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A randomized controlled trial to study the effect of supplemental premilking stimulation on milking performance, teat tissue condition, udder health, and well-being in dairy cows

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ABSTRACT

The objectives of this study were to evaluate the effect of supplemental premilking stimulation, provided after manual stimulation, by means of high-frequency pulsation without reduction of the vacuum in the pulsation chamber on milking performance, teat tissue condition, udder health, and well-being in dairy cows. In a randomized controlled trial, Holstein cows (n = 491) from 1 commercial dairy farm with a thrice-daily milking schedule were assigned to treatment and control groups over a 64-d period. Treatments consisted of a maximum of 20 s of pulsation stimulation at a pulsation rate of 100 (SPS100) or 300 (SPS300) cycles per minute and a pulsation ratio of 25:75. The treatments were applied after completion of manual premilking stimulation upon milking unit attachment. Cows in the control group (CON) received only traditional premilking stimulation by manual forestripping and wiping for 6 s. Milking characteristics were measured with on-farm milk flow meters. Milking machine-induced short-term (swelling at teat base, firmness at teat end, and teat discoloration) and long-term (teat-end callosity) changes to the teat tissue were assessed manually and visually. Composite milk samples were analyzed for SCC. Cow hind-leg activity was assessed with 3-dimensional accelerometers. Fecal 11,17-dioxoandrostanes (11,17-DOA; a group of cortisol metabolites) were determined on wk 4 and 8 to assess the well-being of the cows. Generalized linear mixed models were used to study the effect of treatments on the outcome variables milk yield per milking and milking unit-on time. We observed no meaningful differences among groups for milk yield or milking unit-on time. Least squares means and their 95% CI for cows in the SPS100, SPS300, and CON groups were 13.9 (13.3–14.5), 14.0 (13.4–14.6), and 13.9 (13.3–14.6) kg for milk yield and 218 (212–224), 218 (211–224), and 218 (212–224) s for milking unit-on time, respectively. Compared with cows in the CON group, the odds (95% CI) of short-term changes were 1.30 (0.95–1.78) for the SPS100 group and 1.50 (1.10–2.05) for the SPS300 group. The odds of long-term changes were 0.94 (0.67–1.34) for cows in the SPS100 group and 0.71 (0.49–1.04) for cows in the SPS300 group. We observed no differences in SCC. In reference to the CON group, the hazard ratio (95% CI) in SPS100 and SPS300, respectively, were 0.35 (0.13–0.98) and 1.22 (0.57–2.64) for clinical mastitis, and 0.34 (0.12–0.95) and 1.28 (0.60–2.73) for culling. The LSM (95% CI) of hind-leg activity during milking were 8.3 (6.5–10.5), 10.6 (8.1–13.7), and 9.1 (7.2–11.6) movements per milking for the SPS100, SPS300, and CON groups, respectively. The LSM (95% CI) of fecal 11,17-DOA concentration (ng/g) at the first and second test days, respectively, were 31.1 (28.1–34.2) and 22.3 (19.2–25.4) for the SPS100 group, 26.4 (23.4–29.4) and 25.2 (22.0–28.4) for the SPS300 group, and 24.8 (21.8–27.9) and 25.0 (21.7–28.3) for the CON group. We conclude that applying supplemental stimulation after manual stimulation through the high-frequency pulsation system tested here did not impart additional benefits to the milk harvesting process, teat tissue condition, somatic cell count, or the well-being of the animals.
Key words: bovine, milking performance, bimodality, teat condition

INTRODUCTION

Adequate premilking stimulation is indispensable to harvesting high-quality milk from cows as quickly and completely as possible along with minimizing the impact of the milking machine on teat and udder health. Premilking stimulation comprises some form of tactile stimulation and the timing of the milking unit attachment after initiation of the tactile stimulation (NMC, 2013). Tactile stimulation initiates the release of oxytocin from the pituitary gland into the blood stream via the activation of

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pressure-sensitive mechanoreceptors located in the tip of the teat (Bruckmaier and Blum, 1996). The time delay in the milking unit attachment (i.e., latency period) allows the oxytocin to reach the mammary gland via the bloodstream and stimulate myoepithelial cell receptors. The subsequent contraction of the myoepithelial cells leads to a forceful ejection of the alveolar milk into the duct system of the mammary gland. This makes the alveolar milk available for the milk harvest (Bruckmaier and Blum, 1998).

Current recommendations suggest a minimum duration of tactile stimulation of 10 to 20 s and a latency period between the start of stimulation and milking unit attachment of 90 to 150 s (Reneau and Chastain, 1995; Kaskous and Bruckmaier, 2011; Watters et al., 2012). However, according to a recent study on 64 Michigan dairy herds, 32 herds (50%) applied stimulation for a very short duration of ≤6.3 s (Moore-Foster et al., 2019). This is consistent with data from our own group showing that the mean duration of stimulation applied on New York State dairy farms ranged from 3 to 6 s (Wieland et al., 2021). Consequently, the physiological requirements of dairy cows are not met in most current milking systems. This hampers our ability to elicit the cows' maximum milk ejection capacity, leading to delayed milk ejection. Delayed milk ejection has been associated with poor milking efficiency and reduced milk yield (Bruckmaier, 2005; Erskine et al., 2019). Results from a recent study on a 3,600-cow dairy herd with 3 milking sessions per day showed that cows with moderate (lag time between attachment of milking unit and the estimated milk letdown 30–59 s) and severe (lag time ≥60 s) delayed milk ejection produced 1.8 and 3.1 kg less milk per milking session, respectively, compared with cows without delayed milk ejection (lag time <30 s; Erskine et al., 2019).

Poor milking efficiency, associated with improper premilking stimulation and subsequent delayed milk ejection, is reflected by increased milking duration at a lower milk flow rate (Wieland et al., 2020). Thus, teats from cows with delayed milk ejection are subjected to an increased period of mechanical forces during machine milking, affecting the teat integrity. This is further aggravated by the inverse relationship between milk flow rate and milking vacuum (Bade et al., 2009; Ambord and Bruckmaier, 2010), which escalates the vacuum-induced strain on teat tissues during times of low milk flow rates, causing congestion (Hamann and Mein, 1990). Results from our recent study indicate that more than 50% of cows suffer daily from these milking machine-induced teat tissue alterations (Wieland et al., 2021). Blood circulation is of utmost importance for the teats' defense function against mastitis pathogens (Hamann et al., 1994). Alterations of the teats' circulatory system have therefore been associated with an increased risk of new IMI (Zecconi et al., 1996) and an increased somatic cell count (Zwertvaegher et al., 2013). In addition to their detrimental effect on udder health, these milking machine-induced alterations of the teat tissue can lead to pain, defensive reactions, and restless behavior (Raoult et al., 2021). They are therefore considered to diminish animal well-being (Hillerton et al., 2002), which has become a major public concern (von Keyserlingk et al., 2009).

Traditionally, premilking stimulation has been achieved through tactile stimulation from manual forestripping (i.e., the removal of several streams of milk through manual compression of the teat) and teat cleaning, and on most dairy operations in the United States, this traditional practice is used to initiate the milk ejection reflex of cows (USDA, 2016). With the advancement of milking technology, alternative forms of mechanical stimulation have been tested. These include the use of rotating brushes attached to a robotic arm (Hopster et al., 2002), different rates of pulsation (Weiss and Bruckmaier, 2005; Watters et al., 2015), and normal pulsation at reduced vacuum in the pulsation chamber (Neuheuser et al., 2017), as means to provide mechanical stimulation to the teat. However, despite the advanced technical capabilities inherent to modern parlor systems, these automated premilking stimulation systems have not been adopted by farms, and their potential for providing supplemental stimulation (i.e., in addition to the manual premilking stimulation) to cows remains unexplored. Therefore, our objective was to study the effect of supplemental premilking stimulation through a high-frequency pulsation system on milking performance, teat tissue condition, udder health, and animal well-being in dairy cows. We hypothesized that the application of supplemental stimulation by means of a high-frequency pulsation system would increase milk production, decrease milking duration, improve teat tissue condition and udder health indices, and promote the well-being of the cows.

MATERIALS AND METHODS

This randomized controlled field trial was conducted at a commercial dairy farm located near Ithaca, New York, from July 7 to September 7, 2023. The Institutional Animal Care and Use Committee of Cornell University reviewed and approved the study protocol (protocol no. 2022-0197).

Animals and Housing

The lactating herd consisted of approximately 1,600 Holstein cows. These cows were housed in freestall pens with concrete stalls covered with mattresses and bedded with wastepaper pulp or dry shavings or deep-bedded stalls with fresh sand. The 2 pens we considered in our study were bedded with fresh sand. The cows were fed a total mixed ration. The herd 305-d mature equivalent milk production was 13,716 kg, the rolling mean test-day SCC was 143,000 cells/mL, the monthly incidence of clinical mastitis was 5.0%, the 21-d pregnancy rate was 30.0%, and the culling risk was 36.0%.

Milking System

The farm was equipped with a 60-stall rotary milking parlor (Madero Premium, Madero Dairy Systems, Houston, TX). Cows were milked 3 times per day at 0630, 1430, and 2230 h. The vacuum pump (28 kW) was set to supply a receiver operating vacuum of 44 kPa. The milking unit consisted of the Milking Cluster Classic 300 (GEA Farm Technologies, Bönen, Germany) and a milking liner with a square-shaped barrel (GEA Classic GQ, GEA Farm Technologies). The pulsators (HiFlo, BouMatic, Madison, WI) were set to a rate of 60 cycles/min and a ratio of 65:35. The automatic cluster remover settings were as follows: the minimum amount of time the milking unit was attached was set to 120 s, the cluster remover milk flow rate threshold at which milking was terminated was set to 1.6 kg/min, and the delay between the vacuum shut-off to the claw and the beginning of milking unit retraction was set to 0 s. The milk sweep was inactivated. Before the study, the investigators verified all milking machine settings according to the guidelines outlined by the National Mastitis Council (NMC, 2012).

Milking Routine

The platform speed of the rotary parlor was 9 s/stall, resulting in a rotation time of 540 s. The parlor operation included two 12-h work shifts, each with 3 milking technicians who were assigned to perform the following tasks at 3 different positions: (1) forestripping of teats and application of premilking teat disinfectant; (2) cleaning and drying of teats with a cloth towel; and (3) attachment and alignment of the milking unit. Post-milking teat disinfectant was applied with an automated teat spray robot (TSR, DeLaval International AB, Tumba, Sweden). The different tasks were positioned in stall 1 (station 1), stall 5 (station 2), and stall 12 (station 3). This setup resulted in a stimulation duration (duration of manual forestripping) of ~6 s, and teat wiping and a latency from first tactile stimulus to milking unit attachment (i.e., preparation lag time) of ~99 s.

Treatment Allocation

The 3 central components of the treatment allocation were the dairy farm management software program AfiFarm (Afimilk), electronic on-farm milk flow meters (AfiMilk MPC Milk Meter, Afimilk), and the pulsation system (HiFlo, BouMatic). Using AfiFarm, different machine settings can be applied to individual cows that are housed in the same pen. All cows that were housed in study pens were eligible for enrollment, stratified by lactation number (first, second, and third or greater lactation), stage of lactation (≤100, 101–200, and >200 DIM), average daily milk yield during the previous week, and SCC, and randomly assigned to treatment and control groups using a random number generator (Urbaniak and Plous, 2013). The treatments consisted of supplemental stimulation (in addition to the farm's standard premilking stimulation regimen) by means of high-frequency pulsation at 100 (group **SPS100**) or 300 (group **SPS300**) cycles per minute and a pulsation ratio of 25:75 for a maximum duration of 20 s. The high-frequency pulsation stimulation was started immediately after the attachment of the milking unit. The stimulation duration (up to the maximum value) for an individual cow varied according to the duration at which sufficient milk was harvested to initiate the first dump (corresponding to 200 mL) of the milk flow meter, which then switched the milking unit from stimulation to milking mode. No delay was set for switching from high-frequency to normal milking pulsation. Cows in the control group (**CON**) received only traditional premilking stimulation by means of manual forestripping and wiping of teats according to the farm's standard milking routine. During the stimulation phase, the pulsation chamber and claw vacuums were not reduced. Thus, in contrast to previously tested systems (Weiss and Bruckmaier, 2005; Watters et al., 2015), the milking liner was not kept in the closed position to avoid the milk harvest during the stimulation phase, and teats were exposed to the unaltered claw vacuum.

Sample Size Calculation

At the time of enrollment, 491 study cows were available from the 2 pens. This sample size was sufficient to detect a minimum difference of 1 kg of milk per milking in 2 of the 3 groups at an α level of 0.05 with a power of 0.98. We conducted this calculation based on a reported SD of 3.4 kg (Wieland et al., 2017), a presumed within-cow correlation of 0.5, 186 milking observations (62 d × 3 milking sessions per day), and a repeated-measures ANOVA (G*Power version 3.1.9.7; Faul et al., 2007).

Data Acquisition

Cow Characteristics. The study lasted 64 d. We obtained information on lactation number, stage of lactation, SCC at the last test day (20 d before the start of the study), and average daily milk production 1 wk before the study start from the dairy management software program (DairyComp 305, Valley Agricultural Software, Tulare, CA).

Milking Characteristics. We collected the following milking characteristics during each milking session with the electronic on-farm milk flow meters (AfiMilk MPC Milk Meter, Afimilk) and recorded them using the dairy farm management software AfiFarm (Afimilk): milk yield (i.e., the yield of milk, in kilograms, harvested from start of milking to detachment of the milking unit), milking unit-on time (i.e., the duration, in seconds, from start of milking to detachment of the milking unit), first 15-s milk flow rate, 15 to 30-s milk flow rate, 30 to 60-s milk flow rate, and 60 to 120-s milk flow rate (i.e., average milk flow rate, in kilograms per minute, recorded in the first 15 s, between the first 15 to 30 s, between the first 30 to 60 s, and between the first 60 to 120 s of milking, respectively). All the preceding parameters refer to the time from unit attachment (i.e., push of the start button) and, for the treatment groups, also included the stimulation duration. Thus, the duration of the pulsation stimulation could not be discriminated. Reports from each milking session were created with the dairy farm management software AfiFarm (Afimilk) and exported to a text document (.txt file).

For subsequent analyses, we created a new categorical variable (i.e., bimodality) using the 30 to 60-s milk flow rate in accordance with the protocol described previously by our group (Wieland and Sipka, 2023). Briefly, we simultaneously collected data on the first 15-s milk flow rate, 15 to 30-s milk flow rate, 30 to 60-s milk flow rate, and 60 to 120-s milk flow rate with the on-farm milk flow meter (AfiMilk MPC Milk Meter), as well as data on the presence of bimodality with a portable milk flow meter (Lactocorder, WMB AG, Switzerland) from 91 individual cow milking observations. Using logistic regression models, we studied the association between each milk flow rate and bimodality. We then determined the optimal cut-point of each milk flow rate for predicting bimodality as detected with the Lactocorder by calculating Youden's J statistic of the receiver operating characteristic table. We determined that the 30 to 60-s milk flow rate and a value of 3.9 kg/min provided the best cut-point to predict bimodality, leading to an area under the curve of 0.71. As such, we defined bimodality in the current study to be present if the 30 to 60-s milk flow rate was <3.9 kg/min.

Nonlactating Quarter and Teat Tissue Conditions. Three trained investigators assessed the presence of a nonlactating quarter (author JV) and the teat tissue condition (AS and MW). The presence of a nonlactating quarter was assessed visually during milking on the first day of the study and considered to be present if the teat cup was not attached to the corresponding quarter.

Short-term changes (**STC**) to teat tissue condition induced by the milking machine were assessed visually and through palpation every other week (4 times in total) according to a scoring system that was adopted from Hillerton et al. (2000). Briefly, within ~60 s after milking, the condition of the teat base was evaluated and scored as no visible mark present (score 1), visible mark present (score 2), or significant swelling present (score 3). Second, the teat-end consistency was evaluated and categorized as soft (score 1), firm (score 2), or wedging present (score 3). Third, the color of the teat was visually assessed and discriminated into normal (score 1), red discoloration present (score 2), or blue discoloration present (score 3). We considered an STC to be present if the teat base score was 3, the score of the teat-end consistency was ≥2, or the score of the teat skin color was 3 for 1 or more teats; STC were considered absent otherwise.

Teat-end callosity (hyperkeratosis) was assessed visually on the first day of the study to establish a baseline value and every other week throughout the study (once before and 4 times during the study) according to the scoring system described by Mein et al. (2001). Briefly, teat-end callosity was scored as no callosity ring present (score 1), callosity ring but no roughness present (score 2), callosity ring and roughness present with keratin fronds extending from the teat orifice that range from 1 to 3 mm in length (score 3), or callosity ring present with excessive keratin fronds that are 4 mm or longer (score 4). The presence of hyperkeratosis was considered if 1 or more teats had a score ≥3, whereas hyperkeratosis was absent otherwise.

Milk Sampling and SCC Analysis. Composite milk samples were collected by DHIA service personnel (Dairy One Cooperative Inc., Ithaca, NY) on d 16 and 41. Milk samples were analyzed for SCC (cells/mL) at Dairy One Cooperative Inc. For subsequent analyses, SCC values were $\log_{10}$-transformed (**logSCC**).

Clinical Mastitis Detection and Culling. Clinical mastitis detection was performed by farm personnel during the premilking udder preparation and recorded with the farm management software (Dairy Comp 305). Clinical mastitis was considered to be present if milk from a quarter was abnormal with or without signs of local inflammation of the affected quarter as previously described (Erskine et al., 2003). For subsequent analyses, we defined a case of clinical mastitis as the first case that occurred during the study period. Culling events were also recorded in the farm management software (Dairy Comp 305) and extracted at the end of the study for subsequent analysis.

Vacuum Dynamics. We collected measurements of the vacuum dynamics every other week from a subset of cows using an electronic vacuum measurement device (VaDia, Biocontrol, Rakkestad, Norway). For this purpose, we used 6 VaDia devices and installed them in 6 milking units according to the guidelines provided by the manufacturer. The data were analyzed by 1 trained investigator (MES) using the related software program (version 1.15.0.932, VaDia Suite, BioControl). We determined the average cyclical vacuum fluctuations (assessed for 10 pulsation cycles at 60 s after the start of the peak milk flow period) as a proxy for the average claw vacuum during the peak milk flow period. For this purpose, the start of the peak milk flow period was defined according to the manufacturer's manual (Biocontrol, 2011) and detected as follows: The average teat-end vacuum (i.e., short milk tube vacuum) in 10-s periods after attachment was monitored. When the average vacuum from 1 period to the next declined less than 0.15 kPa, the midpoint of the first (of the 2) periods was indicated as the start of the peak milk flow period. This automated assessment was performed with the "Split" function and was visually confirmed. In addition, we manually evaluated the duration of the high-frequency pulsation stimulation for cows in groups SPS100 and SPS300 using the vacuum dynamics in the short-pulsation tube with the "Delta Time" function in a subset of cows.

Hind-Leg Activity. We obtained hind-leg activity (**HLA**) measurements every other wk from a subset of cows according to the procedure outlined by Raoult et al. (2021). For this, we installed six 3-dimensional accelerometers (MSR Electronics GmbH, Pocking, Germany) on the claw piece of 6 milking units. The time of attachment of the milking unit and detachment of the milking unit were recorded manually. The acceleration data during milking was pulled along with a time stamp using the adjunct software MSR r6.09.01 (MSR Electronics GmbH) to a comma-separated file for each milking observation.

Fecal Glucocorticoid Metabolites. We collected a fecal sample from all cows during the afternoon in wk 4 and 8 as described previously (Bertulat et al., 2013). In brief, ~50 to 80 g of feces were obtained from the rectum with a disposable obstetrical sleeve (VetOne OB Sleeves, #603042, MWI, Boise, ID), placed into sample vials (90 mL, #244010, Parter Medical Products Inc., Carson, CA), stored on ice immediately, transported, and frozen at −20°C until analysis. To extract the fecal glucocorticoid metabolites, we thawed the samples at 4°C. The samples were mixed thoroughly, and 0.5 g of feces was weighed (MXX-123, Denver Instrument Inc., Bohemia, NY), added to 15-mL tubes (15-mL centrifuge tube, #430791, Corning Science Mexico, Reynosa, Mexico), and dispersed in 5 mL of 80% methanol (MX0490-4, EMD Millipore Corporation, Burlington, MA). Subsequently, the samples were vortexed for 30 min with a multitube vortexer (#02-215-450, Thermo Fisher Scientific, Waltham, MA) and then centrifuged at 2,500 × g for 15 min at room temperature (TJ-6, Beckman Coulter Inc., Brea, CA). Last, a 0.5-mL aliquot of the supernatant was transferred into a 1.7-mL tube (VWR Microcentrifuge Tubes, #87003-294, VWR International, Radnor, PA) and dried at 60°C with a heat block (Dry Bath, #BSH1002, Benchmark Scientific Inc., Sayreville, NJ). To determine the concentration of 11,17-dioxoandrostanes (**11,17-DOA**), an 11-oxoetiocholanolone enzyme immunoassay validated for use in cattle (Palme et al., 1999) was performed as previously described (Palme and Möstl, 1997). Concentrations of 11,17-DOA are presented in nanograms per gram of fresh feces.

Analytical Approach

We analyzed the effects of treatment on outcome variables of interest which included milking characteristics (milk yield and milking unit-on time), bimodality, teat tissue conditions (STC and hyperkeratosis), SCC, clinical mastitis, culling, and HLA. Data were maintained in Excel (Microsoft Office Excel 2019; Microsoft Corp., Redmond, WA). Data from cows that were lost to follow-up were included in the analyses up until the dry day or the point of removal from the herd. We performed the statistical analyses with the software program SAS (version 9.4; SAS Institute Inc., Cary, NC).

Baseline Characteristics. We conducted chi-squared tests with PROC FREQ in SAS to determine differences in lactation number (i.e., first, second, and ≥third lactation), stage of lactation (i.e., ≤100, 101–200, and >200 DIM), nonlactating quarter status (presence or absence), and hyperkeratosis status (presence or absence) on the first day of the study among groups. Differences in logSCC from the test before the start of the study, average daily milk yield in the week before the study, and the mean cyclic average vacuum fluctuations among groups were assessed with ANOVA using PROC ANOVA.

Milking Characteristics. Data from the first 2 d of the study were excluded to allow the cows in the treatment groups to adjust to the differences in milking procedures. In the first step, we screened the data for missing and erroneous values by investigating the distributions of milk yield and milking unit-on time. We removed observations with missing values, as well as observations with outliers or probable data errors, by excluding observations with values of <2.5 kg for milk yield and values of >800 s for milking unit-on time. To evaluate the effect of treatment on milking characteristics, milk yield, and milking unit-on time, we fitted 2 separate general linear mixed models with PROC MIXED. Milk yield and milking unit-on time, respectively, were included as the dependent variables. To account for the dependence of milking observations between milking sessions and days within a cow, we included a REPEATED statement. Three covariance structures (autoregressive order 1, compound symmetry, and variance components) were evaluated to model the covariance of the repeated measurement, and the covariance structure that led to the smallest Akaike information criterion was selected. Treatment was forced into each model as a fixed effect. We considered the following independent variables and initially screened them for inclusion in each model through univariable analysis: lactation number (first, second, or ≥third lactation), stage of lactation (≤100, 101–200, or >200 DIM), and logSCC. We included all variables with a P-value <0.20 in this step in the initial models. We calculated Spearman correlation coefficients with PROC CORR to assess collinearity among the eligible variables and considered coefficients >|0.60| to be indicative of collinearity. We performed manual backward elimination, and only variables with a P-value <0.05 remained in the final model. Finally, we assessed 2-way interactions between treatment and the remaining variables individually and retained them in the final model if the P-value was <0.05. Tukey-Kramer's post hoc test was used for multiple comparisons. Finally, we assessed the assumptions of homoscedasticity and normality of residuals by inspecting plots of residuals versus the corresponding predicted values and examining quantile-quantile residual plots for each final model. To satisfy these assumptions, data on the dependent variable milking unit-on time were $\log_{10}$-transformed. The resulting LSM estimates were consequently back transformed and presented as the geometric mean and 95% CI.

Bimodality. To determine differences in the odds of bimodality between groups, we fitted a generalized linear mixed model with a logit link and a binomial distribution using PROC GLIMMIX in SAS. We included cows as a random effect to account for the clustered structure of the data. Treatment was included as a fixed effect. Lactation number (first, second, or ≥third lactation), stage of lactation (≤100, 101–200, or >200 DIM), and the logSCC from the test day before the study were considered independent variables and initially screened for inclusion through univariable analysis. All variables with a P-value <0.20 in this step were included in the initial model. Manual backward elimination was used, and only variables with a P-value <0.05 remained in the final model. Finally, the 2-way interaction between treatment and the remaining variable was evaluated and retained in the model if the P-value was <0.05.

Teat Tissue Condition. To compare differences in STC and teat-end hyperkeratosis among groups, 2 generalized linear mixed models with a logit link and a binomial distribution were fitted with PROC GLIMMIX. We included cow as a random effect to account for the clustered nature of the data and modeled the covariance using the variance components covariance structure. Treatment was entered into the models as a fixed effect. We considered lactation number (first, second, or ≥third lactation), stage of lactation (≤100, 101–200, or >200 DIM), and logSCC from the test day before the study as independent variables and initially screened them for inclusion with univariable analysis. For the dependent variable, teat-end hyperkeratosis, the baseline value (i.e., teat-end hyperkeratosis assessed on the first day of the study) was also included. We included all variables with a P-value <0.20 in univariable analysis in the initial models. Manual backward elimination was used to achieve the final models. Two-way interactions were evaluated and retained in the final model if the P-value was <0.05.

Somatic Cell Count. To compare the logSCC among groups, we generated a general linear mixed model with PROC MIXED in SAS. The model was constructed in accordance with the procedure outlined above and modified as follows: first, we included a REPEATED statement for test day to account for the dependence of the test day data within a cow. Second, in addition to lactation number, and stage of lactation, the logSCC from the test day before the start of the study was also included. Third, to calculate the LSM over time, we included the interaction between treatment and test day.

Clinical Mastitis and Culling. We generated survival curves of the time to clinical mastitis event and time to culling with GraphPad Prism (version 10.1.2, GraphPad Software, San Diego, CA). To explore differences in the risks of clinical mastitis and culling among groups, we constructed 2 separate semiparametric Cox proportional hazards models (Cox, 1972) with PROC PHREG in SAS. The days to occurrence of clinical mastitis and culling were the outcomes of interest and included as the dependent variables. The independent variable of interest was treatment, which was added to each model as a fixed effect. We considered lactation number, stage of lactation, and logSCC from the test day before the start of the study as additional independent variables and initially screened them for inclusion with a univariable analysis. All variables with a P-value <0.20 in the univariable analysis were included in the initial models. Manual backward selection was performed to reach the final model. We retained variables with a P-value <0.05. For the dependent variable time to mastitis, animals that had no mastitis event by the end of the follow-up and animals that went dry or were removed from the herd before a mastitis event were right-censored. For the analysis of time to culling, animals that were dried during the study and those that remained in the herd at the end of the follow-up period were right-censored. We assessed each final model for the proportionality assumption with the ASSESS statement and assumed model fit if none of the predictor variables violated the Supremum test at a level of $P < 0.05$. Finally, we also assessed the post hoc power analyses for clinical mastitis and culling based on our sample size for an α-level of 0.05, using JMP Pro (version 17.2.0, SAS Institute Inc.).

Hind-Leg Activity. For the HLA data, we obtained the lateral accelerations (x-axis) at a frequency of 50 Hz. The acceleration data obtained were processed using R version 4.3.2 (R Core Team, 2023) as described by Raoult et al. (2021) as follows. We eliminated the first 3 s of data from the time of attachment to account for the stabilization of the cluster following milking unit attachment. We used the "slider" package version 0.3.1 (Vaughan, 2023) to obtain the rolling mean acceleration value over a window of 1 s. We screened the values to remove smaller variations and kept larger accelerations by applying the standard deviation filter over the sliding window and obtained the absolute value of the difference between the measured value and the rolling mean of the values measured during the previous second. Subsequently, we removed accelerations smaller than 0.13 *g* (i.e., 1.27 m s^{-2}) to eliminate cluster movements unrelated to HLA (Raoult et al., 2021). To consider the rocking motion of a cluster following a leg movement, consecutive activities that were less than 3 s apart were merged as a single period of activity (Raoult et al., 2021). Finally, we counted the total number of HLA during the milking session for each cow and calculated the HLA per minute by dividing the total number of activities by the milking duration. The final data were log10-transformed to meet the normality assumption and analyzed in SAS. To analyze the total number of HLA, we fitted a generalized mixed model with a log link and a negative binomial distribution using PROC GLIMMIX. The HLA per minute was fitted with a general linear mixed model using PROC MIXED. The following steps were similar for both models. The independent variables under consideration were lactation number, stage of lactation, and SCC. Treatment was forced into the model as a fixed effect. To account for variabilities among cows, a random effect for cow was considered. Models were fitted using manual backward elimination until each variable had a *P*-value ≤0.05. Tukey-Kramer post hoc tests were used for multiple comparisons. We used the "lsmeans" and "ilink" functions to obtain the LSM and corresponding 95% CI.

Fecal Glucocorticoid Metabolites. To determine differences in fecal 11,17-DOA concentrations among groups, we fitted a generalized linear mixed model with PROC MIXED. The independent variables of consideration and the model building were in accordance with the procedures outlined above for the milking characteristics.

RESULTS

Description of the Study Population

A total of 491 cows (164 SPS100, 164 SPS300, and 163 CON cows) were enrolled in the study. The average (mean ± SD) DIM at the day of enrollment was 174 ± 90, ranging from 17 to 421 d. The average (mean ± SD) daily milk yield in the wk before enrollment was 42.0 ± 9.2 and ranged from 7.5 to 66.7 kg. The median SCC was 27,000 cells/mL and ranged from 13,000 to 9,052,000 cells/mL. A nonlactating quarter was observed in 15 cows (3%). A total of 74 cows (15.1%) were identified with hyperkeratosis of 1 or more teats at the first assessment. Baseline characteristics stratified by group are shown in Table 1. During the study, 80 cows were dried off, 18 cows were sold, and 3 cows died. Thus, 390 cows completed the study.

Vacuum Analytics

We obtained a total of 151 vacuum measurements from 138 cows (41 in wk 2; 25 in wk 4; 38 in wk 6; and 47 in wk 8). Six observations with an average cyclical vacuum fluctuations of <30 kPa were excluded, resulting in a total of 145 vacuum measurements that were available for the final analysis. The overall mean (± SD) of the average cyclical vacuum fluctuations was 40.2 ± 1.6 kPa and ranged from 35.6 to 43 kPa. The mean (± SD) cyclical vacuum fluctuations among the 3 groups were 40.2 ± 1.5 kPa in group SPS100, 40.0 ± 1.4 kPa in group SPS300, and 39.8 ± 1.7 kPa in group CON and were not different ($P = 0.50$). The average (mean ± SD, range) stimulation durations (i.e., durations of high-frequency pulsation) were 13.7 ± 2.9 (7.8–19.2) s in group SPS100 and 13.9 ± 3.8 (5.4–19.5) s in group SPS300.

Milking Characteristics

We obtained data from 73,330 cow milkings over the 64-d study period. The exclusion of the first 2 d (2,704 observations) resulted in 70,626 observations. These were inspected for missing and erroneous values. A total of 950/70,626 observations (1.3%) were excluded due to missing or erroneous values, leading to a total of 69,676 individual milking observations that were available for statistical analyses. The average values (mean ± SD) of milk yield per milking were 14.6 ± 3.6 kg, milking unit-on time was 221 ± 45 s, 15-s milk flow rate was 1.8 ± 0.9 kg/min, 15 to 30-s milk flow rate was 4.0 ± 1.6 kg/min, 30 to 60-s milk flow rate was 4.4 ± 1.7 kg/min, and 60 to 120-s milk flow rate was 4.8 ± 1.3 kg/min. We documented bimodality in 27,442/69,676 milking observations (39.4%). Descriptive statistics of the milking characteristics stratified by group are shown in Table 2.

The final model for milk yield per milking included treatment group ($P = 0.96$), lactation number ($P < 0.0001$), and stage of lactation ($P < 0.0001$). The LSM (95% CI) for cows in the SPS100, SPS300, and CON groups were 14.6 (14.2–15.0), 14.6 (14.2–15.0), and 14.6 (14.2–15.0) kg/milking (Figure 1A). The final model for the dependent variable milking unit-on time included treatment group ($P = 0.98$), lactation number ($P < 0.0001$), stage

Table 1. Baseline characteristics of 491 Holstein cows subjected to 3 different regimens[1] of premilking stimulation over a 64-d period

Item[2]	SPS100	SPS300	CON	P-value[3]
Lactation number (n, %)				0.99
1	68 (41.5)	68 (41.5)	68 (41.7)	
2	17 (10.4)	16 (9.8)	16 (9.8)	
≥3	79 (48.1)	80 (48.7)	79 (48.5)	
Stage of lactation (n, %)				0.99
≤100 DIM	39 (23.8)	40 (24.4)	41 (25.1)	
101–200 DIM	57 (34.8)	58 (35.4)	58 (35.6)	
>200 DIM	68 (41.4)	66 (40.2)	64 (39.3)	
LogSCC[4]	4.59 ± 0.53	4.55 ± 0.43	4.58 ± 0.53	0.80
Average milk yield[5] (kg)	41.8 ± 9.2	42.1 ± 9.1	42.2 ± 9.3	0.91
Nonlactating quarter (n, %)				0.48
Present	3 (1.8)	6 (3.7)	6 (3.7)	
Absent	151 (92.1)	142 (86.6)	147 (90.2)	
Not assessed	10 (6.1)	16 (9.7)	10 (6.1)	
Hyperkeratosis (n, %)				0.48
Present	28 (17.1)	19 (11.6)	27 (16.6)	
Absent	122 (74.4)	124 (75.6)	119 (73.0)	
Not assessed	14 (8.5)	21 (12.8)	17 (10.4)	

[1]Cows in the treatment groups (SPS100 and SPS300) received supplemental stimulation of a maximum of 20 s after the premilking udder preparation by means of high-frequency pulsation (at a pulsation ratio of 25:75) at 100 (SPS100) and 300 (SPS300) cycles per minute, respectively. Control cows (CON) received only traditional premilking stimulation by means of manual forestripping and wiping.

[2]The results presented are the mean ± SD, unless otherwise stated.

[3]P-values were derived from Pearson's chi-squared tests and ANOVA for categorical and continuous variables, respectively.

[4]SCC from 3 d before the start of the study, $\log_{10}$-transformed.

[5]Daily average milk yield for the last 7 d before the start of the study.

of lactation ($P < 0.0001$), and logSCC ($P = 0.01$). The LSM (95% CI) for cows in the SPS100, SPS300, and CON groups were 217 (212–224), 218 (211–224), and 218 (212–224) s, respectively (Figure 1B).

The final model for the dependent variable bimodality included treatment group ($P = 0.64$), lactation number ($P < 0.0001$), and stage of lactation ($P = 0.0003$). Compared with cows in the CON group, the odds ratios (**OR**) and 95% CI of bimodality were 0.82 (0.48–1.41) in the SPS100 group and 1.05 (0.62–1.80) in the SPS300 group (Figure 2A).

Teat Tissue Condition

A total of 1,507 cow observations were available for analysis. We recorded STC in 317/1,521 (20.8%) cows (SPS100, 111/521 [21.3%]; SPS300, 118/493 [23.9%]; and CON, 88/507 [17.4%]). The final model for the pres-

Table 2. Descriptive statistics of the milking characteristics[1] from 69,676 milking observations of 491 Holstein cows subjected to 3 different regimens[2] of premilking stimulation over a 64-d period

Item[3]	SPS100	SPS300	CON
Milk yield (kg)	14.6 ± 3.5	14.7 ± 3.8	14.6 ± 3.7
Milking unit-on time (s)	220 ± 46	221 ± 45	223 ± 45
Milk flow rate (kg/min)			
First 15 s	2 ± 0.8	1.8 ± 0.9	1.8 ± 1
15–30 s	4.1 ± 1.7	3.9 ± 1.7	4 ± 1.5
30–60 s	4.5 ± 1.7	4.4 ± 1.7	4.3 ± 1.6
60–120 s	4.8 ± 1.3	4.8 ± 1.3	4.8 ± 1.4
Bimodality (n; %)	8,642/23,560; 36.7	9,580/23,050; 41.6	9,220/23,066; 40.0

[1]Data were obtained with electronic on-farm milk meters (AfiMilk MPC Milk Meter, Afimilk, Kibbutz Afikim, Israel).

[2]Cows in the treatment groups (SPS100 and SPS300) received supplemental stimulation of a maximum of 20 s, after manual stimulation, by means of high-frequency pulsation (at a pulsation ratio of 25:75) at 100 (SPS100) and 300 (SPS300) cycles per minute, respectively. Control cows (CON) received only traditional premilking stimulation by means of manual forestripping and wiping.

[3]The results presented are the mean ± SD, unless otherwise stated.

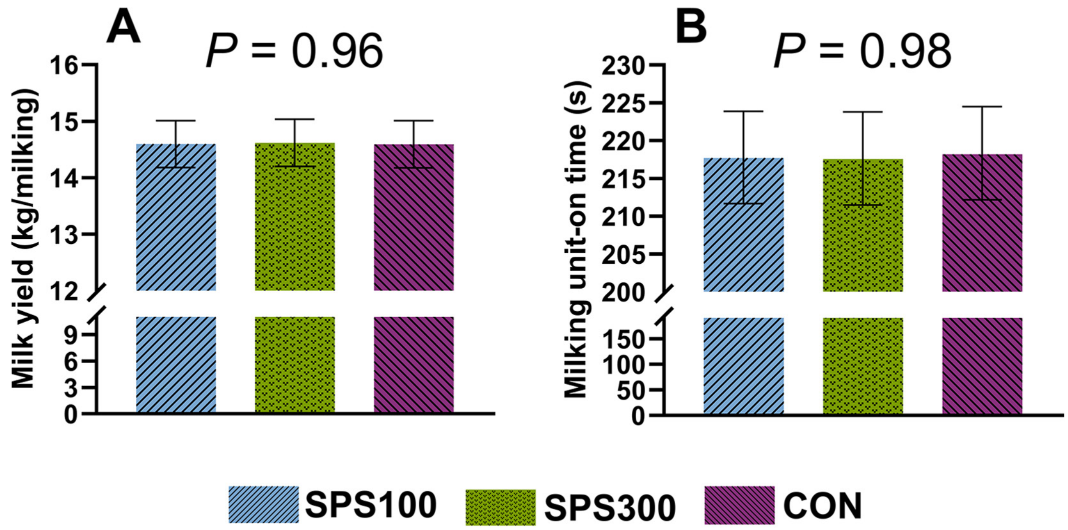

Figure 1. Least squares means derived from general linear mixed models show the effects of 3 different regimens of premilking stimulation on milk yield per milking (A) and milking unit-on time (B). Cows in the treatment groups (SPS100 and SPS300) received supplemental stimulation of a maximum of 20 s, after completion of traditional manual stimulation, by means of high-frequency pulsation (at a pulsation ratio of 25:75) at 100 (SPS100) and 300 (SPS300) cycles per minute, respectively. Control cows (CON) received only traditional premilking stimulation by means of manual forestripping and wiping. Error bars show the 95% CI. *P*-values are shown for the effect of treatment.

ence or absence of STC included treatment ($P = 0.04$), whereas no other variables were retained in the model. Compared with cows in the CON group, the OR (95% CI) of STC were 1.29 (0.94–1.76) in the SPS100 group and 1.50 (1.10–2.04) in the SPS300 group (Figure 2B).

Regarding teat-end hyperkeratosis, 1,521 observations were available for analysis. We missed 8.5%, 12.8%, and 10.4% of cows, respectively, from SPS100, SPS300, and CON cows for evaluation of hyperkeratosis. We documented teat-end hyperkeratosis in 212/1,507 (14.1%) cases (SPS100, 79/518 [15.3%]; SPS300, 57/488 [11.7%]; and CON, 76/501 [15.2%]). The final model for the presence or absence of teat-end hyperkeratosis included treatment ($P = 0.69$) and the baseline value from the start of the study ($P < 0.0001$; Figure 2C). No difference in the odds of hyperkeratosis were observed among the treatment groups. With the CON group as a reference, the OR (95% CI) was 0.89 (0.58–1.38) for cows in the SPS100 group and 0.82 (0.52–1.30) for cows in the SPS300 group. The presence of teat-end hyperkeratosis at the start of the study increased the odds of teat-end hyperkeratosis (OR [95% CI]: 33.76 [23.20–49.13]).

Somatic Cell Count

We obtained a total of 904 test observations from 450 cows for the 2 test days. Of these, 45 SCC observations were missing. Excluding those, a total of 859 individual test observations (450 on test day 1 and 409 on test day 2) from 450 cows were available for SCC analysis. The

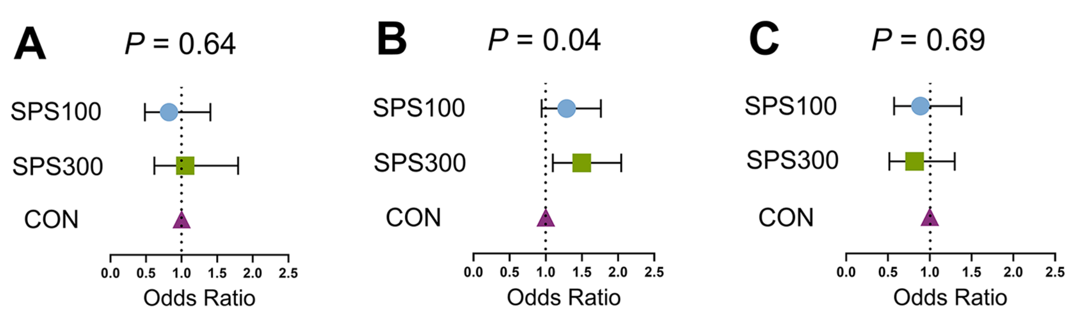

Figure 2. Adjusted odds ratios and 95% CI of bimodality (A), short-term teat tissue changes induced by machine milking (B), and teat-end hyperkeratosis (C) derived from the generalized linear mixed model on the effect of 3 different regimens of premilking stimulation. Cows in the treatment groups (SPS100 and SPS300) received supplemental stimulation of a maximum of 20 s, after completion of traditional manual stimulation, by means of high-frequency pulsation (at a pulsation ratio of 25:75) at 100 (SPS100) and 300 (SPS300) cycles per minute, respectively. Control cows (CON) received only traditional premilking stimulation by means of manual forestripping and wiping. *P*-values are shown for the effect of treatment.

median SCC over the 2 test days was 32,000 cells/mL. The final model included test day (P = 0.49), treatment (P = 0.13), the interaction between test day and treatment (P = 0.11), lactation number (P < 0.0001), stage of lactation (P = 0.04), and the logSCC before the start of the study (P < 0.0001). The LSM (95% CI) for treatment groups over the 2 test days are shown in Figure 3. A one-unit higher logSCC before the start of the study increased the logSCC by 0.67 (0.59–0.74) units.

Clinical Mastitis

We documented a total of 37 (7.5%) clinical mastitis cases (SPS100, 5/164 [3.5%]; SPS300, 17/164 [10.4%]; CON, 15/163 [9.2%]). The average value (mean ± SD) for the study days at which the clinical mastitis cases occurred was 36 ± 22 d (SPS100, 13 ± 6; SPS300, 33 ± 22; and CON, 37 ± 24 d). A total of 454 (92.5%) observations were right-censored. Among these, 78 were due to drying (SPS100, 27; SPS300, 22; and CON, 29); 11 were due to culling of the animals (SPS100, 2; SPS300, 4; and CON, 5); and 365 cows had no mastitis by the end of the study (SPS100, 130; SPS300, 121; and CON, 114). Figure 4A depicts Kaplan–Meier survival analysis of time to clinical mastitis for the 3 groups. The final Cox proportional hazards regression model included treatment (P = 0.05) and logSCC (P = 0.0008). Compared with cows in the CON group, the hazard ratio (**HR**) and 95% CI of clinical mastitis was 0.35 (0.13–0.98) in the SPS100 group and 1.22 (0.57–2.64) in the SPS300 group. A one-unit increase in logSCC increased the hazards of clinical mastitis (HR [95% CI] = 2.48 [1.46–4.20]). We assumed model fit based on the Supremum test ($P \geq 0.06$). The post hoc power analyses, at an α-level of 0.05, revealed a power of 0.55 between the SPS100 and CON groups.

Culling

A total of 21 (4.3%) cows were culled (SPS100, 4/164 [2.4%]; SPS300, 8/164 [4.9%]; CON, 9/163 [5.5%]). The mean (± SD) value of study days at which culling occurred was 57 ± 14 d (SPS100, 16 ± 8; SPS300, 24 ± 9; and CON, 38 ± 18 d). A total of 470 (95.7%) cow observations were right-censored. Among the right-

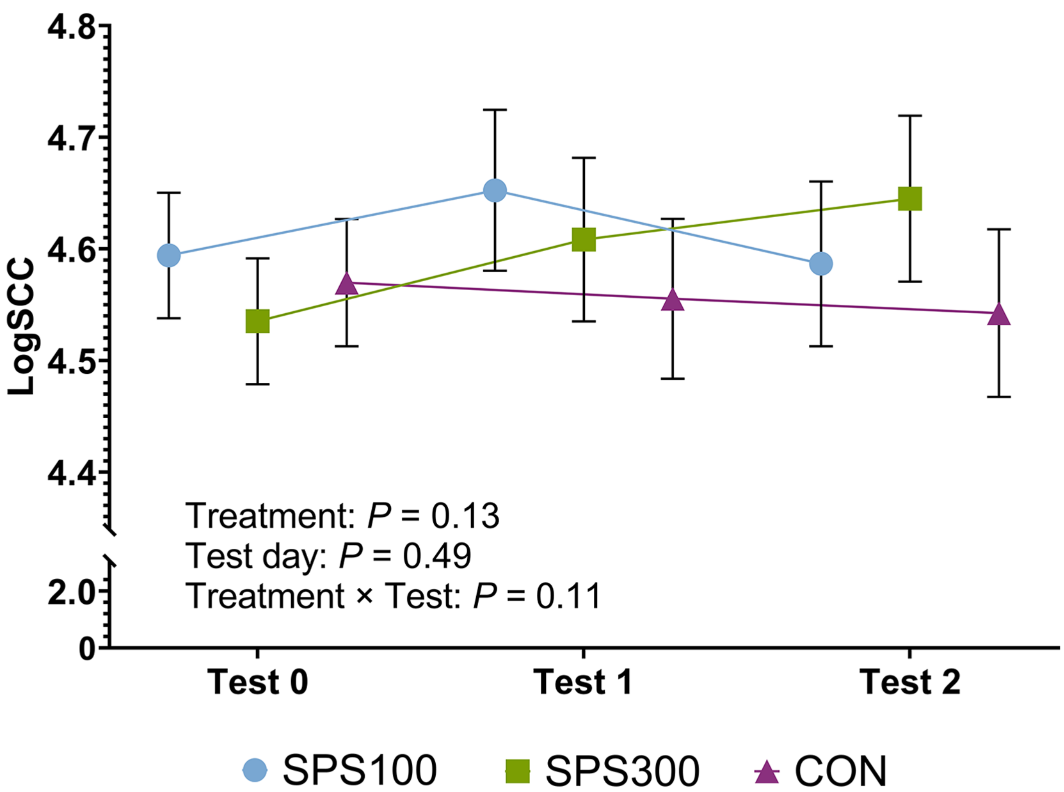

Figure 3. Least squares means of SCC (log_{10}-transformed, logSCC) derived from general linear mixed models on the effect of 3 different regimens of premilking stimulation on SCC (log_{10}-transformed, logSCC). Values at Test 0, Test 1, and Test 2 represent the mean values and 95% CI of the logSCC from the test day 19 d prior, 16 d, and 41 d from the start of the study, respectively. Cows in the treatment groups (SPS100 and SPS300) received supplemental stimulation of a maximum of 20 s, after completion of traditional manual stimulation, by means of high-frequency pulsation (at a pulsation ratio of 25:75) at 100 (SPS100) and 300 (SPS300) cycles per minute, respectively. Control cows (CON) received only traditional premilking stimulation by means of manual forestripping and wiping.

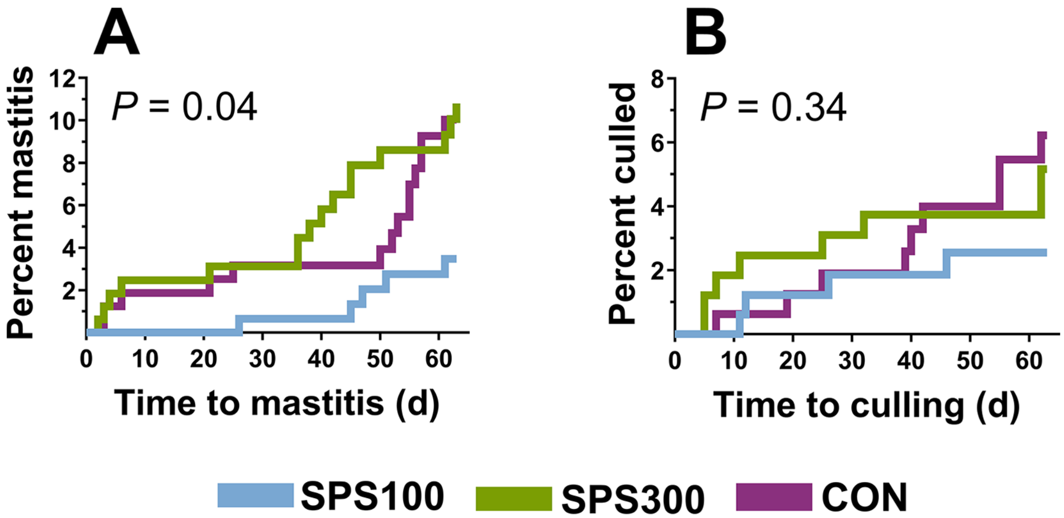

Figure 4. Kaplan–Meier survival analysis of time to clinical mastitis event (A) and culling (B) for 491 Holstein cows subjected to 3 different regimens of premilking stimulation over a 90-d period. Cows in the treatment groups (SPS100 and SPS300) received supplemental stimulation of a maximum of 20 s, after completion of traditional manual stimulation, by means of high-frequency pulsation (at a pulsation ratio of 25:75) at 100 (SPS100) and 300 (SPS300) cycles per minute, respectively. Control cows (CON) received only traditional premilking stimulation by means of manual forestripping and wiping.

censored observations, 80 were due to drying (SPS100, 27; SPS300, 23; and CON, 30), and 390 cows were not culled by the end of the study (SPS100, 133; SPS300, 133; CON, 124). The Kaplan–Meier survival analysis of the time to culling for the 3 groups is depicted in Figure 4B. The final Cox proportional hazards regression model included treatment ($P = 0.04$) and logSCC ($P = 0.0006$). Compared with cows in the CON group, the HR (95% CI) of culling was 0.34 (0.12–0.95) for the SPS100 group and 1.28 (0.60–2.73) for the SPS300 group. A 1-unit increase in logSCC increased the hazards of culling (HR [95% CI], 2.48 [1.47–4.16]). Based on the Supremum test ($P \geq 0.07$), we assumed the model fit. The post hoc power analyses, at an α-level of 0.05, revealed a power of 0.26 between the SPS100 and CON groups.

Hind-Leg Activity

We obtained individual cow HLA from 145 cow milking observations (SPS100, 45; SPS300, 44; CON, 56). The average (mean ± SD, median [range], first and third quartiles) values were 9 ± 7, 7 (1–46), 4 and 11 for the total HLA, and 2.4 ± 1.8, 1.8 (0.2–11.3), 1.1 and 3.2 for HLA per minute. The final models for both total HLA and HLA per minute included the effect of treatment ($P = 0.28$) and lactation number ($P \leq 0.02$). We found no meaningful differences among groups for total HLA or HLA per minute (both $P = 0.28$). Figure 5 illustrates the LSM of HLA among treatment groups. The LSM (95% CI) values of total HLA were 8.3 (6.5–10.5), 10.6 (8.1–13.7), and 9.1 (7.2–11.6) for the SPS100, SPS300, and CON groups, respectively. The LSM (95% CI) values of HLA per minute were 1.8 (1.4–2.3), 2.3 (1.8–3.0), and 2.0 (1.6–2.5) for the SPS100, SPS300, and CON groups, respectively.

Fecal Glucocorticoid Metabolites

We obtained a total of 626 fecal samples (test d 1: 114 SPS100, 111 SPS300, and 111 CON; test d 2: 103 SPS100, 96 SPS300, and 91 CON) that were analyzed for 11,17-DOA concentration. The final model included treatment ($P = 0.51$), lactation number ($P < 0.0001$), test day ($P = 0.01$), and the interaction between treatment and test day ($P = 0.003$). The LSM (95% CI) concentrations of fecal 11,17-DOA (ng/g) at the first and second test days, respectively, were 31.1 (28.1–34.2) and 22.3 (19.2–25.4) for the SPS100 group, 26.4 (23.4–29.4) and 25.2 (22.0–28.4) for the SPS300 group, and 24.8 (21.8–27.9) and 25.0 (21.7–28.3) for the CON group ($P = 0.03$; Figure 6). The first test day 11,17-DOA concentration of SPS100 was higher as compared with CON on the first test day and compared with SPS100 on the second test day ($P \leq 0.03$).

DISCUSSION

Milking Characteristics

In this study, we investigated the effect of high-frequency pulsation stimulation without vacuum alteration, after completion of the traditional practice of premilking

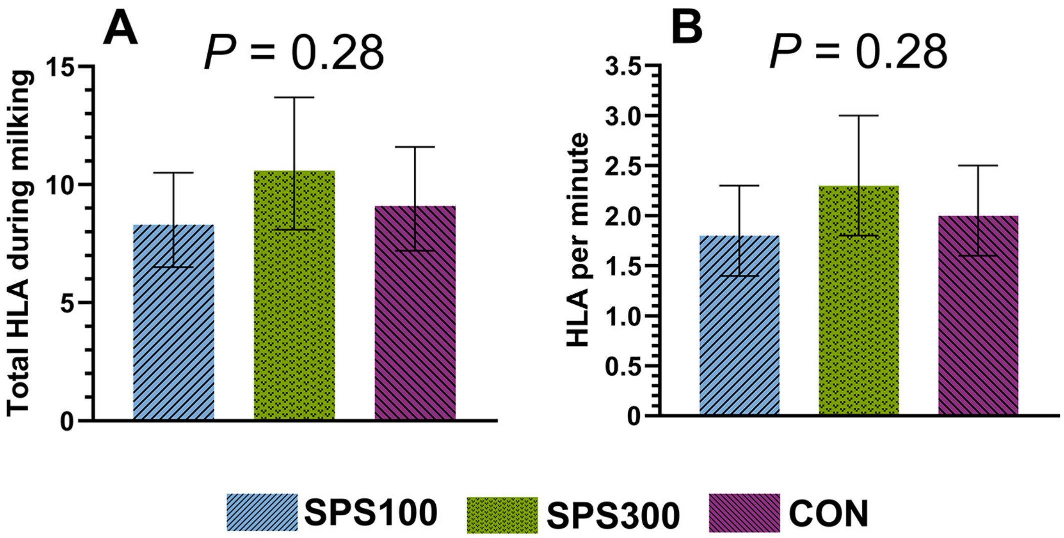

Figure 5. Least squares means derived from generalized mixed models showing the effect of 3 premilking stimulation regimens on the total number of hind-leg activities (HLA) during milking (A) and a general linear mixed model showing the effect of 3 premilking stimulation regimens on HLA per minute (B). Cows in the treatment groups (SPS100 and SPS300) received supplemental stimulation of a maximum of 20 s, after completion of traditional manual stimulation, by means of high-frequency pulsation (at a pulsation ratio of 25:75) at 100 (SPS100) and 300 (SPS300) cycles per minute, respectively. Control cows (CON) received only traditional premilking stimulation by means of manual forestripping and wiping. Error bars show the 95% CI. *P*-values are shown for the effect of treatment.

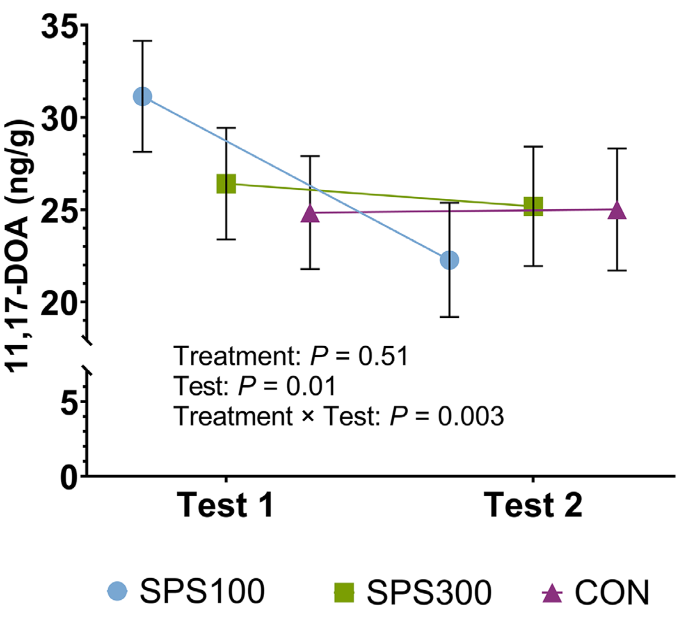

Figure 6. Least squares means derived from general linear mixed models showing the effect of 3 premilking stimulation regimens on 11, 17-DOA during milking during d 16 (Test 1) and d 55 (Test 2) of study. Cows in the treatment groups (SPS100 and SPS300) received supplemental stimulation of a maximum of 20 s, after completion of traditional manual stimulation, by means of high-frequency pulsation (at a pulsation ratio of 25:75) at 100 (SPS100) and 300 (SPS300) cycles per minute, respectively. Control cows (CON) received only traditional premilking stimulation by means of manual forestripping and wiping. Error bars show the 95% CI. *P*-values are shown for the effect of treatment.

udder stimulation regimen, on milking performance, teat tissue condition, udder health, and animal well-being in Holstein dairy cows with a thrice-daily milking schedule. This study extended our previous work where we tested the high-frequency pulsation system to replace the manual stimulation by the human hand (Wieland et al., 2023). Our data showed that the high-frequency pulsation stimulation settings applied in this study did not lead to meaningful differences in milk yield or milking unit-on time compared with the traditional premilking stimulation regimen. This finding is consistent with previous studies involving manual and mechanical premilking stimulation regimens. Sagi et al. (1980) reported no differences in milk yield among the groups when cows were subjected to high-frequency pulsation stimulation (120 cycles/min at a ratio of 30:70) for 60 s, as compared with cows receiving manual or positive pressure stimulation for a similar duration. Upton et al. (2023) reported no difference in milk yield between cows receiving high-frequency pulsation stimulation (120 cycles/min at a ratio of 30:70) for 60 s and cows receiving no premilking stimulation. In a recent study, we evaluated the role of a lower pulsation rate (50 cycles/min at a ratio of 30:70) in conjunction with manual premilking udder preparation and found no differences in the milk yield between groups (Singh et al., 2024).

The absence of meaningful differences in milk yield per milking in previous studies, together with the results described herein, indicate that cows can be milked completely, irrespective of the type and intensity of the premilking udder stimulation provided. We speculate that long-term selective breeding of high yielding Holstein cows for higher milk production evolved the udder in such a way that it facilitates udder emptying, irrespective of the duration and type of premilking stimulation regimen. Recent studies reported no differences in strip yield (residual milk) at the end of milking when different premilking stimulations were applied (Rasmussen et al., 1992; Edwards et al., 2013; Watters et al., 2015), which supports our theory.

We observed no differences in milking unit-on time among groups. A possible explanation could be that the stimulation provided by high-frequency pulsation had no additional effect on milk ejection, compared from the teat stimulation provided by the milking liner at a pulsation rate of 60 cycles/min and a pulsation ratio of 65:35. A previous study by Bruckmaier and Blum (1996) reported the stimulatory effect of the milking machine liner on oxytocin release. The researchers found a similar level of oxytocin release and its maintenance throughout the milking in cows milked by direct attachment of the milking unit at a pulsation rate of 60 cycles/min and a ratio of 65:35, as compared with cows milked upon manual premilking stimulation (Bruckmaier and Blum, 1996). In our study, cows in groups SPS100 and SPS300 were subjected to high-frequency stimulation at a rate of 100 and 300 cycles/min, respectively, for an average duration of 14 s. Thus, one may suggest that cows might have compensated for the time lapsed during stimulation by a fastened milk harvest during milking mode. However, Table 2 indicates that the amount of milk harvested during the first 15 s of milking was not meaningfully different among groups, suggesting that the automated premilking stimulation system did not delay the harvest of milk. Tuor et al. (2022) reported no differences in milking duration when cows were milked with reduced pulsation or reduced pulsation and vacuum with manual premilking stimulation of 5 s, in comparison to manual premilking stimulation for 5 or 15 s. Some previous studies reported a difference in milking unit-on time between cows receiving manual premilking stimulation and those subjected to mechanical stimulation (Sagi et al., 1980; Watters et al., 2015). Sagi et al. (1980) reported longer milking unit-on time when cows were stimulated using high-frequency pulsation (120 cycles/min at a ratio of 30:70) as compared with manual and no premilking stimulation. Watters et al. (2015) found that cows receiving 90 s of high-frequency pulsation with reduced vacuum had the shortest milking duration in comparison to 30 s of mechanical stimulation, 30 or 90 s of manual stimulation, or no premilking stimulation. One must be careful when interpreting the results among studies, as in some studies, the duration between unit attachment and detachment was considered in the calculation of milking duration including the duration of mechanical stimulation (Sagi et al., 1980; Tuor et al., 2022). The same was true in the current study. In contrast, Watters et al. (2015) deducted the duration of high-frequency pulsation from the total milking unit-on time and thus did not include the period of mechanical stimulation. In addition, differences in the stimulation regimens with different preparation lag times and milking machine settings could help explain the differences among studies.

We observed no differences in the odds of bimodality among the groups, suggesting that the high-frequency pulsation system in this study did not meaningfully affect the milk flow rates. Watters et al. (2015) reported a higher incidence of bimodality (17%) in cows that received 90 s of high-frequency pulsation stimulation at reduced vacuum compared with 7% in cows that were subjected to manual forestripping and a preparation lag time of 90 s.

Teat Tissue Condition

We found higher odds of STC in group SPS300 compared with CON cows. We speculate that the increased odds of STC in group SPS300 were due to the inability of the milking liner to properly open and close at a rate of 300 cycles/min during the stimulation phase. This may have led to a higher vacuum-induced strain to the teat tissue and a contemporary lack of effective massage during the d-phase, leading to increased teat congestion. Upton et al. (2023) reported no differences in teat-end congestion when cows were stimulated with high-frequency pulsation for 60 s (120 cycles/min) as compared with cows milked without premilking stimulation. We found no meaningful differences among groups in the odds of hyperkeratosis. Taken together, our data showed that the application of high-frequency pulsation at a pulsation rate of 300 cycles/min with no reduction of the pulsation chamber or claw vacuum, applied in addition to manual premilking stimulation, increased the occurrence of STC, whereas the teat-end condition (i.e., hyperkeratosis) was not affected. However, caution is in order when interpreting the STC results, as we have not established baseline values for STC before the study.

Somatic Cell Count, Clinical Mastitis, and Culling

We observed no differences in logSCC among groups or between test days within groups. The absence of differences in SCC among groups supports the findings of previous studies (Watters et al., 2015; Upton et al., 2023). The lower HR of developing clinical mastitis in SPS100 is difficult to explain, as these results were not supported by those observed for SCC. The post hoc power calculations of clinical mastitis and culling reveled that the study was underpowered for these 2 outcome variables. Therefore, caution is in order when interpreting these results.

Hind-Leg Activity

We did not observe meaningful differences in HLA among the groups. We expected differences in HLA among the groups for two reasons. First, we hypothesized that cows receiving supplemental stimulation through high-frequency pulsation would experience fewer bimodal milk flow curves and thus a lower level and duration of vacuum strain, resulting in gentler milking with less HLA. The second reason for considering HLA in this study was to evaluate if the automated stimulation system tested herein would cause discomfort. At a similar vacuum level applied to all groups, the teats likely experienced similar amounts of forces to impose discomfort, resulting in no meaningful differences in the HLA. We observed an average of 9 HLA per cow, which is consistent compared with Cerqueira et al. (2017) who reported an average number of 6.7 steps and 0.1 kicks per milking. Other factors, such as type of parlor, overmiking durations, temperature of the milking parlor, and SCC, were reported to have an impact on stepping and kicking (Cerqueira et al., 2017).

Fecal Glucocorticoid Metabolite

The rationale for measuring fecal glucocorticoid was to evaluate if the application of high-frequency pulsation may improve animal well-being by reducing stress on the cows. We expected that the application of supplemental stimulation, through mechanical stimulation, at the beginning of milking may facilitate gentle milk harvest through enhanced milk ejection. We were also interested in whether high-frequency pulsation stimulation imposed additional stress during the stimulation phase. Compared with cortisol measurements in blood or saliva, fecal cortisol metabolites reflect cortisol secretions over a longer period of time and are less likely to vary with short-term stressors during the day (Palme, 2019). Previous investigations showed that different short-term, prolonged, or recurrent stressors can lead to an increase in fecal cortisol metabolites (Palme et al., 2000; Möstl et al., 2002). However, our results showed no meaningful differences in fecal 11,17-DOA concentration among the groups, indicating that the high-frequency pulsation stimulation applied in this study did not alter the well-being of the cows. The observed difference between test days 1 and 2 for SPS100 is difficult to explain. A possible limitation to this assessment could be that 11,17-DOA may not be able to detect subtle changes in stress, as other factors such as milk yield are reported to have an impact on plasma cortisol (Vanjonack and Johnson, 1975) and therefore on fecal cortisol metabolites.

Study Limitations and Future Directions

There are certain limitations that must be considered when determining the generalizability of the findings of this study. First, we studied Holstein dairy cows on a single New York dairy that were milked 3 times a day. Thus, our findings may be applicable to commercial dairies in the same region with similar management and milking routines. Second, we were unable to discriminate the milking parameter data between stimulation and milking mode. This hindered our capacity to draw conclusions regarding the parameters of milk flow. Third, the high-frequency pulsation was set to switch to normal milking pulsation after the first dump (200 mL). This may have led to premature switching before the milk ejection reflex was established and a reduced duration of the additional stimulation phase. Fourth, we calculated the sample size based on its ability to detect a difference in milk yield of 1 kg/milking, which is equal to 3 kg/day for a thrice-daily milking schedule. Therefore, we may have failed to detect smaller differences in milk yield if such differences had existed. Finally, our findings reflect the outcome over a timeframe of 64 d. Future research is warranted to determine how mechanical stimulation systems affect udder health and the likelihood of being removed from the herd during the complete course of lactation with a sufficiently large sample size.

CONCLUSIONS

The high-frequency pulsation stimulation applied in this study, after the completion of manual premilking udder preparation, had no meaningful effects on milk yield, milking unit-on time, or the occurrence of bimodality. We attributed the increased odds of STC in the SPS300 group to a higher compressive load on the teat tissue. We observed no meaningful difference in stress level and discomfort among groups. We conclude that applying supplemental stimulation through the high-frequency pulsation system in the setup tested here did not impart additional benefits to the milk harvesting process compared with only manual stimulation. In order to achieve the desired improvement in milking performance and animal well-being, additional modifications in the milking system settings may be necessary, such as lowering the vacuum in the pulsation chamber or milking vacuum during the stimulation phase, as well as expanding the stimulation duration.

NOTES

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Nonstandard abbreviations used: 11,17-DOA = 11,17-dioxoandrostanes; CON = control group; HLA = hind-leg activity; HR = hazard ratio; logSCC = $\log_{10}$-transformed SCC values; OR = odds ratios; SPS100, SPS300 = pulsation treatments at 100 or 300 cycles/min, respectively; STC = short-term changes.

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