, calves in our study receiving a 3-day course of parenteral antimicrobial experienced a large decrease in *Bifidobacterium* compared to untreated healthy calves which demonstrated an increase in *Bifidobacterium* between E1 and E4. The differences associated with antimicrobial use might indicate a destabilization of the gut microbiota. A human neonatal study evaluating the impact of parenteral antimicrobials (ampicillin/gentamicin) on fecal *Bifidobacterium* showed a similar effect to those in our study (33). Using a different study design, Ma and others (7) investigated disturbances to the gut microbiome and reported that the use of antibiotics early in a calf's life delayed the development of microbial diversity. They noted that a gut microbiome with greater stability was more resistant to outside disturbances. In another study, when oxytetracycline was fed at different levels compared to controls, the calf microbiota composition was more affected by time and not antibiotic level (15).

One of our study objectives was to describe the impact of antimicrobial therapy on health outcomes. Across all the pre-weaning follow-up sampling periods (E4, E9, E14), antimicrobial therapy was not associated with post-treatment calf health. Calves classified as sick at enrollment (E1) were more likely to be classified as sick at E4 regardless of E1 treatment group. This suggests that antimicrobial treatment had little or no impact on the course of disease. There was also no evidence that antimicrobial treatment affected health outcomes for those healthy calves that were selected to receive antimicrobials. This trend held true for all the follow-up timepoints as calves classified as sick at one timepoint were associated with being sick at the previous timepoint.

In our study, enrollment health category was associated with *Bifidobacterium* quantity; calves with either uncomplicated diarrhea or classified as sick were associated with the lowest quartile of E1 $\log_{10}$ fecal *Bifidobacterium* relative to healthy

calves. Others have reported a similar finding, i.e., higher levels of *Bifidobacterium* spp. were associated with healthy vs. diarrheic calves. Although in the same study a second farm had other bacterial species associated with health (34), suggesting that microbiota is farm specific. In another study, having diarrhea was associated with a fluctuation in microbial diversity and temporal stability of the fecal microbiota was considered best in healthy calves compared to sick (7). In our study, we were not able to assign cause and effect, i.e., whether abnormal health status was a consequence of lower quantities of *Bifidobacterium* present in feces or whether abnormal health resulted in lower quantities. It is possible that lower fecal DM associated with our classification of health reflected a decreased amount of detected *Bifidobacterium* per gram of feces for calves with low fecal dry matters though the temporal trends we observed in our study were similar to those reported elsewhere (29).

Antimicrobial treatment did not affect either ADG or post-weaning events associated with mortality or days to first calving. There were associations of not finishing milk meals and being classified as sick at E9 and E14 on decreased ADG. There was no consistent finding associated with *Bifidobacterium* change between E4 and E1, though a depressed change was associated with lower ADG. We did not monitor and collect daily health scores on calves between E14 and weaning and could not account for their possible impact on ADG, but it is important to note that recorded sick events at E9 and E14 as well as not completing milk meals between E1 and E9 had impacts on ADG, i.e., sick calves did not appear to catch up to their healthy peers following the early negative pre-weaning events.

In summary, health and treatment decision making on the farm is often subjective particularly when determining whether antibiotic treatment is appropriate for an animal. These data illustrate a misalignment between clinical observations made by investigators and the initiation of antibiotic treatment by farm personnel. This presents an opportunity for calf treaters and veterinarians to develop and evaluate protocols for disease detection. Antimicrobials used in our study accelerated the temporal trend of decreasing *Bifidobacterium* and by themselves had no impact on the course of disease. Although it is unclear if these observations are related, it does suggest that discretionary use of antimicrobial therapy should be guided by veterinary input and monitoring and highlights the need for point of care diagnostics to better define gradients of disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://doi.org/10.7273/k0hn-gy79.

ETHICS STATEMENT

The research protocol was reviewed and approved by the Institutional Animal Care and use Committee of Washington State University (ASAF 04925). All protocols involving calves housed on the commercial dairy farm were authorized by the farm owner, who was aware of all procedures. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

OO'K participated in project design and implementation and contributed to all versions of the manuscript. DM participated in project implementation and contributed to all versions of the manuscript. CM participated in project implementation and contributed to the final version of the manuscript. WS participated in project design and implementation and contributed to all versions of the manuscript. All authors have read and approved of the submitted version of the manuscript.

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Survey on Antimicrobial Drug Use Practices in California Preweaned Dairy Calves

*Emmanuel Okello[1,2], Deniece R. Williams[1], Wagdy R. ElAshmawy[1,3], Jaymes Adams[4], Richard V. Pereira[2], Terry W. Lehenbauer[1,2] and Sharif S. Aly[1,2]**

[1] Veterinary Medicine Teaching and Research Center, School of Veterinary Medicine, University of California, Davis, Tulare, CA, United States, [2] Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, Davis, CA, United States, [3] Department of Internal Medicine and Infectious Diseases, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt, [4] Antimicrobial Use and Stewardship Program, California Department of Food and Agriculture, Sacramento, CA, United States

The California (CA) dairy industry was surveyed in July 2017 to evaluate producers' knowledge and perceptions and antimicrobial drug (AMD) use in preweaned dairy calves following the implementation of the nationwide veterinary feed directive final rule (VFD) in January 2017 and prior to statewide implementation of CA Senate Bill (SB) 27 in January 2018. Together, these regulations require veterinary oversight for all uses of medically important antimicrobial drugs (MIADs) administered to livestock in CA. Survey questionnaire was mailed to 1,361 CA Grade A milk producing dairies and calf ranches across CA resulting in a 12% (169) response. Most respondents (83%) were aware of the VFD and SB 27 changes. Use of antibiotics was perceived as important (77%) in raising preweaned dairy calves and judicious use of antibiotics was ranked as the most important antimicrobial stewardship practice, amongst record keeping, observing withdrawal periods, having a valid Veterinarian-Client-Patient-Relationship (VCPR), and use of alternatives to antibiotics. Treating sick calves was the major indication for AMD use (90.5%); however, few producers reported use of antibiotics to control (12.7%) or prevent disease (11%). Neomycin sulfate, chlortetracycline, oxytetracycline and sulfamethazine were the most used AMD. The respondents reported a decreased use of AMD in milk (10%) and in solid feed (5%), and discontinuation of one or more AMDs used in milk (18.6%) or in solid feed (5%) post-VFD rule implementation in 2017. Most respondents reported keeping treatment records and the information recorded included date (82%), dose (44%) and route (15%) of AMD used. A few respondents reported they had initiated use of alternatives to AMDs, such as vitamins (32.6%), minerals (25.6%), herbal remedies (11.6%) and pathogen specific antibodies (7%), post-VFD. The limited changes noted in AMD use could be attributed to the short period between the implementation of the VFD and the time of the survey. Our study outcomes identified opportunities to improve AMD use practices, including record keeping and use of AMD alternatives, and provides a baseline for future evaluation of the impact of these regulatory changes, as well as guidance for the future recommendations on best practices to promote judicious AMD use.

Keywords: antimicrobial drug use, veterinary feed directive, perception, preweaned dairy calves, California Senate Bill 27

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***Correspondence:**
Sharif S. Aly
saly@ucdavis.edu

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INTRODUCTION

Antimicrobial drugs (AMD) are important compounds used in food animal production for treatment, control, and prevention of bacterial diseases. However, use of AMD is associated with development of antimicrobial resistance (AMR) (1, 2), which is a major concern for both human and animal health worldwide. Many countries have formulated and implemented surveillance programs to monitor AMD use in food animals, as the first step toward promoting their judicious use (3–9). In the United States, cattle production leads the utilization of AMD among the different livestock species. The 2018 FDA report on domestic sales and distribution of AMD for use in cattle (dairy and beef) accounted for the highest percentage (42%) of the total species-specific sales (kg) estimates for antimicrobial drugs approved for use in food animals in the United States (10). Although the U.S. does not currently have an antimicrobial drug use report in livestock that accounts for individuals at risk of being treated and a standard body weight at treatment, such as the "defined daily dose for animals," the annual sales and distribution report has indicated decreased consumption of AMDs among the different livestock species.

In dairy cattle, the most common use of AMD are to prevent and treat mastitis in lactating and dry cows (11, 12), and for treatment or control of enteritis and respiratory diseases in calves (13). Besides direct exposure of dairy calves to AMD for treatment and prevention purposes, additional indirect exposure occurs through feeding non-saleable (waste or hospital) milk that may contain low concentrations of drug residues (14). Waste milk is milk harvested from cows treated with intramammary, oral, or injectable antimicrobials during the withholding period when such milk cannot be sold for human consumption. Waste milk may also contain milk from recently calved cows while they transition from colostrum to normal milk secretion. The practice of feeding waste milk is widespread in the dairy industry, despite research evidence which indicates that it is associated with changes in microbial populations and increased presence of AMR bacteria in calves, compared to calves fed saleable bulk tank milk (15–19).

Understanding the ways in which AMD are used in the dairy industry and estimating the associated risks for AMR is critical for understanding hurdles encountered by the dairy industry to adopt judicious drug use practices necessary to ensure the well-being of food animals and protect both veterinary and public health (20). Previous research has shown that both veterinarians and producers play crucial roles in the use of AMD; among veterinarians, prescription decision-making habits are the major factors that influence the use of AMD (21, 22), while the major drivers for AMD use among producers included the type of cattle operation, disease and animal welfare, economic factors, veterinary consultation, producer's experience and peer support, perceived drug efficacy and drug use regulations (23). Furthermore, raising food animals in a production setting sustainably should employ preventative management practices that modify the environment and host to reduce the risk of disease as a priority before using AMD. Specifically, these preventative strategies should include modifying the environment to reduce stress using proper housing, optimum colostrum management and nutrition, and use of effective vaccines (24, 25). An approach that allows producers to raise and manage food animals sustainably with emphasis on addressing the environment and host factors includes the risk assessment tool for bovine respiratory disease (26).

In the United States, recent regulatory changes were made by the Food and Drugs Administration (FDA) to improve the regulatory oversight of Veterinary Feeds Directive (VFD) drugs while continuing to protect human and animal health. The VFD drugs refer to new animal drugs intended for use in or on animal feed which are limited to use under the professional supervision of a licensed veterinarian (27). The VFD final rule was implemented effective January 1, 2017. Locally in California, Senate Bill (SB) 27 was approved and passed in 2015 as the Livestock: Use of Antimicrobial Drugs Law (California Food and Agriculture Code Sections 14400-14408) (28), here onwards referred to as SB 27. Effective January 1, 2018, SB 27 regulations restricted all uses of medically important antimicrobial drugs (MIADs) to veterinary prescription or VFD only. The term MIADs refers to antimicrobial drugs listed in Appendix A of the federal Food and Drug Administration's Guidance for Industry #152, and include critically important, highly important, and important antimicrobial drugs such as norfloxacin, cephalexin, cefaclor, penicillin, oxacillin, ampicillin, streptomycin, erythromycin, clarithromycin, tetracyclines, vancomycin, chloramphenicol and trimeth/sulfameth (29). Jointly, these regulatory changes increased veterinary oversight in the distribution and use of MIADs in livestock by changing the availability of MIADs from over-the-counter (OTC) to prescription or VFD only.

The purpose of the statewide survey described here was to document AMD use practices for preweaned dairy calves, evaluate producers' knowledge and perception on AMD use, and document any changes in management and AMD use practices following the implementation of VFD rule and prior to the implementation SB-27. In California, newborn dairy calves are either raised on-site at their source dairy or off-site in a calf nursery, commonly known as calf ranches (30). Dairies and calf ranches raise replacement dairy heifers or bulls for dairy beef. The survey targeted both dairies and calf ranches that were raising preweaned calves. The outcome of this survey furthers our understanding of the industry's AMD use practices and perceptions and provides baseline data to guide future evaluation of the rule changes as well as recommendations on best practices to promote judicious use of AMDs.

MATERIALS AND METHODS

Study Design

A survey of the California dairy producers was conducted in July 2017. The questionnaire was pre-tested by the coauthors and several collaborators with in-depth knowledge of the CA dairy industry. The survey questionnaire was comprised of 54 questions grouped into four sections (**Supplementary Material**). Section 1 included questions on the respondent's role on the

farm and herd demographics including location, herd size, cow breeds raised and participation in welfare audit programs; Section 2 was comprised of questions about preweaned calf management practices including housing types, feeding practices, health management protocols such as adding AMD in feed, milk or water and vaccination; Section 3 questions sought information on AMD use in preweaned calves including information sources and decision making on AMD purchased and used on the farm, availability and use of treatment protocols including dose estimation and extra-label AMD use, drugs and health records system, choice of AMD used to treat sick calves, and the extent of veterinarian involvement in on-farm AMD use practices. Section 4 assessed the knowledge and perceptions on AMD stewardship practices as well as awareness of regulations. The study materials were reviewed by the University of California, Davis, Institutional Review Board and granted IRB review exemption approval (IRB ID: 1709653-1).

Surveys and Data Collection

The survey questionnaire was mailed to 1,361 licensed Grade A milk producing dairies and calf ranches in California. In the United States, Grade A milk is the category of milk produced under higher farm sanitary conditions (compared to Grade B) to qualify for sale or consumption as fluid beverage (31). A sample size was not conducted prior to the survey. However, a *post-hoc* sample size and power analysis based on the question on respondent knowledge of AMD regulatory changes was estimated. The mailed survey included a cover letter, questionnaire, an additional form for producers to share their comments about AMD use in preweaned calves or other dairy cattle, a follow up request form, and postage-paid return envelope. The cover letter also provided a web link to the online version of the survey as an alternate response option. Dairies and calf ranches identified to receive the survey were each assigned a confidential index number used only to identify respondents for a second mailing if they had not responded to the first mailing, and to verify the county location of their premises. The second mailing was 4 weeks after the initial mailing and was sent to addresses that did not respond the first time. Similarly, a reminder card was sent 2-weeks after each mailing to those who had not responded at the time of mailing.

Statistical Analyses

Descriptive Statistics

Survey data were analyzed using Stata IC 16 (Stata Corp LLC, College Station, TX USA) and (32). Responses to questions were summarized using descriptive statistics and reported as proportions for categorical variables or means for continuous variables. Uncertainty measures including standard errors and 95% Confidence Intervals (CI) were also reported.

Multiple Factor Analysis

Multiple Factor Analysis (MFA), an extension of the Principle Components Analysis (PCA) was used to explain the variability of the producers responses to the survey on AMD use in preweaned calves on California dairies (33). Principle Components Analysis was achieved through dimensionality reduction of the dataset's variables, both quantitative and categorical (34), through the FactoMineR package in R (35). The MFA was performed based on a subset of 66 variables classified into 12 groups, 11 qualitative groups (65 categorical variables) and one quantitative group (one continuous variables). The first three dimensions of the MFA were used to interpret the percentage of the explained dataset's variance explained. Groups with variance of 0.4 or greater on any of the first three principal components dimensions were retained for interpretation, and within each group, variables with correlation coefficients (coordinates) of 0.4 or greater were retained for interpretation of variability.

RESULTS

Respondents and Their Herd Characteristics

A *post-hoc* sample size based on the question on respondent knowledge of AMD regulations showed that a sample size of 136 respondents is needed assuming that 50% answer yes, 5% level of significance and 80% power in a two-way test, and a 10% response rate out of a total of 1,361 mailed surveys. The power analysis based on the 139 surveys returned and 115 responses indicating knowledge of AMD regulations (82.7%) resulted in a *P*-value of 0.04 and power >99%.

Of the 1,361 mailed surveys, 169 (12%) responses were received including five responses completed online. Among the 169 responses received, 23 were excluded from the analysis either because they were not completed but returned or the premise responded twice, in which case only the first response was considered. Because some respondents did not answer all the questions of the surveys included in the analysis (n = 146), each question was analyzed based on the number of respondents (n) who answered the specific question. During analysis, the respondents' locations were categorized as one of the three milk shed regions of California; Northern California (NCA), Northern San Joaquin Valley (NSJV) and Greater Southern California (GSCA), with the latter being a merger of dairies in the Southern San Joaquin Valley and the limited number of dairies in Southern California (36). Most of the respondents (84 responses; 49.7%) were from GSCA, followed by NSJV (58 responses; 34.2%) and lastly NCA (27; 16%). Among the responses received, five were from calf ranches located in the GSCA. Most of the dairy survey respondents were dairy owners (83.8%, n = 146), and only a smaller proportion were individuals with various roles on the dairy such as herd manager (32%, n = 146), calf manager (12.2%, n = 146) and/or calf feeder (6.2%, n = 146). Responses from certified organic dairy farms (8.9%, n = 146) were excluded from analyses on AMD use, except for the questions on availability and type of VCPR. The mean calf herd size of respondents was 246 preweaned calves, with the predominant breeds being Holsteins (79.2%). **Table 1** summarizes the demographics of the respondent dairies.

TABLE 1 | Summary dairy characteristics.

Dairy characteristic	Mean	SE	*N*	95% Confidence limits	
				Lower	Upper
Herd size					
Preweaned calves	246	43.02	67	160	331.78
Milking cows	1,507	105.12	144	1,299	1,715
Rolling herd average milk production (kg)	11,118	187.07	139	10,748.86	11,488.67
Bulk tank somatic cell count (cells/mL)	245,863	6,703	139	232,608	259,118
Predominant breed (%)					
Holstein	79.21	3.03	142	73.22	85.19
Jersey	11.82	2.27	142	7.34	16.3
Crossbred	7.58	1.87	142	3.88	11.28
Other	0.99	0.35	142	0.05	1.43

The values indicate mean properties of the herds based on the participant responses to the 2017 mail and online survey of California Grade A dairy milk producers on antimicrobial drug use in preweaned dairy calves.

TABLE 2 | Ranking of antimicrobial stewardship practices by survey respondents.

Stewardship practices	Mean	SE	*N*	95% Confidence limits	
				Lower	Upper
Appropriate drug, dose, route, duration	1.91	0.1	134	1.72	2.1
Good record keeping	2.65	0.11	132	2.44	2.86
Observing withdrawal periods	2.73	0.12	131	2.50	2.97
Having current VCPR*	3.03	0.13	133	2.78	3.28
Alternatives to antimicrobial drugs	3.69	0.14	131	3.41	3.96

The five stewardship practices were ranked from 1 (most important) to 5 (least important) based on the respondent's perception. The mean rank for each stewardship practice was calculated based on all the responses by respondents.
**VCPR is veterinarian-client-patient relationship.*

Knowledge, Perception, and Impact of VFD and SB 27 Regulatory Changes

Knowledge on Regulatory Changes

Most of the producers were aware of the VFD rule changes and the then soon-to-be-implemented SB 27. A total of 82.7% ($n = 139$) respondents reported knowledge of the requirement for veterinary prescription for all OTC AMD starting January 1, 2018. This regulatory change was projected by the respondents to affect the producers who were reportedly using OTC AMD according to label (35.7%, $n = 129$) or extra-label (6.6%, $n = 129$). Among the calf ranches, 3 out of the 5 respondents reported knowledge of regulatory changes that were set to start on January 1, 2018 at the time of the survey, and one respondent reported using OTC AMD according to label.

Knowledge and Perception of Antimicrobial Stewardship Practices

The producers' knowledge and perceptions of AMD stewardship practices were assessed by asking respondents to rank the relative importance of five key stewardship practices ranging from most important (1) to least important (5). The responses for each of these practices were then ranked in order based on their mean values. Respondents ranked administration of appropriate AMD, dose, route and duration as the most important (1st), followed by good record keeping (2nd), observing withdrawal periods and drug residue avoidance (3rd), having a current veterinary-client-patient-relationship (VCPR) (4th), and the least important was the use of alternatives to antibiotics (5th). Details of the ranking are summarized in **Table 2**. The same ranking order was observed among the 5 calf ranch respondents.

Perception of Antimicrobial Drugs

The respondents perceived the use of AMD to be extremely important (39%; $n = 64$; CI: 27.67, 51.78) or important (37.5%; $n = 64$; CI: 26.29,50.23) in raising preweaned dairy calves, and only a small percentage indicated that AMD were somewhat important (17.19%; $n = 64$; CI: 9.62, 28.8) or not important (10.9%; $n = 64$; CI: 5.2, 21.58). In the calf ranch responses, AMD use was perceived by respondents as extremely important (two respondents), important (one respondent) or somewhat important (two respondents). The importance of AMD in raising preweaned dairy calves was further emphasized by the fact that 62.5% ($n = 65$; CI: 49.77, 73.71) of respondent dairies predicted increased disease prevalence if AMD use for dairy calves was ceased completely. Similarly, 73.4% ($n = 64$; CI: 60.99, 83.02) and 54.7% ($n = 64$; CI: 42.12, 66.81) of respondent dairies predicted poor animal welfare and decreased performance, respectively, if the use of AMD in preweaned calves was stopped. Only

TABLE 3 | Changes in the use of antimicrobial drugs in preweaned calves on California dairies following the implementation of the Veterinary Feed Directive (VFD) final rule on January 1, 2017 compared to practices during the year 2016.

Changes since Jan 1st 2017	Percent	SE	*N*	95% Confidence limits	
				Lower	Upper
Antimicrobial drugs used in milk or milk replacer					
No changes made	61.0	6.4	59	47.7	72.9
Increased amount or duration	1.7	1.7	59	0.2	11.7
Decreased amount or duration	10.1	4.	59	4.5	21.3
Discontinued 1 or more	18.6	5.1	59	10.5	31
Added 1 or more	1.7	1.7	59	0.2	11.7
Other[a,b]	8.4	3.7	59	3.5	19.2
Antimicrobial drugs used in solid feed					
No changes made	81.7	5	60	69.4	89.7
Increased amount or duration	0		60		
Decreased amount or duration	5	2.8	60	1.6	14.8
Discontinued 1 or more	5	2.8	60	1.6	14.8
Added 1 or more	1.7	1.7	60	0.2	11.5
Other[a]	5	2.8	60	1.6	14.8
Antimicrobial drugs used in water					
No changes made	83.6	4.8	61	71.7	91.1
Increased amount or duration	0		61		
Decreased amount or duration	1.6	1.6	61	0.2	11.3
Discontinued 1 or more	1.6	1.6	61	0.2	11.3
Added 1 or more	4.9	2.8	61	1.5	14.6
Other[a]	6.6	3.2	61	2.4	1.7

[a] *Used antimicrobial drugs occasionally.* [b] *Stopped using uniprim (trimethoprim and sulfadiazine oral antibiotic powder).*

a small proportion of the respondents indicated there would be no effect if AMD use in preweaned calves was stopped (14.1%; $n = 64$; CI: 7.39, 25.24) or were organic producers who were not using antibiotics (7.8%; $n = 64$; CI: 3.21, 17.8). The same response pattern was seen among calf ranches with four in five respondents predicting increased disease prevalence, poor growth, and compromised animal welfare, and only one respondent predicting no effect.

Change in Cost of Antimicrobials

Cost-wise, most of the respondents reported no change in the cost of AMD in their operations following the implementation of VFD regulatory changes (69.7%; $n = 59$; CI: 56.28, 80.12). Majority of the respondents reported no change in the cost of antibiotics following the implementation of VFD changes (70.5%; $n = 61$; CI: 56.28, 80.12), while only a small proportion of the respondents indicated that the cost of AMD had either increased (13.1%; $n = 61$; CI: 6.56, 24.49) or decreased (16.4%; $n = 61$; CI: 8.89, 28.26). Two calf ranch respondents reported an increase in cost of AMD while three respondents reported a decrease in cost.

Change in Antimicrobial Drug Use

Most of the producers reported no change in the use of AMD in milk (61%, $n = 59$), solid feed (81.7%, $n = 60$) or water (8.6%, $n = 61$) for preweaned calves. The details of the specific changes reported in AMD use are summarized in **Table 3**. A small proportion of respondents (10.9%; $n = 55$) reported a decrease in use of OTC AMD labeled for feed that do not fall under VFD requirements (e.g., amprolium); this decrease was mainly due to reduced use of these AMDs in milk. Respondents that reported a decrease in OTC AMD use also reported an increase in calf mortality. A single respondent (1.9%, $n = 54$) reported increased use of OTC AMD labeled for feed that do not fall under the VFD, explaining that reduced use of OTC AMD in milk "resulted in more aggressive use through other routes." Some respondents reported they had initiated use of alternatives to AMD after the VFD requirements were implemented such as herbal remedies (11.6%, $n = 43$) and pathogen specific antibodies derived from sources such as eggs (7%, $n = 43$).

Antimicrobial Drug Use Practices in Preweaned Dairy Calves

Preweaned dairy calves were reportedly exposed directly to AMD for treatment, control, or prevention purposes through parenteral or oral administration, or by adding in milk, solid feed, or water. Indirect exposure to AMDs reportedly occurred through feeding non-saleable or waste milk containing drug residues. Sources of non-saleable milk included recently calved cows that were previously treated with long acting intramammary AMD infusion at dry-off, mastitis cows treated with intramammary or other parenteral AMD or other lactating

cows treated with systemic AMD as treatment for other health conditions.

Exposure of Preweaned Dairy Calves to Antimicrobial Drugs

Indirect exposure of preweaned dairy calves to AMD occurred mainly through feeding of milk sources that presumably contained AMD residues. The mean proportion of liquid diet fed to calves by the responding dairies included non-saleable or waste milk (44.2%; $n = 68$; CI: 34.34, 53.96), saleable or bulk tank milk (28.31%; $n = 68$; CI: 18.73, 37.89), milk replacer (20.56%; $n = 68$; CI: 12.8, 28.32), and other minor sources such as transition cow milk, fortified non-saleable milk and non-fat dry milk powder (3.94%; $n = 68$; CI: −0.21, 8.09). Sources of milk containing AMD residues could have included waste (hospital) milk, as well as colostrum and milk from transition cows treated at dry-off with long acting intramammary (IMM) AMD. Most of the responding dairies treated all cows (77.5%; $n = 142$; CI: 69.77, 83.66) while a few dairies treated cows selectively (4.9%; $n = 142$; CI: 2.35, 10.06) at dry-off with long acting IMM AMD. Dairy reported sources of colostrum fed to calves included pooled colostrum (51.2%; $n = 141$; CI: 43.74, 58.6), individual cow colostrum (34.6%; $n = 142$; CI: 27.47, 41.74) or direct nursing from the dam (5.5%; $n = 139$; CI: 2.28, 8.65). A few respondents reported feeding transition cow milk to preweaned calves 5.42%; $n = 48$; CI: 0.88, 10). Among the calf ranches, 2 out of 5 respondents reported feeding preweaned calves with a liquid diet comprised of either 75 or 100% saleable milk.

Direct exposure of preweaned dairy calves to AMD occurred parenterally or orally in milk, grain, or water. More than half of the respondents (64%; $n = 61$) had a treatment protocol developed by either a veterinarian (50%, $n = 34$), farm owner (17.6%, $n = 34$) or both veterinarian and owner (30.3%, $n = 33$). The availability, content and access to the treatment protocols are summarized in **Table 4**. Only 27.8% ($n = 61$) respondents reported submission of calves for diagnosis, while 30.2% ($n = 61$) used other diagnostic techniques to guide antimicrobial treatment of preweaned calves. Treatment of calves using AMD was reported to mainly follow label-use (78.3%, $n = 60$) and only a small proportion of respondents reported extra-label use (16.7%, $n = 60$) or did not know if antibiotics were being used extra-label (5%, $n = 60$). Generally, most of the respondents reported following label recommendations when estimating treatment dosage (87.1%, $n = 62$) and treatment duration for both parenteral or oral AMD administration (88.1%, $n = 59$) or AMDs added in feed (74.6%, $n = 55$). The major indications for AMD use in calves, methods for estimating treatment dosage and treatment duration are summarized in **Table 5**. Individual treatment of sick calves was the single most important indication for AMD use (90.5%, $n = 63$), whereas use for control of ongoing diseases (12.7%, $n = 63$) or prevent disease in high-risk calves (11.1%, $n = 63$) were minor indications. **Table 6** shows the mean percentage of calves that received different AMD administrations between birth and weaning. Neomycin and oxytetracycline were the most used AMDs administered to more than half of the calves during the preweaning period. The list of common AMDs of choice for treating respiratory diseases and diarrhea or enteritis in preweaned calves is shown in **Table 7**. The most common antimicrobials reportedly used by respondents as first choice treatment for respiratory disease and enteritis were florfenicol (43% respondents) and sulfonamide (24.4% respondents), respectively.

Drug and Treatment Records

Only 32.31% ($n = 65$; CI: 21.86, 44.88) of the respondents kept a drug inventory log on the dairy, but respondents recorded AMD treatment information such as treatment date (82.3%; $n = 62$; CI: 70.34, 90.06), dose (43.6%; $n = 62$; CI: 31.52,56.37), and route (14.5%; $n = 62$; CI: 7.59, 25.99) of administration. Forty percent ($n = 60$, CI: 28.15, 53.15) of the respondents reportedly did not track the antibiotic withdrawal interval in treated calves. The record systems used by respondents who tracked AMD withdrawal, included paper records (31%; $n = 58$; CI: 20.23, 44.39), computer software such as DC 305® (Valley Ag Software, Tulare, CA 93274) (22.4%; $n = 58$; CI: 13.26, 35.3), marking the calf hutch (12.1%; $n = 58$; CI: 5.73,23.65), memory (8.6%; $n = 58$; CI: 3.54, 19.53), or used other methods including chalk and phone (3.6%; $n = 56$; CI: 0.86, 13.71). Besides withdrawal period, other drug related information tracked included drug name (60%; $n = 60$; CI: 46.85, 71.85) quantity at hand (56%; $n = 59$; CI: 42.78, 68.31), supplier (22%; $n = 59$; CI: 13.04, 34.76), expiration date (33%; $n = 60$; 22.34, 46.49), cost (27%; $n = 59$; CI: 170.8, 40.19), manufacturer (22%; $n = 60$; 12.81, 34.24), and purchase date (15%; $n = 59$; CI: 7.97, 27.21).

Veterinarian Client Patient Relationship (VCPR) and Antimicrobial Drug Use

Most of the respondents had a VCPR with a practicing veterinarian (89.2%; $n = 65$; CI: 78.73,94.88) and a minor proportion had a VCPR with a technical services or consulting veterinarian (4.6%; $n = 65$; CI: 1.45,13.72). Three dairies (4.6%; $n = 65$; CI: 1.45,13.72) reported having no VCPR; among these dairies, one was an organic dairy and the remaining two showed evidence of working with a local veterinarian or having a written VCPR which indicated that their response of not having a VCPR was in error or the question was misunderstood. The nature of the VCPR included a written signed agreement (47.5%; $n = 61$; CI: 38.08,60.32), verbal agreement (42.6%; $n = 61$; CI: 30.59,55.6) or being assumed based on veterinary care provided (14.8%; $n = 61$; CI: 7.71, 26.39) or based on a longtime acquaintance between the veterinarian and the producer (1.6%; $n = 61$; CI: 0.22, 11.3). All respondent calf ranches reported having a VCPR which was either written (2 out of 5), verbal (2 out of 5) or not discussed (1 out of 5).

Decision Making on Antimicrobial Drugs Purchased and Used on Farms

The higher proportion of respondents reported that owners made decisions for purchase and stocking of AMD added to feed or water on the dairy (69.4%, $n = 63$), compared to the veterinarian (49.2%; $n = 63$), herd manager (25.4%, $n = 63$), calf manager (20.6%, $n = 63$) and the nutritionist (3.2%, $n = 63$) (**Table 8**). A similar pattern was reported in the decision making to purchase injectable or oral AMD. The calf manager, however, was more

TABLE 4 | Use of treatment protocols for health management in preweaned calves on California dairies.

Treatment protocol (presence, content, access, development, and review)	Percent	SE	N*	95% CI	
				Lower	Upper
Availability and author of written treatment protocols					
Dairies with written or computerized treatment protocol	63.9	62	61	50.9	75.2
Protocol developed by veterinarian	50	8.7	34	33	67
Protocol developed by owner	17.7	6.6	34	7.8	35.2
Protocol developed by owner and veterinarian	30.3	8.1	33	16.6	48.8
Information contained in treatment protocols					
Disease definitions	41	8	39	26.3	57.6
Disease specific treatments	66.7	7.7	39	49.9	80.1
Antimicrobial drug use information contained in treatment protocols					
Dosage	64.1	7.8	39	47.4	78
Duration	59	79.8	39	42.4	73.7
Withdrawal period	51.3	8.1	39	35.3	67
Not sure of details	0				
Other information	5.1	3.6	39	1.2	19.3
Personnel access to protocols					
Owner	79	6.7	38	62.4	89.5
Herd manager	57.9	8.1	38	41.2	73
Office staff	5.3	3.7	38	1.2	19.8
Calf manager	57.9	8.1	38	41.2	73
Calf feeder	36.8	7.9	38	22.6	53.8
Nutritionist	10.5	5.1	38	3.82	25.8
Veterinarian	57.9	8.1	38	41.2	73
Calf treatment crew	21.1	6.7	38	10.5	37.6
Schedule for review of treatment protocols					
Once to twice a year	51.4	8.33	37	34.9	67.5
Every few years	18.9	6.5	37	9	35.6
I don't know	8.1	4.6	37	2.5	23.3
Other[a]	24.3	7.15	37	12.8	41.4

The dairies were surveyed for the availability of written or computerized treatment protocols, content, and employee access to treatment protocols.

[a]*Every month, when needed or when a problem occurs.*

**A total of 61 respondents answered the question on availability and author of written treatment protocols and the rest of the responses are subset of this group.*

commonly the decision maker to treat preweaned calves on their 1st day of illness compared to the veterinarian. The information sources that guided the producer's decision in treating preweaned calves were mainly the veterinarians (84.6%, $n = 65$), previous experience with the drug (66.2%, $n = 65$), pharmaceutical company representative (32.3%, $n = 65$), or product label (27.7%, $n = 65$). Other minor sources of information on AMD included magazines, journals, promotional materials, other producers, local/national meeting, and online materials. Cooperative extension and FARAD (Food Animal Residue Avoidance Databank) were not among the information sources reported (**Table 9**).

The majority of dairy producers who responded to the survey reported that that they consulted veterinarians on disease management decisions (96.8%). Similarly, veterinarians were the main prescribers for AMD used in feed or water (71.4%), besides other personnel such as nutritionists and pharmaceutical veterinarians and sales representatives. Similarly, all the calf ranches consulted the veterinarians on disease management and antimicrobial use. **Table 10** summarizes the role of veterinarians and other livestock health professionals in providing consultancy services on animal disease management and prescription for AMD use.

Health Management Practices

Health management practices explored included colostrum management and vaccination practices. Most of the responding premises did not heat-treat colostrum fed to calves (87.1%; $n = 139$; CI: 80.28, 91.73). Intranasal vaccination against respiratory pathogens was the most used form of vaccine delivery in calves (77.6%; $n = 58$; 64.70, 86.74), administered at a mean age of 5.5 days ($n = 41$); other vaccine types reported included modified live vaccines against pneumonia or diarrhea causing pathogens (52.5%; $n = 58$; CI: 39.54, 65.21) administered at a mean age of 35.5 days ($n = 31$) days, and killed vaccines against pneumonia or diarrhea pathogens (20.7%; $n = 58$; CI: 11.94, 33.43) given at mean age of 19.3 days ($n = 15$).

TABLE 5 | Indications, estimation of dosage and treatment duration for antimicrobial drugs administered to preweaned dairy calves on California dairies.

Antimicrobial drug use in preweaned calves	Percent	SE	*N*	95% Confidence limits	
				Lower	Upper
Indication for antimicrobial drug use*					
Treat sick animals	90.5	3.7	63	80	95.7
Control ongoing disease	12.7	4.2	63	6.4	23.8
Prevent disease in high-risk calves	11.1	4.0	63	5.3	21.9
Other[a]	4.8	2.7	63	1.5	14.2
Estimating dosage*					
Body weight and label dosage	87.1	4.3	62	75.9	93.5
Body weight and experience	3.2	2.3	62	0.8	12.4
Body weight and vet authorization	14.5	4.5	62	7.6	26
Standard dosage by animal category	17.4	4.9	62	9.9	29.7
Level of clinical illness	3.2	2.3	62	0.8	12.4
Different approaches for different drugs	14.5	4.5	62	7.6	26
Other[b]	3.2	2.3	62	0.8	12.4
Estimating treatment duration*					
a) Antimicrobial drugs added to feed					
Follow label instruction	74.6	5.9	55	61.0	84.6
Stop early if animal is cured	20	5.4	55	33.1	-
Extend use if animal still sick	21.8	5.6	55	12.6	35.1
Based on previous results on the farm	16.4	5.0	55	8.6	29.0
Different approaches for different diseases	7.3	3.5	55	2.7	18.3
Other[c]	18.2	5.3	55	9.9	31.1
b) Antimicrobial drugs administered via injection or orally*					
Follow label instruction	88.1	4.3	59	76.7	94.4
Stop early if animal is cured	25.4	5.7	59	15.7	38.4
Extend use if animal still sick	35.6	6.3	59	24.2	48.9
Based on previous results on the farm	13.6	4.5	59	6.8	25.3
Different approaches for different diseases	11.9	4.3	59	5.6	23.3
Other[d]	6.8	3.3	59	17.1	-

N, number of respondents who answered the specific questions.

**Some respondents chose more than one option. [a]Antimicrobial drugs were not used, rarely used or there were no sick calves. [b]Other methods, not specified. [c]Use low levels continuously, do not use AMD, follow veterinarian recommendation, use AMD occasionally. [d]Follow veterinarian recommendation or do not use AMD.*

Multiple Factor Analysis

Multiple factor analysis of responses to 66 survey questions identified six components from which 25 variables explained most of the variability in the survey responses (**Table 11**). The first three dimensions described 21.46% of the variability in the data set. The first dimension explained 9.75% of the variability with most of the variability (65.23%) explained by use of diagnostics to guide treatment with AMD (18.60%), the source of information and decision on AMD (17.95%), treatment protocols and records (17.64%), and the common drugs used for treatment of diarrhea and pneumonia (11.06%). The second dimension explained 6.56% of the variability with calf management practices explaining 16.70% of the variability with the highest correlation 0.642 to pasteurization of milk (yes/no) for pre-weaned calves. The highest variation explained on the third dimension (37.13%) was related to the changes made in AMD use on dairies post-VFD final rule change, with high correlation determined for changes in AMD administered to pre-weaned calves in grain, water, injectables, and milk or milk replacer (0.670, 0.633, 0.629, 0.623, respectively).

DISCUSSION

The survey response rate of 12% in this study was comparable to the 15% response outcome from a previous mailed survey of the same demographics (37). The mean characteristics of the respondent dairies (herd size, rolling herd average, and breed composition) mirrored the state averages for the year 2016 (38), indicating the respondents were representative sample of the dairy farmers in the area. Similarly, majority of the respondents were from GSCA which has the highest number of dairy farms within the state. Most of the producers (82.7%) were aware of the recently implemented regulatory changes in the VFD rules which could have been due to extensive awareness campaigns at both the national and state levels through online resources, fact sheets, and other materials as well as workshop

TABLE 6 | Mean percentage of preweaned dairy calves receiving antimicrobial therapy between birth and weaning with different antimicrobial drugs.

Antimicrobial drug	Mean	SE	*N*	95% CI	
				Lower	Upper
Liquid feed (milk or milk replacer)					
Neomycin sulfate	62.8	15.2	10	28.4	97.1
Chlortetracycline	44	22.9	5	0	100
Neomycin-oxytetracycline	36.3	21.5	4	0	100
Spectinomycin	27.5	22.5	2	0	100
Oxytetracycline	56.7	23.3	3	0	100
Sulfamethazine	29.2	14.6	6	0	66.3
Coccidiostats	100	0	4	–	–
Other	100	–	1		
Solid feed (grain)					
Chlortetracycline	52.5	47.5	2	0	100
Neomycin-oxytetracycline	10	–	1	–	–
Oxytetracycline	70	30	3	0	100
Sulfamethazine	5	–	1	–	–
Coccidiostats	95.6	3.29	16	88.6	100
Water					
Neomycin sulfate	5	–	1	–	–
Chlortetracycline	7.5	2.5	2	0	39.3
Neomycin-oxytetracycline	100	0		–	–
Spectinomycin	50	–	1	–	–
Oxytetracycline	5		1		
Sulfamethazine	75	25	2	0	100
Bacitracin	100	–	1	–	–
Coccidiostats	51.7	17.4	6	7.1	96.3
Other	5.5	4.5	2	0	62.7

The mean percentage was calculated for number of responses (N) for a given antimicrobial compound.

presentations at producer and veterinary meetings. Use of AMD was generally perceived as important in raising calves and the respondents thought calf health and welfare would be negatively affected if AMD were no longer available. The same opinion was expressed among dairy farmers in Tennessee during focus group discussions on the impact of VFD changes on the ability of producers to prevent disease on their herds; these producers indicated that the VFD regulation had limited access to essential AMD which led to increased disease occurrence and deaths particularly among calves, and reduced growth rate (39). It is worth noting that the outcome of this study could have been influenced by the negative perception of surveyed producers toward regulatory changes and may not correlate to actual increase in disease occurrences. Comparatively, the European ban on the use of growth-promoting antimicrobials was mainly associated with increased early postweaning diarrhea in piglets and enteritis in broiler chicken, while minimal or no negative clinical effects on the ban was reported in other animal species (40–42).The increased disease burden in affected animal species resulted in increased use of antimicrobials for treatment and prevention, a challenge that was later addressed by improvements in animal health management and housing (41).

Among the five key AMD stewardship practices stated in the survey (judicious AMD use, good record keeping, having a valid VCPR, observing withdrawal period and using alternatives to AMD), the respondents ranked judicious drug use (appropriate drug, dose, route, and duration of use) as the most important, and the use of alternatives to AMD as the least important. This finding indicates that the respondents appreciated the concept of judicious drug use and highlights other aspects of stewardship, such as disease prevention and the use of alternatives to AMD, which should be the focus of future outreach efforts. Having a valid VCPR was ranked 4th (low), although up to 90% of the respondents had a valid VCPR. It is possible the respondents considered having a valid VCPR to be a regulatory requirement for access to AMDs, rather than a stewardship practice. Most of the respondents reported no change in the use of AMD post-VFD rule changes, although a small proportion indicated a decrease in the use of AMD, primarily for those administered in milk fed to calves. The limited changes following the implementation of the VFD final rule may be attributed to the short time lapse (6 months) between the implementation date and administration of this survey. It is worthwhile though that the key change noted among a few respondents was a reduction in use of antimicrobials. However, the producers that reported decreased use of OTC drugs due to the VFD requirement also indicated a resultant increase in calf mortality. It is possible that respondents who reported increased calf mortality post-VFD were partly

TABLE 7 | Antimicrobial drugs used to treat respiratory disease and diarrhea in preweaned calves after January 1, 2017 on California dairies.

First choice antimicrobial drug			Second choice antimicrobial drug		
Respiratory disease ($n = 49$)	**Number of respondents**	**Percent**	**Respiratory disease ($n = 33$)**	**Number of respondents**	**Percent**
Ampicillin	1	2.0	Ampicillin	1	3.0
Ceftiofur	8	16.3	Enrofloxacin	5	15.2
Enrofloxacin	4	8.2	Enrofloxacin and Flor[c]	2	6.1
Florfenicol	21	42.9	Florfenicol	8	24.2
Gamithromycin	2	4.1	Oxytetracycline	2	6.1
Oxtetracycline	4	8.2	Penicillin	2	6.1
Penicillin	1	2.0	Tildipirosin	1	3.0
Tildipirosin	1	2.0	Tilmicosin	1	3.0
Tulathromycin	5	10.2	Tulathromycin	11	33.3
Tylosin	1	2.0			
None*	1	2.0			
Diarrhea ($n = 45$)			**Diarrhea ($n = 17$)**		
Adsorbent*	1	2.2	Ceftiofur	3	17.7
Ampicillin	4	8.9	Enrofloxacin	2	11.8
Ceftiofur	3	6.7	Florfenicol	1	5.9
Enrofloxacin	1	2.2	Oxytetracycline	2	11.8
Florfenicol	1	2.2	Penicillin	2	11.8
Neomycin	2	4.4	Sulfonamide	3	17.7
Oxytetracycline	1	2.2	Tulathromycin	1	5.9
Penicillin	1	2.2	Penicillin and Ceftiofur	1	5.9
SMZ/Bismuth/Charcoal*	1	2.2	Ampicillin	1	5.9
Salt solutions*	3	6.7	Others[b,*]	1	5.9
Spectinomycin	2	4.4			
Sulfamethoxazole	1	2.2			
Sulfonamide and Ceftiofur	1	2.2			
Sulfonamide	11	24.4			
Others[a,b*]	12	26.7			

The first and second choice antimicrobial drugs (AMD) used to treat respiratory disease and diarrhea are shown as number of respondents who answered that they used this specific AMD.
[a] Bismuth subsalicylate, [b] ivermectin, [c] Enrofloxacin and Florfenicol.
**Non-antimicrobial compounds.*

reliant on AMD prophylaxis for disease prevention prior to VFD implementation. In addition, the survey respondents could have been biased in their responses by a perceived negative effect of the regulatory changes on AMD use. Opportunities exist to improve and manage calf health through vaccination, use of diagnostics to guide treatment decisions, and use of supportive therapy when AMD use is not justified, such as cases of viral enteritis. Indeed, very respondents reported use of salt solutions as the first-choice treatment for diarrhea, and less than one-third of the respondents either reported submission of calves for diagnosis or use of other diagnostic methods to guide treatment of preweaned calves. Furthermore, there is need for future on-farm studies to generate data on-farm changes in AMD use in preweaned calves post-VFD and the associated impact on calf health and mortality. In addition, longitudinal studies that investigate implementation of stewardships practices that reduce unnecessary use of AMDs and the animal health and welfare outcomes are needed.

Feeding calves colostrum or non-saleable (waste) milk containing AMD residues, as reported by most respondents, constituted potential sources for indirect exposure of preweaned calves to AMD. Previous studies on California dairies reported detectable concentrations of at least one AMD compound in 15 out of 25 waste milk samples tested (43). The presence of AMD in waste milk could potentially contribute to development of AMR (2). Since waste milk is a valuable feed source for calves (44), stewardship efforts should focus on strategies to reduce residues in waste milk to mitigate the potential risk of AMR development. Although most dairies pasteurized waste milk prior to feeding to calves, pasteurization is not effective in removing residues, and calves fed pasteurized waste milk were shown to have increased presence of AMR gut bacteria compared to calves fed milk replacers (16, 45). Recent research evidence shows that alkalinization of milk to pH 10 and spiked with ceftiofur sodium resulted in 96% degradation of the initial drug concentration (46), and is thus a potential strategy to treat waste milk before feeding to calves. Such a strategy would increase wider use of waste milk for feeding calves as a low-cost diet alternative and reduce costs associated with its disposal otherwise.

TABLE 8 | Decision making on antimicrobial drugs (AMD) purchased and used to treat sick calves on California dairies.

Person making decision	Percent	SE	*N*	95% Confidence limits	
				Lower	Upper
Purchase of AMD added in feed*					
Owner	69.35	5.9	63	56.5	79.77
Veterinarian	49.2	6.35	63	36.83	61.68
Herd manager	25.4	5.53	63	15.97	37.89
Calf manager	20.63	5.14	63	12.19	32.75
Nutritionist	3.17	2.22	63	0.76	12.24
Purchase Injectable or oral AMD*					
Owner	65.67	5.84	67	53.27	76.25
Veterinarian	46.27	6.14	67	34.47	58.5
Herd manager	31.34	5.71	67	21.18	43.68
Calf manager	25.37	5.36	67	16.2	37.42
Nutritionist	1.49	1.49	67	0.2	10.31
Other[a]	1.49	1.49	67	0.2	10.31
Treatment of preweaned calves on 1st day of illness*					
Owner	50.06	6.16	66	43.65	67.76
Veterinarian	28.79	5.62	66	18.96	41.13
Herd manager	21.21	5.07	66	12.81	33.04
Calf manager	45.45	6.18	66	33.63	57.81
Nutritionist	0				
Other[b]	1.52	1.52	66	0.2	10.47

The dairy owner and the veterinarian were reported to play a major role in deciding on which AMD are purchased and stocked on the dairy, as well as the choice of the AMD used to treat calves on the 1st day of illness. N, total number of respondents who answered the specific question.
**Some respondents chose more than one option. [a]Son of calf-feeder. [b]Calf-feeder.*

Treatment of calf diseases was the main cause of direct exposures of calves to AMD. This finding is consistent with the outcome of a previous survey of Tennessee cattle producers which showed that treatment of clinical disease and animal welfare were some of the key drivers for AMD use (23). With regards to the specific AMD types, the highest mean percentage of calves that were administered a given AMD type was reported for tetracycline and neomycin added in milk, grain, and water. In the United States, tetracycline and neomycin are among the drugs currently labeled for treatment of diarrhea (scours) (47, 48), which is the most common preweaning calfhood disease (49). Some respondents listed non-antimicrobial compounds amongst AMD administered treat preweaned calves indicating the need for awareness to correctly identify drug classes. Whereas, most of the respondents reported keeping treatment records, treatment date was the only common information recorded, and only a few respondents recorded additional information such as the drug dose, duration of treatment and route of administration. In addition, only half of the respondents used permanent computer software or paper records, the remaining respondents relied on hutch markings, memory, used chalk or did not keep records. Record keeping is one of the key elements of AMD stewardship, and future efforts to promote antimicrobial stewardship for preweaned calves should address this area.

Some of the reported uses of AMD in preweaned calves indicated usages that are not permitted by FDA. Among these uses were a few responses that indicated administration of spectinomycin or coccidiostats in milk or milk replacer. None of these products has label directions for use in milk or milk replacer, and extra-label use (ELDU) of any animal or human drug in or on animal feed is not permitted by FDA (50). By contrast, ELDU of AMD administered in water is permissible provided that the other requirements for ELDU established by FDA are properly met, which includes supervision of such use by a licensed veterinarian with a VCPR. The single reported use of enrofloxacin as an AMD choice for treatment of scours or diarrhea would be a violation of a specific FDA regulation which prohibits ELDU of this AMD in food-producing animals (51). The few responses for these uses associated with regulatory violations may be attributed to the tendency of written survey respondents to give answers they feel is correct rather than the actual practice or may represent errors by respondents in completing the survey items; otherwise, these responses indicate the ongoing need for veterinarians and producers to be vigilant of current regulations and to be in compliance with those requirements to ensure a safe food supply of animal origin.

Having a valid VCPR constitutes the regulatory and operational basis of interaction between veterinarians and their clients in provision of health care for their animals (52). Up to 90% of responding dairies reported having a VCPR. Dairies that reported not having a VCPR indicated otherwise in their responses to other questions. It is therefore most likely that 100% of the respondents had a VCPR; however, outreach efforts

TABLE 9 | Information sources for antimicrobial drugs used to treat preweaned calves on California dairies.

	Percent	SE	*N*	95% Confidence limits	
				Lower	Upper
Previous experience	66.2	5.91	65	53.55	76.81
Product label	27.7	5.59	65	17.98	40.09
Sales representative	32.3	5.85	65	21.86	44.88
Websites	1.5	1.54	65	0.2	10.62
Promotional materials	9.2	3.62	65	4.11	19.42
FARAD (Food Animal Residue Avoidance Databank)	0	–	–	–	–
Cooperative extension	0	–	–	–	–
Veterinarian	84.6	4.51	65	73.35	91.66
Other producers	16.9	4.69	65	9.47	28.39
Magazines and Journals	12.3	4.1	65	6.16	23.09
Local/national meetings	3.1	2.16	65	0.74	11.88
Other[a]	12.3	4.1	65	6.16	23.09

The respondents were reportedly reliant on the veterinarian and previous experience with a drug as sources of information on AMD used to treat calves. The respondents typically relied on more than one information source. N, number of respondents who answered the questions.
[a]Do not use antibiotics.

TABLE 10 | Consultation and prescription of antimicrobial drugs used to manage diseases in preweaned calves on California dairies.

Decision on disease management	Percent	SE	*N*	95% CI	
				Lower	Upper
Producers' consultant					
Veterinarian	96.8	2.3	62	87.6	99.2
Nutritionist	17.7	4.9	62	9.9	29.7
Pharmaceutical Co. vet/nutritionist	12.9	4.3	62	6.5	24.1
Pharmaceutical Co. Sales rep.	12.9	4.3	62	6.5	24.1
Other[a]	1.6	1.6	62	0.2	11.1
Who prescribes (or authorizes) antimicrobials in feed or water					
Veterinarian	71.4	6.1	56	57.9	82
Nutritionist	7.1	3.5	56	2.6	18
Pharmaceutical Co. vet/nutritionist	0		56		
Pharmaceutical Co. Sales rep.	1.8	1.8	56	0.2	12.3
Other[b]	16.1	5	56	8.4	28.6

The respondents mainly consulted the veterinarians on the type of AMD used to treat preweaned dairy calves. Similarly, the veterinarians were the principal personnel prescribing AMD for dairy calves. N, number of respondents who answered the question.
[a]Milk replacer and grain company consultant. [b]Used containing antimicrobials (supplied by company).

are further needed to inform a small percent of CA dairies on what constitutes the creation and maintenance of a VCPR with a veterinarian. Maintaining a valid VCPR allows the veterinarian to be in the best position to provide advice on AMD use decisions on farms (53). The decisions to purchase AMD for the dairy and treat sick animals with a specific AMD influence drug use on farms. In this survey, producers had a greater influence on the decision to purchase drugs, followed by veterinarians. However, the veterinarians were the major source of information that guided the producer's decisions, besides producers' experience with the drug, pharmaceutical sales representatives, and drug label information. Our finding is in concordance with previous studies that identified veterinary advice was the primary reason for choosing AMD by farmers in New Zealand (21).

Six components explained most of the variability in the survey responses. Knowledge of these components provide insights into management practices that can be the focus for stewardship interventions and outreach. The first major component identified pertained to the changes made in the use of antimicrobials following implementation of the VFD final rule, including discontinued use of one or more AMD or reduced the amount or duration of use. Such changes could have been the direct consequence of the VFD rule restricting use of AMD. Only a small proportion of respondents had started the use of alternatives to AMD, and as such future studies on stewardship should explore barriers and motivations for use of AMD alternatives. The rest of the identified components were key features of AMD

TABLE 11 | Component variables explaining variability in antimicrobial drugs (AMD) in preweaned dairy calves on California dairies.

Identified components	Variation Percent (%)	Component variables	Correlation
1. Change in the use of antimicrobial drugs following the implementation of Veterinary Feed Directive (VFD) in 2017	37.13	Antimicrobials used on solid feed	0.670
		Antimicrobials used in water	0.633
		Injectable antimicrobials	0.629
		Antimicrobials used in milk or milk replacer	0.623
		Producer considers AMD importance in raising calves	0.521
		Antibiotic drug costs since VFD final rule	0.477
		Started use of alternatives to antimicrobials	0.475
2. Use of diagnostics to guide treatment decision	18.60	Submission of calves to diagnostic labs	0.674
		Use of diagnostic techniques to guide treatment	0.491
		Frequency of animal health monitoring by veterinarian	0.468
3. Source of information and decision on AMD for feed, milk, water, oral, and injectable	17.95	Decision to use injectable or oral antimicrobials (Vet/Non-vet) [a]	0.618
		Source of information on antimicrobials (Vet/ Non-vet)[a]	0.582
		Decision to use any antimicrobials (Vet/Non-vet)[a]	0.573
		Method of estimating the drug dosage (Vet/Non-vet)[a]	0.552
		Decision to treat on 1st day of illness (Vet/Non-vet)[a]	0.548
		Decision on second choice antimicrobial (Vet/Non-vet)[a]	0.510
4. Antimicrobial use protocols and records	17.64	Estimation treatment duration for any antimicrobial (Label/Others)	0.613
		Tracked treatment information (Date/Route/Dose/None)	0.574
		Estimation of treatment duration for injectable antimicrobials (Label/Others)	0.506
		Tracked antibiotic withdrawal interval (Yes/No)	0.446
		Treatment protocols components (Vaccinations/ Disease Definition/Treatment)	0.432
		Method of tracking treatments (Computer/Paper/Hutch/Memory/Chalk)	0.436
		Keep drug inventory log (Yes/No)	0.425
5. Common drugs to treat pneumonia and diarrhea	11.06	First choice antibiotic for treatment of Pneumonia (1-florfenicol, 2- 3rd generation cephalosporins, 3-Tulathromycin, Gamithromycin and Tildipirosin, 4-Oxytetracycline, 5-Pencillin and Ampicillin, 6-Tylosin, 7-Enrofloxacin)	0.511
6. Calf feed management	16.70	Milk fed to preweaned calves (Pasteurized/ Not)	0.642

The multiple factor analysis identified six components with 25 component variables with correlation >0.40 at the first three dimensions. The first three dimensions of MFA explained 21.46% of the variability (dimension 1 = 9.75%; dimension 2 = 6.56%; dimension 3 = 5.15%). [a]Varaibility due to veterinarian making decision compared to non-veterinarian.

management practices (disease diagnosis, AMD use practices, record keeping, information sources and decision of AMD use, and health management practices). In the health management component, heat treatment of colostrum fed to calves was the single most important variable to explain the variability between dairies. Variation in the colostrum management was possibly associated with farm size as shown in a previous survey of Pennsylvania dairy farmers in which larger farms were more likely to have the equipment for colostrum heat treatment (54).

One limitation of the current study was the small number of responses ($n = 5$) received form the calf ranch producers. Similarly, the number dairy respondents were relatively low, although the response rate was comparable to the outcome of previous survey conducted among the same demographics. The low response could be attributed to the survey fatigue and challenges inherent in the discussions and reporting of antimicrobial drug use among food animal producers in general.

CONCLUSION

Following the implementation of the VFD final rule on January 1, 2017, more than one third of the producers had made changes in the use of AMD, most notably by reducing the amount or duration of use or discontinuing the use of one or more AMD added in liquid diet or on solid feeds. The limited changes noted in AMD use could have been due to the short period between the implementation of VFD and conducting the survey. Most respondents reported a greater involvement of the herd veterinarian, compared to nutritionists or pharmaceutical sales representatives, in informing producers about the use of AMDs. Whereas, most producers had knowledge of the VFD and SB 27, opportunities exist to improve AMD use practices, including record keeping, using AMD alternatives, and improved farm management practices to reduce disease burden and need for AMD use.

The current survey outcomes allow immediate assessment of the impact of VFD final rule implementation and provides

baseline data for future evaluation of the impact of VFD as well as SB 27 regulatory changes. The knowledge gained from this study is a valuable resource that could guide future recommendations for best health management practices and promote antimicrobial stewardship efforts.

DATA AVAILABILITY STATEMENT

Data collected for this study is protected under California Food and Agriculture Code 14407. Requests for raw data may be made to the authors, who will consult with the California Department of Food and Agriculture (CDFA) on fulfilling the request.

AUTHOR CONTRIBUTIONS

SA and EO designed the study, authored the first draft of the survey questions, and conducted the survey. SA, EO, DW, TL, RP, and JA reviewed and edited the survey. EO, SA, and WE analyzed the survey data. EO wrote the first draft of the manuscript. All authors edited and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.636670/full#supplementary-material

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Description of the Characteristics of Five Bedding Materials and Association With Bulk Tank Milk Quality on Five New York Dairy Herds

*Valeria M. Alanis[1], Michael Zurakowski[2], Deb Pawloski[2], Tiago Tomazi[1], Daryl V. Nydam[1] and Paula A. Ospina[3]**

[1] Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, United States, [2] Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Cobleskill, NY, United States, [3] Lechear Limited Liability Company, King Ferry, New York, NY, United States

Environmental mastitis represents a major challenge on dairy farms where contagious pathogens are controlled by improved milking procedures. Therefore, research focused on the environment is important to improve udder health programs. The objectives of this prospective and descriptive study were to (1) describe bedding bacterial counts, pH, and dry matter (DM) of five different bedding types (organic: manure solids, straw, paper fiber; inorganic: sand, recycled sand) and (2) explore the association between bedding bacterial counts with bulk tank milk quality. This study took place within five conveniently selected commercial dairy herds, each with a predominant bedding material in lactating pens. Bedding samples (used $n = 237$; fresh $n = 53$) were collected monthly from July 2018 to July 2019 following a standard operating procedure (SOP) to minimize sampling variability. Additionally, a bulk tank (BT) milk sample ($n = 40$) was collected on the same day unless milk had been picked up prior to arrival. Both BT and bedding samples were submitted to the laboratory for culture and bacterial identification and quantification of *Streptococcus* spp, coliforms, and non-coliforms as well as detection of several pathogens of mastitis importance. Somatic cell count was evaluated in BT samples. Within bedding type, the correlation between bedding characteristics and bacterial counts in bedding was evaluated using Pearson correlation. Within bedding type, the correlation between bacterial counts in bedding samples and bacterial counts in BT were determined. The Kruskal–Wallis test was used to evaluate the bacterial count by bedding type and to evaluate BT somatic cell count differences based on bedding type. In fresh bedding, bacterial counts were generally higher for manure solids for all bacterial groups compared with other materials. In used samples, organic materials had the highest levels of all bacterial groups. The proportion of samples with detectable organisms of mastitis importance varied within and among herds in both bedding and BT samples throughout the study period. In bedding samples, a higher DM content had the lowest levels of bacterial growth compared with those with lower DM content. Most bedding samples were on the alkaline side within a pH range of 8–11. No relationship between bacterial counts and pH was observed. No associations between BT bacteria counts and bedding bacterial counts were observed. No association between bulk tank

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***Correspondence:**
Paula A. Ospina
paulao@lechear.com

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somatic cell counts based on bedding type were observed. Despite using an SOP for bedding sampling in an effort to consistently collect samples, we still observed a large amount of variability both within and among bedding samples. This variability may have obscured any potential association between BT milk quality and bedding type.

Keywords: bacteria counts, milk quality, environmental mastitis, bulk tank milk, bedding material

INTRODUCTION

As a multifactorial disease, bovine mastitis is one of the most complex, frequent, and costly diseases of dairy herds associated with decreased milk yield and quality (1–4). Research shows that coliform and *Streptococcus* spp pathogens cause impactful milk losses (3–5) and that these losses vary between primiparous and multiparous cows. Raw milk with high somatic cell count (SCC) often has higher lipolysis and proteolysis than in low SCC milk and also has effects in pasteurized milk, such as decreasing shelf life and sensory defects, including rancidity, bitterness, and astringency (6). In the last years, there have been some changes in the distribution and patterns of mastitis in dairy herds in developed countries with an important decrease of cows with contagious forms of mastitis but persistent environmental forms (7–10).

Coliforms (including *Escherichia* spp, *Klebsiella* spp, and other Gram-negative bacteria), Streptococcus species (including *Streptococcus uberis* and *Streptococcus dysgalactiae*), and non-aureus *Staphylococcus* are among the most common environmental bacteria causing mastitis in U.S. dairy herds (USDA, 2014). This distribution of mastitis pathogens was also identified in a recent study from eight commercial herds in New York (11). Additionally, cows with at least one clinical mastitis case due to environmental pathogens, such as *E. coli*, *Klebsiella* spp, and *T. pyogenes*, have greater risks of culling (12) compared with non-mastitic cows. Further, Gram-negative cases increased the risk of mortality as stated in a study from 30,233 lactations in cows of seven dairy farms in New York State (13).

These environmental mastitis pathogens have been isolated from bedding materials, soil, rumen, feces, vulva, lips, nares, and feed samples (14–17), which demonstrates their nearly ubiquitous risk to environmental and teat end contamination. Like any other types of bacteria, they require appropriate moisture, temperature, and nutrients to live. Appropriate conditions are often present on dairy farms to allow bacterial numbers to increase. Therefore, the number of these bacteria on teat skin is a reflection of the cow's exposure to the contaminating environment (18). Bedding material itself has physical and biochemical properties that support bacterial growth along with external factors that influence it (19).

Extensive research demonstrates that both heifers and cows need 12–14 h of lying daily and that they prioritize it over other activities (20, 21). Considering this strong behavioral need to rest, a fundamental issue to consider is bedding materials that provide adequate cushion and also that can reduce udder and teat exposure to environmental pathogens. Exposure to these pathogens when the cow lies down could result in intramammary infections with a possible mastitis outcome (18). Several studies show that bacteria can be transferred between the lying surface and the teats (22–25). Because environmental pathogens are highly influenced by management practices, such as the housing system, cow comfort, manure collection method, proper bedding, and pen cleanliness (26, 27), one of the most difficult dairy farm challenges is to minimize the level of exposure to environmental mastitis pathogens at the teat level between milkings to maintain good udder hygiene.

Few studies focus on the association between bedding material and bulk tank (BT) milk quality (i.e., bacterial load and somatic cell counts). Among these few studies, there have been few consistent results. One prospective study using data from BT test results from 325 dairy herds in Wisconsin using the same bedding in all pens during the two-year study period (28) shows that total bacterial counts in the BT were not associated with bedding type, but bulk milk somatic cell score (BTSLS) was lower for farms using inorganic materials.

A cross-sectional study using data from 125 herds in the United Kingdom (29) show no significant differences between bedding material in bacterial counts in milk for any of the organisms studied and no significant correlations between bacterial load in used bedding and milk. More recently, another cross-sectional study using data from 167 herds from 17 states in the United States (30) shows a wide variation of pathogen load in bedding among farms with organic material bedding showing the highest coliform levels compared with inorganic materials and manure solids showing the highest counts for streptococci-like organisms. They establish a guide for monitoring bedding hygiene in commonly used organic and inorganic bedding. Looking at another aspect of milk quality, research focused on food safety shows that bedding management practices (e.g., re-bedding frequency, raking frequency) were associated with mesophilic and thermophilic spore levels, and used organic bedding spore levels were positively related to those in BT milk (31).

The objectives of this prospective and descriptive study with repeated measures were to (1) describe the variability in bedding bacterial counts, pH, and dry matter (DM) of five different bedding types (manure solids, sand, straw, paper fiber, and recycled sand) and (2) explore the association between bedding bacterial counts with BT milk quality in five conveniently selected New York dairy farms using one of five bedding materials in lactating pens.

MATERIALS AND METHODS

Herd Selection and Sample Collection

Five commercial dairy herds in central New York State with an average herd size of approximately 1,400 cows (ranging from 838 to 2,050) were conveniently selected based

on the willingness of the producers to participate and the proximity of the herds to the Quality Milk Production Services laboratory (QMPS) at the Animal Health Diagnostic Center, Cornell University (Ithaca, New York). Each herd used a predominant bedding material for lactating pens: manure solids (MS), paper fiber (PF), straw (ST), recycled sand (RS), or sand (SD).

All herds used Dairy Comp 305 (DC305; Ag Valley Software) as the management software. Participating herds used a well-established milking routine, and every case of mastitis was identified by trained on-farm personnel, who collected all milk samples from all quarters with visibly abnormal milk, stored in a refrigerator (≅4°C), and saved information in DC305. These milk samples were submitted to the QMPS laboratory for culture and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) identification. These herds also had a regular DHIA testing program (monthly individual SCC and linear score) and were fed a balanced total mixed ration (TMR).

Farms were visited once monthly for a period of 6 to 12 months from July 2018 to July 2019 by the same observer. The sample collection period among the herds varied in one herd because they changed bedding type mid-study. At each visit, used and fresh bedding samples were collected as well as a BT sample and a DC305 backup.

Herd Bedding Practices

The herd using RS used a modified plug-flow aerobic digestion system with recirculation and mixing and a multistage SD separation system. The herd using MS used a screw press as a manure separation system. Herds using PF, SD, and ST purchased the material and stored it in a clean and dry storage area inside the herd.

They were also asked to notify investigators of any changes to these management practices during the study.

Bedding Samples

The samples were collected once a month from lactation pens. A standard operating procedure (SOP) was followed to minimize sampling variability. The day that the fresh bedding was due to be applied and after the routine cleaning, used bedding from three to five stalls from each pen was collected. Wearing clean disposable gloves, samples were collected from a 60 cm x 60 cm area, avoiding any manure spots, where the udder would touch the stall after scraping 3 cm off the top of the bedding material. Samples were transferred to a new one-quart storage freezer bag.

Using new and clean disposable gloves, fresh bedding samples were collected after asking the worker to dump extra bedding material in five stalls distributed throughout the pen. Fresh bedding was collected from the top of this pile to form a combined sample. The sample was transferred to a new one-quart storage freezer bag.

Each used and fresh sample bag was labeled with the herd name, pen number, and date. Samples were placed in ice coolers, transported the same day within 2 h after sampling, and frozen at−18°C for up to 4 weeks for analysis at QMPS.

BT Milk Samples

Unless milk had been picked up prior to arrival, the same day bedding was sampled, one BT sample was collected directly from the BT using a clean and sanitized dipper into a 10-ml vial. Sampling was performed following the Dairy Practices Council (DPC) guidelines (i.e., mechanically agitate the milk for at least 5 min until sufficient homogeneity is obtained and 10 min for tanks larger than 1,500 gallons). Each vial was labeled with the herd name and date. Samples were placed in ice coolers at 1°C, transported the same day within 2 h after sampling, and frozen at−18°C for up to 4 weeks for analysis at QMPS.

Laboratory Analysis and Bacteria Quantification

Frozen bedding and BT samples were submitted for bacterial identification and quantification for *Streptococcus* spp, coliforms, and non-coliforms at QMPS as well as detection of other pathogens associated with mastitis.

Bedding Samples

Frozen samples were allowed to thaw at refrigeration temperature (2°C−8°C) for one to 4 h, depending on the bedding material to be analyzed. The sample was placed into a large, clean, zip-type bag that allowed thorough mixing and breaking up of any clumps. For ST samples, pieces were cut into approximately 2.5 cm in length using sterile scissors. Using a weight-verified scale, bedding material was weighed 10 ± 1% (9.90–10.10) grams into a stomacher bag (MS, SD, and PF) or sterile vial (RS, ST) by taking small subsamples from at least three random locations within the mixed sample. Then, 90 ml of sterile PBS was added to the 10-g test sample and mixed for 2 min using a stomacher set at blending speed 2 (two strokes/second) or vortex for 40 s at setting 7 (1,800 rpm) for vials. Approximately 10 ml of this suspension was decanted into an empty sterile dilution tube. This was the 10^{-1} dilution. The 10^{-2} dilution was made by vortexing the 10^{-1} dilution for a minimum of 4 s and removing 1 ml using a micropipette and adding it to 9 ml of PBS. This dilution process continued until the 10^{-5} dilution.

BT Samples

Frozen samples were allowed to thaw at refrigeration temperature 2°C−8°C and mixed thoroughly by shaking. The 10^{-1} dilution was made by removing 1 ml and adding it to 9 ml of PBS and vortex for a minimum of 5 s after a vortex has been achieved. This dilution process continued until the 10^{-2} dilution.

Plate Inoculation and Incubation Parameters (Bedding and BT Samples)

For each bedding and BT sample, 50 μl of each dilution was inoculated on different selective media. Edwards media was inoculated to test for *Streptococcus* spp and "streptococci-like" organisms. MacConkey media was inoculated to test for coliforms and non-coliforms. Hayflick media was inoculated with 50 μl of used bedding material from dilutions 10^{-2}, 10^{-3}, and 10^{-4} to test for mycoplasma and placed in a CO_2 incubator. For BT samples, trypticase soy agar with 5.0% blood and 0.1%

esculin media was inoculated to test for total count of all organisms (TBC).

In addition to the organisms that were quantified, the following organisms of mastitis significance were identified and counted as detected or not detected: *Staphylococcus aureus*, *Streptococcus agalactiae*, *E. coli*, *Klebsiella* spp, *Serratia* spp, *Pasteurella* spp, *Pseudomonas* spp, *Prototheca* spp, *Trueperella pyogenes*, yeast, mold, other fungi, and other microorganisms (*Listeria* spp, *Nocardia* spp, and *Salmonella* spp). Experienced technicians in microbiology used visual cues and biochemical tests (NMC, 2017) along with colony morphology of the plate to identify these pathogens. The presence of even one colony would be considered as detected.

Plates were incubated at 35°C−38°C. After 18–24 h of incubation, plates were observed using standard microbiology procedures. At 18–24 h, the lactose-positive, Gram-negatives colonies were counted and *E. coli* and *Klebsiella* were observed and recorded. Plates were placed back in the incubator at 35°C−38°C for an additional 18–24 h.

Bacteria Counts Calculation

Plates were removed from the incubator, and the number of colony-forming units (CFU; CFU/g for bedding samples and CFU/ml for BT samples) counted by an experienced laboratory technician from the dilution plate (up to 10^{-5} for bedding samples and up to 10^{-2} for BT samples) that presented 25–250 colonies whenever possible. All counts and the dilution plate were recorded in an internal form. The formulas used are as follows:

Bedding:

((A(1000/B)∗9)/C)/(D/E)

$$\left(\frac{\left(\frac{A\ CFU}{50\ \mu L}\right) * \left(\frac{1000\ \mu L}{1\ mL}\right)}{\left(\frac{10\ g}{90\ mL}\right)}\right) * C * \left(\frac{E}{D}\right)$$

$$= \left(\left(\left(\left(\frac{A\ CFU}{50\ \mu L}\right) * \left(\frac{1000\ \mu L}{1\ mL}\right)\right) * \left(\frac{90\ mL}{10\ g}\right)\right) * \left(10^{n}\right)\right) * \left(\frac{E}{D}\right)$$

$$= X\ \frac{CFU}{g}$$

Where:
A = number of colonies (CFU)
B = inoculation volume = 50 µl
C = dilution factor, n (10^{-n})
D = dry weight (g)
E = wet weight (g)

% moisture

((A+B)-C)∗100/10

Where:
A = empty dish
B = bedding weight (added to the dish to go into the oven)
C = after drying (dish + bedding)

BT:

A(1000/B)/C

Where:
A = number of colonies (CFU)
B = inoculation volume (µl)
C = dilution value of the plate counted or dilution factor, n (10^{-n}).

Moisture Content (DM Content) Estimation

The drying dish was weighed. The scale was tared and 10 ± 1% (9.90–10.10) g of bedding material was added and evenly spread. The dish containing the 10 g of bedding was placed into the oven and dried for at least 4 h at 100 ± 10°C. After drying, the sample was weighed, and the total weight to two decimals was recorded.

pH Estimation

A flip-top vial was placed on the scale and tared and 10.00 g of bedding material was added by taking small subsamples from at least three random locations within the mixed sample. Next, 90 ml of deionized water was added using a 100-ml graduated cylinder and mixed well. The pH probe from a pH meter was verified with appropriate buffers (7 and 10 buffers for calibration as most bedding material fit that range). If a bedding material ended up with a lower pH after calibration with the 7 and 10 buffer, the pH meter was recalibrated using a 4 and 7 buffer. This probe was placed into the mixture, and pH was recorded to two decimals.

Somatic Cell Count

BT milk SCCs (BTSCC) were analyzed using a DeLaval cell counter (DCC). The DCC analyses were performed using samples at 10°C−40°C following the manufacturer's instructions. To transform BTSCC into BTSLS the following equation was applied: BTSLS = log2 (BTSCC/100) + 3.

Statistical Analysis

Data collected and laboratory results were transferred to Excel spreadsheets (Microsoft Corp; Redmond, WA). Data were imported into R version 4.0.3 (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA) to perform statistical analysis and to create the appropriate plots. All graphical representations were made using the ggplot2 package. The normality of continuous variables (i.e., bacteria counts) was visually assessed with density plots and quantile-quantile plots. These were not normally distributed; therefore, bacteria count values greater than zero were log10 transformed. When no bacteria were identified, a value of log10+1 CFU/g for bedding and log10+1 CFU/ml for BT was used, assuming that at least 10 CFU were present in a given sample. The decision to use this arbitrary value was due to the potential losses on each dilution before having the final count. An additional outcome was created in which the counts of each bacterial group isolated (*Streptococcus* spp, coliforms, and non-coliforms) in bedding samples were summed. This new outcome was named sum bacterial count (SBC).

Within bedding type, the correlation between bedding characteristics and bacterial counts in bedding were evaluated

using Pearson correlation. For bacterial count analysis in bedding samples, the Kruskal–Wallis test was used to evaluate the differences based on bedding type running the kruskal.test function. When appropriate (meaning following a Kruskal–Wallis test at $P < 0.05$), Dunn's multiple comparison test among the five bedding materials and Bonferroni correction were used as a *post hoc* nonparametric test running the dunn.test function. Correlations between bedding characteristics (pH and DM) and bacterial counts were determined using the Pearson correlation coefficient running the cor.test function. Within bedding type, the correlation between bacterial counts in bedding samples and bacterial counts in BT were determined. The Kruskal–Wallis test was used to evaluate the bacterial count by bedding type and to evaluate BT somatic cell count differences based on bedding type.

For bacterial count analysis in BT samples and to evaluate differences between BTSLS based on bedding type; the Kruskal–Wallis test was used to evaluate the differences based on bedding type running the kruskal.test function. When appropriate (meaning following a Kruskal–Wallis test at −<0.05), Dunn's multiple comparison test among the five bedding material and Bonferroni correction were used as a *post hoc* nonparametric test running the dunn.test function. The proportion of bedding and BT samples with detectable organisms from the list of pathogens of mastitis importance was also described. Correlation between bedding bacterial counts and bacterial counts was determined using the Pearson correlation coefficient running the cor.test function with the average value of used bedding samples per time point.

RESULTS

Study Herds

The mean number of lactating cows was 1,400, and the daily mean milk production was 37 kg. The mean BTSCC was 130,000 cells/ml. All farms used a consistent milking routine with pre-dipping, foremilk stripping, and wiping teats with either cloth (MS, PF, RS, and SD) or paper towels (ST). All farms used iodine-based disinfectant solutions for pre-dipping and post-dipping. Basic farm descriptors, design, and management of bed descriptors are displayed in **Table 1**. Additionally, the results of cow positioning, bedding quantity, and quality can also be found in **Table 1**. Generally, most cows had adequate positioning (>70% except the MS herd with 25%).

Bacterial Counts in Bedding Samples

All collected samples were evaluated in the laboratory. Although the goal was to collect 12 fresh samples (one representative stall per month from each herd bedding type) and 60 used samples (five representative stalls per month from each herd bedding type) from 12 monthly visits ($n = 360$ total samples), only a total of 290 bedding samples (used $n = 237$; fresh $n = 53$) were collected for final analysis. The difference in the number of samples was due to lack of bedding available to sample (18 visits among herds) or equipment malfunction (12 visits among herds) on the follow-up visit. Due to cold storage space and laboratory time limitations, the number of used bedding samples collected from each farm visit was changed to three in the second half of

TABLE 1 | Herd characteristics from five conveniently selected New York dairy herds using one of five bedding materials in lactating pens.

Herd	Bedding type	Milking cows (n)[a]	DIM[b]	Average milk (kg)[c]	Average SCC[d]	Type of stalls	Re-bedding frequency[e]	Rake/groom bedding surface frequency	Milking frequency per day	Milking parlor type/Number of stalls	Towel material	Dry-off routine
A	Manure solids	2,050	189	41.7	143	Deep beds	Daily	3x daily	3x	Rotatory/80	Cloth	Blanket
B	Paper fiber	1,214	184	39.4	166	Mattress	Twice a week	2x daily	2x	Parallel/Double 18	Cloth	Blanket
C	Straw	838	161	32.2	211	Concrete	Daily	2x daily	2x	Parallel/Double 12	Paper	Blanket
D	Recycled sand	1,750	172	40.3	187	Deep beds	Weekly	3x daily	3x	Parallel/Double 17	Cloth	Selective
E	Sand	1,170	169	39.9	148	Deep beds	Weekly	3x daily	3x	Parallel/Double 18	Cloth	Blanket

[a] *Lactating cows monthly average.*
[b] *Average days in milk.*
[c] *Average of total daily milk produced (kg).*
[d] *Test day average somatic cell count (SCC; ×1,000 cells/mL) over the year of study.*
[e] *Frequency of adding new bedding material to resting area and stalls.*

the study. Last, we started sampling in the ST herd later compared with the other herds, which affected the final number of samples, and this herd changed bedding midway through the study, which severely limited the number of used samples of this bedding type. Thus, inferences from ST should be interpreted in light of the small number of observations. Comparatively the fresh samples were not as strongly impacted. The final analysis consisted of MS = 54 (used $n = 44$; fresh $n = 10$), PF = 86 (used $n = 70$; fresh $n = 16$), ST = 24 (used $n = 18$; fresh $n = 6$), RS = 74 (used $n = 60$; fresh $n = 14$), and SD = 52 (used $n = 45$; fresh $n = 7$).

Bacterial counts ($\log_{10}$ CFU/g) from fresh and used samples during the entire study period are summarized in **Figure 1**. The ST samples showed a wider variation on all bacterial counts compared with the other bedding types. The SD bedding type had four fresh samples with no detectable levels of *Streptococcus* spp and no detectable levels of coliforms.

There was a clear increase in bacterial counts in used bedding samples compared with fresh samples for all bedding materials. *Streptococcus* spp, coliform, and non-coliform counts in inorganic materials (RS and SD) were generally lower than in organic materials (MS, PF, and ST). For example, coliform counts were different between all bedding types, being the highest on ST, then equally highest on MS and ST, and equally lowest on RS and SD (MS vs. SD $P < 0.0001$, MS vs. RS $P < 0.0001$, ST vs. SD $P < 0.0001$, ST vs. RS $P < 0.0001$). All pairwise comparisons are shown in **Figure 1**. A similar relationship was seen with SBC counts in which inorganic materials were approximately 1 $\log_{10}$ less than the organic materials.

The variability between used samples collected on the same day is illustrated in **Figure 2**.

Detection of Specific Bacteria in Bedding

A summary of the proportion of bedding samples in which bacteria were positively identified is shown in **Figure 3** (i.e., if bacteria were not detected in fresh or used bedding these bacteria are not included in the figure).

DM Content and pH

The percentage of DM content and pH values for fresh and used bedding samples during the entire study period are shown in **Figure 4**. Generally, inorganic bedding samples were dryer than organic. Regarding pH values, fresh samples were on the alkaline side within a range of 8–11 except for ST, with acidic values (5.8 ± 1.4). For used bedding samples, all materials were in the alkaline range of 8–9. Relationships between DM content and bacterial count in fresh and used samples are shown in **Figures 5**, **6**, respectively. For example, correlation analysis showed a negative linear relationship between DM content and bacterial count in used samples: SBC ($r = -0.61$, $P < 0.001$), *Streptococcus* spp ($r = -0.60$, $P < 0.001$), coliforms ($r = -0.56$, $P < 0.001$) and for non-coliforms ($r = -0.53$, $P < 0.001$), suggesting drier bedding material had lower bacterial counts.

BT Bacterial Counts

On several visits ($n = 15$), the BT had recently been picked up, and a BT sample was not available. A total of 40 BT samples were collected for the final analysis: MS ($n = 11$), PF ($n = 7$), RS ($n = 8$), SD ($n = 8$), and ST ($n = 6$).

The bacterial groups evaluated in BT samples are summarized in **Figure 7**. Dunn's test with Bonferroni correction for multiple comparisons indicated that coliform counts on the ST herd (0.19) were observed to be different from those on RS (2.24) ($P = 0.04$) although it is important to notice that only one BT sample from this herd had detected levels of this bacterial group. In the other bacterial groups among herds based on the Kruskal–Wallis test, the *p*-values were *Streptococcus* spp ($P = 0.19$), *Staphylococcus* spp ($P = 0.08$), TBC ($P = 0.57$).

A correlation analysis of the average of coliforms and *Streptococcus* spp counts in used bedding samples and those counts in BT was performed, and the results showed a limited association with values of−0.09 ($P = 0.5$) and 0.06 ($P = 0.6$), respectively.

Detection of Specific Bacteria in BT

A summary of the proportion of BT samples with detectable pathogens of mastitis importance are illustrated in **Figure 8** (i.e., those without detectable organisms are not displayed).

BT Somatic Cell Linear Score

The overall BTSLS among herds was 3.54, ranging from 2.80 to 5.35 (**Figure 9**). The *p*-value for the Kruskal–Wallis test for the differences observed among bedding materials and BTSLS was 0.13.

DISCUSSION

This study describes characteristics (i.e., bacteria counts, pH, and DM) for fresh and used bedding samples as well as bacterial counts and SCCs from BT samples. These samples were collected, following a strict sampling SOP, monthly over 1 year from five conveniently selected New York dairy farms. Each farm used one of five bedding materials in lactating pens. In addition to describing these characteristics, this sampling scheme allowed us to demonstrate the variability within bedding samples in the same farm.

The first objective of this study was to describe bedding bacterial counts, pH, and DM. It is known that bedding material (especially organic material) can support bacterial growth due to contained nutrients, and even inorganic bedding once soiled with feces, urine, or any other cow secretion can grow bacteria. Our results confirm this with bacterial counts higher in used samples compared with fresh samples, which agrees with what has been stated by other research groups (29, 30, 32, 33). Evaluating these bedding characteristics is important because organic bedding material has been associated with higher bacterial load (30, 32) and with higher bacterial counts on teat skin (22, 23). Our results on bacterial counts were generally highest for MS on fresh bedding samples for all bacterial groups, which is similar to what is described by other researchers (23, 30). Particularly, coliforms counts were not different between RS, ST, and PF. As for used samples, we observed that organic materials supported the highest levels of all bacterial groups (**Figure 1**). In herds bedding with inorganic bedding material, *Streptococcus* spp levels

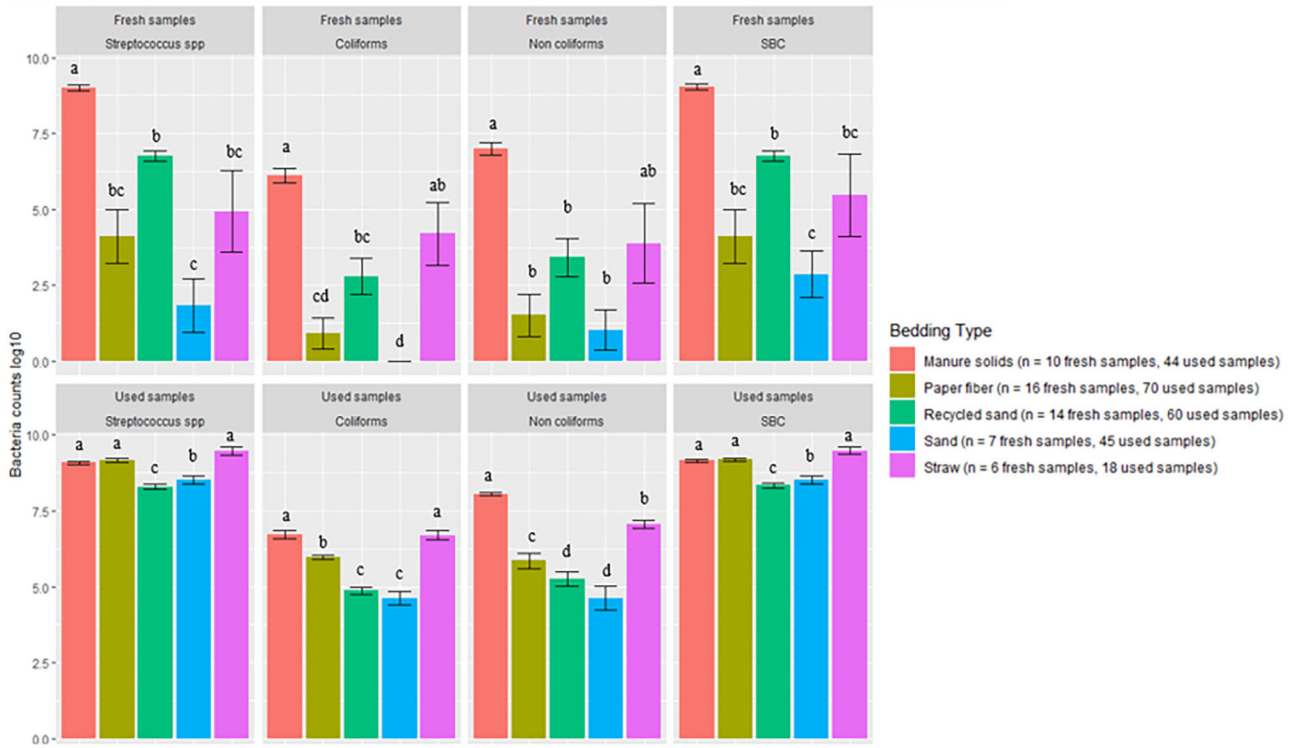

FIGURE 1 | Average bacterial counts (log10 CFU/g) for fresh and used bedding samples collected from July 2018 to July 2019 from five conveniently selected New York dairy herds using one of five bedding materials in lactating pens. One fresh bedding sample and three to five used bedding samples were collected monthly following an SOP at each visit (unless there was no bedding available due to lack of supply or equipment malfunction on the follow-up visit). Error bars represent SD. The same letters are not different at $P \leq 0.01$ (P-values adjusted for multiple contrasts). SBC (sum bacterial count) = Streptococcus spp, coliforms and non-coliforms summed.

were lowest in RS compared with SD, but similar to previous research (34), there was no difference in the number of coliforms and non-coliforms.

Several organisms of mastitis importance were not quantified but rather their presence evaluated because we focused on the pathogens that we can manage in bedding. In other words, we manage all *Streptococcus* species as a whole, but we cannot specifically manage *Streptococcus uberis* or *Streptococcus dysgalactiae*. *E. coli* was detected in only 50% and 54% of MS fresh and used bedding samples, respectively, which was surprising given that *E. coli* is known to exist in high quantities in feces. Apparently, in the herd studied here that bedded with MS, the manure and bedding processing procedures reduced *E. coli* to levels below detection. However, another fecally shed organism, *Klebsiella* spp, was found in 100% of fresh and used MS samples (**Figure 3**), suggesting that, at least on this farm, the manure processing and bedding procedures did not eliminate *Klebsiella* spp, leaving it as a risk factor for intramammary infections.

In our study, DM content was higher for RS and SD (∼92%) in fresh samples, similar to Canadian farms (35) and within the ranges reported for used samples (∼95%) by Zdanowicz, Patel, and Kristula (22, 30, 34). These values seem to have low variability across studies. Fresh MS had a DM average content of 39.5% (34.2–47.0%), similar to the values reported by Robles (35). However, a different study (30) reports a much wider range (21.4 – 96.3%) in samples collected from 17 states across the United States. That variability might be explained by different MS processing techniques (i.e., digested, compost, or fresh) and possibly due to the sampling variability (e.g., time in relation to when were applied). In our study, used MS samples had a higher DM content (57.8%, range of 40.6–74.6%) compared to MS fresh samples. This observation has been reported by others (35–37).

The relationship of DM content and bacterial growth suggest that drier bedding material impedes bacterial growth for all bacterial groups in all bedding types. The correlations between these variables are similar to the ones reported by Zdanowicz (22). As a result, a high percentage of DM (e.g., as for RS or SD) supported the lowest levels of growth of *Streptococcus* spp, coliforms, and non-coliforms compared with those bedding materials with a lower percentage of DM (**Figures 5**, **6**).

Regarding pH values, most of the bedding material samples were on the alkaline side within a range of 8–11 except for ST with acidic values (5.8 ±1.4) in fresh samples (**Figure 4**). This is similar to those reported in other research (30). This can be of importance when controlling some bacteria species that do not multiply well in low-pH environments (19).

Our results show that, even following a standardized sampling protocol, the bacterial count distribution in used samples within the same day of sampling had a noticeable variation, especially in PF, RS, and ST materials. The MS and SD appeared to have

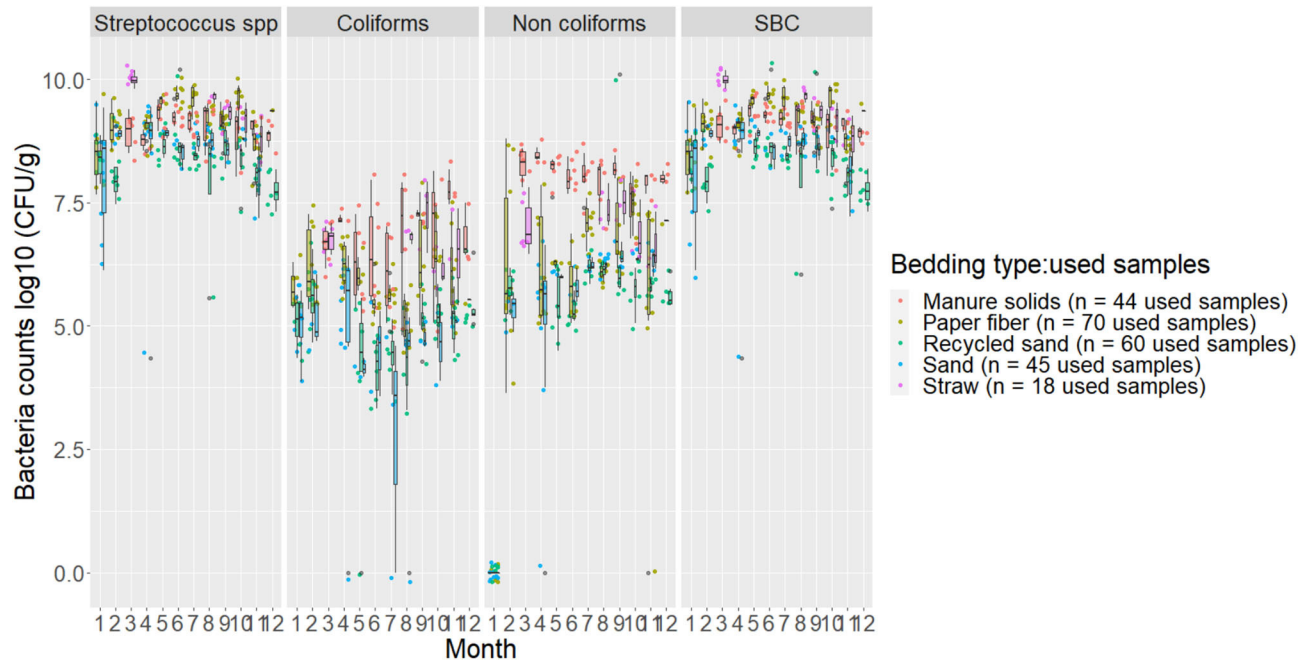

FIGURE 2 | Boxplots showing 25th, 50th (median), and 75th percentiles of the distribution of bacteria counts (log10 CFU/g) for used bedding samples collected from July 2018 to July 2019 from five conveniently selected New York dairy herds using one of five bedding materials in lactating pens. Used bedding samples (three to five) were collected monthly following an SOP at each visit (unless there was no bedding available due to lack of supply or equipment malfunction on the follow-up visit).

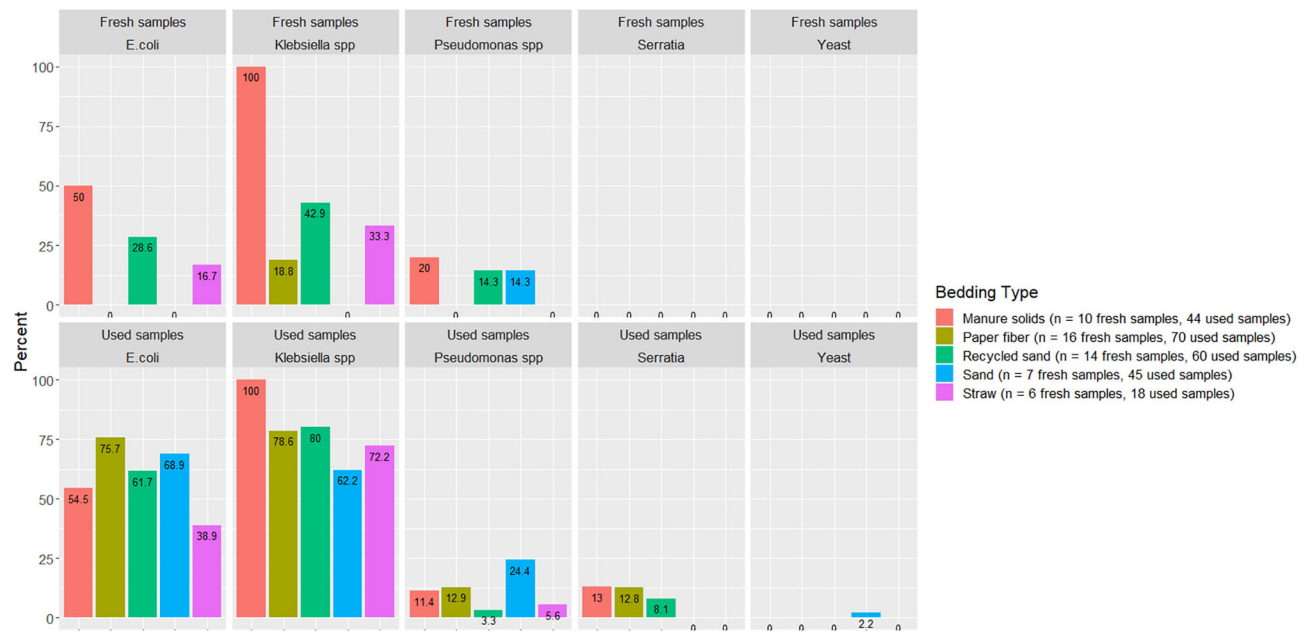

FIGURE 3 | Proportion of used or fresh bedding samples with detectable organisms of mastitis importance. Fresh and used bedding samples collected from July 2018 to July 2019 from five conveniently selected New York dairy herds using one of five bedding materials in lactating pens. One fresh bedding sample and three to five used bedding samples were collected monthly following an SOP at each visit (unless there was no bedding available due to lack of supply or equipment malfunction on the follow-up visit).

counts that were more constant within the same day of sampling although they differed throughout the study period (**Figure 2**). This suggests that using summarized data, such as averages might not be a good way to analyze bedding bacteria because one might lose a lot of important information about the variability. This is important to consider when evaluating bedding samples and

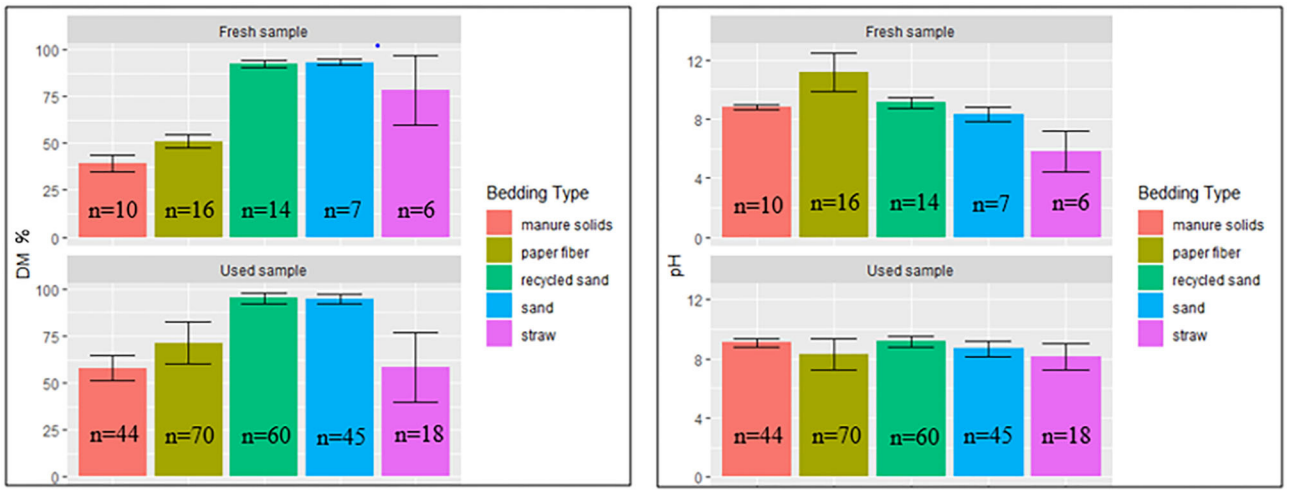

FIGURE 4 | Average DM content (% DM) and pH values (Error bars represent SD) for fresh and used bedding samples collected from July 2018 to July 2019 from five conveniently selected New York dairy herds using one of five bedding materials in lactating pens. One fresh bedding sample and three to five used bedding samples were collected monthly following an SOP at each visit (unless there was no bedding available due to lack of supply or equipment malfunction on the follow-up visit).

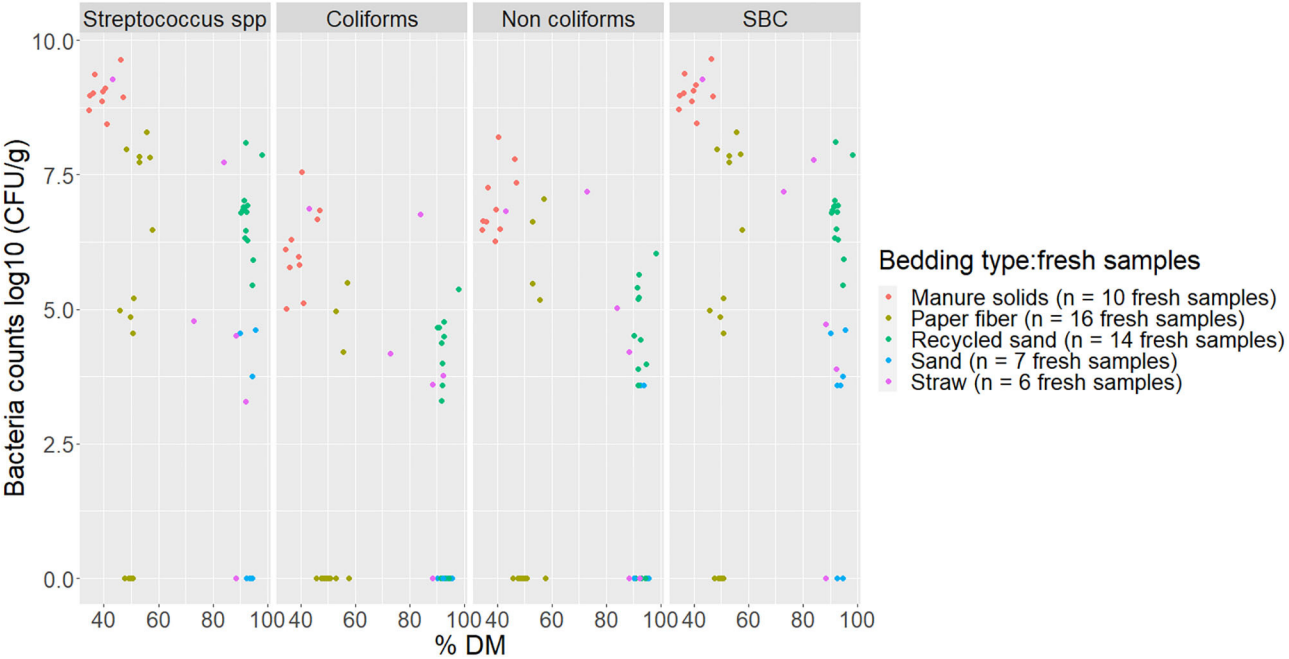

FIGURE 5 | Scatterplot of DM content (% DM) vs. bacteria counts (log10 CFU/g) by bacteria group from fresh samples collected from July 2018 to July 2019 from five conveniently selected New York dairy herds using one of five bedding materials in lactating pens. One fresh bedding sample was collected monthly following an SOP at each visit (unless there was no bedding available due to lack of supply or equipment malfunction on the follow-up visit). When no bacteria were identified, a value of $\log_{10} + 1$ CFU/g was given, assuming that at least 10 CFU were present. SBC (sum bacterial count) = *Streptococcus* spp, coliforms and non-coliforms summed.

a specific outcome and when using only a few samples from a specific point in time in an attempt to describe bedding data.

The second objective was to evaluate the association between bedding type with milk quality. When evaluating the association between BT bacteria and bedding bacteria counts, our results show the greatest difference was in coliforms in the RS and ST bedding (**Figure 7**). However, ST is also the bedding from the farm that was only present for 6 months of the study, so these findings should be interpreted with caution. Other studies show a similar lack or marginal association between BT total bacterial count and bedding type (27, 28), respectively. Bradley reported a marginal difference, and it was higher for farms using

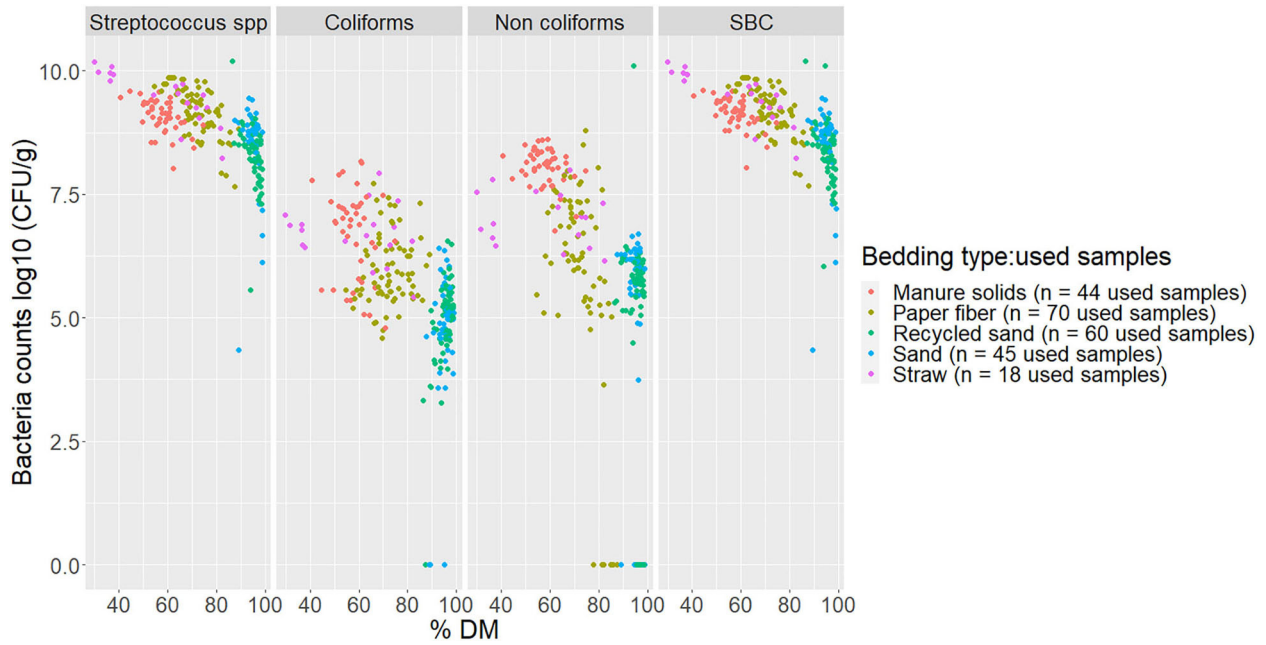

FIGURE 6 | Scatterplot of DM content (% DM) vs. bacteria counts (log10 CFU/g) by bacteria group and bedding type in used bedding samples collected from July 2018 to July 2019 from five conveniently selected New York dairy herds using one of five bedding materials in lactating pens. Three to five used bedding samples were collected monthly following an SOP at each visit (unless there was no bedding available due to lack of supply or equipment malfunction on the follow-up visit). When no bacteria were identified, a value of $\log_{10} + 1$ CFU/g was given, assuming that at least 10 CFU were present. SBC (sum bacterial count) = *Streptococcus* spp, coliforms and non-coliforms summed.

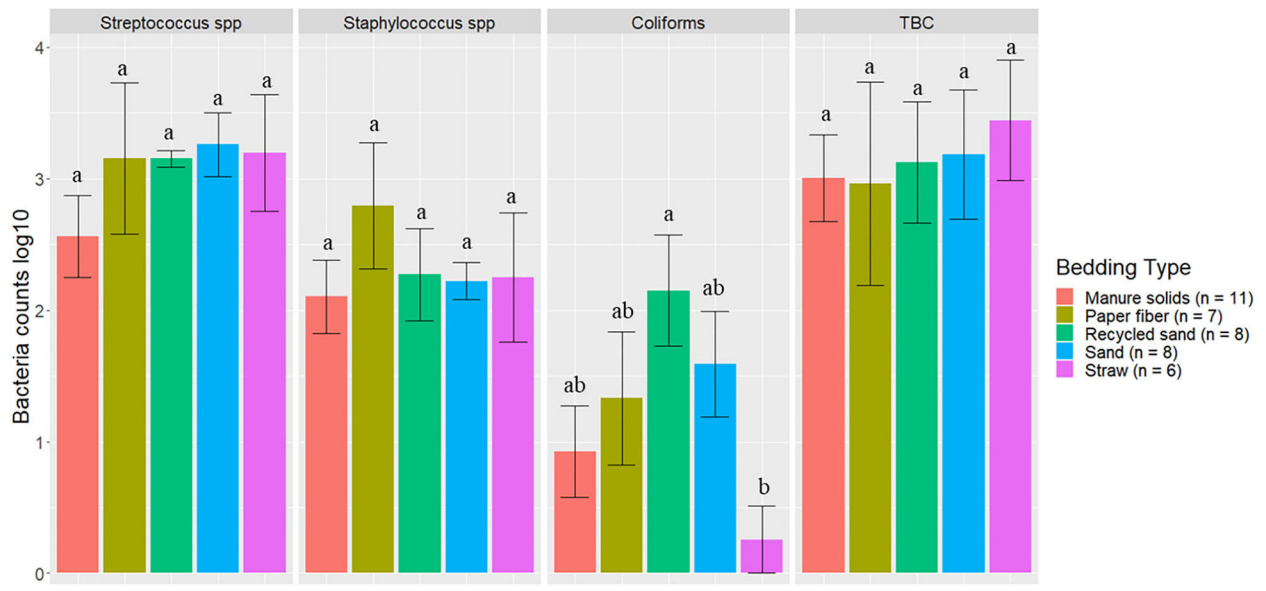

FIGURE 7 | Average bacteria counts (log10 CFU/ml) in milk samples collected monthly (unless milk had been picked up prior to arrival for follow up visit) from the BT after mechanically agitating the milk for at least 5 min until sufficient homogeneity is obtained from five conveniently selected New York dairy herds using one of five bedding materials in lactating pens. Error bars represent SD. TBC = total bacteria count. When no bacteria were identified, a value of $\log_{10} + 1$ CFU/ml was given, assuming that at least 10 CFU were present.

recycled MS bedding, followed by wood products, ST, and SD. The detected organisms of mastitis importance varied across BT samples; surprisingly, *E. coli* was detected in only 9.1% of samples from the MS herd, whereas it was 87.5% in RS farm (**Figure 8**). We did not find an association between bedding type and BTSLS (**Figure 9**).

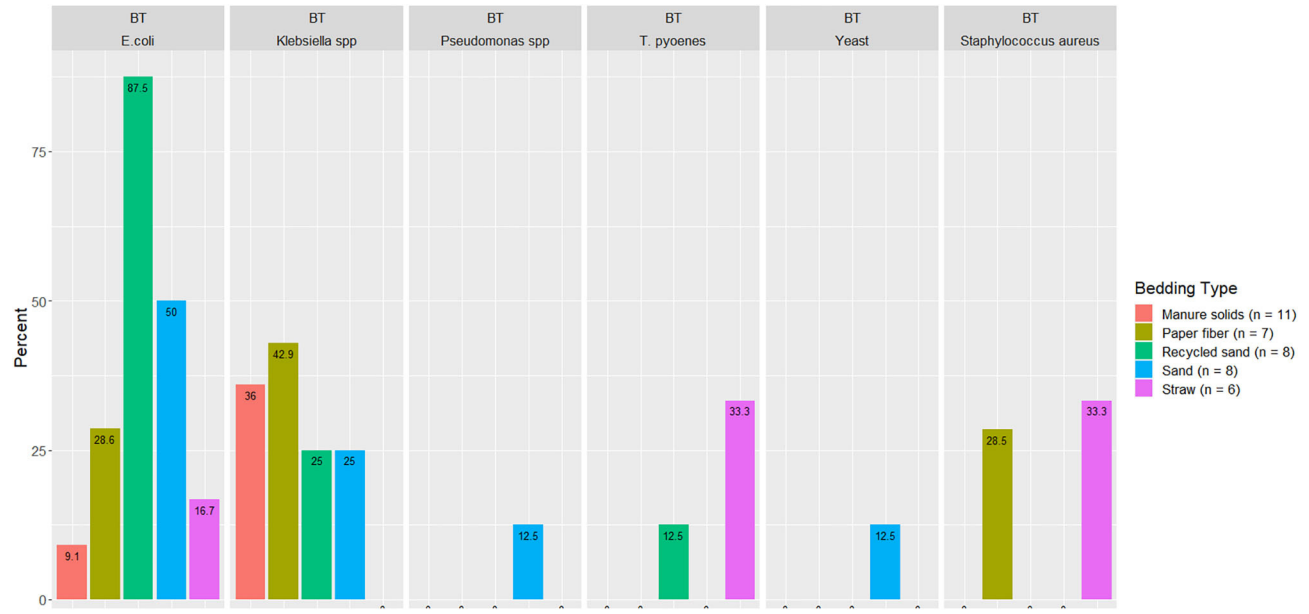

FIGURE 8 | Displayed only the proportion from BT milk samples with detectable organisms of mastitis importance. Milk samples collected monthly (unless milk had been picked up prior to arrival for follow up visit) from the BT after mechanically agitating the milk for at least 5 min until sufficient homogeneity is obtained from five conveniently selected New York dairy herds using one of five bedding materials in lactating pens.

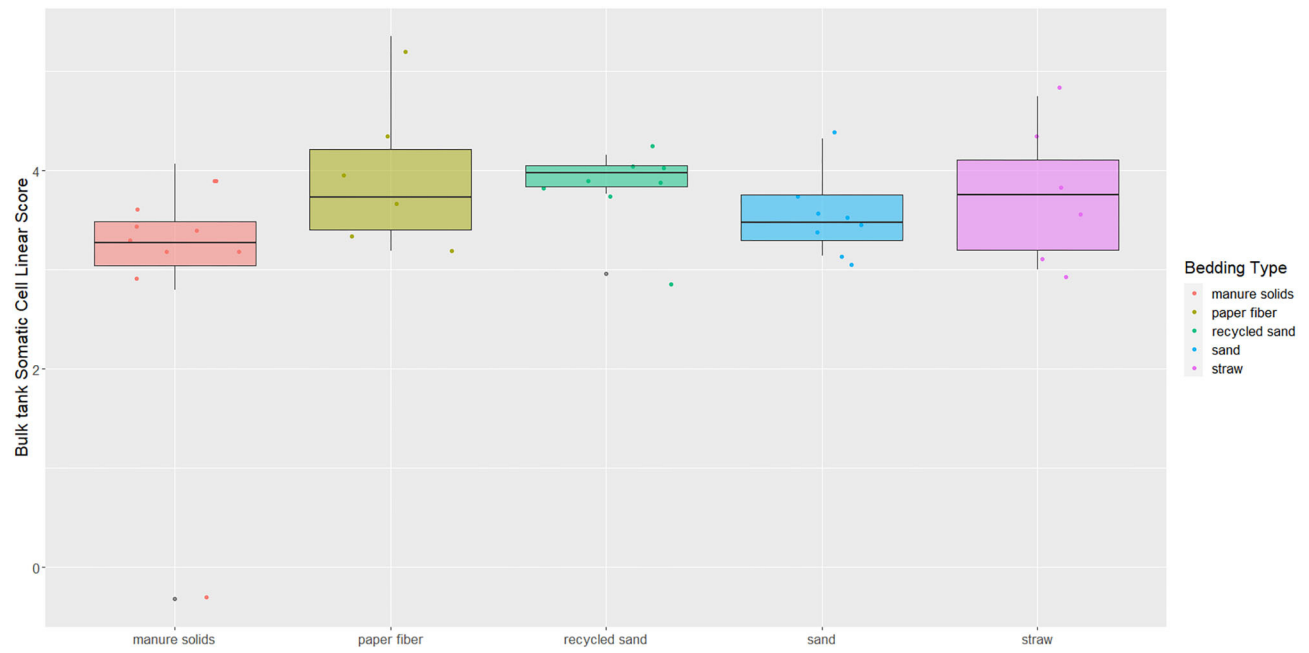

FIGURE 9 | Boxplots showing 25th, 50th (median), and 75th percentiles of somatic cell score in milk samples monthly collected (unless milk had been picked up prior to arrival for follow up visit) from the BT after mechanically agitating the milk for at least 5 min until sufficient homogeneity is obtained from five conveniently selected New York dairy herds using one of five bedding materials in lactating pens. Milk samples analyzed using DCC to get SCCs and transformed into somatic cell scores (BTSLS) by applying the following equation: BTSLS = log2 (BTSCC/100) + 3.

It is important to note that other cross-sectional bedding studies used only two points in time during different seasons (winter and summer) and did not take into account the variability during an extended period. Even though these researchers showed the variability among farms, they did not take into account the variability in bacterial counts within the same farm,

on the same day of sampling, or even how the sampling method can affect these parameters.

STRENGTHS AND LIMITATIONS

This was a descriptive study that prospectively evaluated bedding bacterial counts over time. The two main strengths of this study are the consistent sampling SOP and serial sampling of bedding through time. These features can reduce the variability in sample procurement and improve the understanding of bedding bacteria count variability among sampling times.

However, missing bedding and BT samples decreased the number of complete evaluations. Another possible limitation is the use of frozen samples, which can result in possible measurement error in bacterial counts. Although Homerosky (38) reports a decrease in Gram-negative and coliform bacteria counts after freezing, the QMPS laboratory did not find any difference in bacteria counts. In the aforementioned data from QMPS, bacteria counts were evaluated weekly from 20 bedding samples and did not show a significant difference between each day for up to 21 days (M. Zurakowski, unpublished data).

Finally, this study only involved five herds, each with one bedding type. Thus, only one experimental unit per bedding type was included in this analysis, and this limits the ability to generalize the findings to other farms using these types of bedding material. Nonetheless, our results show that even conducting repeated sampling within a farm, there was a significant variation in the bacterial count within the sampling day and throughout the study period (monthly samples). These findings indicate that results from studies evaluating the association between bedding material and bulk tank bacterial load should be interpreted with caution, especially if a single or few sample collections were carried out over time. That may be a concern even in studies enrolling several herds per bedding material.

The herds enrolled in our study were well-managed and conveniently selected; therefore, our findings should not be generalized to herds with different management practices and different bedding processing. Differences in management practices in each herd may likely influence the bedding bacterial counts and the association between bedding type and BT parameters. However, it is important to mention that the main objective of this study was to report the variability in bacterial counts within the farms over time and its association with the bacterial load present in the BT milk. The association assessment between bedding bacterial counts and particular herd management practices was not in the scope of the study.

CONCLUSIONS

Bedding sample results can be difficult to interpret because bacteria counts in bedding are not easily linked to bacteria counts in BT or milk quality. Results from this study show that there is a lot of variability in bedding samples even when collected under strict SOP guidelines. In bedding samples, a higher DM content had the lowest levels of bacterial growth compared with those with lower DM content. No associations between BT bacteria counts and bedding bacterial counts were observed. No association between BTSCC based on bedding type were observed. Despite using an SOP for bedding sampling in an effort to consistently collect samples, we still observed a large amount of variability both within and among bedding samples. This variability may have obscured any potential association between BT milk quality and bedding type.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

VA and PO conceived the presented idea. PO directed the project. VA performed all sampling. MZ and DP performed the laboratory analysis. VA analyzed the data. TT, DN, and PO encouraged VA to investigate different ways to analyze the data. VA interpreted the results and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Feeding Pre-weaned Calves With Waste Milk Containing Antibiotic Residues Is Related to a Higher Incidence of Diarrhea and Alterations in the Fecal Microbiota

*Martina Penati[1], Giulia Sala[1], Filippo Biscarini[2], Antonio Boccardo[1], Valerio Bronzo[1], Bianca Castiglioni[2], Paola Cremonesi[2], Paolo Moroni[1,3], Davide Pravettoni[1] and Maria Filippa Addis[1]**

[1] Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Lodi, Italy, [2] Institute of Agricultural Biology and Biotechnology, National Research Council (CNR), Milan, Italy, [3] Quality Milk Production Services, Animal Health Diagnostic Center, Cornell University, Ithaca, NY, United States

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Georgios Oikonomou, University of Liverpool, United Kingdom

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***Correspondence:**
Maria Filippa Addis
filippa.addis@unimi.it

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The cows receiving antibiotics for intra-mammary infection (IMI) produce milk that cannot be marketed. This is considered waste milk (WM), and a convenient option for farmers is using it as calf food. However, adding to the risk of selecting resistant bacteria, residual antibiotics might interfere with the gut microbiome development and influence gastrointestinal health. We assessed the longitudinal effect of unpasteurized WM containing residual cefalexin on calf intestinal health and fecal microbiota in an 8-week trial. After 3 days of colostrum, six calves received WM and six calves received bulk tank milk (BM) for 2 weeks. For the following 6 weeks, all 12 calves received milk substitute and starter feed. Every week for the first 2 weeks and every 2 weeks for the remaining 6 weeks, we subjected all calves to clinical examination and collected rectal swabs for investigating the fecal microbiota composition. Most WM calves had diarrhea episodes in the first 2 weeks of the trial (5/6 WM and 1/6 BM), and their body weight was significantly lower than that of BM calves. Based on 16S rRNA gene analysis, WM calves had a lower fecal microbiota alpha diversity than that in BM calves, with the lowest p-value at Wk4 ($p < 0.02$), 2 weeks after exposure to WM. The fecal microbiota beta diversity of the two calf groups was also significantly different at Wk4 ($p < 0.05$). Numerous significant differences were present in the fecal microbiota taxonomy of WM and BM calves in terms of relative normalized operational taxonomic unit (OTU) levels, affecting five phyla, seven classes, eight orders, 19 families, and 47 genera. At the end of the trial, when 6 weeks had passed since exposure to WM, the phyla Bacteroidetes, Firmicutes, and Saccharibacteria were lower, while Chlamydiae were higher in WM calves. Notably, WM calves showed a decrease in beneficial taxa such as *Faecalibacterium*, with a concomitant increase in potential pathogens such as *Campylobacter*, *Pseudomonas*, and *Chlamydophila* spp. In conclusion, feeding pre-weaned calves with unpasteurized WM containing antibiotics is related to a higher incidence of neonatal diarrhea and leads to significant changes in the fecal microbiota composition, further discouraging this practice in spite of its short-term economic advantages.

Keywords: calf, microbiome, milk, antibiotic residues, gut microbiome, mastitis

INTRODUCTION

Waste milk (WM) includes low-quality colostrum, transition or post-colostral milk, milk from cows treated for mastitis and other diseases, milk with high somatic cell count (SCC), and other unsalable milk (1). According to European food safety regulations (such as EC Regulation 853 of 2004), this milk is not allowed for direct human consumption or processing into dairy products, with no specific provisions for other uses. Given the clear economic and practical advantages, WM is widely used by farmers as calf food (1, 2). Nevertheless, several countries are issuing guidelines discouraging this practice (i.e., European Commission notice 2015/C 299/04) (1), as the potential presence of anti-microbial residues may increase the risk of maintaining and spreading antimicrobial resistance gene pools in the dairy farm and the environment (3, 4) and expose newborn calves to intestinal diseases (5–7). A further potential issue is the interference of antibiotics and microbial pathogens with the gut microbiome's physiological development in growing calves, with possible consequences on their future health and production performances (5–7).

When antibiotics are administered to adult individuals with a mature gut microbiome, microbial diversity has been shown to decrease significantly, but resilience mechanisms slowly restore the original condition once antibiotics are removed (8). On the other hand, exposure to antimicrobials at an early age may lead to permanent shifts in microbial composition and functions with consequent long-term metabolic alterations (9–12). Therefore, adding to the increased risk of selecting antimicrobial resistance traits, feeding calves with milk containing antimicrobials in the first weeks of life might compromise their intestinal microbiome development impacting gut immunity, gastrointestinal well-being, and ability to metabolize nutrients efficiently (13, 14).

Given its relevance for the dairy industry, previous studies have assessed the impact of WM on calf health and the gut microbiome (3, 13, 14), investigating subtherapeutic levels of antibiotics spiked into milk (14) or milk replacer (13, 15) and pasteurized WM with antibiotic residues at unknown concentrations (3, 16, 17). These studies demonstrated that short-term changes in the microbial taxonomy do occur following WM ingestion, but these are generally limited to disruptions that do not go beyond the genus level (14). However, these studies investigated low or undetermined antibiotic residues and assessed only the time frame of WM feeding.

With these premises, we assessed the impact of WM obtained from cows receiving intra-mammary cefalexin on calf intestinal health and on fecal microbiota diversity and taxonomy during 2 weeks of feeding and after up to 6 weeks after the removal of WM from the diet. To reduce variability, colostrum and WM were standardized and characterized before feeding them to calves. The two-step, 8-week trial included 12 dairy calves enrolled in a commercial farm and managed with standard procedures. For the first 2 weeks, six calves received WM, and six received bulk tank milk (BM); for the following 6 weeks, all calves received the same weaning diet with milk whey and starter feed. Every week for the first 2 weeks and then biweekly for the following 6 weeks, we carried out a complete clinical evaluation and collected fecal swabs for investigating the fecal microbiota composition.

MATERIALS AND METHODS

Farm Description and Ethics Statement

The study was performed on a commercial dairy farm in Northern Italy with a long-standing collaboration with the University of Milan. The farm included 390 lactating Italian Friesian cows. The herd was accredited free from infectious bovine rhinotracheitis (IBR), vaccinated for neonatal diarrhea agents [Rotavec Corona®, MSD Animal Health S.r.l., Segrate (MI), Italy], and type-1 and type-2 bovine viral diarrhea virus (BVDV) (Bovela®, Boehringer Ingelheim, Milan, Italy). The farm was followed by our University Hospital Clinic and was selected for its very low prevalence of neonatal calf diarrhea (NCD) in the previous 3 months (<1% of cases between newborn calves). The research protocols were reviewed and approved by the Institutional Committee for Animal Care of the University of Milan (protocol number 78_2018). The trial was carried out between March 2019 and June 2019.

Design of the Feeding Trial and Sample Collection

The trial structure is illustrated in **Figure 1**. Twelve consecutive born male calves were enrolled at birth between March 11 and April 22, 2019. The calves were separated from the dam immediately after birth and received 3 L of the same standardized first colostrum within 6–8 h, followed by 2 L after 8–12 h. During the second and third days of life, calves were fed two times daily with 2.5 L of the same standardized second-day and third-day transition milk (TM), respectively. Colostrum and TM preparation and administration procedures are detailed in section Colostrum, Transition Milk, Waste Milk, and Bulk Tank Milk.

Starting from the fourth day of life, six calves were allocated to the BM group and six to the WM group according to the order of birth. For 2 weeks (Wk0–Wk2; **Figure 1**), BM calves were fed twice a day with 2 L of fresh unpasteurized BM, while WM calves were fed twice a day with 2 L of an unpasteurized WM lot that was prepared, standardized, and characterized before the beginning of the trial. For the following 6 weeks (Wk2–Wk8; **Figure 1**), all calves were fed twice a day with 6 L of a commercial milk replacer (Emme Erre Flash 22,5, Tredi Italia S.r.l., Cremona, Italy), and pelleted starter feed (Fly Start, Cortal Extrasoy S.p.A., Cittadella, PD, Italy) was available *ad libitum*. In the first 2 weeks, the calves were housed in individual hutches, while in the last 6 weeks, they were kept in two separated collective pens, one for each experimental group. The WM preparation and administration procedures, as well as the composition of WM, BM, and milk replacer, are detailed in section Colostrum, Transition Milk, Waste Milk, and Bulk Tank Milk.

At birth and on the third day (Wk0), 10th day (Wk1), 17th day (Wk2), 31st day (Wk4), 45th day (Wk6), and 59th day of life (Wk8), all calves were submitted to a complete clinical examination (18) as detailed in section Clinical Examination and

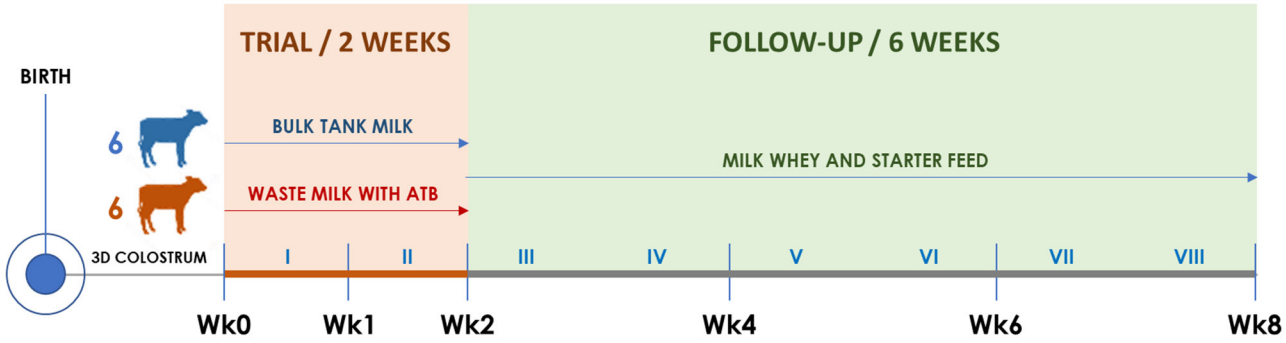

FIGURE 1 | Timeline of the trial illustrating calf groups, diets, and sampling times. Roman numerals indicate weeks. The timing of clinical visits and rectal swab collection is shown according to the trial week, as follows. Wk0: third day of life; Wk1: 10th day of life; Wk2: 17th day of life; Wk4: 31st day of life; Wk6: 45th day of life; Wk8: 59th day of life.

Calf Growth Measurements. At each time point, duplicate rectal swabs were collected, refrigerated, brought to the laboratory within 12 h, and stored at −20°C until DNA extraction.

Colostrum, Transition Milk, Waste Milk, and Bulk Tank Milk

To eliminate possible variables related to colostrum or TM, a pooling strategy was applied as follows. Six liters of good-quality colostrum (Brix >22%) were milked from each of 10 different cows and stored in 500-ml bottles, 12 for each cow. The bottles were identified as colostrum, labeled with the cow number, and frozen at −20°C. Then, 6 L of the second and third milking of the same cows (TM) were again collected in 500-ml bottles, 12 for each cow. The bottles were identified as second-day or third-day TM, respectively, labeled with the cow number, and frozen at −20°C. For colostrum administration, the 3-L morning feeding of each calf was prepared by defrosting and pooling six 500-ml aliquots belonging to cows 1–6, while the 2-L afternoon feeding was prepared by defrosting and pooling four 500-ml aliquots belonging to cows 7–10. The aliquots were gently thawed in a water bath at 45°C for 30 min, mixed, and administered at 35–40°C by oroesophageal tubing. The second-day TM and third-day TM were prepared by mixing aliquots 1–5 for the morning dose (2.5 L for each calf) and aliquots 6–10 for the afternoon dose (2.5 L for each calf) of the respective TM. In this way, all calves received the same colostrum and TM before the start of the feeding trial.

WM was obtained from five cows affected by chronic mastitis (A–E), selected based on a previous bacteriological culture result according to the National Mastitis Council (NMC) guidelines (19). Ten microliters of milk was spread on blood agar plates (5% defibrinated sheep blood), incubated at 37°C, and examined after 24 and 48 h. Colonies were identified based on size, Gram stain, morphology, and hemolysis pattern. The SCC was determined using an automated counter (Bentley Somacount 150, Bentley Instruments, Chaska, MN, USA). The milk collected from the five cows had the following characteristics in terms of SCC and isolated bacteria: cow A, SCC 312,000 cells/ml, *Bacillus* spp.; cow B, SCC 901,000 cells/ml, Non-aureus staphylococci (NAS); cow C, SCC 239,000 cells/ml, *Staphylococcus aureus*; cow D, SCC 5,045,000 cells/ml, *Bacillus* spp.; cow E, SCC 454,000 cells/ml, NAS.

The five cows were subjected to the intramammary administration of 210 mg cefalexin monohydrate (Rilexine 200 T lactation, Virbac S.r.l.) in each quarter for four consecutive milkings, and the milk was collected at each following milking time for a total of 336 L. All the milk was maintained in a refrigerated tank for 36 h from the first to the fourth milking, mixed, aliquoted in 2-L aluminum bags (Perfect Udder® bags, Dairy Tech Inc.) and stored at −20°C until needed. This collection, mixing, and aliquoting procedure ensured the generation of a uniform pooled WM. WM bags were gently thawed in a water bath at no more than 45°C for 45 min and fed to calves at a temperature ranging from 35 to 40°C. BM was collected fresh from the commercial milk tank.

WM and BM were subjected to the determination of total fat, protein, and lactose according to the ISO 9622:2013 (IDF 141) methods and tested for the presence of inhibitors by the Delvotest® SP NT (DSM). WM was further evaluated in triplicate by liquid chromatography–high-resolution mass spectrometry (LC-HRMS) for antibiotic residue detection and quantitation as described by Chiesa et al. (20).

The commercial milk replacer contained milk whey, whey proteins, vegetable oils (coconut, palm), hydrolyzed wheat protein, pregelatinized wheat flour, dextrose, butyric acid esters added with vitamins, oligo-elements, and stabilizers of the intestinal flora *Enterococcus faecium DSM 7134* and *Lactobacillus rhamnosus DSM 7133* at 1×10^9 CFU/kg. The powder was reconstituted according to the manufacturer instructions (125 g/L of powder).

Clinical Examination and Calf Growth Measurements

Clinical examination and calf growth measurements were performed at the six experimental time points (Wk0–Wk8; **Figure 1**) by an expert bovine practitioner (GS). At 24 h from birth and on the third day of life, the serum total protein concentration (STP) of each calf was measured to assess the

correct transfer of passive immunity (21). A blood sample was collected in a 9-ml tube without anticoagulant from the jugular vein. Samples were allowed to clot, centrifuged at 20°C for 10 min at 900 g, and the STP was measured with a handle refractometer. The calf growth rate was estimated using a heart-girth measuring tape pulled snuggly around the thorax, just caudal to the forelimbs. Obtained measurements were then used to estimate body weight (BW) following the equation proposed by Heinrichs et al. (22). Diarrhea was defined when a calf had visibly watery feces (fecal consistency that permitted feces to run through slightly opened fingers). When a diarrhea episode was detected, fecal samples were collected and submitted to routine diagnostic tests at the local animal health institution (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna) for the main agents of NCD: rotavirus and coronavirus by real-time PCR and bacteriological agents by culture.

DNA Extraction and Generation of 16S rDNA Data

Rectal swabs were thawed, and DNA was extracted using a QIAmp DNA Stool kit (Qiagen, Hilden, Germany) according to the manufacturer instructions with a minor modification. The rectal swabs were dissolved in 1 ml Buffer ASL and shaken at 1,000 rpm (Mixing Block MB-102, CaRlibiotech S.r.l. Rome, Italy) continuously until the stool samples were homogenized. DNA quality and quantity were assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the isolated DNA was stored at −20°C until use.

Bacterial DNA was amplified by targeting the V3–V4 hypervariable regions of the 16S rRNA gene (23). PCR amplification of each sample was performed in a 25-μl volume. A total of 12.5 μl of KAPA HIFI Master Mix 2× (Kapa Biosystems, Inc., MA, USA) were used. Then, 0.2 μl of each primer (100 μM) was added to 2 μl of genomic DNA (5 ng/μl). Blank controls (no DNA template) were also included. Amplification and library quantification were carried out as described previously (24).

Bioinformatic Processing

Demultiplexed paired-end reads from 16S rRNA-gene sequencing were first checked for quality using FastQC (25) for an initial assessment. Forward and reverse paired-end reads were joined into single reads using the C++ program SeqPrep (26). After joining, reads were filtered for quality based on (i) maximum three consecutive low-quality base calls (Phred <19) allowed; (ii) fraction of consecutive high-quality base calls (Phred >19) in a read over total read length ≥0.75; (iii) no "N"-labeled bases (missing/uncalled) allowed. Reads that did not match all the above criteria were filtered out. All remaining reads were combined in a single FASTA file for the identification and quantification of operational taxonomic units (OTUs). Reads were aligned against the SILVA closed reference sequence collection release 123, with 97% cluster identity (27, 28) applying the CD-HIT clustering algorithm (29). A predefined taxonomy map of reference sequences to taxonomies was then used for taxonomic identification along the main taxa ranks down to the genus level (domain, phylum, class, order, family, and genus). By counting the abundance of each OTU, the OTU table was created and then grouped at each phylogenetic level. OTUs with total counts lower than 10 in fewer than two samples were filtered out. All the above steps, except the FastQC reads quality check, were performed with the Quantitative Insights into Microbial Ecology (QIIME) open-source bioinformatics pipeline for microbiome analysis (30). More details on the command lines used to process 16S rRNA-gene sequence data can be found in Biscarini et al. (31).

The 16S rRNA-gene sequencing reads were processed with the QIIME pipeline (30) used to estimate most diversity indices. The Abundance-based Coverage Estimator (ACE) index and sample-based rarefaction were estimated using Python (https://github.com/filippob/Rare-OTUs-ACE.git) and R (https://github.com/filippob/sampleBasedRarefaction) scripts. Plots were generated using the ggplot2 R package (32). Additional data handling and statistical analysis were performed with the R environment for statistical computing (33) and with Microsoft Excel.

Alpha and Beta Diversity Indices

The fecal microbiota diversity was assessed within (alpha diversity) and across (beta diversity) samples. All indices (alpha and beta diversity) were estimated from the complete OTU table (at the OTU level), filtered for OTUs with more than 10 total counts distributed in at least two samples. Besides the number of observed OTUs directly counted from the OTU table, within-sample microbial richness, diversity, and evenness were estimated using the following indices: Chao1 and ACE for richness; Shannon, Simpson, and Fisher alpha for diversity (34–38); Simpson E and Pielou J (Shannon evenness) for evenness (39). The across-sample microbiota diversity was quantified by calculating Bray–Curtis dissimilarities (40). Prior to calculating the Bray–Curtis dissimilarities, OTU counts were normalized for uneven sequencing depth by cumulative sum scaling CSS (41). Among-groups (BM vs. WM) and pairwise Bray–Curtis dissimilarities were evaluated non-parametrically using the permutational analysis of variance (999 permutations) (42). Details on the calculation of the mentioned alpha and beta diversity indices can be found in **Supplementary File 1** and in Biscarini et al. (43).

Statistical Analysis

The differences between feeding groups were evaluated with SPSS 25.0 (IBM). The distribution of continuous variables was analyzed with the Shapiro–Wilk test. Since the distribution was not normal, data were compared with a non-parametric Mann–Whitney *U*-test. Categoric variables were compared with contingency tables and with the Fisher's exact test (2 × 2 tables), calculating the odds ratio. Statistical significance was considered for $p < 0.05$.

For the microbiome analysis, differences between groups (WM, BM) along time points in terms of OTU abundances and alpha diversity indices were evaluated with a linear model of the following form:

$$\text{y\_ij} = \text{mu} + \text{treatment\_j} + \text{e\_ij} \quad (1)$$

where y_ij is the abundance (counts) or index value for each taxonomy (OTU) and alpha diversity metric in animal I

belonging to treatment group j, treatment_j is either WM or BM, and e_ij are the residuals of the model. From model (1), *p*-values were obtained to identify those OTUs and alpha diversity indices that were significantly different between treatments along the six time points of the experiment/trial. Alpha diversity indices: value = mu + group + e, within time point.

RESULTS

Composition of Waste Milk and Bulk Tank Milk

WM had the following gross composition: SCC 450,000 cells/ml; fat 3.7%; protein 3.6%; lactose 4.7%; microbial inhibitors: present. According to HPLC-MS/MS (20), WM had a residual cefalexin concentration of 727 ppb (727 ng/ml). The mean ± SD composition of BM, based on the routine 10-day measurements received by the farm during its use in the trial, was the following: SCC 284,000 ± 38,742.74 cells/ml; fat 4.23% ± 0.06; protein 3.60% ± 0.00; lactose 4.97 ± 0.06; microbial inhibitors: absent.

Clinical Findings

During the first 2 weeks of the trial, five out of six (83.33%) WM calves and one out of six (16.67%) BM calves had at least one diarrhea episode. Diarrhea occurred without general impairment of clinical conditions (calves stood securely, presented a strong suckle reflex, and dehydration was <3–5%) (44). Diarrheic calves were treated with oral rehydration solution (ORS) containing 4 g sodium chloride, 20 g dextrose, 3 g potassium bicarbonate, and 3 g sodium propionate between milk feedings, as described by Boccardo et al. (44). According to Constable guidelines (45), antibiotic treatment was omitted because clinical conditions were not severe, no bacterial pathogens of NCD were detected by fecal analysis, and all calves presented an adequate transfer of passive immunity [BM group: 60 g/L of STP, 25% interquartile range (IQR) 58.5 g/L, 75% IQR 61.5 g/L; WM group: 64 g/L of STP, 25% IQR 57.5 g/L, 75% IQR 69 g/L]. During the study period, there were no mortality cases.

At Wk0, the calves enrolled in the BM and WM groups had estimated median weights of 45.41 (25% IQR 43.27; 75% IQR 47.32) and 41.94 (25% IQR 40.61; 75% IQR 48.04), respectively. The difference in weight between the two calf groups at the beginning of the trial was not statistically significant ($p = 0.29$). At Wk1, the difference in estimated weight was significant ($p < 0.05$) and remained so until the end of the trial (Wk8), when the BM and WM groups had estimated median weights of 85.24 (25% IQR 78.50; 75% IQR 86.50) and 69.99 (25% IQR 62.69; 75% IQR 76.81), respectively.

Impact of Waste Milk on Fecal Microbiota Diversity

Sequencing of the V3–V4 regions in the bacterial 16S rRNA-gene produced a total of 7,744,670 reads (joined R1–R2 paired-end reads), with an average of 107,564 reads per sample (12 calves × 6 time points = 72 samples). After quality filtering, 1,438,378 sequences were removed, leaving 6,306,292 sequences for subsequent analyses (81.3% overall average retention rate, maximum 86%, minimum 66.3%). **Supplementary Table 1** reports the average retention rate and the number of sequences per treatment and time point: the number of sequences ranged from a minimum of 61,592 (±33,344) in the BM group at Wk1 to a maximum of 139,889 (±94,526) in the BM group at Wk4. The initial number of OTUs identified was 10,835; after filtering out OTUs with <10 counts in at least two samples, 3,264 distinct OTUs remained. **Supplementary Figure 1** reports the sequence-based and sample-based rarefaction curves generated from the OTU table before filtering (10,835 OTUs), where the observed number of OTUs detected was plotted, respectively, as a function of the number of reads (up to 75,000) in each sample and of the number of samples. Both curves tend to plateau asymptotically, indicating that sequencing depth and the number of samples were adequate. Deeper sequencing or addition of any other sample would not significantly increase the number of new OTUs discovered.

Alpha Diversity

Figure 2A illustrates the alpha (within-sample) diversity indices in the fecal microbiota of the two calf groups during the trial, after correcting for baseline. Index values are averages per group, expressed as differences from values at baseline (Wk0). At Wk1, alpha diversity increased in both groups, although slightly less in WM calves. At Wk2, all diversity indices increased in BM and decreased in WM. The difference between groups was further amplified at Wk4, 2 weeks after removing WM from the diet. The two groups reached similar levels at Wk6. At Wk8, the microbiota diversity decreased in both groups, although slightly more in BM. **Figure 2B** illustrates the significance values for all alpha diversity indices at all the experimental time points. At Wk4, the difference between WM and BM was statistically significant ($p < 0.05$) for all alpha diversity indices, indicating a substantial negative impact on the fecal microbiota diversity that persisted for at least 2 weeks after removing the antibiotic-containing WM from the diet. Equitability and Simpson evenness were significantly different also at Wk1 and Wk2 ($p < 0.05$), respectively.

Beta Diversity

Figure 3 illustrates the first two dimensions from the (non-metric) multidimensional scaling of the Bray–Curtis dissimilarity matrix, clustering samples by treatment (top left), time point (bottom left), and by treatment-and-time point (right). While the two groups (WM and BM) overlapped extensively, the fecal microbiota evolved by changing significantly during the first 8 weeks of life ($p = 0.0069505$, from PERMANOVA between time points, 999 permutations). Concerning beta diversity between treatments at each time point, the BM and WM groups were separated at Wk4 (**Figure 4**, right), in line with the alpha diversity results (**Figures 2A,B**).

Impact of Waste Milk on the Fecal Microbiota Taxonomy

Figure 4 summarizes all the statistically significant taxonomy changes observed in the fecal microbiota. The changes occurring in WM calves compared to BM calves are illustrated in a heatmap as relative normalized OTU levels for each time point. Normalized OTU levels are detailed in

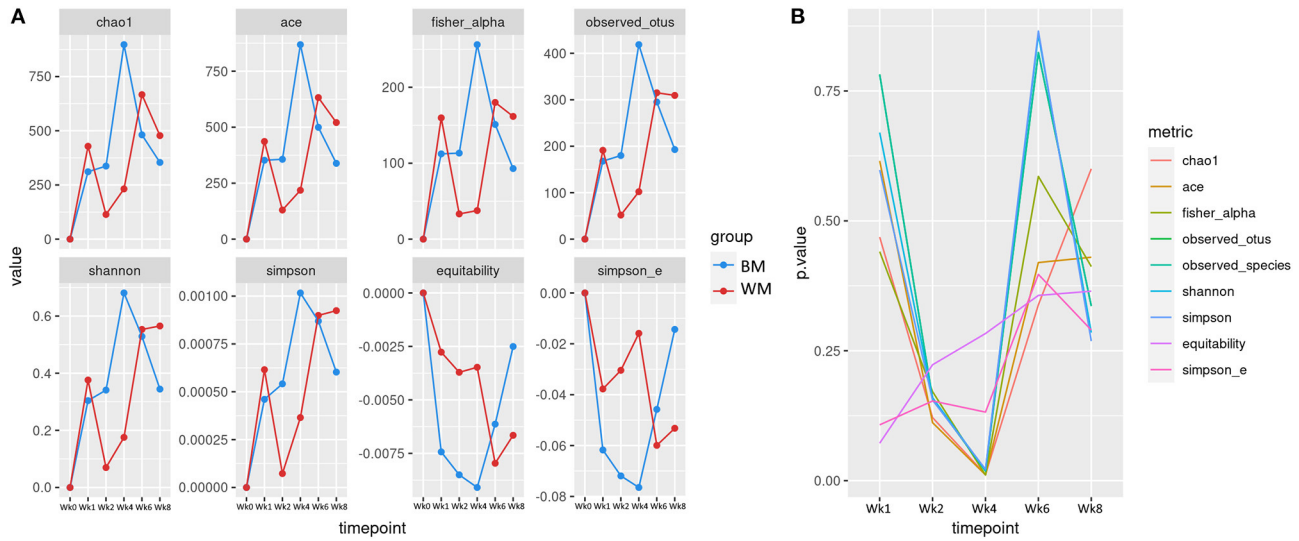

FIGURE 2 | (A) Alpha diversity indices at the different trial time points for the two groups: bulk tank milk (BM) and waste milk (WM). Index values are indicated as differences from baseline (Wk0 = 0). **(B)** Statistical significance of alpha diversity indices at the various time points.

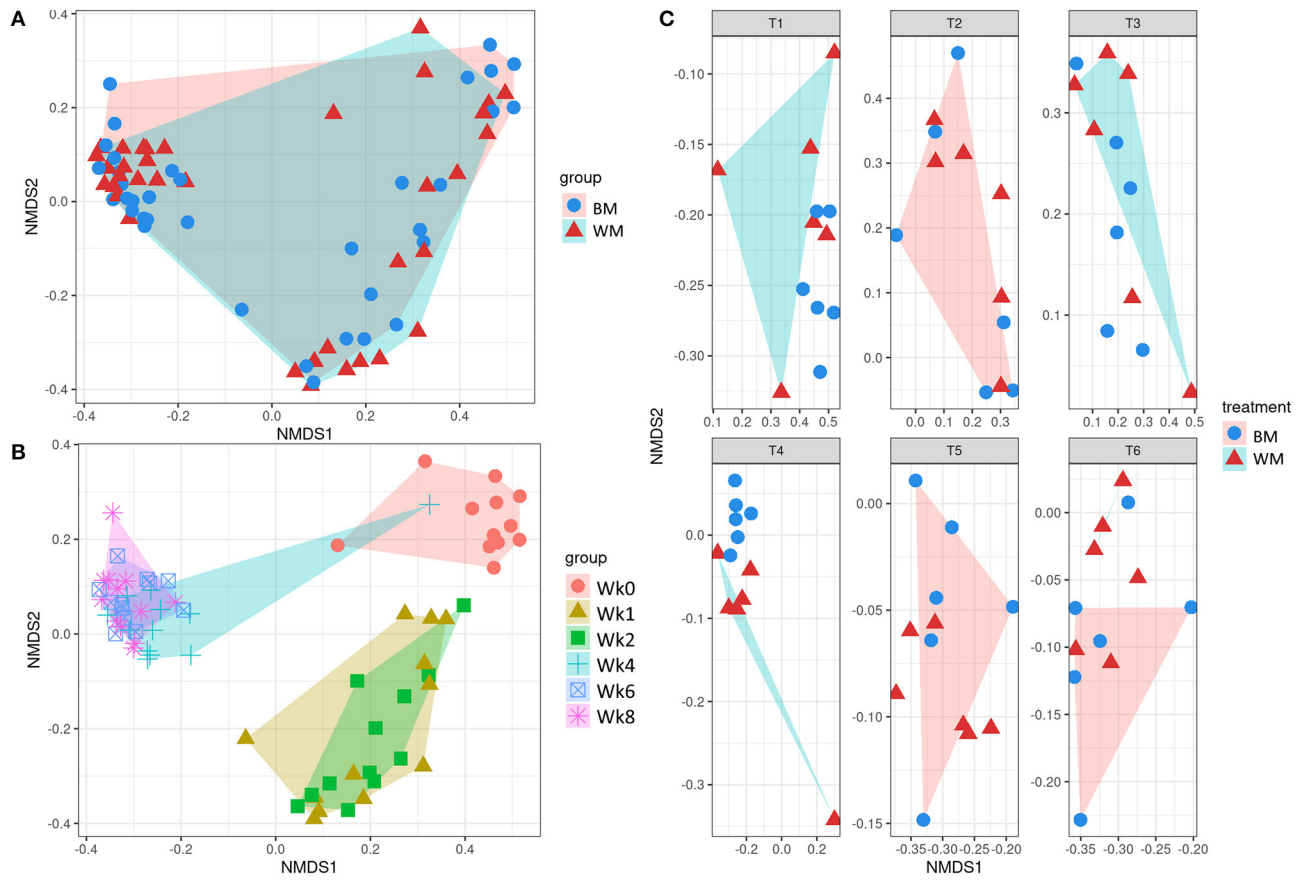

FIGURE 3 | Beta diversity according to treatment **(A)**, time point **(B)**, and treatment-by-time point **(C)**. The legends indicate the color codes and symbols used for the different sample groups [blue circles and pink shading, bulk tank milk (BM); red triangles and turquoise shading, waste milk (WM)] and time points (pink circle, Wk0; brown triangle, Wk1; green square, Wk2; turquoise cross, Wk4; blue box, Wk6; pink asterisk, Wk8).

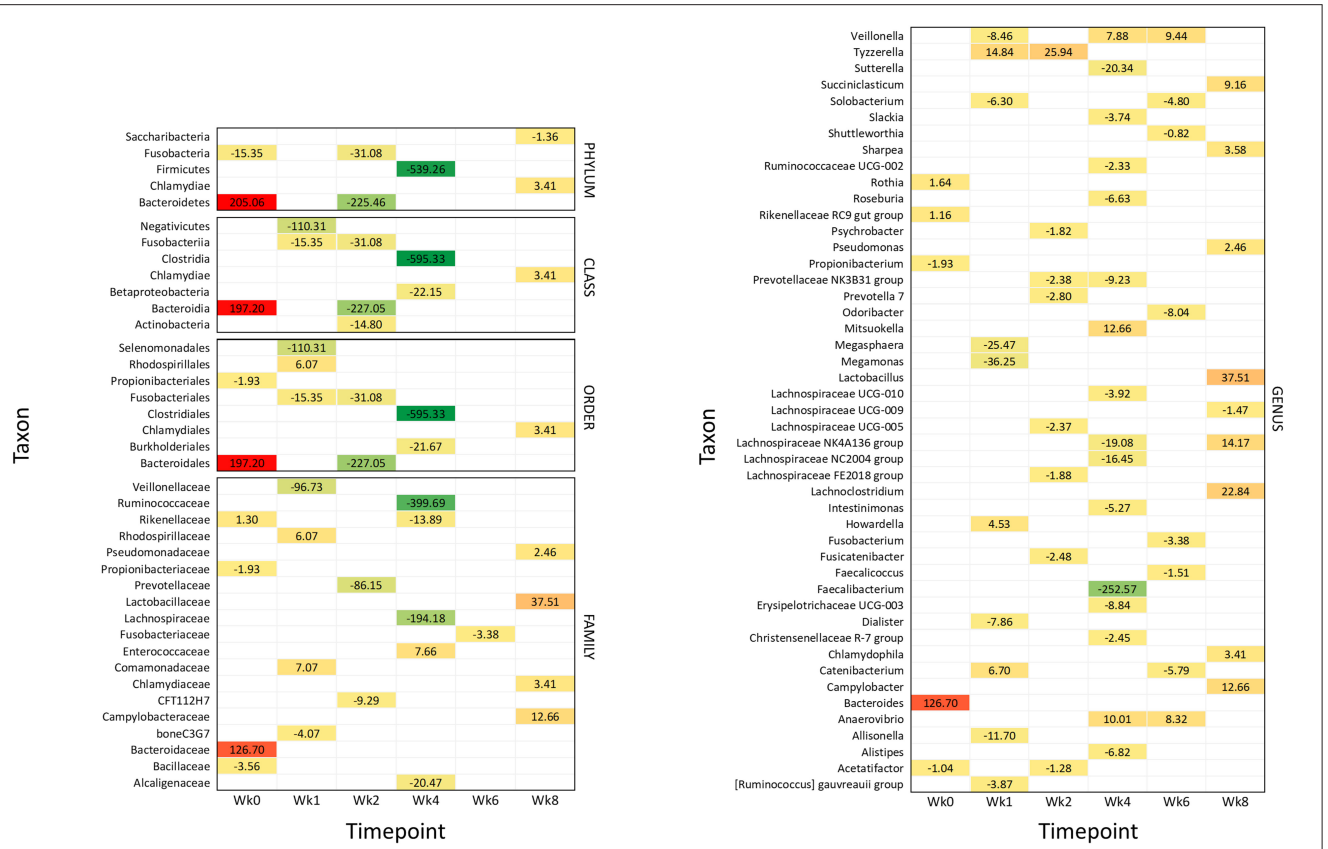

FIGURE 4 | Significantly different taxonomic groups in the fecal microbiota in the two calf groups at the different time points. The results are reported as a heatmap where red indicates a decrease and green indicates an increase of the normalized operational taxonomic unit (OTU) value in waste milk (WM) calves vs. bulk milk (BM) calves for each taxon at the different time points. The normalized OTU values and the statistical significance of the differences are reported in **Supplementary Figures 2**, **3**, respectively.

Supplementary Figure 2, while significant values are illustrated in **Supplementary Figure 3**. As a general observation, and in agreement with the alpha diversity and beta diversity results, most differential taxa were less abundant in WM than in BM calves at all time points, except for the last time point, at Wk8.

Wk0 (Age: 3 Days)

At 4 days of life, the phylum Bacteroidetes was significantly more abundant in WM calves; this was reflected in the class Bacteroidia, order Bacteroidales, family Bacteroidaceae, and genus *Bacteroidetes*. The family Rikenellaceae with the related genus *Rikenellaceae* RC9 gut group and *Rothia* were also more abundant. On the other hand, the phylum Fusobacteria and the order Propionibacteriales with the genus *Propionibacterium* were less abundant, together with the family Bacillaceae and the genus *Acetatifactor*.

Wk1 (Age: 10 Days)

After 1 week of WM feeding, several taxa showed a significantly lower abundance in WM calves compared to BM calves. These included the two classes Fusobacteria and Negativicutes, the two orders Fusobacteriales and Selenomonadales, the two families boneC3G7 and Veillonellaceae, and the seven genera [*Ruminococcus*] *gauvreauii* group, *Allisonella*, *Dialister*, *Megamonas*, *Megasphaera*, *Solobacterium*, and *Veillonella*. On the other hand, the order Rhodospirillales and the related family Rhodospirillaceae were more represented, together with Comamonadaceae. The three genera *Catenibacterium*, *Howardella*, and *Tyzzerella* were also higher.

Wk2 (Age: 17 Days)

After 2 weeks of WM feeding, numerous taxa were less abundant in WM vs. BM calves: the two phyla Bacteroidetes and Fusobacteria; the three classes including the related Bacteroidia and Fusobacteriia, together with Actinobacteria; the two related orders Bacteroidales and Fusobacteriales; the two families CFT112H7 and Prevotellaceae; and the seven genera *Acetatifactor*, *Fusicatenibacter*, *Lachnospiraceae* FE2018 group and UCG-005, *Prevotella* 7, *Prevotellaceae* NK3B31 group, and *Psychrobacter*. Only the genus *Tyzzerella* was higher in WM vs. BM calves.

Wk4 (Age: 31 Days)

The most significant differences between WM and BM calves were observed 2 weeks after the removal of WM from the diet, in line with the alpha diversity and beta diversity

results. Numerous taxa were less abundant in WM calves, while only few were more abundant. The most dramatic difference was seen for the phylum Firmicutes and the related class Clostridia, order Clostridiales, family Ruminococcaceae, and genera *Faecalibacterium* and *Ruminococcaceae* UCG-002. Less abundant were also the class Betaproteobacteria with the order Burkholderiales; the three families Alcaligenaceae, Lachnospiraceae, and Rikenellaceae; and the 11 genera *Alistipes*, *Christensenellaceae* R-7 group, *Erysipelotrichaceae* UCG-003, *Intestinimonas*, *Lachnospiraceae* NC2004 group, *Lachnospiraceae* NK4A136 group, *Lachnospiraceae* UCG-010, *Prevotellaceae* NK3B31 group, *Roseburia*, *Slackia*, and *Sutterella*. Only the family Enterococcaceae was higher in WM calves, together with the three genera *Anaerovibrio*, *Mitsuokella*, and *Veillonella*.

Wk6 (Age: 45 Days)

Four weeks after removing WM from the diet, the family Fusobacteriaceae and the six genera *Catenibacterium*, *Faecalicoccus*, *Fusobacterium*, *Odoribacter*, *Shuttleworthia*, and *Solobacterium* were lower in WM vs. BM calves. On the other hand, the two genera *Anaerovibrio* and *Veillonella* were higher.

Wk8 (Age: 59 Days)

Six weeks after removing WM from the diet, the abundance of several taxonomic groups was still different in WM vs. BM calves. In contrast with all the previous time points, however, most differential taxa were significantly higher in WM calves, as follows: the phylum Chlamydiae with the related class Chlamydiae, order Chlamydiales, family Chlamydiaceae, and genus *Chlamydophyla*, the family Campylobacteriaceae with the related genus *Campylobacter*, the family Lactobacillaceae with the related genus *Lactobacillus*, the family Pseudomonadaceae with the related genus *Pseudomonas*, together with the genera *Lachnoclostridium*, *Lachnospiraceae* NK4A136 group, *Sharpea*, and *Succiniclasticum*. Only the phylum Saccharibacteria and the genus *Lachnospiraceae* UCG-009 were less abundant in WM calves at this time point.

DISCUSSION

Using WM for feeding calves seems a convenient perspective for the farmer for economic and practical issues, including its disposal, and because of its nutritional qualities. However, as highlighted by numerous researchers and reported in a recent European Food Safety Authority (EFSA) opinion paper, feeding calves with milk containing antibiotic residues presents a significant risk for the development of antimicrobial resistance (1). Another relevant issue is the action on the developing calf gut microbiome, with the potential reduction of overall diversity and the selective inhibition of antibiotic-sensitive microbial groups. Possible consequences are an increased susceptibility to intestinal diseases and the establishment of a dysbiosis with adverse effects on animal health and welfare in later life (1). Gut health results from multiple factors that maintain a disease-free status, and, in this respect, the gut microbiome is crucial (46). Dysbiosis, an imbalance in the gut microbiome, is associated with numerous gastrointestinal and autoimmune diseases (47, 48) and is typically characterized by a reduction in microbial diversity with the loss of beneficial microorganisms and the proliferation of pathobionts (49–51). The general principles governing resilience and dysbiosis seem to apply to most mammals (52–54), but further studies are required to unravel species-specific differences in consideration of the significant differences in the anatomy and physiology of digestion.

Study Strengths and Limitations

A relevant advantage of this study was the administration of standardized colostrum, TM, and WM, together with WM characterization in terms of antibiotic concentration and nutrient content. In this way, there were no differences in colostrum quality among calves or calf groups, and the composition and antibiotic content of WM remained the same throughout the trial. However, some limitations were also present.

For ethical and practical reasons, the number of calves enrolled in the trial was limited to six per group, and calves were enrolled sequentially, first in the BM and then in the WM group. To offset these issues, the trial was carried out in a reduced time frame, and stringent statistics were applied to highlight the most relevant differences between the groups.

We observed some differences in BM and WM calves' fecal microbiota at the beginning of the trial. Newborn calves have an unstable microbiota, as in the first day of postnatal life, the microbial community's relative composition changes dramatically (55). Therefore, even minimal variations in the hour of sampling in relation to the hour of birth may have led to this result. However, the dramatic changes occurring within 24 h from birth are followed by a relevant increase in the bacterial load, reducing the impact of the time of delivery and reinforcing the reliability of the study findings.

Another point to consider is that WM from cows with mastitis likely had a different milk microbiota in itself than BM. Therefore, the different microbiota in calves fed with WM could have resulted from the microbes being ingested (or the ecological change these microbes created); the study design model used here did not allow us to dissect the effect of drug residues from other factors that differed between WM and BM, such as milk composition and milk microbiota effect on fecal microbiota (56). Furthermore, we cannot rule out a possible influence of the ORS on the WM calves' fecal microbiota (57).

The 16S rRNA gene analysis approach provides information only on bacteria. However, the gut microbiota also includes archaea, protozoa, viruses, algae, and fungi that play crucial roles and participate in maintaining eubiosis (58, 59). For instance, while bacterial communities recover mostly 30 days after heavy perturbations such as an antibacterial treatment, the fungal community may shift from mutualism toward competition (60). Investigations by metagenomics or metaproteomics would also include the non-bacterial components of the calf hindgut microbiome and highlight possible functional profile alterations accompanying the taxonomy changes (61–63). Additionally, results from 16S rRNA-gene sequencing may vary to some extent depending on the software (e.g., QIIME version) and parameters used to process and analyze the data. For instance, the robustness of results to the Phred filtering threshold has been indicated

(31), and more comprehensive sensitivity analyses to computer packages and parameters would shed light on these aspects.

Our study was carried out on male calves for animal value issues and ethical aspects due to female calves' more extended life expectancy. Long-term effects in the dairy farm are of interest mainly for what concerns female calves, and therefore gender effects may have to be evaluated more carefully. The breed might also play a role in resilience to intestinal microbiota perturbations (64, 65).

Finally, first-generation cephalosporins are widely used for the intra-mammary treatment of clinical mastitis and are therefore one of the antibiotic classes most likely to be found in WM from cows with bacterial mastitis (66, 67). However, the types and concentrations of antimicrobials in a farm can vary considerably according to management variables and time of the year (13). Some effects observed here might be antimicrobial-dependent, and the presence of other antibiotics in WM, broad-spectrum antibiotics, or the same antibiotics at different concentrations may lead to different results (68). Furthermore, the pasteurization of WM might lead to different results by reducing the microbial load and removing the influence of the WM microbiome. On the other hand, the concentration of antibiotic residues is not changed significantly by pasteurization (69).

Impact on Calf Diarrhea Incidence and Weight Gain

During the 2 weeks of WM feeding, we observed a significant increase in calf diarrhea incidence. Mitigation of pre-weaned calf mortality is a substantial challenge of the modern cattle industry, and enteric problems are among the major causes of newborn calf death (7, 70). When considering the limitations on prophylactic antimicrobial use (71), it is urgent to minimize the factors that favor the onset of diarrhea and compromise pre-weaned calf gut health, including administration of WM from mastitic cows. A related observation was the negative effect on calf growth. This reduced growth might lead to a slowed start of the animal's productive life (72) and discourages the use of WM also for feeding veal calves. Our results, differ from those of previous reports on this topic. Aust et al. (69) observed that animals fed with WM had a similar growth rate to those fed with milk powder. However, this might be related to the very high incidence of diarrhea observed in our study in the first 2 weeks. The development of juvenile diarrhea is notoriously associated with reduced calf growth (72).

Alterations in Diversity and Taxonomy of the Microbiota at the Different Time Points

WM feeding led to a dramatic loss in the fecal microbiota's alpha diversity compared to BM. The difference was already evident at Wk2 and highest at Wk4, both concerning richness and uniformity. Therefore, the adverse effects of WM in pre-weaned calves persisted and increased even under a diet with milk replacer containing probiotics integrated with pelleted starter feed, which should instead have led to an increase in the number of bacterial phylotypes in the calf gut (7). Notably, increased microbiome diversity is associated with increased weight gain and a lower incidence of diarrhea in healthy calves at the fourth week of life (73, 74).

Numerous taxa showed significant changes in abundance in calves fed with WM vs. BM, starting from the beginning of the trial and up to 6 weeks after removing WM from the diet. The significant differences observed in the fecal microbiota of WM calves might result from the selective action of cefalexin on some bacterial groups, with a resulting alteration in the microbial equilibria resulting in dysbiosis. On the other hand, the significantly higher incidence of diarrhea in the first weeks of life, due to the elevated antibiotic concentration in WM, could have been responsible for disrupting the microbial ecosystem and the consequent incomplete recovery of the healthy stable state (53).

At Wk1, *Veillonella* was already decreased in WM calves, in agreement with Van Vleck Pereira et al. (14), who observed that *Veillonella* was the only genus significantly decreased in calves fed milk with drug residues at week 1. Their study, however, analyzed WM spiked with low amounts of antibiotics and assessed their effects only during WM feeding. In our study, after 2 and 4 weeks of removing WM from the diet, *Veillonella* increased compared to BM calves. This is undesirable since *Veillonella* produces toxic compounds by fermenting proteins and is negatively associated with short-chain fatty acid (SCFA) production and gut health (75). Also at Wk1, the genus *Tyzzerella* was higher in WM than that in BM calves. Previous studies in humans found a significant increase of *Tyzzerella* and *Tyzzerella 4* in Crohn's disease patients, indicating that this might be a negative occurrence (76). In line with this, another study demonstrated that this genus is overrepresented in patients with an unhealthy diet (77). Other beneficial taxa were decreased, such as *Megamonas* (3), which is also involved in the production of SCFA. SCFAs are crucial for intestinal tissue metabolism and epithelium development and are absorbed into the bloodstream, providing energy for calf metabolism and growth (78).

At Wk2, at the end of the WM feeding period, the Bacteroidetes phylum was significantly less abundant in WM than BM calves. During the pre-weaning period, the rectal microbiota is composed mainly of Firmicutes and Bacteroidetes (79); such a relevant change at this state indicates a strong impact of antibiotics on the microbial equilibria in the calf gut. This agrees with the observations of Maynou et al. (13). In their study, most of the antimicrobials used to treat the cows from which WM originated belonged to the β-lactam family and were mainly cephalosporins. Other studies did not observe disruptions at the phylum level (14). However, this might be due to the higher antibiotic concentration in our WM.

At Wk4, 2 weeks after removing WM from the diet, the phylum Firmicutes was dramatically lower in WM calves than BM calves, and *Faecalibacterium* was the genus with the highest difference in abundance between the groups in the whole study. *Faecalibacterium prausnitzii*, the only known species in this genus, is strongly associated with positive effects on calf health and performance, including the reduction of diarrhea incidence and related mortality rate as well as increased weight gain (80), often together with *Roseburia* that was also less abundant in WM calves (81). These two bacteria are prototypical anti-inflammatory components of the gut microbiota and SCFA

producers, especially butyrate, and *Faecalibacterium* represents one of the most abundant bacteria encountered in the feces of healthy animals (82). Calves with a higher abundance of *Faecalibacterium* at a very young age show higher daily weight gain and a lower incidence of diarrhea (74). The whole Firmicutes phylum, mainly concerning the class Clostridia and the order Clostridiales, was dramatically less abundant in WM calves at Wk4. Dysbiosis is characterized by changes entailing a decreased prevalence of Clostridia (obligate anaerobes) (83, 84). Studies in mice showed that a lower relative abundance of Clostridia is associated with intestinal inflammation (54, 85).

At Wk8, when 6 weeks had passed since exposure to the cefalexin-containing WM, alpha diversity was higher for the first time in WM calves than that in BM calves. However, this was accompanied by an increased carriage of taxa associated with veterinary and zoonotic diseases, including *Campylobacter*, *Chlamydophila*, and *Pseudomonas* (86–89), with relevant consequences on calf health but also in terms of public health, as campylobacteriosis is the most important bacterial food-borne disease in the developed world (90, 91). *Campylobacter* employs many survival strategies and can survive over an extended time in the ruminant gut (91), and its association with *Pseudomonas* may further enhance its survival capabilities (92).

In a general perspective, the increased presence of potential pathogens at the end of the trial, 6 weeks after exposure to the antibiotic-containing WM, may also suggest a status of failing resilience and reduced colonization resistance, that is, the microbiota's competitive exclusion capacities (53, 93). In this respect, the microbiota of WM calves was also more affected by the probiotics contained in the milk substitute, as they showed a significant increase in Enterococcaceae (Wk4, the only increased bacterial taxon above the genus at this time point) and Lactobacillaceae (Wk8, the most intense change observed in terms of increased taxa). In other words, 2 and 6 weeks after receiving WM with antibiotics, the WM calves' gut microbiome was more susceptible to changes due to microorganisms administered with food; that is, the gut microbiome of WM calves was less resilient.

The phylum Saccharibacteria was one of the few taxa decreased in WM vs. BM calves at Wk8. Saccharibacteria, formerly known as TM7 (94), increase in the mature rumen (95), are more abundant in older animals (96), and are part of the core rumen community in lactating dairy cows (97). This further suggests that feeding calves with antibiotic-containing WM may lead to long-term disruptions of the gut microbiota physiology.

CONCLUSION

The microbiota plays a crucial role in the development and function of the gastrointestinal tract and gut health (7). It is essential for the proper development of the intestinal epithelium and of the mucus layer (98, 99), the formation of lymphoid structures (100), and the differentiation of immune cells (50, 101). Feeding pre-weaned calves with unpasteurized WM containing residual antibiotics might compromise these processes, impairing gut health and medium-term growth performances. The negative influences observed in the short term on alpha diversity, beta diversity, and taxonomy, together with the longer-term consequences on microbial taxa relevant for ruminal digestive processes and intestinal health, indicate that WM from cows treated with antibiotics should not be given to young calves.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the EBI European Nucleotide Archive repository, accession number PRJEB42855.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Committee for Animal Care of the University of Milan (protocol number 78_2018). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MP participated in the feeding trial, data analysis, data interpretation, and manuscript drafting. GS and AB contributed to the feeding trial, clinical monitoring of calves, sample collection, and clinical data analysis and interpretation. PC, BC, and FB contributed to the 16S data generation, analysis, and visualization. VB contributed to the bacteriological culture of milk, selection of cows, and data interpretation. PM and DP contributed to the study conception and design and data interpretation. MA contributed to the study conception, design and coordination, data interpretation and visualization, and manuscript drafting. All authors contributed to the revision and approval of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.650150/full#supplementary-material

Supplementary File 1 | Metataxonomic pipeline command lines.

Supplementary Figure 1 | Rarefaction curves. The figures show the observed number of detected OTUs plotted as a function of the number of reads in each sample and of the number of samples.

Supplementary Figure 2 | Normalized OTU values observed for all taxa showing statistically significant differences in abundance between WM and BM calves. The results are reported as a heatmap where red indicates the highest and green indicates the lowest normalized OTU value observed for each taxon at the different time points.

Supplementary Figure 3 | Statistical significance of the differences in normalized OTU abundances between WM and BM calves reported as a heatmap. Intensity of the red color increases with statistical significance.

Supplementary Table 1 | Average number of sequences per treatment and time point.

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Investigating the Use of Dry Matter Intake and Energy Balance Prepartum as Predictors of Digestive Disorders Postpartum

*Johanny Pérez-Báez[1], Carlos A. Risco[2], Ricardo C. Chebel[2], Gabriel C. Gomes[2], Leandro F. Greco[3], Sha Tao[3], Izabella M. Toledo[3], Bruno C. do Amaral[3], Marcos G. Zenobi[3], Natalia Martinez[3], Geoffrey E. Dahl[3], Jorge A. Hernández[2], Jessica G. Prim[2], José Eduardo P. Santos[3,4] and Klibs N. Galvão[2,4]**

[1] Escuela de Medicina Veterinaria, Facultad de Ciencias Agronómicas y Veterinarias, Universidad Autónoma de Santo Domingo, Santo Domingo, Dominican Republic, [2] Department of Large Animal Clinical Sciences, D. H. Barron Reproductive and Perinatal Biology Research Program, University of Florida, Gainesville, FL, United States, [3] Department of Animal Sciences, University of Florida, Gainesville, FL, United States, [4] D. H. Barron Reproductive and Perinatal Biology Research Program, University of Florida, Gainesville, FL, United States

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***Correspondence:**
Klibs N. Galvão
galvaok@ufl.edu

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One objective was to evaluate the association of dry matter intake as a percentage of body weight (DMI%BW) and energy balance (EB) prepartum and postpartum, and energy-corrected milk (ECM) postpatum with digestive disorders postpartum. For this, ANOVA was used, and DMI%BW, EB, and ECM were the outcome variables, and left displaced abomasum (LDA), indigestion, and other digestive disorders (ODDZ) were the explanatory variables. The main objective was to evaluate prepartum DMI%BW and EB as predictors of digestive disorders. For this, logistic regression was used, and LDA, indigestion, and ODDZ were the outcome variables and DMI%BW and EB were the explanatory variables. Data from 689 cows from 11 experiments were compiled. Left displaced abomasum was not associated with prepartum DMI%BW or EB. Postpartum data were normalized to the day of the event (day 0). Cows that developed LDA had lesser postpartum DMI%BW on days −24, −23, −12, −7 to 0 and from days 1 to 8, 10 to 12, and 14 and 16, lesser postpartum EB from days −7 to −5, −3 to 0, and 12, and lesser postpartum energy-corrected milk on days −19, −2, −1, 0, 7, 9, 10, 15, and 17 relative to diagnosis than cows without LDA. Cows that developed indigestion had lesser prepartum DMI%BW and EB than cows without indigestion, and lesser postpartum DMI%BW on days −24, −1, 0, 1, and 2, and greater DMI%BW on day 26, lesser ECM on days −24, −2, −1, 0, 1, and 2 relative to diagnosis. Postpartum EB was not associated with indigestion postpartum. Cows that developed ODDZ had lesser prepartum DMI%BW on day −8 and from days −5 to −2, lesser prepartum EB on day −8 and from days −5 to −2, and lesser postpartum DMI%BW than cows without ODDZ. Each 0.1 percentage point decrease in the average DMI%BW and each Mcal decrease in the average EB in the last 3 days prepartum increased the odds of having indigestion by 9% each. Cutoffs for DMI%BW and EB during the last 3 days prepartum to predict

indigestion were established and were ≤1.3%/day and ≤0.68 Mcal/day, respectively. In summary, measures of prepartum DMI%BW and EB were associated with indigestion and ODDZ postpartum and were predictors of indigestion postpartum, although the effect sizes were small.

Keywords: dry matter intake, energy balance, digestive disorders, predictive models, dairy cows

INTRODUCTION

The transition period in dairy cows is characterized by changes in the dry matter intake (DMI). Dairy cows start to decrease their DMI during the last 10 days of gestation, with a pronounced decrease during the last 3–4 days prepartum (1–3). There is an increase in DMI during the first weeks postpartum, although it does not meet the energy requirements for maintenance and milk production; therefore, dairy cows experience a negative energy balance that leads to an increase in non-esterified fatty acids and beta-hydroxybutyrate in blood (4–6).

The postpartum period is also characterized by an increase in the incidence of diseases and disorders that affect the welfare, production, reproduction, and longevity of cows in the herd. Previous research has shown that digestive disorders (i.e., left displaced abomasum (LDA), indigestion, diarrhea, rumen stasis, or bloat) are associated with delayed resumption of ovarian cyclicity (7), decreased fertility (8), and decreased milk yield (9), thus causing economic losses to the herd.

Several studies have investigated how dry matter and energy restriction prepartum influence dry matter intake and energy balance postpartum, with mixed results. Some studies showed that cows that were feed restricted during the dry period had increased DMI and energy intake in the first 3 weeks postpartum (10), some studies showed mixed results (11–13), and some studies did not show any significant improvements for the feed-restricted groups (14, 15). The literature investigating the association between DMI or energy balance (EB) prepartum and digestive disorders postpartum is more limited. One study showed that cows that were feed restricted during the dry period had lesser incidence of LDA postpartum than cows that were fed *ad libitum* (16). Furthermore, Ospina et al. (17) observed an association between high blood non-esterified fatty acids (NEFA) prepartum and LDA postpartum. In addition, indigestion (described later) has been associated with loss of BCS during the dry period (18); therefore, it is likely that cows with digestive disorders postpartum would have experienced a more severe drop in DMI and EB prepartum. Therefore, the hypothesis of this study is that cows with digestive disorders postpartum would have experienced a greater reduction in DMI as percentage of body weight (DMI%BW) or EB prepartum and would have a lesser DMI%BW or EB postpartum than cows without digestive disorders (**Figure 1**). One objective was to evaluate the association of DMI%BW and EB prepartum and postpartum, and energy-corrected milk (ECM) postpatum with digestive disorders [indigestion, LDA, and other digestive disorders (ODDZ; described later)]. The main objective was to evaluate the use of prepartum DMI%BW and EB as predictors of digestive disorders postpartum.

MATERIALS AND METHODS

Experimental Design, Housing, and Sample Size

A retrospective longitudinal study was performed using the data from a total of 689 cows (236 primigravid and 453 multigravid) from 11 different experiments conducted at the University of Florida dairy unit, located in the city of Hague, Florida. This was a convenience sample; therefore, no *a priori* sample size calculation was performed. For continuous variables, 122 cows affected with indigestion (described later; **Table 1**) would provide sufficient power to detect statistical differences with an effect size of 0.25 (e.g., difference in DMI%BW of 0.25 and SD of 1), alpha of 0.05, and beta of 0.2. With a sample size of 26 to 27 cows (LDA and ODDZ; **Table 1**), only differences with an effect size of 0.5 would be found statistically significant. Individual experiments were approved by the University of Florida Animal Research Committee.

The University of Florida dairy unit milked ∼500 Holstein cows twice daily with a rolling herd average of ∼10,500 kg/cow. The freestall beds and walking alleys were cleaned twice daily. Clean and dry sand was added on the top of the freestall beds twice weekly. Fans with misters and sprinklers over the feed line were present in the barns and activated when environmental temperatures rose above 18°C. Primiparous and multiparous cows were housed separately. Cows were vaccinated and treated for common diseases or disorders according to the standard operating procedures developed with participation of the veterinarians from the University of Florida, College of Veterinary Medicine, Food Animal Reproduction, and Medicine Service.

The experiments were conducted from 2007 to 2017. Six of the 11 experiments were conducted during the hot months (June to October) to evaluate the effect of evaporative cooling during the dry period on production measures (25–30). For these experiments, cows were provided with shade only or with shade plus evaporative cooling with fans and sprinklers. The average environmental temperature during the 3 weeks before calving for these experiments was 26.9°C ± 2.0°C and a temperature humidity index (THI) of 77.7 ± 2.8. Herein, we maintained the categorization of heat stress abatement applied in these six previous experiments resulting in cows categorized as hot with evaporative cooling ($n = 108$) or hot without evaporative cooling ($n = 106$). In the remaining studies, prepartum cows were

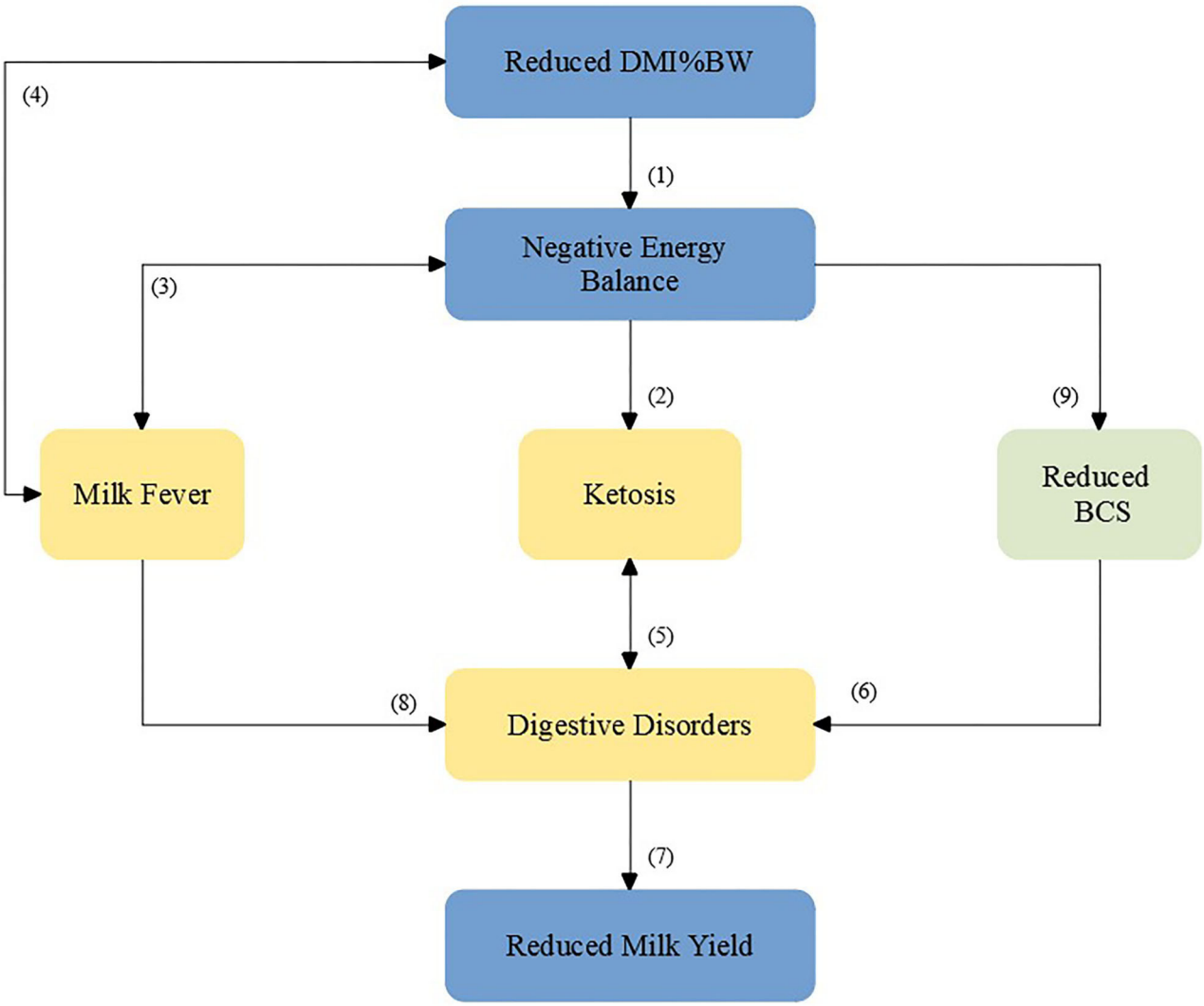

FIGURE 1 | Causal diagram showing the relationship of dry matter as percentage of body weight (DMI%BW) and energy balance with digestive disorders and its consequences. Based on the work of the following: (1) Hammon et al. (19); (2) Ospina et al. (17); (3) Martinez et al. (20); (4) Martinez et al. (21); (5) Raboisson et al. (22); (6) Chebel et al. (18); (7) Raizman et al. (23); (8) Madison and Troutt (24); (9) Hayirli et al. (1).

enrolled from December to May with an average environmental temperature of 17.6°C ± 3.4°C, and a THI of 63.8 ± 8.9 (31–35), and cows were provided evaporative cooling with fans and sprinklers when temperatures rose above 20°C. Fans and sprinklers were turned on and off automatically based on thermostat reading. Fans stayed on while environmental temperature exceeded 20°C, but sprinklers were on cycles of 1 min on and 3 min off. Cows enrolled in the experiments from December to May could still be exposed to heat stress. As far as we know, a heat stress THI cutoff for the dry period has not been established; therefore, we chose a prepartum cutoff of THI ≥70 as the midpoint between the traditional (72) and revised (68) THI cutoffs for lactating dairy cows (36, 37). Hence, we categorized cows as hot with evaporative cooling when the average THI during the last 3 weeks prepartum was ≥70 ($n = 126$ cows) and cool when the average THI for the last 3 weeks prepartum was <70 ($n = 349$). Hence, to account for any conditional effect of heat abatement, the variable heat stress abatement was created: cool, hot without evaporative cooling, and hot with evaporative cooling. The following formula was used to calculate the THI, according to (38):

THI = 0.8° ambient temperature + [(relative humidity/100) × (ambient temperature – 14.3)] + 46.4

The meteorological data obtained from The Weather Underground, Inc. (39) for the city of Hague, Florida was used to calculate THI.

Measurement of Dry Matter Intake

Cows had their daily DMI recorded using a system with individual feeding gates (Calan Gates, American Calan Inc.,

TABLE 1 | Frequency table of digestive disorders during the first 28 days postpartum in 689 cows.

Disorder	Frequency	Percentage (%)	DPP (range)
Digestive disorders	175	25.4	10 (0–28)
Left displaced abomasum	26	3.7	11 (2–27)
Indigestion	122	17.1	11 (1–28)
Other digestive disorders	27	4.5	
Sand impaction	3	0.4	4 (3–4)
Cecal dilatation	1	0.1	21 (–)
Diarrhea	18	3.2	16 (1–13)
Bloat	3	0.3	6 (4–7)
Constipation	2	0.2	2 (–)

DPP, average of days postpartum when the disorder was diagnosed.

Northwood, NH). For this study, we used DMI collected from days −21 to −1 prepartum and from days 1 to 28 postpartum. Dry matter intake on the day of calving (day 0) was not included because of inconsistent DMI measurements due to parturition itself and due to pen moves from the prepartum pen to the postpartum pen. Chemical composition of diets of each experiment included in this study is on **Supplementary Table 1**.

Milk Yield and Energy-Corrected Milk

Cows were milked twice a day, and milk production was recorded automatically using milk meters (AfiFlo; S.A.E. Afikim). Data for milk components such as concentrations of fat, true protein, and lactose were available either daily ($n = 356$ cows) or weekly ($n = 120$ cows). For cows sampled weekly, daily measurements were estimated by interpolation. Milk fat percentage decreases linearly from weeks 1 to 4 of lactation (40); therefore, interpolation would be an acceptable method for estimating daily fat percentage. As an example, when fat percentage was available for day 7 (Fat % = 3.12) and day 14 (Fat % = 3.55) postpartum, fat percentage on each subsequent day from days 7 to 14 was calculated using the formula: Fat percentage (Fat %) subsequent day = [(Fat % day 14 – Fat % day 7)/7] + Fat % previous day. For day 8, Fat % day 8 = [(3.55 – 3.12)/7] + Fat % day 7 = 0.06 + 3.12 = 3.18%. The ECM was calculated as follows, derived from the Nutrient Requirements of Dairy Cattle [NRC, (41)]:

ECM = [(0.3246 × kg of milk) + (12.86 × kg of fat) + (7.04 × kg of protein)].

Energy Balance

The EB was calculated using NRC (2001) equations for energy requirements as follows:

For prepartum EB:

EB prepartum = Net energy of lactation (NEL) intake – (NEL pregnancy + NEL maintenance)

For postpartum EB:

EB postpartum = NEL intake – (NEL maintenance + NEL milk)

where NEL intake, NEL maintenance, NEL pregnancy, and NEL milk were calculated as follows:

NE intake = DMI × NEL of the diet

NEL maintenance = (BW 0.75×0.08)

NEL pregnancy = [(0.00318 × day of gestation – 0.0352) × (calf BW/45)]/0.218.

NEL milk = (9.35 × milk yield × fat percentage/100) + (5.35 × milk yield × protein percentage/100) + (3.95 × milk yield × lactose percentage/100).

Health Disorders

Detailed paper and electronic health records were recorded for each cow. Each cow underwent scheduled complete physical examinations by a trained herdsman or by a veterinarian from the College of Veterinary Medicine Food Animal Reproduction and Medicine Service (FARMS) at the University of Florida on d 4, 7, and 12 postpartum. Furthermore, the attitude of cows was monitored daily pre- and postpartum, and milk yield was monitored postpartum. Any cow showing signs of depression, inappetence, lethargy, altered stride, inflammation of the mammary gland, or a drop >10% in milk yield underwent a physical examination by a trained herdsman or by a FARMS veterinarian. The veterinarians from FARMS performed physical examinations and provided supervision and training of herd personnel performing clinical diagnosis and treatment of postpartum cows at least once a week. Additionally, FARMS veterinarians were called to assist or confirm clinical diagnosis or treatment of postpartum cows throughout the weekdays and weekends. Only health events occurring during the first 28 days in milk were used in this study. We first retrieved the electronic health records, and then confirmed the information using the paper health records. Cows with mismatched information or with a disease diagnosis prepartum were excluded from the study. The health disorders recorded were ketosis, digestive disorders, calving disorders (dystocia, twins, stillbirths), retained placenta, metritis, and mastitis. Digestive disorders included LDA, indigestion, and ODDZ such as sand impaction, cecal dilatation, diarrhea, bloat, and constipation. Left displaced abomasum was diagnosed by a characteristic ping over the 9th to 13th ribs on the left side and was confirmed during surgery. None of the cows followed were diagnosed with right displaced abomasum. Indigestion was diagnosed in cows with undigested feces (presence of large amount of undigested fiber and grain in feces), scant pasty malodorous feces, rumen stasis (<1 rumen contraction/min), or a combination of two or more of these signs. Sand impaction was diagnosed during surgery in some cows that were suspected to have LDA. Cecal dilatation was diagnosed *via* rectal palpation and was characterized by a caudal displacement of the dilated cecum as previously described (42). Cows with cecal dilatation usually present with abnormal demeanor, decreased ruminal motility, scant feces, and colic. Cecal dilatation may evolve into volvulus and lead to death (42). Cecal dilatation was corrected surgically. Diarrhea was diagnosed in cows with watery feces that would sift through bedding (43). Bloat was diagnosed in cows with gas-distended rumen. Constipation was diagnosed in cows with very dry feces. The diagnosis of some of the clinical signs of digestive disorders such as undigested feces, scant pasty malodorous feces, and constipation can be subjective; therefore, a potential for misdiagnosis exists. Detailed

TABLE 2 | Association of pre- (−21 to −1 day) and postpartum (1 to 28 days) dry matter intake as percentage of body weight (DMI%BW), energy balance (EB), and energy-corrected milk (ECM) with left displaced abomasum (LDA) postpartum according to multivariable analysis.

	Prepartum		*p*-Value			Postpartum		*p*-Value		
	LDA	No LDA	LDA	Day	LDA × day	LDA	No LDA	LDA	Day	LDA × day
DMI%BW	1.47 ± 0.09	1.59 ± 0.02	0.15	<0.01	0.99	2.00 ± 0.22	2.53 ± 0.22	0.11	<0.01	<0.01
EB (Mcal/day)	0.4 ± 0.9	2.4 ± 0.2	0.03	<0.01	0.99	−11.9 ± 1.7	−8.6 ± 1.7	0.28	<0.01	<0.01
ECM (kg/day)	–	–	–	–	–	23.9 ± 4.4	34.3 ± 4.4	0.17	<0.01	<0.01

Day, day relative to parturition; LDA × day, interaction between left displaced abomasum and day.

information about calving and uterine disorders and ketosis and mastitis are presented in Pérez-Báez et al. (2, 3). Cows suffering from ketosis, digestive disorders, metritis, or mastitis were treated according to the farm standard operating procedure[1].

Statistical Analysis

To evaluate the association of prepartum and postpartum DMI%BW and EB with digestive disorders, we analyzed the data using ANOVA for repeated measures using the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC). The data were divided into two periods, prepartum and postpartum. For prepartum, the outcome variables were prepartum DMI%BW or EB, and the explanatory variable was one of the three digestive disorders (LDA, indigestion, ODDZ), and they were modeled separately; cows that developed LDA were compared with cows that did not develop LDA. Cows that did not develop LDA could have developed any other disorder. Cows that developed indigestion were compared with cows that did not develop indigestion. Cows that did not develop indigestion could have developed any other disorder. Cows that developed ODDZ were compared with cows that did not develop ODDZ. Cows that did not develop ODDZ could have developed any other disorder. Other studies have used healthy cows as the comparison group (44). However, this would introduce selection bias; therefore, this could artificially increase the differences in the measures of DMI between the groups and inflate the estimates in a prediction model. The models included the fixed effects of digestive disorder of interest (yes vs. no), parity (primigravid vs. multigravid), BCS in the last week prepartum (<3.75 vs. ≥3.75), day relative to calving (prepartum: days −21 to −1), heat stress abatement (cool vs. hot without evaporative cooling vs. hot with evaporative cooling), and the interaction between the digestive disorder of interest and day relative to calving. Cow was nested within experiment as a random effect. First-order autoregressive, compound symmetry, and unstructured covariance structures were tested, and the first-order autoregressive was selected because it resulted in the smallest Aikaike's information criterion.

As an example, the initial model to evaluate the association between prepartum DMI%BW and LDA was:

DMI%BW prepartum = LDA + day + heat stress abatement + BCS + parity + LDA × day + LDA × season + LDA × BCS + LDA × parity + cow (experiment).

The disorder of interest was forced into the model, but other variables were removed from the model by stepwise backward elimination according to Wald statistics criterion when $p > 0.05$. When an interaction was detected, the mean separation was assessed using the SLICE option in the MIXED procedure, and multiple comparisons were performed using the Tukey-Kramer adjustment method in SAS. All models were tested for multicollinearity using the GLM procedure of SAS, and all variables had a variance inflation factor of <5, therefore, indicating no multicollinearity. It is important to note that these analyses were used to test for associations and by no means can be used to infer causation.

To evaluate the use of prepartum DMI%BW and EB as predictors of digestive disorders, each disorder was considered the dependent variable and DMI%BW and EB as independent variables. These data were analyzed by logistic regression with the GLIMMIX procedure of SAS. The objective was to assess if measures of prepartum DMI%BW or EB were associated with the odds of digestive disorders. In this case, each disease or disorder was the dependent variable and the measures of prepartum DMI%BW or EB were assessed separately in different models as independent variables. For this purpose, the variable average DMI%BW or EB in the last 14, 7, and 3 days prepartum and reduction from days −8 to −1 and −4 to −1 were created. Univariable and multivariable models were performed. The univariable models included cow nested within experiment as a random variable. Measures of DMI%BW or EB with $p < 0.20$ were selected for inclusion in the multivariable logistic regression models. Multivariable models also included parity (primigravid vs. multigravid), prepartum BCS [<3.75 vs. ≥3.75 (45)], and heat stress abatement (cool vs. hot without evaporative cooling vs. hot with evaporative cooling), and cow nested within experiment as a random effect. Two-way interaction terms of measures of DMI%BW and EB with $p \leq 0.05$ and other covariates were tested. A stepwise backward elimination was performed and explanatory variables with $p > 0.05$ according to the Wald statistics criterion were removed from the model.

When a measure of DMI%BW or EB prepartum had $p \leq 0.05$, we assessed their contribution to the predictive ability of the logistic regression model containing other covariates by comparing the area under the curve (AUC) of a receiver operating characteristic curve (ROC) of the model with and without the measures of DMI%BW or EB using the

[1]https://vetmed-extension.sites.medinfo.ufl.edu/files/2012/01/WEB_VERSSION_-2011-Dairy-Unit-Complete-SOPs-vers-11-07-01-with-Title-page.pdf

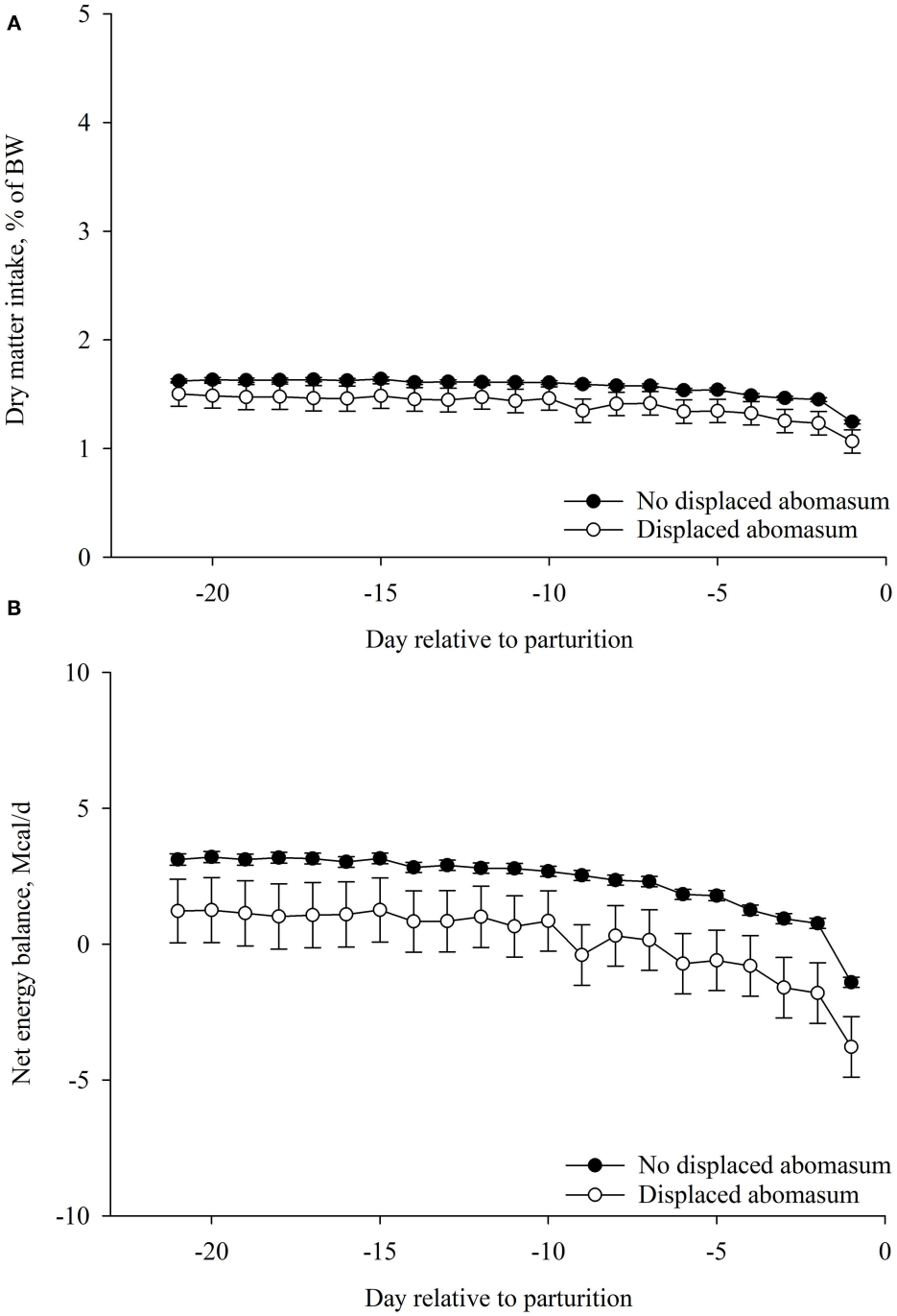

FIGURE 2 | Association of left displaced abomasum postpartum ($n = 26$) with **(A)** dry matter intake (DMI, %BW) and **(B)** net energy balance (EB, Mcal/day) during the prepartum period (from −21 to −1 day relative to parturition). Values are least square means ± SEM. Prepartum DMI (%BW): left displaced abomasum, $p = 0.15$; day relative to parturition, $p < 0.01$; and the interaction between left displaced abomasum and day, $p = 0.99$. Prepartum net energy balance: left displaced abomasum, $p = 0.03$; day relative to parturition, $p < 0.01$; and the interaction between left displaced abomasum and day, $p = 0.99$. *$p \leq 0.05$.

ROCCONTRAST statement of the LOGISTIC procedure of SAS as previously reported (46). The AUC ≤0.50 was considered non-informative, AUC between 0.50 and 0.70 was considered with low accuracy, AUC between 0.70 and 0.90 was considered accurate, and AUC between 0.9 and 1.0 was considered highly accurate (47). Finally, we determined cutoff values for measures of DMI%BW and EB prepartum with $p \leq 0.05$ for predicting digestive disorders postpartum using ROC, and the cutoff with

TABLE 3 | Effect of the average DMI as a percentage of body weight (DMI%BW) and the average energy balance (EB) in the last 3 days prepartum on postpartum left displaced abomasum (LDA), indigestion, and other digestive disorders in the first 28 days postpartum.

	DMI%BW			EB (Mcal/day)		
Disorder	**OR[a]**	**95% CI**	***p*-Value**	**OR[b]**	**95% CI**	***p*-Value**
LDA	1.03	0.95–1.13	0.45	1.03	0.94–1.12	0.58
Indigestion	1.09	1.04–1.15	<0.01	1.09	1.04–1.14	<0.01
ODDZ	1.08	0.99–1.18	0.07	1.08	0.99–1.16	0.08

ODDZ, other digestive disorders include sand impaction, cecal dilation, bloat, diarrhea, and constipation.

[a] The odds ratio (OR) represents a 0.1 percentage point decrease in the average DMI%BW in the last 3 days prepartum, when the average DMI%BW ranged from 0.27% to 2.90%, with an interquartile range from 1.03 to 1.70%.

[b] The OR represents a unit decrease in the average EB in the last 3 days prepartum, when the average EB ranged from −16.72 to 25.12 Mcal/day, with an interquartile range from −0.82 to 5.47 Mcal/day.

the greatest Youden's J statistic which combines the values for sensitivity and specificity was chosen. The sensitivity, specificity, and overall accuracy of applying the cutoff to predict digestive disorders were calculated. Statistical significance was considered when $p \leq 0.05$.

For postpartum, data were collected for the first 28 days postpartum and were organized to evaluate the association of DMI%BW, EB, and ECM relative to the day of diagnosis (i.e., days −2, −1, 0 (day of diagnosis), 1, and 2). Therefore, cows diagnosed on day 3 postpartum had 2 days of data before diagnosis and 25 days of data after diagnosis, whereas cows diagnosed on day 26 postpartum had 2 days of data after diagnosis and 25 days of data before diagnosis. Cows that had at least one digestive disorder were matched with cows that did not have the digestive disorder being analyzed but they could have any other disorder. Cows were matched on study number, heat stress abatement treatment, and parity group. Only one cow without a disorder was selected for each cow with a disorder; therefore, if more than one cow fit the matching criteria, an online random selector program (i.e., https://miniwebtool.com/random-picker/) was used to select the matching cow. In this analysis, the outcome variables were postpartum DMI%BW, EB, and ECM, and the explanatory variable was one of the three digestive disorders (LDA, indigestion, ODDZ), and they were modeled separately. The models included the fixed effects of digestive disorder of interest (yes vs. no), day relative to diagnosis, and the interaction between the digestive disorder of interest and day relative to diagnosis. Similar to prepartum data, cow was nested within experiment as a random effect. First-order autoregressive, compound symmetry, and unstructured covariance structures were tested, and the first-order autoregressive was selected because it resulted in the smallest Aikaike's information criterion.

As an example, the initial model to evaluate the association between postpartum DMI%BW and LDA was:

DMI%BW postpartum = LDA + day of diagnosis + LDA × day of diagnosis + cow (experiment).

RESULTS

The frequencies of each digestive disorders diagnosed during the first 28 days postpartum are shown in **Table 1**.

Association of Prepartum DMI%BW and EB With LDA

Prepartum DMI%BW was not associated with LDA postpartum ($p < 0.15$; **Table 2**; **Figure 2A**). Cows that had LDA had lesser prepartum EB ($p = 0.03$) compared with cows that did not have LDA (**Table 2**; **Figure 2B**).

Prepartum DMI%BW and EB as Predictors of LDA

The average DMI%BW and EB during the last 3 days prepartum were not explanatory variables for LDA (**Table 3**). Of the variables evaluated, parity was the only predictor of LDA postpartum. Multigravid cows had 9.3 times the odds of developing DA postpartum compared with primigravid cows (OR, 9.3; CI, 2.1–41.7; $p < 0.01$).

Association of Postpartum DMI%BW, EB, and ECM With LDA

The association between LDA and DMI%BW, EB, and ECM were dependent on time (**Table 2**). Cows that had LDA postpartum had lesser DMI%BW than cows that did not develop LDA on days −24, −23, −12, −7 to 0 and from days 1 to 8 and 10 to 12, 14, and 16 relative to diagnosis (**Figure 3A**). Cows that had LDA postpartum had lesser EB than cows that did not develop LDA from days −7 to −5, −3 to 0, and 12 relative to diagnosis (**Figure 3B**). Cows that had LDA postpartum had lesser ECM on days −19, −2, −1, 0, 7, 9, 10, 15, and 17 relative to diagnosis, compared with cows that did not develop LDA (**Figure 3C**).

Association of Prepartum DMI%BW and EB With Indigestion

Cows that developed indigestion had lesser prepartum DMI%BW ($p < 0.01$) compared with cows that did not develop indigestion (**Table 4**; **Figure 4A**). Cows that developed indigestion had lesser prepartum EB ($p < 0.01$) compared with cows that did not developed indigestion (**Table 4**; **Figure 4B**).

Prepartum DMI%BW and EB as Predictors of Indigestion

Of the variables evaluated, the average of DMI%BW and EB during the last 3 days prepartum, body condition score, and heat stress abatement were predictors of indigestion postpartum.

For each 0.1 percentage point decrease in the average DMI%BW in the last 3 days prepartum, the odds of having indigestion increased by 9% (OR, 1.09; CI, 1.04–1.15), and for each Mcal decrease in the average EB in the last 3 days prepartum, the odds of having indigestion increased by 9% (OR, 1.09; CI, 1.04–1.14; **Table 3**). The average DMI%BW ranged from 0.27 to 2.90%, with an interquartile range from 1.03 to 1.70%, and the average EB ranged from −16.72 to 25.12 Mcal/day, with an interquartile range from −0.82 to 5.47 Mcal/day.

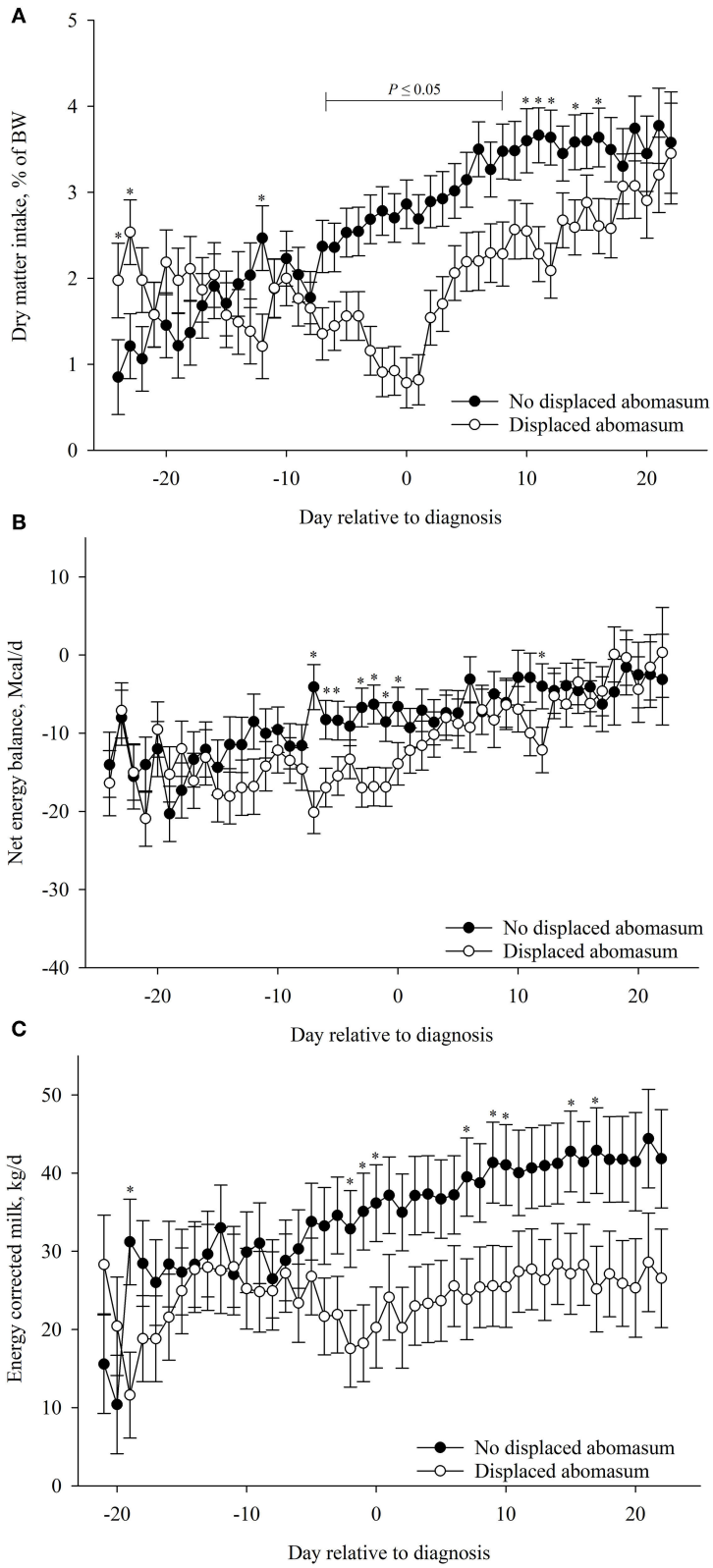

FIGURE 3 | Association of left displaced abomasum postpartum ($n = 26$) with **(A)** dry matter intake (DMI, %BW), **(B)** net energy balance (EB, Mcal/day), and **(C)** energy-corrected milk (ECM, kg/day) during the postpartum period (from −24 to 22 days relative to diagnosis). Values are least square means ± SEM. Postpartum DMI (%BW): left displaced abomasum, $p = 0.11$; day relative to parturition, $p < 0.01$; and the interaction between left displaced abomasum and day, $p < 0.01$. Postpartum EB: left displaced abomasum, $p = 0.28$; day relative to parturition, $p < 0.01$; and the interaction between left displaced abomasum and day, $p < 0.01$. ECM: left displaced abomasum, $p = 0.17$; day relative to parturition, $p < 0.01$; and the interaction between left displaced abomasum and day, $p < 0.01$. *$p \leq 0.05$.

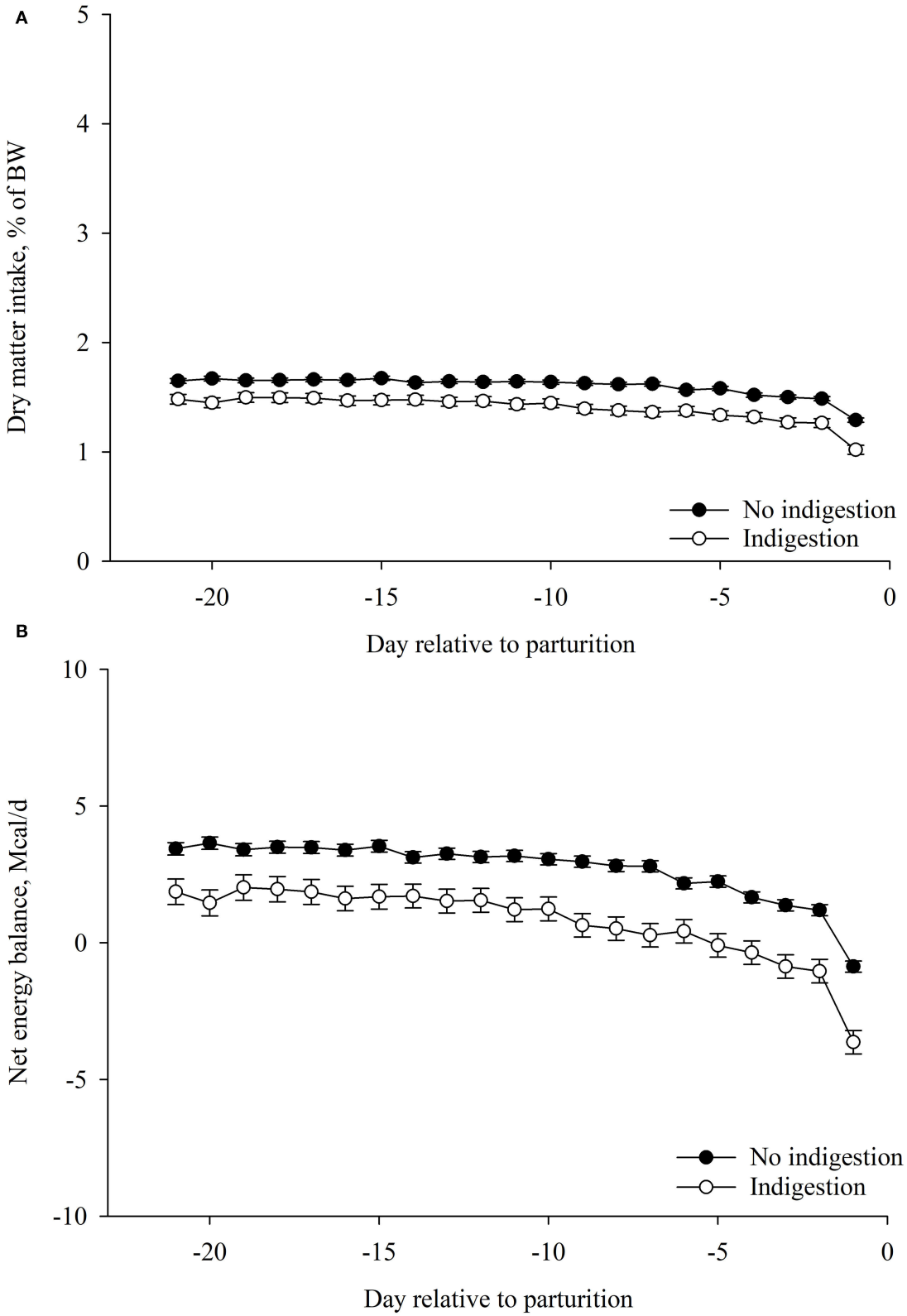

FIGURE 4 | Association of indigestion postpartum ($n = 118$) with **(A)** dry matter intake (DMI, %BW) and **(B)** net energy balance (EB, Mcal/day) during the prepartum period (from −21 to −1 day relative to parturition). Values are least square means ± SEM. Prepartum DMI (%BW): indigestion, $p < 0.01$; day relative to parturition, $p < 0.01$; and the interaction between indigestion and day, $p = 0.09$. Prepartum net energy balance: indigestion, $p < 0.01$; day relative to parturition, $p < 0.01$; and the interaction between indigestion and day, $p = 0.04$. $^{*}p \leq 0.05$.

TABLE 4 | Association of pre- (−21 to −1 days) and postpartum (1 to 28 days) dry matter intake as percentage of body weight (DMI%BW), energy balance (EB), and energy-corrected milk (ECM) with indigestion (Ind) postpartum according to multivariable analysis.

	Prepartum		*p*-Value			Postpartum		*p*-Value		
	Ind	No Ind	Ind	Day	Ind × day	Ind	No Ind	Ind	Day	Ind × day
DMI%BW	1.43 ± 0.03	1.63 ± 0.02	<0.01	<0.01	0.09	2.42 ± 0.07	2.37 ± 0.08	0.58	<0.01	<0.01
EB (Mcal/day)	0.74 ± 0.4	2.7 ± 0.2	<0.01	<0.01	0.04	−7.7 ± 0.7	−8.00 ± 0.8	0.80	<0.01	0.38
ECM (kg/day)	–	–	–	–	–	32.1 ± 1.3	32.8 ± 1.3	0.72	<0.01	<0.01

Day, day relative to parturition; Ind × day, interaction between indigestion and day.

Cows with high BCS had increased odds of developing indigestion postpartum compared with cows with low BCS (OR, 2.2; CI, 1.5–3.6). Cows in cool weather had increased odds of developing indigestion postpartum compared with cows under heat stress with evaporating cooling (OR, 1.76; CI, 1.1–2.9); whereas, there was no difference in the odds of developing indigestion for cows under heat stress without evaporating cooling compared with cows under heat stress with evaporating cooling (OR, 1.25; CI, 0.61–2.5).

When the average DMI%BW and EB in the last 3 days prepartum were included individually in the indigestion-predicting models containing BCS and heat stress abatement, the AUC increased from 0.60 (CI, 0.56–0.65) to 0.64 (CI, 0.60–0.69) and the AUC were different ($p < 0.05$) between the models.

The average DMI%BW and EB during the last 3 days prepartum produced cutoffs ($p < 0.01$) to predict indigestion, which were ≤1.3 DMI%BW and ≤0.68 EB (**Table 5**).

Association of Postpartum DMI%BW, EB, and ECM With Indigestion

The association of postpartum DMI%BW with indigestion was dependent of time ($p < 0.01$; **Table 4**). Cows that had indigestion had lesser postpartum DMI%BW than for cows that did not develop indigestion on days −24, −1, 0, 1, and 2 and greater DMI%BW on day 26 relative to diagnosis (**Figure 5A**). Postpartum EB was not associated ($p = 0.80$) with indigestion (**Table 4**; **Figure 5B**). The association of ECM with indigestion was dependent of time ($p < 0.01$; **Table 4**). Cows that had indigestion had lesser ECM than cows that did not develop indigestion on days −24, −2, −1, 0, 1, and 2 (**Table 4**; **Figure 5C**).

Association of Prepartum DMI%BW and EB With ODDZ

The association of prepartum DMI%BW with ODDZ was dependent of time ($p < 0.01$; **Table 6**). Cows that had ODDZ had lesser prepartum DMI%BW on day −8 and from days −5 to −2 compared with cows that did not develop other digestion disorders (**Figure 6A**). The association of prepartum EB with ODDZ was dependent of time ($p < 0.01$; **Table 6**). Cows that had ODDZ had lesser prepartum EB on day −8 and from days −5 to −2 compared with cows that did not develop other digestion disorders (**Figure 6B**).

Prepartum DMI%BW and EB as Predictors of ODDZ

The average DMI%BW and EB during the last 3 days prepartum were not explanatory variables for ODDZ (**Table 3**). Of the variables evaluated, parity, BCS, and heat stress abatement were the only predictors of ODDZ postpartum. Multigravid cows had 3.8 times increased odds of developing ODDZ postpartum compared with primigravid cows (OR, 3.8; CI, 1.08–13.5). Cows with high BCS had decreased odds of developing ODDZ postpartum compared with cows with low BCS (OR, 0.36; CI, 0.14–0.93). Cows in cool weather had decreased odds of developing ODDZ postpartum compared with cows under heat stress with evaporating cooling (OR, 0.35; CI, 0.14–0.86); whereas, there was no difference in the odds of developing ODDZ in cows under heat stress without evaporating cooling compared with cows under heat stress with evaporating cooling (OR, 0.97; CI, 0.37–2.5).

Association of Postpartum DMI%BW, EB, and ECM With ODDZ

Cows that developed ODDZ had lesser postpartum DMI%BW ($p = 0.04$) compared with cows that did not develop ODDZ (**Table 6**; **Figure 7A**). Postpartum EB and ECM were not associated ($p > 0.40$) with ODDZ (**Table 6**; **Figures 7B,C**).

DISCUSSION

In this study, we showed that cows that developed LDA, indigestion, and ODDZ had decreased DMI%BW or EB during the transition period. Furthermore, the average DMI%BW and EB in the last 3 days prepartum were predictive of indigestion, although the effect sizes were small.

As previously stated, there is scant literature on the association of DMI%BW and EB prepartum with digestive disorders postpartum. Janovick et al. (16) showed that cows that were feed restricted prepartum had lesser incidence of LDA postpartum than cows that were fed *ad libitum*. Feed-restricted cows also had decreased lipid mobilization, decreased lipid accumulation in the liver, and decreased ketosis incidence postpartum. Interestingly, feed-restricted cows had decreased circulating concentrations of leptin prepartum, which could have helped maintain DMI pre- and postpartum; therefore, improving metabolism and health. Furthermore, increased NEFA prepartum has been determined to be a risk factor for LDA postpartum (17), which indicated that prepartum DMI%BW and EB could have been negatively impacted in cows that later developed LDA. Herein, we saw that

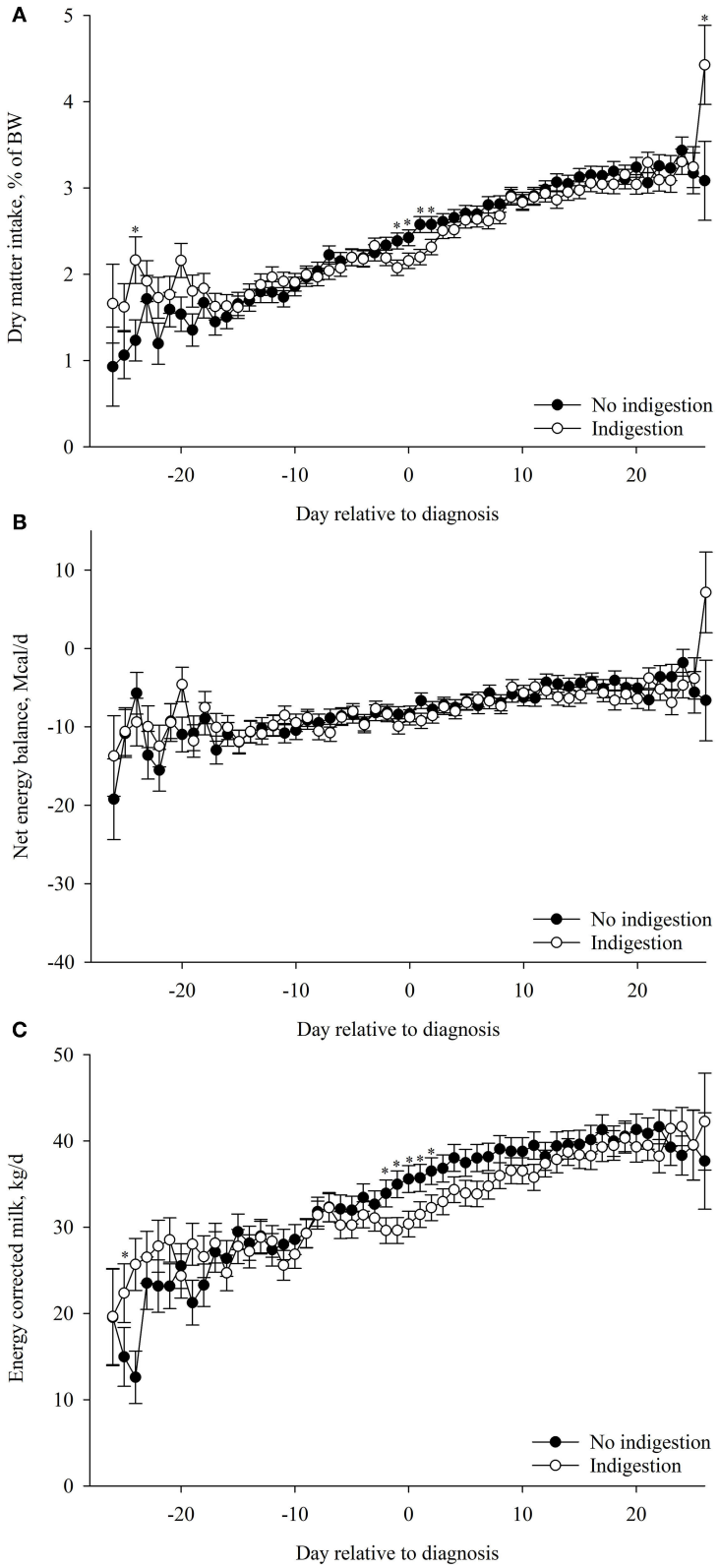

FIGURE 5 | Association of indigestion postpartum ($n = 118$) with **(A)** dry matter intake (DMI, %BW), **(B)** net energy balance (EB, Mcal/day), and **(C)** energy-corrected milk (ECM, kg/day) during the postpartum period (from −26 to 26 days relative to diagnosis). Values are least square means ± SEM. Postpartum DMI (%BW): indigestion, $p = 0.58$; day relative to parturition, $p < 0.01$; and the interaction between indigestion and day, $p < 0.01$. Postpartum EB: indigestion, $p = 0.80$; day relative to parturition, $p < 0.01$; and the interaction between indigestion and day, $p = 0.38$. ECM: indigestion, $p = 0.72$; day relative to parturition, $p < 0.01$; and the interaction between indigestion and day, $p < 0.01$.*$p \leq 0.05$.

TABLE 5 | Cut-offs of dry matter intake DMI as percentage of BW (DMI%BW) and energy balance (EB) to predict indigestion postpartum.

	Cut-off	Se (%)	Sp (%)	PPV (%)	NPV (%)	Acc (%)	AUC	*p*-Value
DMI%BW	≤1.3	65	55	23	88	57	0.61	<0.01
EB (Mcal/day)	≤0.68	74	48	23	90	53	0.62	<0.01

Se, sensitivity; Sp, specificity; PPV, positive predicted value; NPV, negative predictive value; Acc, accuracy; AUC, area under the curve.

TABLE 6 | Association of pre- (−21 to −1 day) and postpartum (1 to 28 days) dry matter intake as percentage of body weight (DMI%BW), energy balance (EB), and energy-corrected milk (ECM) with other digestion disorders (ODDZ) postpartum according to multivariable analysis.

	Prepartum		*p*-Value			Postpartum		*p*-Value		
	ODDZ	No ODDZ	ODDZ	Day	ODDZ × day	ODDZ	No ODDZ	ODDZ	Day	ODDZ × day
DMI%BW	1.55 ± 0.07	1.59 ± 0.02	0.51	<0.01	<0.01	2.23 ± 0.15	2.68 ± 0.15	0.04	<0.01	0.37
EB (Mcal/day)	1.9 ± 0.7	2.4 ± 0.2	0.53	<0.01	<0.01	−7.4 ± 1.7	−5.4 ± 1.7	0.40	<0.01	0.53
ECM (kg/day)	–	–	–	–	–	30.5 ± 1.9	34.3 ± 1.9	0.57	<0.01	0.96

ODDZ, other digestive disorders that include sand impaction, cecal dilation, bloat, diarrhea, and constipation; Day, day relative to parturition; ODDZ × day, interaction between other digestive disorders and day.

cows that developed LDA had lesser EB prepartum compared with cows that did not develop LDA, and numerically lesser DMI%BW. Nonetheless, neither EB nor DMI%BW could be used to predict LDA postpartum. Therefore, our interpretation is that maintaining DMI and EB prepartum is important for preventing LDA but cannot be used to predict LDA postpartum. This is likely a result of the multifactorial nature of LDA development.

During postpartum, cows that developed LDA had decreased DMI%BW before and on the day of diagnosis, and this decrease continued during the first 2 weeks after diagnosis. Edwards and Tozer (2004) showed that cows with LDA increased activity in the last 10 days prior to diagnosis which could mean less time eating at the feed bunk, therefore, lower DMI%BW before the onset of LDA. Energy balance was also reduced in cows with LDA and indigestion which might be a consequence of lower DMI and the onset of lactation. Furthermore, this decrease in DMI and increase in NEFA can lead to subclinical ketosis which is a risk factor for LDA (17) and consequently exacerbate the decrease in postpartum DMI%BW and EB (3, 48). Energy-corrected milk in cows that developed LDA was lesser from day −2 relative to diagnosis and continued to be decreased up to day 17 after diagnosis. In agreement to our results, Edwards and Tozer (49) showed that milk yield for cows with LDA starts to decline ∼3 days before diagnosis and continued to decrease during the first 7 days after diagnosis compared with healthy cows.

Cows with indigestion had decreased prepartum DMI%BW and EB. Furthermore, DMI%BW and EB in the last 3 days prepartum were significant predictors for indigestion postpartum, although the contribution to the prediction was modest. This indicates that DMI%BW and EB prepartum are predictors of indigestion postpartum, but their contribution is minor when accounting for other variables such as BCS and heat stress abatement. A limitation of the current study is that we did not perform external validation of our predictive models; therefore, future validation studies are needed. Herein, we calculated EB prepartum but others have used BCS change prepartum as a proxy for EB and found that cows that had loss of BCS prepartum had greater incidence of indigestion and uterine disease postpartum (18). In addition, we determined cutoffs for DMI%BW and EB to see if they could be used solely as a predictor of indigestion postpartum, and the cutoffs resulted in low to moderate sensitivity, specificity, overall accuracy, and AUC. Therefore, although significant, these cutoffs are of limited applicability. In summary, DMI%BW and EB prepartum are significant but minor contributors to indigestion development postpartum and cannot be used reliably to identify cows that will develop indigestion postpartum.

During postpartum, we showed that cows with indigestion decreased postpartum DMI%BW 1 day before diagnosis, and the decrease continued during the 3 days after diagnosis. (50) showed that there is a decrease in rumination in cows that developed indigestion at least 5 days before clinical diagnosis, indicating that ruminal activity and therefore a decrease in DMI%BW and EB occurred before the onset of clinical diagnosis postpartum. Energy-corrected milk in cows that developed indigestion was lesser from day −2 relative to diagnosis and continued to decrease up to day 2. Similar to our results, Kirchman et al. (9) showed that cows diagnosed with indigestion had decreased milk yield on the day of diagnosis compared with healthy cows. In addition, other studies where indigestion was lumped with other digestive disorders, milk yield was shown to decrease before and after disease diagnosis compared with healthy cows (49, 51).

Cows with ODDZ also had lesser prepartum DMI%BW and EB in the last 5 days prepartum. However, the average of DMI%BW and EB during the last 3 days prepartum were not predictors of ODDZ. In addition, during postpartum, we showed that cows with ODDZ had lesser DMI%BW compared with cows that did not developed ODDZ during postpartum, and most of the differences occurred around the time to diagnosis. Previous research showed that cows that developed digestive disorders, which included indigestion and LDA, had increased

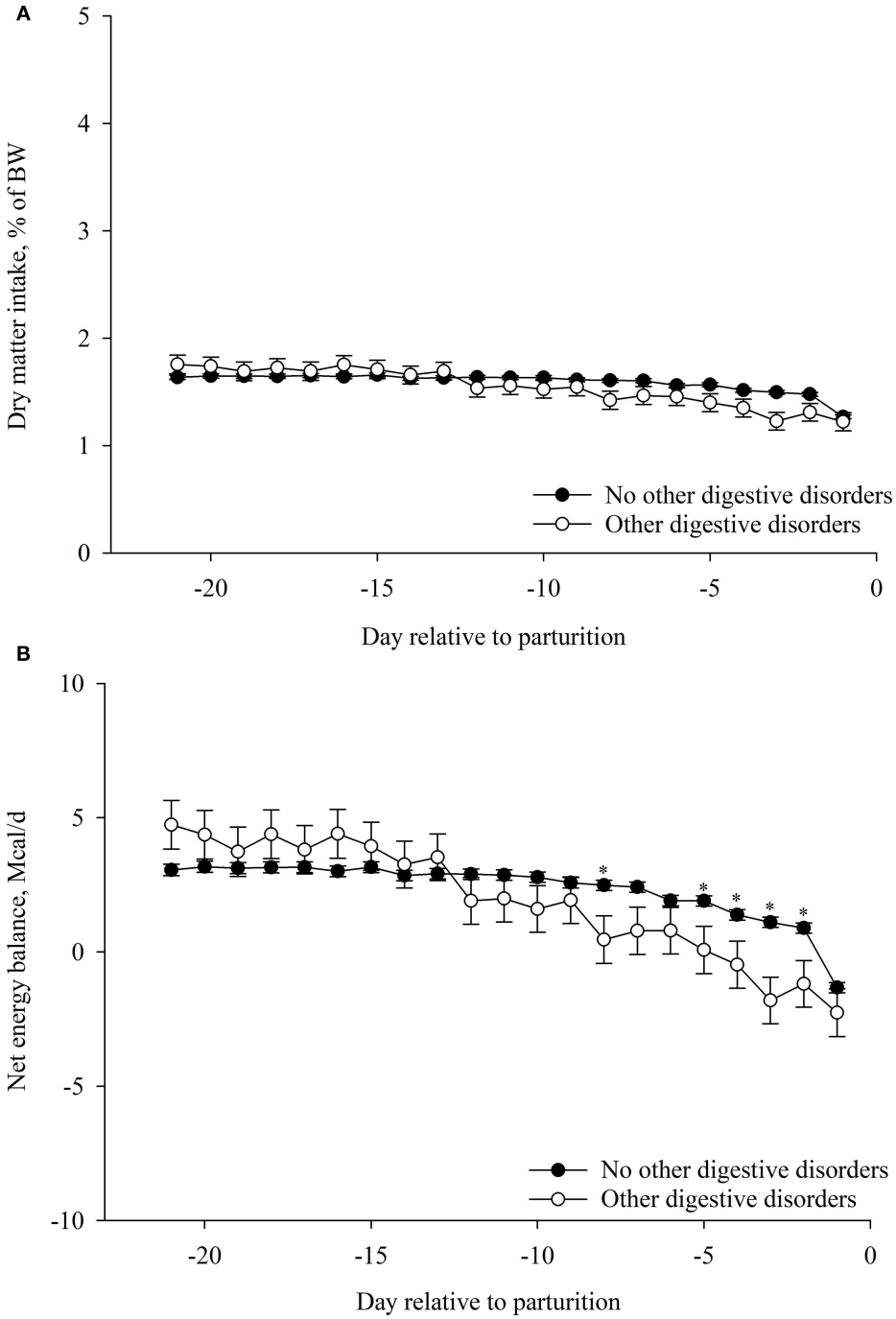

FIGURE 6 | Association of other digestive disorders postpartum ($n = 31$) with **(A)** dry matter intake (DMI, %BW) and **(B)** net energy balance (EB, Mcal/day) during the prepartum period (from −21 to −1 day relative to parturition). Values are least square means ± SEM. Prepartum DMI (%BW): other digestive disorders, $p = 0.51$; day relative to parturition, $p < 0.01$; and the interaction between other digestive disorders and day, $p < 0.01$. Prepartum net energy balance: other digestive disorders, $p = 0.53$; day relative to parturition, $p < 0.01$; and the interaction between other digestive disorders and day, $p < 0.01$. *$p \leq 0.05$.

activity at 8 days prior to disease diagnosis postpartum compared with healthy cows (49). Hence, if cows that developed digestive disorders spent more time walking, they probably spent less time eating before disease diagnosis. After disease diagnosis, their activity was lesser than healthy cows, which could mean they spend more time laying down and not eating. Unfortunately, they did not evaluate rumination data. The results of this study and previous studies (2, 3) indicate that maintaining DMI%BW during the last days of prepartum could reduce postpartum disorders.

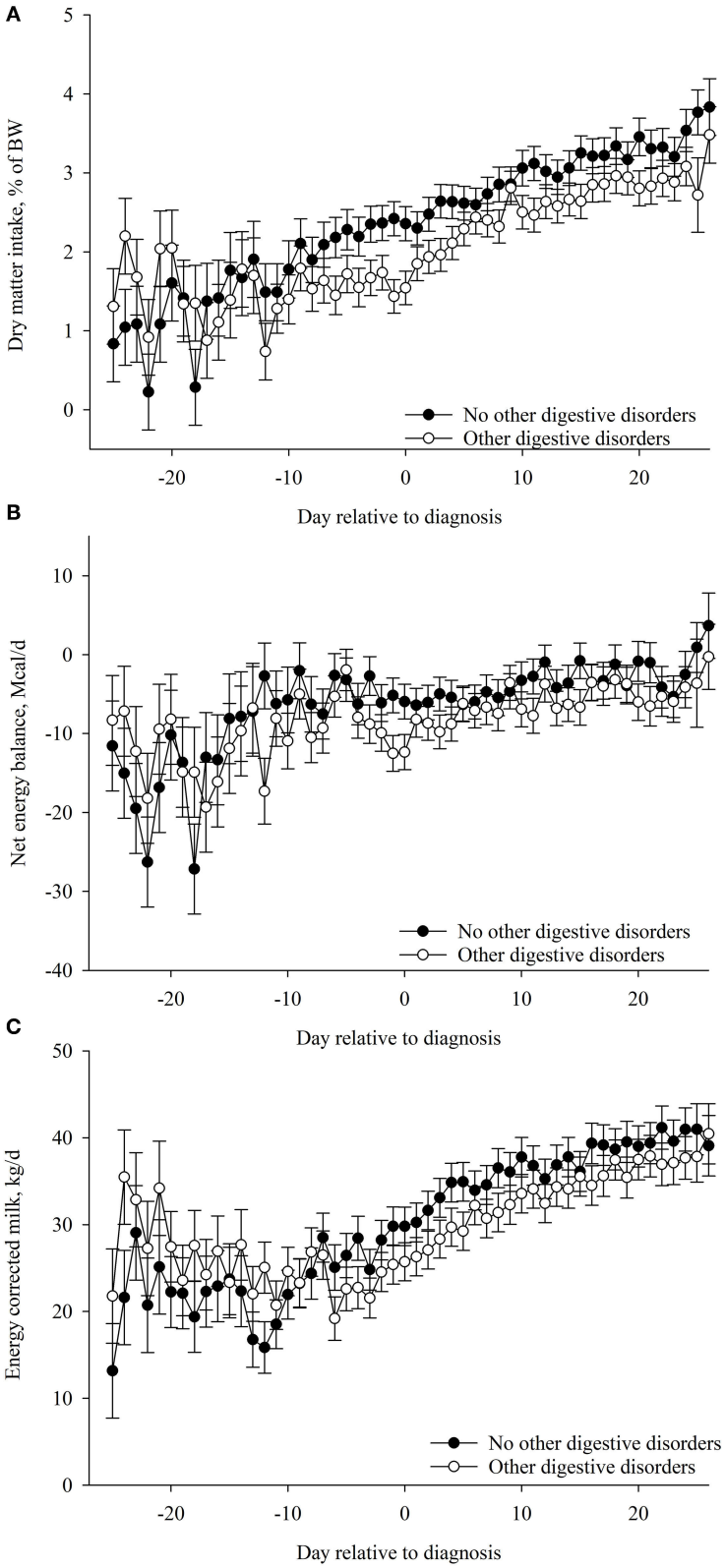

FIGURE 7 | Association of other digestive disorders postpartum ($n = 31$) with **(A)** dry matter intake (DMI, %BW), **(B)** net energy balance (EB, Mcal/day), and **(C)** energy-corrected milk (ECM, kg/day) during the postpartum period (from −25to 25 days relative to diagnosis). Values are least square means ± SEM. Postpartum DMI (%BW): other digestive disorders, $p = 0.04$; day relative to parturition, $p < 0.01$; and the interaction between other digestive disorders and day, $p = 0.37$. Postpartum EB: other digestive disorders, $p = 0.40$; day relative to parturition, $p < 0.01$; and the interaction between other digestive disorders and day, $p = 0.53$. ECM: other digestive disorders, $p = 0.57$; day relative to parturition, $p < 0.01$; and the interaction between other digestive disorders and day, $p = 0.96$.*$p \leq 0.05$.

An interesting finding of this study is that ODDZ were associated with reduced ECM. These results are different from what has been reported by others. (52) showed that cows with digestive disorders, excluding diarrhea and DA, had decreased milk production from −4 to 35 days relative to the day of diagnosis compared with cows that did not have the disease event. Edwards and Tozer (49) observed that cows that developed disease postpartum (i.e., at least one of the following: ketosis, RP, milk fever, LDA, indigestion, acidosis, and bloating, reduced feed intake or hardware disease) produced an average of 2.1 kg/day less milk than healthy cows. In this case, the effect of having a digestive disorder cannot be isolated from other diseases or disorders. Indeed, we have observed that cows that had calving disorders, metritis, and clinical mastitis postpartum had decreased milk yield, whereas cows that had ketosis had increased milk yield compared with cows that did not have those diseases or disorders (2, 3). Others have looked at management factors pre- and postpartum that may affect milk production and found that the most important non-dietary factors that affected milk production were age at first calving, presence or absence of feed refusals, number of free stalls per lactating cow, and whether feed was pushed up in the feed bunk (53). These findings show that several factors not accounted for in this study could have affected milk yield.

In conclusion, this study showed that digestive disorders such as indigestion and ODDZ were associated with prepartum DMI%BW and EB whereas LDA was associated with prepartum EB. The average DMI%BW and EB in the last 3 days prepartum were significant explanatory variables for indigestion, and the average DMI%BW and EB in the last 3 days prepartum increased the predictive ability of indigestion although the effect sizes were small. Prepartum cutoffs for DMI%BW and EB to predict indigestion postpartum were established, although with low sensitivity, specificity, and overall accuracy. In addition, LDA, indigestion, and ODDZ were associated with postpartum DMI%BW whereas LDA was associated with EB relative to the day of diagnosis. In summary, DMI%BW and EB prepartum are associated with digestive disorders and are significant but minor contributors to the risk of indigestion postpartum.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee, University of Florida.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.645252/full#supplementary-material

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Conflict of Interest: GG, LG, and NM were employed by the companies Merck Animal Health, Kemin Industries Inc, and Zoetis (United States), respectively.

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